Inhibitor-induced HER2-HER3 heterodimerisation promotes proliferation through a novel dimer interface

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Abstract While targeted therapy against HER2 is an effective first-line treatment in HER2\textsuperscript{+} breast cancer, acquired resistance remains a clinical challenge. The pseudokinase HER3, heterodimerisation partner of HER2, is widely implicated in the resistance to HER2-mediated therapy. Here, we show that lapatinib, an ATP-competitive inhibitor of HER2, is able to induce proliferation cooperatively with the HER3 ligand neuregulin. This counterintuitive synergy between inhibitor and growth factor depends on their ability to promote atypical HER2-HER3 kinase domain heterocomplex formation. This dimer exists in a head-to-head orientation distinct from the canonical asymmetric active dimer. The associated clustering observed for these dimers predisposes to neuregulin responses, affording a proliferative outcome. Our findings provide mechanistic insights into the liabilities involved in targeting kinases with ATP-competitive inhibitors and highlight the complex role of protein conformation in acquired resistance.
Introduction

The epidermal growth factor receptor (EGFR) family of receptor tyrosine kinases plays a major role in proliferative signalling in a variety of cancers (Baselga and Swain, 2009; Yarden and Pines, 2012). Apart from EGFR (also known as ErbB1), the family consists of the orphan receptor HER2 (ErbB2), the pseudokinase HER3 (ErbB3), and HER4 (ErbB4). Overexpression of HER2 is an oncogenic driver in approximately 20% of all breast cancers (Lovekin et al., 1991; Owens et al., 2004; Slamon et al., 1987). The high clinical relevance of these receptors has made them a target for directed therapy with both antibodies and small molecule kinase inhibitors. In the case of HER2+ breast cancer, the monoclonal antibody trastuzumab (Herceptin) and its cytotoxic drug-conjugated derivative trastuzumab-emtansine (Kadcyla), the monoclonal antibody blocking HER2-HER3 dimerisation pertuzumab (Perjeta), and the small molecule kinase inhibitor lapatinib (Tykerb/Tyverb) have been successful in the clinic (Blackwell et al., 2010; Cameron et al., 2017; Diéras et al., 2017; Geyer et al., 2006; Krop et al., 2017; CLEOPATRA Study Group et al., 2015; EMILIA Study Group et al., 2012).

While HER2 itself has no known ligand, HER3 binds the growth factor neuregulin (N RG, also known as heregulin or HRG) to induce heterodimerisation and signalling (Sliwkowski et al., 1994). HER3 has been implicated in therapeutic resistance to HER2-targeted therapy through a variety of mechanisms, including receptor rephosphorylation, HER3 overexpression and increased NRG production (reviewed in [Claus et al., 2014]). In terms of cellular signalling in response to HER-family kinase inhibition, HER3-mediated buffering through the Akt/PKB signalling axis has been shown to be an important factor in therapeutic resistance ( Sergina et al., 2007).

The dimerisation of EGFR family members is a fluid process mediated by interaction dynamics in practically every domain of the receptor. For EGFR, the ligand-bound, active dimer shows an upright, back-to-back extracellular domain (ECD) interaction where both receptors have bound ligand, although singly-bound dimers can also occur ( Garrett et al., 2002; Liu et al., 2012; Ogiso et al., 2002). Although HER2 has no known ligand, it natively adopts this upright, dimerisation-ready ectodomain conformation ( Garrett et al., 2002). On the intracellular side, formation of the active kinase domain dimer is critically affected by the conformation of the juxtamembrane domain (JMD) ( Jura et al., 2009a; Thiel and Carpenter, 2007). The kinase domains associate in an asymmetric dimer, which resembles the CDK/cyclin-like asymmetric dimer interface (Jeffrey et al., 1995; Zhang et al., 2006). In this canonical dimer, one kinase (the ‘activator’) allows the dimerisation partner (the ‘receiver’) to adopt an active conformation and become catalytically active. These various conformations have also been observed in near-complete receptors using negative stain electron microscopy ( Mi et al., 2011). Of note in these receptor dimer formations was the lack of active, asymmetrical kinase domain interactions when the receptor was bound to the ATP-competitive inhibitor lapatinib ( Mi et al., 2011). Although these interactions have mainly been described in the context of EGFR homodimerisation, they remain a template for the interactions of the rest of the EGFR family. The conformation of the active kinase domain interaction has been validated for EGFR-HER3 and HER2-HER3 ( Jura et al., 2009b; Littlefield et al., 2014; van Lengerich et al., 2017).

A multitude of studies, using a variety of techniques, have confirmed that EGFR-family receptors can form higher order oligomers, and that the exact nature of these oligomers is modulated by a variety of conditions, including receptor density, ligand presence, ligand type and temperature-dependent membrane behaviour (Clayton et al., 2005; Clayton et al., 2007; Huang et al., 2016; Nagy et al., 2010; Needham et al., 2016; Saffarian et al., 2007; van Lengerich et al., 2017; Yang et al., 2007; Zhang et al., 2017). Against the backdrop of such a multitude of association modes, it is clear that conformational dynamics and structural rearrangements are an integral regulator of protein behaviour in the EGFR family.

We have shown previously that within a kinase, in this case PKCε, occupation of the nucleotide binding pocket with ATP (or an inhibitor) is a major determinant of protein behaviour, conferring the structural stability required for protein–protein interactions to occur and priming sites to be stably phosphorylated ( Cameron et al., 2009). Similar effects have been observed in several additional
kinases, including PKB/Akt, IRE1, and AMPK (Okuzumi et al., 2009; Papa et al., 2003; Ross et al., 2017; Wang et al., 2012).

A notable example of nucleotide binding pocket occupation inducing behaviour independent of catalysis has been described for the RAF family, originally in cRAF, where the inhibitor SB 203580 paradoxically induced activity (Eyers et al., 1998). More recently, a similar phenomenon has been shown in BRAF, where the small molecule kinase inhibitor vemurafenib blocks the oncogenic mutant V600E, but stabilises the wild type protein, promoting downstream proliferative signalling (Hatzivassiliou et al., 2010; McKay et al., 2011; Poulikakos et al., 2010; Thevakumaran et al., 2015). Within the EGFR family, we and others have shown previously that quinazoline inhibitors can cause homodimer formation of EGFR, and EGFR-MET heterodimerisation, by stabilising particular kinase domain conformers (Arteaga et al., 1997; Bublil et al., 2010; Lichtner et al., 2001; Ortiz-Zapater et al., 2017).

The structural, conformational role that nucleotide pocket occupation can fulfil is particularly interesting in the context of pseudokinases, which have lost their catalytic activity. Sequence analysis shows that many pseudokinases retain several of the conserved residues involved in ATP-binding (Boudeau et al., 2006; Claus et al., 2013). In vitro analysis of the pseudokinome showed that many pseudokinases have nucleotide binding capability (Murphy et al., 2014).

In the case of these ATP-binding pseudokinases, where nucleotide binding does not elicit phosphotransfer, the structural stability conferred by ATP binding may be integral to protein function. This has been observed for the pseudokinase STRAD, which requires ATP binding to sustain a heterotrimeric complex with LKB and MO25 (Zeqiraj et al., 2009a; Zeqiraj et al., 2009b). Similarly, in the pseudokinase FAM20A ATP-binding, albeit in a non-canonical orientation, is essential for stabilising the FAM20A/FAM20C complex (Cui et al., 2015; Cui et al., 2017). ATP binding is a structural requirement for the JAK2 JH2 V617F mutant to promote pathogenic signalling (Hammarén et al., 2015). In the pseudokinase MLKL, ATP-binding pocket occupation is essential for membrane translocation and its role in necroptotic signalling (Hildebrand et al., 2014; Murphy et al., 2013).

HER3 is able to bind ATP (crystallised as PDB ID 3KEX, 3LMG), as well as the Src/ABL inhibitor Bosutinib (PDB ID 4OTW) (Levinson and Boxer, 2014; Davis et al., 2011; Jura et al., 2009b; Murphy et al., 2014; Shi et al., 2010). Considering the importance of HER3 as a conformational
partner in the HER2-HER3 heterodimer, and the established importance of ATP-binding for complex formation in other pseudokinases, the role of nucleotide binding pocket occupation in HER3 function warrants investigation.

Here, we have integrated the study of kinase-autonomous conformational effects of nucleotide binding pocket occupation with that of HER2-HER3 heterointeraction modalities and downstream proliferative phenotypes in response to drug treatment. We show that nucleotide pocket occupation in both HER2 and the pseudokinase HER3 is of great conformational importance for kinase domain heterodimerisation and subsequent proliferative signalling. In HER2+ breast cancer cells this leads to an unexpected synergy between the HER3 ligand NRG and the HER2 inhibitor lapatinib, by which their concomitant binding promotes proliferation in 2D and 3D culture systems. Lapatinib is able to promote heterodimerisation between the kinase domains of full-length HER2 and HER3 in cells. However, this dimer interface is different from the canonical active EGFR-family dimer, and it is necessary for the lapatinib/NRG combinatorial proliferative phenotype. Both the lapatinib-induced heterodimer and the cooperative proliferation effects depend strongly on the ability for the pseudokinase HER3 to bind ATP. Consistent with the model, occupying the pseudokinase HER3 with the Src/Abl inhibitor bosutinib stabilises the pseudokinase domain to the extent that it actually promotes HER2-HER3 heterodimerisation and downstream proliferation.

Results

Lapatinib-NRG co-treatment shows a synergistic effect on proliferation, dependent on HER3 ATP binding

The sensitivity of a variety of oncogene-addicted cell lines to small molecule kinase inhibitors can be counter-acted by the addition of growth factors (Wilson et al., 2012). This includes the case of lapatinib-treated HER2+ breast cancer cell lines, where NRG is seen to mediate a rescue of drug toxicity (Novotny et al., 2016; Wilson et al., 2012). Using different experimental procedures, we have investigated further these competing effects of lapatinib and NRG on the proliferative behaviour of HER2+ breast cancer cells.

In SKBR3, BT474, AU565, and HCC1419 cells treated with a range of lapatinib concentrations for 72 hr, the addition of 10 nM NRG rescues the drug-induced cytotoxicity except at very high drug concentrations (Figure 1a, Figure 1—figure supplement 1a–c).

Interestingly, in the case of the SKBR3, BT474 and AU565 cell lines, low concentrations of lapatinib (~40–400 nM) are able to enhance proliferation in conjunction with 10 nM NRG by 25–30% compared to growth factor alone (Figure 1a, Figure 1—figure supplement 1a–b). A partial response of this cooperative phenotype is observed in ZR75 and HCC1419 cells (Figure 1—figure supplement 1c–d). This phenotype in SKBR3 cells, while observed previously, has gone unremarked (Novotny et al., 2016; Wilson et al., 2012). We corroborated our results with a cell counting assay, in which SKBR3 cells were treated for 72 hr with 250 nM lapatinib or vehicle ±10 nM NRG (Figure 1b). The emergent effect of lapatinib plus NRG depends on lapatinib sensitivity. Two breast cancer cell lines with low lapatinib sensitivity, MCF7 and HCC1569, show low inhibitor-growth factor cooperation (Figure 1—figure supplement 1e–f). The growth phenotype in ZR75 may be partially explained by its HER4 expression, considering that NRG is also a ligand for HER4 (Figure 1—figure supplement 1g).

Although HER3 has been shown to bind lapatinib in vitro with very low affinity (Kd = 5.5 μM) (Davis et al., 2011), the synergistic behaviour between lapatinib and NRG occurs in cells at a ~50 x lower dose than the in vitro Kd, indicating that any binding of lapatinib to HER3 would likely be minor under these conditions. Using a thermal shift assay (TSA), which measures a shift in the thermal stability of a protein after ligand/inhibitor binding in vitro, we also show that lapatinib does not strongly bind HER3 as compared to ATP and a panel of other inhibitors (Figure 2a, see further below).

While EGF treatment rescued SKBR3 cells from the effects of low-concentration lapatinib treatment, synergistic growth effects such as those observed with lapatinib-NRG co-treatment were not observed for lapatinib-EGF co-treated SKBR3 or BT474 cells (Figure 1—figure supplement 1h–i). Although NRG is also a growth factor ligand for HER4, protein levels of HER4 in SKBR3 cells are very low (Figure 1—figure supplement 1g). Additionally, lapatinib is a strong inhibitor of both EGFR and...
HER4 (Davis et al., 2011). Taken together, these data seem to exclude a significant role for EGFR and HER4 in the synergistic growth observed for lapatinib-NRG co-treatment. Moreover, transient knockdown of HER3 with two different siRNA oligonucleotides shows a modest, but consistent reduction in the proliferative effect of ligand-inhibitor co-treatment, implicating HER3 as the relevant growth factor-binding receptor for this NRG response (Figure 1—figure supplement 1j).

The proliferative effects of lapatinib and NRG on SKBR3 cells were also observed in 3D spheroid cultures. As seen in 2D culture systems, in 3D spheroid culture the addition of NRG to lapatinib-treated cells rescues SKBR3 cells from lapatinib-induced cytotoxicity/cytostasis (Figure 1c, Figure 1—figure supplement 1k–l). Lapatinib and NRG share a cooperative effect on the induction of proliferation in 3D spheroid cultures, where spheroid size is greater for inhibitor-ligand co-treatment conditions than for those treated with growth factor alone.

The irreversible inhibitor neratinib binds the same inactive conformation as lapatinib and with similar binding affinity (Davis et al., 2011). However, neratinib is an irreversible inhibitor and forms a covalent bond with HER2<sub>C805</sub>, a residue conserved in EGFR and HER4 but not HER3. Neratinib-NRG co-treatment did not show the synergistic proliferative phenotype observed with lapatinib-NRG, in
either a cell counting assay, or in 3D spheroid formation (Figure 1—figure supplement 2a–d). Similarly, the induction of HER2 and HER3 phosphorylation seen in western blot analysis of lapatinib-NRG co-treated 3D spheroids was absent in neratinib-NRG co-treatment (Figure 1—figure supplement 1l, Figure 1—figure supplement 2d). This indicates that the proliferative phenotype observed for lapatinib is likely to necessitate a dynamic, reversible inhibitor binding. Collectively, the data from both 2D and 3D cultures show that there is a counterintuitive synergy between the HER2 inhibitor lapatinib and the HER3 ligand NRG in driving the proliferation of SKBR3 cells. This prompted us to examine the potential for novel allosteric regulation of HER2-HER3 heterotypic interactions by both ligand and inhibitors.
HER3 nucleotide pocket occupation is of structural importance

To study the effects of ATP binding on HER3 function, we aimed to both stabilise and destabilise the pseudokinase nucleotide-binding pocket. This would allow us to investigate the importance of the structural role that nucleotide binding pocket occupation has been shown to play in several (pseudo)kinases.

To separate the structural and trace catalytic roles that ATP-binding could fulfill in HER3, we used the ATP-competitive Src/Abl inhibitor bosutinib, which has been shown to bind strongly to HER3 but not to other EGFR family members (Levinson and Boxer, 2014; Davis et al., 2011). We compared bosutinib to a small panel of EGFR family inhibitors as well as an additional Src inhibitor, dasatinib, and measured HER3 thermal stability by TSA (Figure 2a, Figure 2—figure supplement 1a). In line with previous observations, we confirmed that HER3 strongly binds bosutinib. Significantly, lapatinib was not able to provide a noticeable thermal shift, which corresponds to previously published results indicating HER3 does not bind lapatinib with high affinity (Davis et al., 2011). While lapatinib was able to confer strongly increased thermal stability to HER2, bosutinib was not (Figure 2b). This is in line with previously published data that indicates HER2 is not a strong bosutinib binder (Davis et al., 2011).

We hypothesised that bosutinib might be able to aid proliferation in a cellular context by stabilising the nucleotide binding pocket of HER3 and helping sustain dimer formation, analogous to vemurafenib-bound behaviour of BRAF. In a 2D proliferation assay, SKBR3 cells treated with bosutinib over 72 hr show a dose dependent induction of proliferation without additional NRG stimulation (Figure 2—figure supplement 1b). This proliferative effect is sustained in eight-day treatments in 3D spheroid cultures (Figure 2c, Figure 2—figure supplement 1d,e). The ability of bosutinib to induce SKBR3 cell proliferation appears to be an EGFR-family mediated event, as lapatinib treatment can curtail its effects in a dose-dependent manner (Figure 2—figure supplement 1e).

In order to destabilise the HER3 nucleotide binding pocket we made the triple mutant HER3KGG. HER3G742 was mutated to methionine to hinder ATP α-phosphate coordination, which by itself has been shown to reduce HER3 mant-ATP binding affinity (Shi et al., 2010). To obstruct ATP binding further, double aspartates were introduced in the glycine-rich loop (HER3G716D/G718D) to mimic the pseudokinase-specific aspartate residue observed in the glycine-rich loop of VRK3 (Scheeff et al., 2009), adding a negative charge in the area where the ATP phosphates would normally sit. Introduction of this ATP-binding deficient HER3KGG mutant into MCF7 cells shows abrogation of ligand-induced trans-phosphorylation of HER3 by HER2 (Figure 2d). SKBR3 cells ectopically expressing HER3wt or HER3KGG show a differential proliferative behaviour upon lapatinib ± NRG treatment. This indicates a critical role for HER3 ATP binding in order to sustain inhibitor-growth factor cooperative proliferation (Figure 2—figure supplement 1f).

The bosutinib binding of HER3wt, HER3KGG, and the proposed drug de-sensitised HER3T787M (Levinson and Boxer, 2014; Dong et al., 2017), was investigated using an in-cell thermal shift assay (CETSA) (Jafari et al., 2014; Reinhard et al., 2015). Where wild type HER3 showed increased thermal stability in cells in the presence of 50 nM bosutinib, HER3KGG did not (Figure 2—figure supplement 1g). Ectopic expression of wild type HER3, but not HER3KGG or HER3T787M, enhances bosutinib-mediated proliferation, indicating this behaviour is driven by bosutinib binding to HER3 directly (Figure 2e). Both HER3KGG and HER3T787M showed normal localization to the plasma membrane, as measured by flow cytometry, indicating that these mutations did not compromise the receptor and its traffic to the plasma membrane (Figure 2—figure supplement 2).

The HER3KGG and bosutinib results indicate that nucleotide pocket occupation in HER3 is essential for its ability to sustain a proliferative signalling pathway under distinct circumstances: in the acute response to growth factor, in promoting ligand-inhibitor cooperative proliferation and even after treatment with a HER3-binding inhibitor. This indicates a critical structural role for HER3 ATP-binding pocket occupation in its ability to sustain heterointeractions and proliferation. Considering the proliferative effects observed with the HER3-binding inhibitor bosutinib, our results also suggest that any residual transferase activity HER3 retains does not appear to be important in these responses in vivo unless we invoke a hit-and-run mechanism of action for bosutinib on HER3 which would seem unlikely.
Lapatinib binding induces HER2-HER3 heterodimerisation

The stability conferred to a protein kinase by small molecule inhibitor binding has been shown to play an important role in the promotion of protein–protein interactions. We investigated the potential role of lapatinib to similarly promote HER2-HER3 heterodimerisation by stabilising particular protein conformations in HER2 using a FRET-FLIM approach. We measured drug-induced heterodimerisation of HER2 and HER3, as we have done previously in the case of drug-induced dimerisation of the EGF receptor (Bubil et al., 2010; Coban et al., 2015).

At endogenous protein levels in SKBR3 cells, we observe lapatinib-driven HER2-HER3 heterodimerisation to levels similar to those seen with NRG (Figure 3a). Interestingly, the lapatinib-induced dimerisation occurs in the absence of exogenously added NRG, indicating a HER2-HER3 dimer that is driven primarily through intracellular domain interactions. MCF7 cells, which express low levels of endogenous HER2 and HER3 compared to SKBR3, also display lapatinib-induced heterodimerisation of ectopically expressed GFP-HER2wt and HA-HER3wt (Figure 3b).

As discussed above, occupation of the nucleotide binding pocket in HER3 is of importance for its ability to sustain proliferation. This is also reflected in the case of lapatinib-induced heterodimer formation, where the introduction of the nucleotide pocket compromised HER3KGG mutant strongly disrupts inhibitor-promoted heterodimerisation (Figure 3c). In line with the proliferative effects described above, bosutinib was also able to directly promote heterodimerisation between HER2 and HER3 (Figure 3d).

Using stochastic optical reconstruction microscopy (STORM), we analysed receptor clustering in SKBR3 cells. Treatment with either NRG, lapatinib, or bosutinib showed a shift in cluster population size compared to control, implying the formation of higher-order oligomers rather than dimers (Figure 3e,f). The exact HER2-HER3 stoichiometry in these drug-treated oligomers remains elusive, because these experimental conditions allowed us to count only cluster size for either HER2 or HER3, not both at the same time. Therefore, it is expected that the observed HER3 clusters also contain uncounted HER2 receptors, and vice versa, as evident in the FRET-FLIM data.

Disruption of the active HER2-HER3 interface

The active signalling dimer in the EGFR family adopts an asymmetric orientation, in which there is a distinct division of labour in the activator-receiver pairing. One kinase (the activator kinase) does not phosphorylate substrates, but binds in a way that helps its heterodimerisation partner (the receiver kinase) in adopting an active conformation. The receiver kinase is then capable of substrate phosphorylation. Originally described for EGFR homodimerisation, and similar to the cyclin/CDK binding mode (Jeffrey et al., 1995; Zhang et al., 2006), this canonical active dimerisation interface has been reported across the EGFR family including the heterodimerisation of HER3, which can only perform the activator role (Jura et al., 2009b; Littlefield et al., 2014; van Lengerich et al., 2017). Mutations that disrupt this active interface in both the activator and receiver partner kinases are well-documented and are schematically highlighted (Figure 4a, Figure 4—video 1).

In the case of the active, activator/receiver interface, HER3 buttresses the inward orientation of the HER2 α-C helix, leaving no space for the HER2 α-C helix to adopt the ‘out’ orientation characteristic of the inactive conformation. We modelled the potential effects of HER2 α-C helix positioning on lapatinib binding to test whether canonical activator/receiver orientation (in which the HER2 α-C helix is pushed inwards) would give sufficient space to still accommodate lapatinib. Our modelling showed that, for a HER2 α-C helix in the active, ‘in’ position, lapatinib binding results in a potential steric clash with HER2E770M774 (Figure 4—figure supplement 1a,b). A general decrease of the nucleotide binding pocket volume from 756 Å³ to 232 Å³ (calculated using SURFNET v1.5 (Laskowski, 1995)) supports these predictions.

To further test whether the lapatinib-induced HER2-HER3 is adopting the canonical activator/receiver orientation, we used FRET-FLIM to investigate lapatinib-induced dimer formation. The I714Q mutation in HER2, which renders the receptor receiver-impaired, disrupted the lapatinib-driven HER2-HER3 association, indicating it is retained in the lapatinib-induced dimer interface (Figure 4b). However, the reciprocal activator-impaired mutation in HER3 (HER3V945R) did not disrupt lapatinib-mediated heterodimerisation, although it efficiently suppressed the canonical active dimer after ligand-induced heterodimerisation (Figure 4c).
It is surmised that the inhibitor binding is able to robustly induce a heterodimer between HER2 and HER3, which is distinct from the canonical active heterodimer induced after growth factor stimulation. The orientation of this non-canonical lapatinib-driven heterodimer retains HER2\textsuperscript{I714} in the...
Figure 4. The lapatinib-induced HER2-HER3 dimer is distinct from the active, asymmetric HER2-HER3 dimer orientation. (a) Schematic representation and molecular model of HER2-HER3 active, asymmetric kinase domain dimer orientation. Inset denotes interaction interface. (b) MCF7 cells were transfected with vectors encoding HER2<sup>wt</sup>-GFP or HER2<sup>I714Q</sup>-GFP and HER3<sup>wt</sup>-HA. Cells were treated as described in Figure 3 and HER2-HER3 association was measured by FRET-FLIM. (c) MCF7 cells were transfected with vectors encoding HER2-GFP and HER3<sup>wt</sup>-RFP or HER3<sup>V945R</sup>-RFP. Cells were incubated as described above, and treated with DMSO, lapatinib or NRG prior to fixation. Data represents mean±SEM. *p<0.05, **p<0.01, ***p<0.001; ****p<0.0001 by one-way ANOVA. Scale bars represent 5 μm. Corresponding data and statistics available as Figure 4—source data 1. Molecular model for the interaction in (a) available as Figure 4—source data 2.

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The following video, source data, and figure supplements are available for figure 4:

Source data 1. Numerical data and statistics relating to Figure 4.
DOI: https://doi.org/10.7554/eLife.32271.020

Source data 2. PDB structure file of molecular interaction model in Figure 4a.
DOI: https://doi.org/10.7554/eLife.32271.021

Figure supplement 1. Model of lapatinib binding in HER2 inactive and active conformations shows a potential steric clash.
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Figure supplement 1—source data 1. PDB structure file of inhibitor docking model in Figure 4—figure supplement 1a.
DOI: https://doi.org/10.7554/eLife.32271.018

Figure supplement 1—source data 2. PDB structure file of inhibitor docking model in Figure 4—figure supplement 1b.
DOI: https://doi.org/10.7554/eLife.32271.019

Figure 4—video 1. Interface view of the molecular model of an active HER2-HER3 heterodimer, with HER2<sup>I714</sup> and HER3<sup>V945R</sup> highlighted.
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dimer interface, giving us a starting point for in silico molecular modelling to investigate potential dimer conformations distinct from the well-described active dimer.

**Lapatinib drives a novel HER2-HER3 heterodimerisation interface**

In the case of type II kinase inhibitors such as lapatinib, the inhibitor stabilises an inactive conformation of the kinase domain, where the α-C helix is tilted outwards. As HER3 lacks the conserved glutamate residue in the α-C helix, HER3<sup>3742</sup> is unable to form the salt bridge normally observed in active kinase domain structures (Huse and Kuriyan, 2002). The HER3 ATP-bound conformation therefore does not show a classical active conformation with the α-C helix tilted inward (Jura et al., 2009b; Shi et al., 2010), but instead resembles the inactive conformation seen in kinases bound to type II inhibitors such as lapatinib. Because lapatinib-bound HER2 and ATP-bound HER3 adopt similar conformations, there is a possibility that the lapatinib-induced, inactive dimer is oriented symmetrically.

In the crystal lattices of EGFR and HER3 kinase domains, two different symmetrical interaction interfaces have been observed (Jura et al., 2009a; Jura et al., 2009b). We used molecular modelling to investigate the potential for HER3 and lapatinib-bound HER2 to adopt either of these conformers (Figure 5a–b, Figure 5—figure supplement 1a–b). HER2<sup>2714</sup> is present in the interaction interface of both the EGFR-like, staggered orientation, as well as in the head-to-head, HER3-like orientation. This falls in line with the FRET-FLIM data in Figure 4 that suggests the retained presence of the HER2<sup>2714</sup> residue in the lapatinib-induced dimer interface.

On the basis of these models, we designed pairs of mutations in HER2 that would exclusively disrupt one of the potential heterodimer orientations (Figure 5—figure supplement 1, Figure 5—video 1 and 2). For the EGFR-like, staggered dimer we substituted two hydrophobic residues on HER2 with two positively charged residues, HER2<sup>2748R/V750F</sup>, which should lead to repulsion from the positively charged residues, K998 and K999, lying on the HER3 side of the interface.

Likewise, for the HER3-like, head-to-head dimer, we predicted that the HER2<sup>N764R/K765F</sup>L700F mutant would disrupt the dimerisation interface. The substitution of an asparagine residue (HER2<sup>N764</sup>) with a positively charged arginine should lead to repulsion from a positively charged HER3 residue (HER3<sup>L700</sup>), lying within a radius of 4 Å and opposite to HER2<sup>N764</sup>, therefore causing severe disruption of the HER3-like dimer interface. Furthermore, the substitution of a lysine residue (HER2<sup>K765</sup>) with a bulky, hydrophobic residue such as phenylalanine should generate clashes at this HER2-HER3 interface.

These dimer interface mutants were introduced into our FRET-FLIM assay for investigation of the lapatinib-induced heterodimerisation conformer (Figure 5c). The HER2<sup>N764R/K765F</sup> mutant disrupted heterodimerisation upon lapatinib binding, whereas HER2<sup>2748R/V750F</sup> showed no difference in heterodimer formation. These mutational FRET/FLIM data is consistent with our model that the lapatinib-induced HER2-HER3 heterodimer adopts a symmetrical, head-to-head orientation, similar to the one observed in the HER3 kinase domain crystal lattice (Jura et al., 2009b) (Figure 5b).

**Head-to-head HER2-HER3 dimerisation is required for inhibitor-induced proliferation**

Having presented modelling and FRET/FLIM data consistent with an orientation of the lapatinib-induced HER2-HER3 dimer being distinct from the active activator/receiver dimer interface, we sought to identify which type of HER2-HER3 interaction caused the NRG-lapatinib co-stimulatory growth observed in 2D proliferation assays.

In these assays, we did not ectopically introduce the HER2<sup>N764R/K765F</sup> mutant because, firstly, it might also disrupt the active, asymmetrical HER2-HER3 heterodimer interface and secondly, SKBR3 cells have vast numbers of endogenous HER2 receptors that would hinder analysis of the behaviour of ectopically expressed HER2<sup>N764R/K765F</sup>. Instead we identified HER3<sup>L700F</sup> as the reciprocal mutant to HER2<sup>N764R/K765F</sup> (Figure 6a, Figure 6—video 1). We introduced HER3<sup>L700F</sup> into SKBR3 cells to investigate the role of the head-to-head, symmetric dimer interface in the lapatinib-NRG synergistic proliferation described above. While the HER3<sup>V945F</sup> active dimer mutant did not disrupt drug-growth factor cooperative proliferation, the HER3<sup>L700F</sup> mutant did (Figure 6d–e). Both HER3<sup>L700F</sup> and HER3<sup>V945F</sup> were expressed on the cell surface, as measured by flow cytometry (Figure 2—figure supplement 2). Combined, this indicates that the inhibitor-induced heterodimer of HER2 and HER3 is consistent with a head-to-head, symmetrical conformation, and it plays an important role in the
Figure 5. The lapatinib-induced HER2-HER3 dimer is in a symmetric orientation. (a) Lapatinib-bound HER2 (blue, lapatinib in yellow) and ATP analogue-bound HER3 (green, AMP-PNP in pink) were modelled in an EGFR-like symmetric dimer orientation (Jura et al., 2009a). Inset highlights the interaction interface. The schematic representation shows active dimer interface residues HER2\textsuperscript{I714} and HER3\textsuperscript{V945}, as well as the two residues in HER2 unique to this interface for further mutational analysis. (b) Lapatinib-bound HER2 in the HER3-like head-to-head symmetric dimer orientation (Jura et al., 2009b). Dimer-specific residues are highlighted in the schematic. (c) MCF7 cells were transfected with vectors encoding HER2\textsuperscript{wt}-GFP, HER2\textsuperscript{N764R/K765F}-GFP or HER2\textsuperscript{I748R/V750R}-GFP and HER3\textsuperscript{wt}-HA. Cells were incubated for 24 hr, and inhibited with 10 \textmu M lapatinib for 1 hr, prior to fixation and staining with anti-HA antibody conjugated to Alexa-546. Data represented as mean ±SEM. ****p<0.0001, as analysed by one-way ANOVA. Scale bars 5 \mu m corresponding data and statistics available as Figure 5—source data 1. Molecular model for the interactions in (a) and (b) available as Figure 5—source data 2 and 3. Residues marking the dimer interface of the lapatinib-induced HER2-HER3 heterodimer, in either the EGFR-like or HER3-like modelled conformations, including the per-residue solvent accessible surface area (in Å\textsuperscript{2}) are available as Figure 5—source data 4. DOI: https://doi.org/10.7554/eLife.32271.023

The following video, source data, and figure supplement are available for figure 5:

Source data 1. Numerical data and statistics relating to Figure 5.
DOI: https://doi.org/10.7554/eLife.32271.025

Source data 2. PDB structure file of molecular interaction model in Figure 5a.
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Source data 3. PDB structure file of molecular interaction model in Figure 5b.
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Source data 4. Table with modelled interface residues, including the per-residue solvent-accessible surface area in Å\textsuperscript{2}.

Figure 5 continued on next page
synergistic proliferative effects of lapatinib and NRG. Although this conformation has been described from the HER3 kinase domain (Jura et al., 2009b), to our knowledge it is the first time a functional role has been ascribed to heterodimers consistent with this interface in cells.

Discussion

The conformational dynamics of HER2-HER3 heterodimerisation are an important consideration for evaluating existing and future targeted therapy intervention strategies against HER2+ breast cancer and other HER family driven cancers. Here, we show that the HER2 inhibitor lapatinib is paradoxically able to promote proliferative behaviour in HER2+ breast cancer cells when administered in the presence of the HER3 ligand NRG. The synergy between growth factor and inhibitor requires an intricate, multi-step cascade of conformational events.

Lapatinib itself is able to promote heterodimerisation between the kinase domains of HER2 and HER3, stabilising an orientation consistent with a symmetric, head-to-head kinase domain heterodimer that is distinct from the canonical, asymmetric, head-to-tail active kinase domain orientation that occurs throughout the EGFR family. An analogous interface has previously been observed in the HER3 kinase domain crystal lattice (Jura et al., 2009b); here, we have provided modelling and cellular evidence of a heterodimer with an interface consistent to the one observed in the HER3 kinase domain crystal lattice. Sequestering HER2 and HER3 in these inactive, lapatinib-bound heterodimers was of benefit to NRG-mediated proliferative signalling. Our results, in which inhibitor binding drives dimer formation that boosts signalling and proliferation, shows some parallels with the inhibitor-induced signalling phenotypes in the RAF-family (Eyers et al., 1998; Hatzivassiliou et al., 2010; McKay et al., 2011; Poulikakos et al., 2010; Thevakumaran et al., 2015).

While the FRET-FLIM analysis of the lapatinib-induced dimerisation was not able to differentiate between heterodimers or higher order oligomers, our clustering data shows that lapatinib is likely to induce higher order oligomers. Because of the modelled symmetrical nature of these lapatinib-induced dimers, in which both lapatinib-bound HER2 and HER3 would be conformationally available as ‘activator’ receptors for additional oligomerization partners, it is not inconceivable they may act as nucleation points for larger oligomeric signalling platforms. Such signalling arrays, in which mutual cooperativity increases signaling output, have been proposed for EGFR oligomers (Huang et al., 2016).

The addition of ligand potentially causes rearrangements within these platforms through the ligand-induced conformational ballet of multi-level interactions between the various extracellular and intracellular domains of EGFR family receptors (reviewed in (Lemmon et al., 2014)). The formation of lapatinib-induced oligomeric platforms may facilitate a transition into active signalling heterodimers upon ligand binding, due to the availability of dimerisation partners in immediate proximity within these drug-induced oligomer platforms.

Both the lapatinib-induced HER2-HER3 heterodimerisation and the downstream lapatinib-NRG synergetic effects on proliferation depended on the ability of HER3 to bind ATP. Although usually classified as a pseudokinase, HER3 has been shown to retain a measure of autophosphorylation activity (not transphosphorylation) under specific circumstances (Shi et al., 2010). We show HER2-HER3 heterodimerisation and downstream proliferative effects can be elicited by the addition of the HER3-binding inhibitor bosutinib, indicating that nucleotide binding pocket occupation performs a...
structural role that is critical to HER3 function, and apparently independent of any retained catalytic activity. Observing increased heterointeractions and cellular proliferation due to inhibition of an activity-deficient kinase is a strong indication of the importance of ATP-binding in certain pseudokinases, and the necessity of pocket-occupied structural conformers in sustaining protein–protein interactions and subsequent downstream signalling output.

Because of the importance of HER3 in HER2-targeted therapy resistance, its conserved ATP binding raised the possibility of targeting HER3 with ATP-competitive kinase inhibitors. Our data show, however, that stabilisation of the HER3 kinase domain with an ATP-competitive kinase inhibitor can have a stimulating effect on HER2⁺ breast cancer cell proliferation. This indicates that the development of small molecule targeted therapy against HER3 for use in HER2⁺ breast cancer needs to be directed away from stabilising the HER3 ATP binding pocket occupied conformer and rather towards...
stabilising the apo, inactive conformer. An exception to this might be the development of irreversible, adamantane-linked inhibitors of HER3 that target the receptor for proteosomal degradation (Xie et al., 2014).

The substantial effect that lower doses of lapatinib have on proliferation in the presence of growth factor may have an impact on the establishment of lapatinib-resistance in vivo. This is in accordance with the observation from xenograft models that resistance occurs much more readily if lapatinib is administered continuously at low doses than if it’s used intermittently at high dose (Amin et al., 2010). Increased production of growth factors (including NRG) is a well-described resistance mechanism against HER2-targeted therapy (reviewed in [Claus et al., 2014]). NRG production by the microenvironment has also been shown to play a role in metastatic spread of ovarian cancer cells that express high levels of HER3 (Pradeep et al., 2014). High expression levels of NRG in HER2+ breast cancer patients showed a strong correlation with disease recurrence (Xia et al., 2013). Several somatic mutations in HER3 observed in cancer fall within the extracellular domain and have a potential effect on ligand-binding affinity (Jaiswal et al., 2013). These mutations may exacerbate the inhibitor-growth factor synergistic behaviour reported here.

Our results provide a potential molecular mechanism for the disappointing results observed in a recent Phase III study of lapatinib used in an adjuvant setting (ALTTO trial) (Piccart-Gebhart et al., 2016). The lapatinib-only arm of this study was terminated prematurely, and the effects observed in the adjuvant setting for both lapatinib-trastuzumab co-treatment and trastuzumab treatment followed by lapatinib were not significant. These clinical results indicate there are complicating factors in hindering lapatinib efficacy in patients, which may involve the expression levels of HER3 and NRG stimulation by a complex tumour microenvironment. The complex relationships between distinct protein conformation dynamics, formation of oligomeric assemblies, the availability of ligand, and the various effects on downstream signalling all need to be considered when applying targeted therapy to avoid potentially unexpected enhanced cancer cell proliferation after inhibitor treatment.

Materials and methods
Reagents and antibodies
NRG1 was purchased from PeproTech. Lapatinib was a kind gift from Professor György Kéri (Vichem Chemie Research Ltd Hungary). Bosutinib was purchased from LC Labs. Total HER2, HER3, PKB, HER2 pY877, HER3 pY1289, PKB pS473 and ERK1/2 pT202/pY204 antibodies were purchased from Cell Signaling Technology, anti-α-tubulin from Sigma, total ERK1/2 from Merck, and Alexa Fluor-488 conjugated anti-HER3 antibody from R&D systems.

Cell culture and plasmid transfection
MCF7 and ZR75 cells were cultured in DMEM supplemented with 10% FCS, SKBR3 cells were grown in McCoy’s medium supplemented with 10% FCS. BT474, AU565, HCC1419, and HCC1569 cells were grown in RPMI with 10% FCS. For BT474 cells 10 μg/ml bovine insulin was included in the culture medium.

Cells were transfected with plasmid DNA using FuGENE 6, FuGENE HD (Roche), or Lipofectamine LTX (Thermo Fisher Scientific) according to the manufacturer’s protocol.

All cell lines were sourced from the Francis Crick Institute’s Cell Services facility, where they were tested negative for mycoplasma and authenticated via STR profiling.

Proliferation assays
For 2D proliferation assays, cells were plated at 1 x 10^4 cells/well in a 96-well plate. The following day they were subjected to treatment for 72 hr, followed by addition of CellTiter-Glo reagent (Promega) and measured on an EnVision plate reader (Perkin Elmer). CellTiter-Glo data were normalised to the growth factor-null/inhibitor-null untreated control. This caused some growth factor-treated plots to start at above-baseline levels, which is an indication of the proliferative effect that growth factor treatment had in these cells.

For 3D spheroid assays, 3 x 10^3 cells were plated in a 96-well, round-bottom, ultra-low attachment plate (Corning) in the presence of 1% Matrigel (Corning). After three days of growth, an equal volume of 2x media containing treatment conditions was added and refreshed every three days for a
total of eight days of treatment. Phase contrast images were taken using a Zeiss Axiovert 40 CFL microscope with a Zeiss 5x A-plan objective and analysed using ImageJ.

**Flow cytometry**

SKBR3 cells were transfected with RFP-HER3 mutants for 48 hr. Cells were pre-treated with 0.5 mM EDTA to facilitate removal from the substrate and stained for HER3 extracellular expression using Alexa Fluor-488 conjugated anti-HER3 antibody (R&D systems, clone 66223) as per manufacturer’s instructions. Briefly, cells were blocked using mouse IgG (Santa Cruz Antibodies) for 15 min at room temperature, followed by incubation with conjugated antibody for 30 min at room temperature in the dark. Cells were washed in PBS, 0.5% BSA, 0.1% sodium azide three times before flow cytometric analysis using a BD Fortessa instrument (BD). Results were analysed using the Flo-Jo software.

**FRET determination by FLIM measurements**

Fluorescence resonance energy transfer (FRET) is used to quantify direct protein–protein interactions and post-translational modifications. Processing of cells for FRET determination by FLIM has been previously described (Barber et al., 2009; Parsons and Ng, 2002). FLIM was performed using time-correlated single-photon counting (TCSPC) with a multiphoton microscope system as described previously (Peter et al., 2005). For experiments measuring endogenous protein, FRET pairs were Cy5-conjugated anti-HER2 IgG, and Alexa546-conjugated anti-HER3 IgG. For exogenous protein measurements, FRET pairs were HER2-GFP and HER3-HA with an anti-HA IgG, tagged with a Cy3 fluorophore. FRET efficiency between the donor and acceptor bound proteins was calculated with the following equation in each pixel and averaged per cell: FRET eff = 1-tau(DA)/tau(control) where tau(DA) is the lifetime displayed by cells co-expressing the donor and acceptor, whereas tau (control) is the mean donor (GFP) lifetime, measured in the absence of the acceptor.

**Modelling HER2-HER3 dimers**

We modelled the HER2-HER3 dimer by comparative homology modelling using a multiple templates approach. The active, asymmetric HER2-HER3 dimer was modelled using the crystal structure of the active EGFR kinase domain (PDB ID 2GS2) (Zhang et al., 2006) and one chain of the crystal structure of the HER3 homodimer (PDB ID 3KEK) (Jura et al., 2009b) as templates. To build the EGFR-like, inactive, symmetric dimer we have used the crystal structure of the EGFR homodimer (PDB ID 3GT8) (Jura et al., 2009a), the crystal structure of EGFR complexed with lapatinib (PDB ID 1XKK) (Wood et al., 2004) and only one chain of the crystal structure of the HER3 homodimer (PDB ID 3KEK) (Jura et al., 2009b). To build the HER3-like dimer, we have used the HER3 homodimer structure (PDB ID 3KEK) (Jura et al., 2009b), the crystal structure of EGFR lapatinib-bound (PDB ID 1XKK) (Wood et al., 2004) and the crystal structure of the inactive EGFR AMP-PNP bound (PDB ID 2GS7) (Zhang et al., 2006). The sequence alignment used to build the model has been created by using PRALINE with the homology-extended alignment strategy (Simossis et al., 2005). We generated 200 three-dimensional models using the MODELLER package (Sali and Blundell, 1993). The selected models were chosen on the basis of the MODELLER objective function’s DOPE score.

The volume of the HER2 ATP binding pocket was calculated with the SURFNET 1.5 package (Laskowski, 1995), where the cavity regions in a protein are built up by fitting a probe sphere of 1.4 Å into the spaces between atoms.

The structural alignment was performed using the multi-seq tool of the VMD 1.9.1 package (Humphrey et al., 1996), and measurement of interaction surface buried residues was performed using POPScomp (Kleinjung and Fraternali, 2005).

**Receptor clustering assays**

STORM imaging and cluster analyses are described in more detail at Bioprotocol (Roberts et al., 2018). SKBR3 cells were treated with either 14 nM Lapatinib or 41 nM Bosutinib. HER2 and HER3 Affibody ligands were used to label the non-activated states of the receptors (HER2 from Affibody Inc. and plasmid encoding the HER3 affibody was a gift from John Löflom, protein made in house and shown to bind specifically to HER3 receptors) and NRG-β1 (Peprotech) was used to stimulate the cells. The conjugation of dyes (Invitrogen) to HER2 and HER3 ligands was done in house and the ratio of dye:ligand was confirmed to be ~1:1. The NRG-dye conjugate has been shown to be as
active as the unlabelled protein. We incubated cells in 100 nM HER2Affibody-Alexa488 + 50 nM HER3Affibody-Alexa647 or 100 nM HER2Affibody-Alexa488 + 10 nM NRG-Alexa647 ± drug for 1 hr. Cells were chemically fixed using 4% paraformaldehyde (EMS solutions)+0.5% glutaraldehyde (Sigma-Aldrich) diluted into ice-cold PBS.

Samples were imaged using a Zeiss Elyra super-resolution microscope to stochastically excite the Alexa488 and Alexa647 fluorophores bound to the receptors in the cells and to image single molecules. Imaging was done in TIRF mode using a 100x oil immersion objective lens. We used a 405 nm laser line to aid fluorophore blinking and 488 nm or 640 nm laser lines to excite the fluorophores, alternating the lasers to image the two receptors independently every 300 frames, over a total of ~10,000 frames. The exposure time was 20 ms. A minimum of two replicates of each sample were imaged generating at least 12 regions (25.6 μm x 25.6 μm) covering at least one cell per region. The Zen software localised the single molecule spots in the cells, a threshold was set to discard background spots and the co-ordinates of the positive localisations (typically 30,000 + for HER2 and 5,000 + for HER3 per region) were passed into the Bayesian cluster identification algorithm (Rubin-Delanchy et al., 2015).

The clustering algorithm expects the background and clusters to be uniformly distributed over a rectangular ROI (Rubin-Delanchy et al., 2015). The analysed images mainly showed single cells. Of interest are the HER2 and HER3 receptors in the cell membrane, which were visible as a circular shape. In order to conform with the prerequisites of the clustering algorithms, rectangular regions have been manually selected that tightly cover the cell membrane using the most suitable angles (assessed by visual inspection). The whole cell membrane has been covered in this way. The data selected by these regions have been rotated so that the sides of the rectangles became parallel to the coordinate axis. The result was used as input for the clustering algorithm and the algorithm was applied as described by the protocol. The complete lists of molecules per cluster that have been produced by the algorithm were used for the presentation.

**Recombinant HER3 KD purification**

The baculoviral HER3 kinase domain construct was kindly provided by Prof. Mark Lemmon, University of Pennsylvania. Sf21 cells at 1 x 10⁶ cells/ml were infected with P3 virus (7 x 10⁷ pfu/ml) at an MOI of 1.0 and allowed to grow for three days. The cells were lysed in lysis buffer containing protease inhibitors, 1 mM DTT and 2 mM BME. The lysate was clarified by centrifugation and incubated with NiNTA resin (Qiagen) for 30 mins at 4°C, after which the resin was washed extensively with buffer containing 50 mM Hepes (pH 7.6), 300 mM NaCl, 2 mM BME, 5% glycerol, 10 mM imidazole. HER3 was eluted in the same buffer with 200 mM imidazole added.

Each elution was centrifuged at 10,000 rpm to remove any precipitate or resin and applied to a S200 gel filtration column in 50 mM HEPES (pH 7.6), 300 mM NaCl, 2 mM BME, 2.5% glycerol.

**Thermal shift assay (TSA)**

Thermal shift assays were carried out as described in (Niesen et al., 2007). Briefly, in a 96-well RT-PCR plate (Life Technologies) 1 μg HER3 kinase domain/well was incubated with 1 μM inhibitor or 200 μM ATP/10 mM MgCl₂ (as indicated) for 30 mins at 4°C in the presence of Sypro Orange dye (Sigma). HER2 TSA experiments were performed in a 384-well RT-PCR plate (Thermo Fisher Scientific). 0.5 μg of HER2 kinase domain/well was incubated with 1 μM lapatinib, 1 μM bosutinib, or 200 μM ATP/10 mM MgCl₂ for 20 mins at 4°C. HER3 measurements were taken on an Applied Biosystems 7500 Fast Real-Time PCR machine, and HER2 measurements on an Applied Biosystems Quant Studio 7 PCR machine. Data were trimmed and a Boltzmann sigmoidal curve fitted in GraphPad Prism 6. The inflection point of the Boltzmann sigmoidal was taken as the Tₘ. Thermal shift ΔTₘ values were obtained by subtracting the Tₘ value of the kinase domain alone control.

**Western blot analysis**

Cells were plated at 0.5 x 10⁵ cells/well in 24-well plates. Cells were lysed in 1x sample buffer (containing 1 mM DTT), sonicated and centrifuged. After centrifugation, the lysates were subjected to SDS-PAGE and analyzed by western blotting.
Cellular thermal shift assay (CETSA)

CETSA was performed with COS7 cells transfected with HER3\textsuperscript{wt}-RFP, HER3\textsuperscript{T787M}-RFP or HER3\textsuperscript{KGG}-RFP plasmids as described in (Jafari et al., 2014; Reinhard et al., 2015). Briefly, COS7 were treated with DMSO or 50 nM bosutinib for 1 hr at 37°C. Cells were washed with PBS, detached and washed again twice with cold PBS. Cell pellets were resuspended in cold PBS with protease inhibitors (Roche) and 100 μl of each cell suspension was transferred into 0.2 ml PCR tubes. PCR tubes were heated for 3 min at 42°C or 50°C in a thermal cycler (DNA Engine DYAD, MJ research, Peltier thermal cycler) and incubated at room temperature for 3 min. Tubes were then immediately transferred onto ice, 35 μl of cold PBS 1.4% NP-40 with protease inhibitors were added and tubes were snap-frozen. Samples were then subjected to two freeze-thaw (at 25°C) cycles and cell lysates were centrifuged at 20,000 g for 1 hr at 4°C. Supernatants were carefully removed and analysed by western blot.

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**References**

Amin DN, Sergina N, Ahuja D, McMahon M, Blair JA, Wang D, Hann B, Koch KM, Shokat KM, Moasser MM. 2010. Resiliency and vulnerability in the HER2-HER3 tumorigenic driver. *Science Translational Medicine* 2:16ra7. DOI: https://doi.org/10.1126/scitranslmed.3000389, PMID: 20371474

Arteaga CL, Ramsey TT, Shawver LK, Guyer CA. 1997. Unliganded epidermal growth factor receptor dimerization induced by direct interaction of quinazolines with the ATP binding site. *Journal of Biological Chemistry* 272:23247–23254. DOI: https://doi.org/10.1074/jbc.272.37.23247, PMID: 9287333

Barber PR, Ameer-Beg SM, Gilbey J, Carlin LM, Keppler M, Ng TC, Vojnovic B. 2009. Multiphoton time-domain fluorescence lifetime imaging microscopy: practical application to protein-protein interactions using global analysis. *Journal of the Royal Society Interface* 6:S93–S105. DOI: https://doi.org/10.1098/rsif.2008.0451.focus

Baselga J, Swain SM. 2009. Novel anticaner targets: revisiting ERBB2 and discovering ERBB3. *Nature Reviews Cancer* 9:463–475. DOI: https://doi.org/10.1038/nrc2656

Blackwell KL, Burststein HJ, Storniolo AM, Rugo H, Sledge G, Koeher M, Ellis C, Casey M, Vukelja S, Bischoff J, Baselga J, O’Shaughnessy J. 2010. Randomized Study of Lapatinib Alone or in Combination With Trastuzumab in Women With ErbB2-Positive, Trastuzumab-Refactory Metastatic Breast Cancer. *Journal of Clinical Oncology* 28:1124–1130. DOI: https://doi.org/10.1200/JCO.2008.21.4437

Boudeau J, Miranda-Saavedra D, Barton GJ, Alessi DR. 2006. Emerging roles of pseudokinases. *Trends in Cell Biology* 16:443–452. DOI: https://doi.org/10.1016/j.tcb.2006.07.003

Bubill EM, Pines G, Patel G, Fruhwirth G, Ng T, Yarden Y. 2010. Kinase-mediated quasi-dimers of EGFR. *The FASEB Journal* 24:4744–4755. DOI: https://doi.org/10.1096/fj.10-16199, PMID: 20682838

Cameron AJM, Escribano C, Saurin AT, Kostelecky B, Parker PJ. 2009. PKC maturation is promoted by nucleotide pocket occupation independently of intrinsic kinase activity. *Nature Structural & Molecular Biology* 16:624–630. DOI: https://doi.org/10.1038/nsmb.1606

Claus et al. eLife 2018;7:e32271. DOI: https://doi.org/10.7554/eLife.32271 19 of 23
11 years' follow-up of trastuzumab after adjuvant chemotherapy in HER2-positive early breast cancer: final analysis of the HERceptin Adjuvant (HERA) trial. The Lancet 389:1195–1205. DOI: https://doi.org/10.1016/S0140-6736(16)32516-2

Claus J, Cameron AJM, Parker PJ. 2013. Pseudokinase drug intervention: a potentially poisoned chalice. Biochemical Society Transactions 41:1083–1088. DOI: https://doi.org/10.1042/BST20130078

Claus J, Patel G, Ng T, Parker PJ. 2014. A role for the pseudokinase HER3 in the acquired resistance against EGFR- and HER2-directed targeted therapy. Biochemical Society Transactions 42:831–836. DOI: https://doi.org/10.1042/BST20140043

Clayton AHA, Walker F, Orchard SG, Henderson C, Fuchs D, Rothacker J, Nice EC, Burgess AW. 2005. Ligand-induced dimer-tetramer transition during the activation of the cell surface epidermal growth factor receptor-A multidimensional microscopy analysis. Journal of Biological Chemistry 280:30392–30399. DOI: https://doi.org/10.1074/jbc.M504770200

Clayton AHA, Tavarnesi ML, Johns TG. 2007. Unligated epidermal growth factor receptor forms higher order oligomers within microclusters on A431 cells that are sensitive to tyrosine kinase inhibitor binding. Biochemistry 46:4589–4597. DOI: https://doi.org/10.1021/bi700002b, PMID: 17381163

Cleopatra Study Group, Swain SM, Baselga J, Kim SB, Ro J, Semiglazov V, Campone M, Ciruelos E, Ferrero JM, Schneeweiss A, Heesons S, Clark E, Ross G, Benyunes MC, Cortés J. 2015. Pertuzumab, trastuzumab, and docetaxel in HER2-positive metastatic breast cancer. New England Journal of Medicine 372:724–734. DOI: https://doi.org/10.1056/NEJMoa1413513, PMID: 25693012

Cober O, Zanetti-Dominguez LC, Matthews DR, Rolfe DJ, Weitsman G, Barber PR, Barbeau J, Devauges V, Kampmeier F, Winn M, Vojnovic B, Parker PJ, Lidke KA, Lidke DS, Ameer-Beg SM, Martin-Fernandez ML, Ng T. 2015. Effect of phosphorylation on EGFR dimer stability probed by single-molecule dynamics and FRET/FLIM. Biophysical Journal 108:1013–1026. DOI: https://doi.org/10.1016/j.bpj.2015.01.005

Cui J, Xiao J, Tagliabracci VS, Wen J, Rahdar M, Dixon JE. 2015. A secretory kinase complex regulates extracellular protein phosphorylation. eLife 4:e06120. DOI: https://doi.org/10.7554/eLife.06120

Cui J, Zhu Q, Zhang H, Cianfroco MA, Leschziner AE, Dixon JE, Xiao J. 2017. Structure of Fam20A reveals a pseudokinase featuring a unique disulfide pattern and inverted ATP-binding. eLife 6:e23990. DOI: https://doi.org/10.7554/eLife.23990, PMID: 28432788

Davis MI, Hunt JP, Herrgard S, Ciceri P, Wodicka LM, Pallaes G, Hocker M, Treiber DK, Zarrinkar PK. 2011. Comprehensive analysis of kinase inhibitor selectivity. Nature Biotechnology 29:1046–1051. DOI: https://doi.org/10.1038/nbt.1990

Diéras V, Miles D, Verma S, Pegram M, Welslau M, Baselga J, Krop IE, Blackwell K, Hoersch S, Xu J, Green M, Gianni L. 2017. Trastuzumab emtansine versus capecitabine plus lapatinib in patients with previously treated HER2-positive advanced breast cancer (EMILIA): a descriptive analysis of final overall survival results from a randomised, open-label, phase 3 trial. The Lancet Oncology 18:732–742. DOI: https://doi.org/10.1016/S1470-2045(17)30312-1

Dong C-L, Guo F-C, Xue J. 2017. Computational insights into HER3 gatekeeper T768I resistance mutation to bosutinib in HER3-related breast cancer. Medicinal Chemistry Research 26:1926–1934. DOI: https://doi.org/10.1007/s00044-017-1901-2

EMILIA Study Group, Verma S, Miles D, Gianni L, Krop IE, Welslau M, Baselga J, Pegram M, Oh DY, Diéras V, Guardino E, Fang L, Lu MW, Olsen S, Blackwell K. 2012. Trastuzumab emtansine for HER2-positive advanced breast cancer. New England Journal of Medicine 367:1783–1791. DOI: https://doi.org/10.1056/NEJMoa1209124, PMID: 23020162

Eyers PA, Craxton M, Morricel N, Cohen P, Goedert M. 1998. Conversion of SB 203580-insensitive MAP kinase family members to drug-sensitive forms by a single amino-acid substitution. Chemistry & Biology 5:321–328. DOI: https://doi.org/10.1016/S1074-5521(99)90170-3

Garrett TPJ, McKern NM, Lou M, Ellerman TC, Adams TE, Lovrecz GO, Zhu H-J, Walker F, Frenkel MJ, Hoyne PA, Jonissen RN, Nice EC, Burgess AW, Ward CW. 2002. Crystal structure of a truncated epidermal growth factor receptor extracellular domain bound to transforming growth factor α. Cell 110:763–773. DOI: https://doi.org/10.1016/S0092-8674(02)00940-6

Geyer CE, Forster J, Lindquist D, Chan S, Romieu CG, Pienkowski T, Jagiello-Gruszfeld A, Crown J, Chan A, Kaufman B, Skarlos D, Campone M, Davidson N, Berger M, Oliva C, Rubin SD, Stein S, Cameron AJM, Parker PJ. 2013. Pseudokinase drug intervention: a potentially poisoned chalice. New England Journal of Medicine 368:2733–2743. DOI: https://doi.org/10.1056/NEJMoa1304320

Hammarén HM, Ungureanu D, Grisouard J, Skoda RC, Hubbard SR, Silvennoinen O. 2015. ATP binding to the pseudokinase domain of JAK2 is critical for pathogenic activation. PNAS 112:4642–4647. DOI: https://doi.org/10.1073/pnas.1423201112

Hatzivassiliou G, Song K, Yen I, Brandhuber BJ, Anderson DJ, Alvarado R, Ludlam MJ, Stokoe D, Gloor SL, Viges G, Morales T, Aliagas I, Liu B, Sideris S, Hoeflisch KP, Jaiswal BS, Seshagiri S, Koeppen H, Belvin M, Friedman LS, et al. 2010. RAF inhibitors prime wild-type RAF to activate the MAPK pathway and enhance growth. Nature 464:431–435. DOI: https://doi.org/10.1038/nature08833

Hildebrand JM, Tanzer MC, Lucet IS, Young SN, Spall SK, Sharma P, Pierotti C, Garnier J-M, Dobson RCJ, Webb AI, Tripaydonis A, Babon JJ, Mulcair MD, Scanlon MJ, Alexander WS, Wilks AF, Czabotar PE, Lessene G, Murphy JM, Silke J. 2014. Activation of the pseudokinase MLKL unleashes the four-helix bundle domain to induce membrane localization and necroptotic cell death. PNAS 111:15072–15077. DOI: https://doi.org/10.1073/pnas.1408987111
Huang Y, Bharill S, Karandur D, Peterson SM, Marita M, Shi X, Kaliszewski MJ, Smith AW, Isacco EF, Kuriyan J. 2016. Molecular basis for multimerization in the activation of the epidermal growth factor receptor. eLifE 5: e14107. DOI: https://doi.org/10.7554/eLife.14107, PMID: 27017828

Humphrey W, Dalke A, Schulten K. 1996. VMD: visual molecular dynamics. Journal of Molecular Graphics 14:33–38. DOI: https://doi.org/10.1016/0267-7855(96)00018-5, PMID: 8744570

Huse M, Kuriyan J. 2002. The conformational plasticity of protein kinases. Cell 109:275–282. DOI: https://doi.org/10.1016/s0092-8674(02)00741-9, PMID: 12015977

Jafari R, Amlqvist H, Axelsson H, Ignatiouschenko M, Lundbäck T, Nordlund P, Martinez Molina D. 2014. The cellular thermal shift assay for evaluating drug target interactions in cells. Nature Protocols 9:2100–2122. DOI: https://doi.org/10.1038/nprot.2014.138, PMID: 25101824

Jaiswal BS, Kljavin NM, Stawiski EW, Chan E, Parikh C, Durinck S, Chaudhuri S, Pujara K, Guillory J, Edgar KA, Janakiraman V, Scholz RP, Bowman KK, Lorenzen M, Li H, Wu J, Yuan W, Peters BA, Kan Z, Stinson J, et al. 2013. Oncogenic ERBB3 mutations in human cancers. Cancer Cell 23:603–617. DOI: https://doi.org/10.1016/j.ccr.2013.04.012, PMID: 23680147

Jeffrey PD, Russo AA, Poljak K, Gibbs E, Hurwitz J, Massagué J, Pavletich NP. 1995. Mechanism of CDK activation revealed by the structure of a cyclinA-CDK2 complex. Nature 376:313–320. DOI: https://doi.org/10.1038/376313a0, PMID: 7630397

Jura N, Endres NF, Engel K, Deindl S, Das R, Lamers MH, Wemmer DE, Zhang X, Kuriyan J. 2009a. Mechanism for activation of the EGF receptor catalytic domain by the juxtamembrane segment. Cell 137:1293–1307. DOI: https://doi.org/10.1016/j.cell.2009.04.025, PMID: 19563760

Jura N, Shan Y, Cao X, Shaw DE, Kuriyan J. 2009b. Structural analysis of the catalytically inactive kinase domain of the human EGF receptor 3. PNAS 106:21608–21613. DOI: https://doi.org/10.1073/pnas.0912101106, PMID: 20007378

Kleijnjung J, Fraternali F. 2005. POPSCOMP: an automated interaction analysis of biomolecular complexes. Nucleic Acids Research 33:W342–W346. DOI: https://doi.org/10.1093/nar/gki369

Krop IE, Kim S-B, Martin AG, LoRusso PM, Ferrero J-M, Badovinac-Crnjevic T, Hoersch S, Smitt M, Wildiers H. 2017. Trastuzumab emtansine versus treatment of physician’s choice in patients with previously treated HER2-positive metastatic breast cancer (TH3RESA): final overall survival results from a randomised open-label phase 3 trial. The Lancet Oncology 18:743–754. DOI: https://doi.org/10.1016/s1470-2045(17)30313-3

Laskowski RA. 1995. SURFNET: A program for visualizing molecular surfaces, cavities, and intermolecular interactions. Journal of Molecular Graphics 13:323–330. DOI: https://doi.org/10.1016/0267-7855(95)00073-9

Lemmon MA, Schlessinger J, Ferguson KM. 2014. The EGFR family: not so prototypical receptor tyrosine kinases. Cold Spring Harbor Perspectives in Biology 6:a020768. DOI: https://doi.org/10.1101/cshperspect.a020768, PMID: 24619165

Levinson NM, Boxer SG. 2014. A conserved water-mediated hydrogen bond network defines bosutinib’s kinase selectivity. Nature Chemical Biology 10:127–132. DOI: https://doi.org/10.1038/nchembio.1404, PMID: 24292070

Lichtner RB, Menrad A, Sommer A, Klar U, Schneider MR. 2001. Signaling inactive epidermal growth factor receptor/ligand complexes in intact carcinoma cells by quinazoline tyrosine kinase inhibitors. Cancer research 61:5790–5795.

Littlefield P, Liu L, Mysore V, Shan Y, Shaw DE, Jura N. 2014. Structural analysis of the EGFR/HER3 heterodimer reveals the molecular basis for activating HER3 mutations. Science Signaling 7:ra114. DOI: https://doi.org/10.1126/scisignal.2005786, PMID: 25468994

Liu P, Cleveland TE, Bouyain S, Byrne PO, Longo PA, Leahy DJ. 2012. A single ligand is sufficient to activate EGFR dimers. PNAS 109:10861–10866. DOI: https://doi.org/10.1073/pnas.1201114109

Lovekin C, Ellis IO, Locker A, Robertson JF, Bell J, Nicholson R, Gullick WJ, Elston CW, Blamey RW. 1991. c-erbB-2 oncoprotein expression in primary and advanced breast cancer. British Journal of Cancer 63:439–443. DOI: https://doi.org/10.1038/bjc.1991.101, PMID: 1672254

McKay MM, Ritt DA, Morrison DK. 2011. RAF Inhibitor-Induced KSR1/B-RAF Binding and Its Effects on ERK Cascade Signaling. Current Biology 21:563–568. DOI: https://doi.org/10.1016/j.cub.2011.02.033

Mi L-Z, Lu C, Li Z, Nishida N, Walz T, Springer TA. 2011. Simultaneous visualization of the extracellular and cytoplasmic domains of the epidermal growth factor receptor. Nature Structural & Molecular Biology 18:984–989. DOI: https://doi.org/10.1038/nsmb.2092

Murphy JM, Czabotar PE, Hildebrand JM, Lucet IS, Zhang J-G, Alvarez-Diaz S, Lewis R, Lalaoui N, Metcalf D, Webb A, Young SN, Varghese LN, Tannahill GM, Hatchell EC, Majewski IJ, Okamoto T, Dobson RCJ, Hilton DJ, Babon JJ, Nicola NA, et al. 2013. The Pseudokinase MLKL Mediates Necroptosis via a Molecular Switch Mechanism. Immunity 39:443–453. DOI: https://doi.org/10.1016/j.immuni.2013.06.018

Murphy JM, Zhang Q, Young SN, Reese ML, Bailey FP, Eyers PA, Gregoire S, Hammar M, Silvernoven OA, Varghese LN, Chen K, Tripathy A, Jura N, Fukuda K, Qin J, Nimchuk Z, Mudgett MB, Elowe S, Kuriyan J, Liu P, et al. 2014. A robust methodology to subclassify pseudokinases based on their nucleotide-binding properties. Biochemical Journal 457:323–334. DOI: https://doi.org/10.1042/BJ20131174

Nagy P, Claus J, Jovin TM, Ampt-Jovin DJ. 2010. Distribution of resting and ligand-bound ErbB1 and ErbB2 receptor tyrosine kinases in living cells using number and brightness analysis. PNAS 107:16524–16529. DOI: https://doi.org/10.1073/pnas.1002642107

Needham SR, Roberts SK, Arkhipov A, Mysore VP, Tyan CJ, Zanetti-Domingues LC, Kim ET, Losasso V, Korovesis D, Hirsch M, Rolfe DJ, Clarke DT, Winn MD, Lageardipour A, Clayton AHA, Pike LJ, Perani M, Parker
PJ, Shan Y, Shaw DE, et al. 2016. EGFR oligomerization organizes kinase-active dimers into competent signalling platforms. Nature Communications 7:13307. DOI: https://doi.org/10.1038/ncomms13307

Niesen FH, Berglund H, Vedadi M. 2007. The use of differential scanning fluorimetry to detect ligand interactions that promote protein stability. Nature Protocols 2:2212–2221. DOI: https://doi.org/10.1038/nprot.2007.321

Novotny CJ, Pollari S, Park JH, Lemmon MA, Shen W, Shokat KM. 2016. Overcoming resistance to HER2 inhibitors through state-specific kinase binding. Nature Chemical Biology. 12:923–930. DOI: https://doi.org/10.1038/nchembio.2171

Ogiso H, Ishitani R, Nureki O, Fukai S, Yamana M, Kim J-H, Saito K, Sakamoto A, Inoue M, Shirouzu M, Yokoyama S. 2002. Crystal Structure of the Complex of Human Epidermal Growth Factor and Receptor Extracellular Domains. Cell 110:775–787. DOI: https://doi.org/10.1016/S0092-8674(02)00963-7

Okuzumi T, Fiedler D, Zhang C, Gray DC, Aizenstein B, Hoffman R, Shokat KM. 2009. Inhibitor hijacking of Akt activation. Nature Chemical Biology 5:484–493. DOI: https://doi.org/10.1038/nchembio.183

Ortiz-Zapater E, Lee RW, Owen W, Weitsman G, Fruhwirth G, Dunn RG, Neat MJ, McCaughan F, Parker P, Ng T, Santos G. 2017. MET-EGFR dimerization in lung adenocarcinoma is dependent on EGFR mutations and altered by MET kinase inhibition. PLoS One 12:e0170798. DOI: https://doi.org/10.1371/journal.pone.0170798; PMID: 28141869

Owens MA, Horton BC, Da Silva MM. 2004. HER2 Amplification Ratios by Fluorescence In Situ Hybridization and Correlation with Immunohistochemistry in a Cohort of 6556 Breast Cancer Tissues. Clinical Breast Cancer 5:63–69. DOI: https://doi.org/10.3816/CBC.2004.n.011

Papa FR, Zhang C, Shokat KM, Walter P. 2003. Bypassing a Kinase Activity with an ATP-Competitive Drug. Science 302:1533–1537. DOI: https://doi.org/10.1126/science.1090031

Parsons M, Ng T. 2007. Intracellular coupling of adhesion receptors: molecular proximity measurements. Methods in Cell Biology 69:261–278. DOI: https://doi.org/10.1016/S0091-679X(02)69017-X

Peter M, Ameer-Beg SM, Hughes MKY, Keppeler MD, Prag S, Marsh M, Vojnovic B, Ng T. 2005. Multiphoton-FLIM Quantification of the EGFP-mRFP1 FRET Pair for Localization of Membrane Receptor-Kinase Interactions. Biophysical Journal 88:1224–1237. DOI: https://doi.org/10.1529/biophysj.104.050153

Piccart-Gebhart M, Holmes E, Baselga J, de Azambuja E, Dueck AC, Viale G, Zúñezska JA, Goldhirsch A, Armou A, Pritchard KI, McCullough AE, Dolci S, McFadden E, Holmes AP, Tonghua L, Eidtmann H, Dinh P, Di Cosimo S, Harbeck N, Tjulandin S, et al. 2016. Adjuvant Lapatinib and Trastuzumab for Early Human Epidermal Growth Factor Receptor 2–Positive Breast Cancer: Results From the Randomized Phase III Adjuvant Lapatinib and/or Trastuzumab Treatment Optimization Trial. Journal of Clinical Oncology 34:1034–1042. DOI: https://doi.org/10.1200/JCO.2015.62.1797

Poulikakos PI, Zhang C, Bollag G, Shokat KM, Rosen N. 2010. RAF inhibitors transactivate RAF dimers and ERK signalling in cells with wild-type BRAF. Nature 464:427–430. DOI: https://doi.org/10.1038/nature08902

Pradeep S, Kim SW, Wu SY, Nishimura M, Chaluvely-Raghavan P, Miyake T, Pecot CV, Kim S-J, Choi HJ, Bischoff FZ, Mayer JA, Huang L, Nick AM, Hall CS, Rodriguez-Aguayo C, Zand B, Dalton HJ, Arumugam T, Lee HJ, Han HD, et al. 2014. Hematogenous Metastasis of Ovarian Cancer: Rethinking Mode of Spread. Cancer Cell 26:77–91. DOI: https://doi.org/10.1016/j.ccr.2014.05.002

Reinhard FBM, Eberhard D, Werner T, Franken H, Childs D, Doce C, Savitski MF, Huber W, Bantscheff M, Savitski MM, Drewes G. 2015. Thermal proteome profiling monitors ligand interactions with cellular membrane proteins. Nature Methods. 12:1129–1131. DOI: https://doi.org/10.1038/nmeth.3652

Roberts S, Hirsch M, McAstea A, Zanetti-Domingues L, Clarke D, Claus J, Parker P, Wang L, Martin-Fernandez M. 2018. Cluster analysis of endogenous HER2 and HER3 receptors in SKBR3 cells. Bio-Protocol 8:e3096. DOI: https://doi.org/10.21769/BioProtoc.3096

Ross FA, Hawley SA, Auclier FI, Gowans GJ, Atrih A, Lamont DJ, Hardie DG. 2017. Mechanisms of Paradoxical Activation of AMPK by the Kinase Inhibitors SU6656 and Sorafenib. Cell Chemical Biology 24:813–824. DOI: https://doi.org/10.1016/j.chembiol.2017.05.021

Rubin-Delanchy P, Burn GL, Grifiﬁé J, Williamson DJ, Heard NA, Cope AP, Owen DM. 2015. Bayesian cluster identiﬁcation in single-molecule localization microscopy data. Nature Methods 12:1072–1076. DOI: https://doi.org/10.1038/nmeth.3612

Saffarian S, Li Y, Elson EL, Pike LJ. 2007. Oligomerization of the EGF receptor investigated by live cell ﬂuorescence intensity distribution analysis. Biophysical Journal 93:1021–1031. DOI: https://doi.org/10.1529/biophysj.107.105494; PMID: 17496034

Sali A, Blundell TL. 1993. Comparative protein modelling by satisfaction of spatial restraints. Journal of Molecular Biology 234:779–815. DOI: https://doi.org/10.1006/jmbi.1993.1626; PMID: 8254673

Scheff ED, Eswaran J, Bunkoczi G, Knapp S, Manning G. 2009. Structure of the pseudokinase VRK3 reveals a degraded catalytic site, a highly conserved kinase fold, and a putative regulatory binding site. Structure 17:128–138. DOI: https://doi.org/10.1016/j.str.2008.10.018; PMID: 19141289

Sergina NV, Rausch M, Wang D, Blair J, Hannon B, Shokat KM, Moasser MM. 2007. Escape from HER-family tyrosine kinase inhibitor therapy by the kinase-inactive HER3. Nature 445:437–441. DOI: https://doi.org/10.1038/nature05474; PMID: 17206155

Shi F, Telesco SE, Liu Y, Radhakrishnan R, Lemmon MA. 2010. ErbB3/HER3 intracellular domain is competent to bind ATP and catalyze autophosphorylation. PNAS 107:7692–7697. DOI: https://doi.org/10.1073/pnas.1002753107; PMID: 20351256

Simossis VA, Kleinjung J, Heringa J. 2005. Homology-extended sequence alignment. Nucleic Acids Research 33:816–824. DOI: https://doi.org/10.1038/ncomms13307; PMID: 15699183
Slamon DJ, Clark GM, Wong SG, Levin WJ, Ullrich A, McGuire WL. 1987. Human breast cancer: correlation of relapse and survival with amplification of the HER-2/neu oncogene. Science 235:177–182. DOI: https://doi.org/10.1126/science.3798106, PMID: 3798106

Sliwkowski MX, Schaefer G, Akita RW, Lofgren JA, Fitzpatrick BD, Nuijens A, Fendly BM, Cerione RA, Vandlen RL, Carraway KL. 1994. Coexpression of erbB2 and erbB3 proteins reconstitutes a high affinity receptor for heregulin. The Journal of Biological Chemistry 269:14661–14665. PMID: 7514177

Thevakumar N, Lavoie H, Critton DA, Tebben A, Mariner A, Sicheri F, Therrien M. 2015. Crystal structure of a BRAF kinase domain monomer explains basis for allosteric regulation. Nature Structural & Molecular Biology 22:37–43. DOI: https://doi.org/10.1038/nsmb.2924, PMID: 2543793

Thiel KW, Carpenter G. 2007. Epidermal growth factor receptor juxtamembrane region regulates allosteric tyrosine kinase activation. PNAS 104:19238–19243. DOI: https://doi.org/10.1073/pnas.0703854104, PMID: 18042729

van Lengerich B, Agnew C, Puchner EM, Huang B, Jura N. 2017. EGF and NGF induce phosphorylation of HER3/ERBB3 by EGFR using distinct oligomeric mechanisms. PNAS 114:E2836–E2845. DOI: https://doi.org/10.1073/pnas.1617994114, PMID: 28320942

Wang L, Perera BG, Hari SB, Bhathara B, Backes BJ, Seeliger MA, Schürer SC, Oakes SA, Papa FR, Maly DJ. 2012. Divergent allosteric control of the IRE1α endoribonuclease using kinase inhibitors. Nature Chemical Biology 8:982–989. DOI: https://doi.org/10.1038/nchembio.1094, PMID: 23086298

Wilson TR, Fridlyand J, Yan Y, Penuel E, Burton L, Chan E, Peng J, Lin E, Wang Y, Sosman J, Ribas A, Li J, Moffat J, Sutherlin DP, Koeppen H, Merchant M, Neve R, Settleman J. 2012. Widespread potential for growth-factor-driven resistance to anticancer kinase inhibitors. Nature 487:505–509. DOI: https://doi.org/10.1038/nature11249, PMID: 22763448

Wood ER, Truesdale AT, McDonald OB, Yuan D, Hassell A, Dickerson SH, Horne E, Lackey K, Allgood KJ, Rusnak DW, Shewchuk L. 2004. A unique structure for epidermal growth factor receptor bound to GW572016 (Lapatinib): relationships among protein conformation, inhibitor off-rate, and receptor activity in tumor cells. Cancer Research 64:6652–6659. DOI: https://doi.org/10.1158/0008-5472.CAN-04-1168, PMID: 15374980

Xia W, Petricoin EF, Zhao S, Liu L, Osada T, Cheng Q, Wulfkuhle JD, Gurbani D, Yang X, Gallagher RI, Bacus S, Vickers SJ, Sliwkowski MX, Slamon DJ, Clark GM, Wong SG, Levin WJ, Ullrich A, McGuire WL. 1987. Human breast cancer: correlation of relapse and survival with amplification of the HER-2/neu oncogene. Science 235:177–182. DOI: https://doi.org/10.1126/science.3798106, PMID: 3798106

Zeqliraj E, Filippi BM, Deak M, Alessi DR, van Aalten DM. 2009a. Structure of the LKB1-STRAD-MO25 complex reveals an allosteric mechanism of kinase activation. Science 326:1707–1711. DOI: https://doi.org/10.1126/science.1178377, PMID: 19892943

Zeqliraj E, Filippi BM, Goldie S, Navratišova I, Boudeau J, Deak M, Alessi DR, van Aalten DM. 2009b. ATP and MO25alpha regulate the conformational state of the STRADalpha pseudokinase and activation of the LKB1 tumour suppressor. PLoS Biology 7:e1000126. DOI: https://doi.org/10.1371/journal.pbio.1000126, PMID: 19513107

Zhang X, Gureasko J, Shen K, Cole PA, Kuriyan J. 2006. An allosteric mechanism for activation of the kinase domain of epidermal growth factor receptor. Cell 125:1137–1149. DOI: https://doi.org/10.1016/j.cell.2006.05.013, PMID: 1677603

Zhang R, Fruhwirth GO, Coban O, Barrett JE, Burgoyne T, Lee SH, Simonson PD, Baday M, Khodolenko BN, Futter CE, Ng T, Selvin PR. 2017. Probing the heterogeneity of protein kinase activation in cells by super-resolution microscopy. ACS Nano 11:249–257. DOI: https://doi.org/10.1021/acsnano.6b05356, PMID: 27768850