Enhanced dimerization drives ligand-independent activity of mutant EGFR in lung cancer

Christopher C. Valley¹, Donna J. Arndt-Jovin², Narain Karedla³, Mara P. Steinkamp¹, Alexey I. Chizhik³, William S. Hlavacek⁴, Bridget S. Wilson¹, Keith A. Lidke⁵ and Diane S. Lidke¹

Author affiliations:
¹Department of Pathology and Cancer Research and Treatment Center, University of New Mexico, Albuquerque, NM, USA
²Laboratory of Cellular Dynamics, Max Planck Institute for Biophysical Chemistry, Göttingen, Germany
³III. Institute of Physics, Georg-August University of Göttingen, Göttingen, Germany
⁴Theoretical Biology and Biophysics Group, Theoretical Division, Los Alamos National Laboratory, Los Alamos, NM, USA
⁵Department of Physics and Astronomy, University of New Mexico, Albuquerque, NM, USA

Corresponding author: Diane Lidke, DLidke@salud.unm.edu

Running title: Kinase mutations drive EGFR dimerization

Abbreviations: EGFR – epidermal growth factor receptor; EGF – epidermal growth factor; RTK – receptor tyrosine kinase; NSCLC – Non-small cell lung carcinoma; QD – quantum dot; TKI – tyrosine kinase inhibitor; HMM – Hidden Markov Model; dSTORM – direct stochastic optical reconstruction microscopy; ACP – acyl carrier protein; FRET – Förster resonance energy transfer; FLIM – fluorescence lifetime imaging microscopy; HA – hemagglutinin tag
Mutations within the epidermal growth factor receptor (EGFR/erbB1/Her1) are often associated with tumorigenesis. In particular, a number of EGFR mutants that demonstrate ligand-independent signaling are common in non-small cell lung cancer (NSCLC), including kinase domain mutations L858R (also called L834R) and exon 19 deletions (e.g. ΔL747-P753insS) which collectively make up nearly 90% of mutations in NSCLC. The molecular mechanisms by which these mutations confer constitutive activity remain unresolved. Using multiple sub-diffraction-limit imaging modalities, we reveal the altered receptor structure and interaction kinetics of NSCLC-associated EGFR mutants. We applied two-color single quantum dot tracking to quantify receptor dimerization kinetics on living cells and show that, in contrast to wild type EGFR, mutants are capable of forming stable, ligand-independent dimers. Two-color super-resolution localization microscopy confirmed ligand-independent aggregation of EGFR mutants. Live cell FRET measurements revealed that the L858R kinase mutation alters ectodomain structure such that unliganded mutant EGFR adopts an extended, dimerization-competent conformation. Finally, mutation of the putative dimerization arm confirmed a critical role for ectodomain engagement in ligand-independent signaling. These data support a model in which dysregulated activity of NSCLC-associated kinase mutants is driven by coordinated interactions involving both the kinase and extracellular domains that lead to enhanced dimerization.
INTRODUCTION

The epidermal growth factor receptor (EGFR/erbB1/HER1) is a member of the erbB family of receptor tyrosine kinases (RTKs) that play a critical role in a number of physiological processes and are additionally implicated in the progression and prognosis of certain cancer types (Lemmon and Schlessinger, 2010). Additionally, EGFR is the target for numerous therapeutic strategies in such cancers, however patients often acquire resistance to such therapies. Therefore, there exists the need for novel approaches to targeting EGFR, which in turn requires the characterization of the structural and biophysical mechanisms by which EGFR signaling—both physiological and pathological—is initiated within the cell.

The current structural model of physiological EGFR activation includes ligand-induced, receptor-mediated dimerization of the ectodomain (Ogiso et al., 2002; Schlessinger, 2002), which is propagated across the plasma membrane via interactions within the transmembrane (Arkhipov et al., 2013; Endres et al., 2013) and juxtamembrane domains (Thiel and Carpenter, 2007; Jura et al., 2009; Red-Brewer et al., 2009), resulting in allosteric activation of the kinase domain via asymmetric dimerization (Zhang et al., 2006). Many aspects of this model, however, are based upon crystal structure or biochemical data of fragments of the entire protein. In such a system, any structural coupling (Lu et al., 2010; Mi et al., 2011) between various receptor domains is lost, as are the complexities involving the receptor interaction with the local environment (i.e. plasma membrane), which contribute both to receptor autoinhibition (Bremer et al., 1986; McLaughlin et al., 2005; Coskun et al., 2011) and the stabilization of kinase activity (Mi et al., 2008). Furthermore, many aspects of this model remain under debate, including ligand occupancy (Macdonald and Pike, 2008; Alvarado et al., 2010), receptor structure/orientation (Kästner et al., 2009; Kozer et al., 2011; Tynan et al., 2011), receptor oligomerization (Clayton et al., 2005; Kozer et al., 2013, 2014), and receptor aggregation and supramolecular organization (Carraway et al., 1989; Keating et al., 2008; Abulrob et al., 2010; Needham et al., 2013, 2014). Therefore, there is an obvious need to study the structure of the receptor—including both receptor conformation and receptor interactions—within the plasma membrane environment.
Recent imaging technologies have captured EGFR behavior in living cells, providing quantitative measures of receptor interactions (Chung et al., 2010; Low-Nam et al., 2011; Valley et al., 2014), phosphorylation (Sako et al., 2000), and conformational states (Ziomkiewicz et al., 2013). In previous work, we developed single molecule imaging techniques to visualize and quantify EGFR dynamics and dimerization on living cells (Lidke et al., 2005; Low-Nam et al., 2011). We showed that wild type EGFR dimers that form in the absence of ligand are short-lived and that receptor dimers are stabilized upon ligand binding (Low-Nam et al., 2011). In other studies, Ziomkiewicz et al. used FRET measurements to study changes in EGFR ectodomain conformation and demonstrated ligand-induced structural rearrangements that are consistent with stabilization of the extended, dimer-competent conformation (Ziomkiewicz et al., 2013). These results in live cells are consistent with dimerization being the initiating event in signal transduction, as predicted by structural and biochemical studies.

EGFR is often dysregulated in cancer by mutation or amplification. Specific mutations within the EGFR kinase domain are common in non-small cell lung cancer (NSCLC), including a somatic mutation resulting in a single amino acid substitution at leucine 858 to arginine (EGFR-L858R, or -L834R in the mature protein numbering) as well as in-frame deletions within exon 19 (e.g. EGFR-ΔL747-P753insS). Together, these mutations make up nearly 90% of EGFR-mutant NSCLC tumors (Sharma et al., 2007; Pao and Chmielecki, 2010) and correlate with sensitivity to tyrosine kinase inhibitors (TKIs) (Lynch et al., 2004; Paez et al., 2004; Pao et al., 2004). Expressed in model cells, EGFR kinase mutants, including EGFR-L858R and EGFR-ΔL747-P753insS, transform Ba/F3 and other cells via ligand-independent receptor activity at the cell surface and additionally confer sensitivity to TKIs (Greulich et al., 2005; Jiang et al., 2005; Choi et al., 2007).

Although it is generally accepted that these NSCLC-associated mutations give rise to constitutive and unregulated activity, the precise molecular and biophysical mechanism(s) by which EGFR mutants initiate signaling from the plasma membrane remain unresolved. Crystal structures of the isolated EGFR-L858R kinase (Yun et al., 2007) provided evidence that such activating mutations disrupt autoinhibitory interactions within the kinase, and this relief of autoinhibition leads to an increase in the activity of the purified, isolated kinase in vitro (Carey et al., 2006; Zhang et al., 2006; Yun et al., 2007). Further studies demonstrated a clear requirement for asymmetric kinase dimerization of certain NSCLC-associated EGFR mutants, including
L858R (Cho et al., 2013), which preferentially adopts the receiver conformation within the asymmetric kinase dimer (Red-Brewer et al., 2013) by suppressing local disorder within the N-lobe region of the kinase dimer interface (Shan et al., 2012).

While it is plausible, if not probable, that such mutations which shift the equilibrium of the kinase domain to the active conformation will promote kinase dimerization, it remains unknown whether such a driving force is sufficient to facilitate dimerization of the full length receptor. Recent work using purified, near full-length EGFR demonstrated that NSCLC kinase mutant L858R readily dimerizes in the absence of ligand (Wang et al., 2011). However, the plasma membrane is known to play a critical role in the negative regulation of EGFR (Bremer et al., 1986; McLaughlin et al., 2005; Coskun et al., 2011) and likely dictates conformational coupling between various receptor domains, evident from the altered ligand binding and functional stability depending on the local environment (Mi et al., 2008; Wang et al., 2011).

Here, using multiple sub-diffraction-limit imaging modalities, we reveal the altered behavior of NSCLC-associated EGFR mutants in living cells. We directly show that NSCLC-mutant EGFR form stable dimers even in the absence of ligand and that unliganded mutant EGFR adopts an extended, dimerization-competent conformation. Furthermore, we demonstrate that, while the NSCLC-associated mutations are located in the kinase domain, ectodomain interactions via the well-characterized dimerization arm are necessary for efficient ligand-independent activity. Collectively, these results show that dysregulated activity of NSCLC-associated kinase mutants is driven by coordinated interactions involving multiple receptor domains that lead to enhanced dimerization.

RESULTS

Ligand-independent phosphorylation of NSCLC-associated EGFR mutants is highly dependent on receptor density in the membrane. We stably expressed EGFR with an N-terminal HA-tag (HA-EGFR) in CHO cells, which lack endogenous EGFR. Transfected cells expressed either the wild type EGFR (EGFR-WT), EGFR-L858R, or EGFR-ΔL747-P753insS.
Figure 1 compares the levels of EGFR phosphorylation on tyrosine 1068. In contrast to the WT receptor, expression of EGFR-L858R or EGFR-ΔL747-P753insS resulted in measurable receptor phosphorylation in the absence of ligand (Figure 1A). As previously reported (Choi et al., 2007), both mutant forms of EGFR retain the capacity for ligand-induced activation as measured by receptor phosphorylation, albeit reduced compared to EGFR-WT. Sensitivity to the EGFR-selective inhibitor PD153035 (Bos et al., 1997) is consistent with receptor phosphorylation independent of endogenous kinases in CHO cells (Figure 1A).

As expected for a stable cell line, expression of EGFR is heterogeneous in transfected CHO cells. Confocal imaging of cells double-labeled with pan-reactive EGFR antibodies (anti-HA) and phospho-specific antibodies (anti-pY1068) revealed the relationships between local EGFR expression level and phosphorylation state (Figure 1B). In the absence of ligand, EGFR-WT exhibits barely measurable phosphorylation of tyrosine 1068 (Figure 1B). In contrast, results for the cells expressing mutant EGFR show significant levels of phosphorylation. Control responses in the presence of ligand are consistent with western blotting results (Figure S1). Quantification of these images confirmed that the NSCLC mutants show a robust, ligand-independent phosphorylation response with the level of overall receptor phosphorylation dependent on increasing receptor expression (Figure 1C).

Considering the two possible mechanisms for increased ligand-independent phosphorylation of EGFR mutants, and to determine if one mechanism might dominate, we fit the data in Figure 1C to a monomer-dimer equilibrium model assuming either (i) higher intrinsic kinase activity (Zhang et al., 2006; Yun et al., 2007) (See Supplementary Note, Table S3 - Model 1) or (ii) increased dimerization affinity (Shan et al., 2012) (See Supplementary Note, Table S3 - Model 2). As shown Figure 1C, however, both models yielded equal goodness of fit (compare the solid and dashed curves, see also Figure S2). We also used computational modeling to explore alternative mechanisms of EGFR dimerization, including dimerization via multiple ectodomain conformations and both ectodomain- and endodomain-mediated interactions, and found that such mechanisms yield the same functional relationship between EGFR abundance and EGFR phosphorylation (see Supplementary Note for a detailed description of all models). Therefore, both augmented kinase activity and altered dimerization sufficiently describe the data, and further experiments were needed to delineate the contribution of either mechanism. Given that EGFR-WT activation is driven by ligand-induced dimerization (Burgess et al., 2003; Chung
et al., 2010; Low-Nam et al., 2011), and mutant activity relies on asymmetric kinase dimerization (Cho et al., 2013; Red-Brewer et al., 2013), we tested the strong possibility that NSCLC mutations enhance ligand-independent receptor dimerization.

**EGFR kinase mutations facilitate receptor dimerization and decrease receptor mobility in the native plasma membrane.** We applied two-color SPT methods to directly visualize and quantify EGFR diffusion and dimerization in live cells (Lidke et al., 2005; Low-Nam et al., 2011; Steinkamp et al., 2014). Receptors were tracked using spectrally distinct quantum dots (QDs), QD585 and QD655, conjugated to either EGF (Lidke et al., 2004; Low-Nam et al., 2011) or a monovalent anti-HA Fab fragment (Steinkamp et al., 2014); these probes track the liganded and unliganded receptor, respectively. QDs are robust SPT probes that provide high brightness and photostability, without impairing the diffusion properties or physiological response of membrane receptors (Dahan et al., 2003; Lidke et al., 2004; Chung et al., 2010; Low-Nam et al., 2011; Schwartz et al., 2015). Dimer events are imaged by independent localization of single molecules in the two spectral channels with high spatial (~20 nm) and temporal (50 ms) resolution (Low-Nam et al., 2011). As shown schematically in Figure 2A, combinations of these probes enabled the tracking of different dimer species: unliganded dimers (0-EGF:2-EGFR), ligand-bound dimers (2-EGF:2-EGFR), and singly-liganded dimers (1-EGF:2-EGFR). Critically, labeling of EGFR with anti-HA-QDs did not cause activation of the receptor in the absence of EGF, nor did it prevent receptor activity or dimerization in the presence of saturating dark EGF (Figure S3).

Figure 2B-D shows examples of repeated dimerization events between receptor pairs with varying dimer stability. The unliganded EGFR-WT exhibited transient dimers (Figure 2B and Movie S1), whereas the ligand-bound EGFR-WT formed longer-lived dimers (Figure 2C and Movie S2). In contrast to EGFR-WT, both mutants demonstrated long-lived interactions in the absence of ligand (Figure 2D and Figure S4; Movie S3 and S4). Dimerization kinetics were estimated using a three-state Hidden Markov Model (HMM) as previously described (Low-Nam et al., 2011). The HMM utilizes the distance between two spectrally distinct QDs as the experimental observable and globally fits all the data in a single condition using a maximum likelihood estimation to extract the rate constants for transitions between dimer and non-dimer
(i.e. hidden) states. Therefore, while the labeled receptors may be undergoing interactions with unlabeled proteins, the off-rates are only calculated from observed two-color dimerization events. Based on calculated transition rates, the Viterbi algorithm can be used to identify the dimerization state for a pair of receptors at each point within a time series (Figure 2B-D, lower panel). The resulting dimer off-rates calculated over many dimer interactions are shown in Figure 2E and summarized in Table S1. Consistent with our previous work (Low-Nam et al., 2011), EGFR-WT receptors formed short-lived dimers in the absence of ligand (dimer off-rate, $k_{\text{off}} = 0.31 \text{ s}^{-1}$) that shifted to longer-lived dimers with ligand bound ($k_{\text{off}} = 0.12 \text{ s}^{-1}$). The singly-ligated EGFR-WT dimer had an intermediate off-rate. While others have reported EGF unbinding rates on the order of our measurement time, but much longer than our dimer lifetime (Hendriks et al., 2003; Mattoon et al., 2004), we have not observed significant losses of EGF-QD during our time courses. Even though this could lead to a small underestimate of the dimer off-rate, the interpretation of the relative differences between conditions is unaffected. Importantly, homodimers composed of EGFR-L858R or EGFR-ΔL747-P753insS mutants were stable regardless of ligand occupancy, with off-rates similar to ligand-bound EGFR-WT (Figure 2E). These results indicate that kinase mutations drive the formation of stable dimers in the absence of EGF.

Our previous studies showed that EGF-bound EGFR forms stable dimers that exhibit markedly reduced mobility (Low-Nam et al., 2011). We found that the EGFR mutants, both in the presence and absence of ligand, displayed mobility more similar to ligand-bound EGFR-WT (Figure 2F). Receptor mobility was quantified by calculating the distribution of squared displacements and is shown as a cumulative probability plot in Figure 2G. As expected, a significant shift to slower mobility is seen for ligand-bound EGFR-WT. Decreased mobility is dependent on receptor phosphorylation and reflects the complexities of signaling-mediated changes, such as recruitment of downstream proteins and changes in the local membrane environment. The EGFR mutants exhibited a similar reduction in mobility, even in the absence of ligand. The average diffusion coefficients are given in Figure 2H (see also Table S1). Upon ligand binding, both mutants slowed further to a degree comparable to that of ligand-bound EGFR-WT, consistent with a further increase in receptor interactions driven by ligand binding. We interpret the reduced mobility of unliganded mutant receptors to be a function of their increased number of dimer events, as well as the recruitment of signaling proteins.
Two-color super-resolution microscopy confirms ligand-induced EGFR aggregation and ligand-independent EGFR mutant aggregation. To further support the conclusion that EGFR-L858R and EGFR-ΔL747-P753insS mutants dimerize more readily than EGFR-WT, we used two-color direct stochastic optical reconstruction microscopy (dSTORM) (Heilemann et al., 2008) to visualize the distribution of EGFR in the plasma membrane (Figure 3A, B). Receptor proximity across multiple samples was quantified using localization-based cross-correlation analysis of Alexa647- and Cy3B-labeled EGFR. As plotted in Figure 3C, the correlation function for unliganded EGFR-WT is ~1 across all distances, indicating a nearly random distribution of receptors in the plasma membrane.

In contrast to the near random distribution of resting EGFR, stimulation with saturating EGF induced a strong peak below 50 nm in the correlation function, indicating clustering of receptors at short distances that is consistent with the conventional model for ligand-induced dimerization (Schlessinger, 2002; Burgess et al., 2003). Analysis of reconstructed images for EGFR-L858R or EGFR-ΔL747-P753insS without EGF addition are shown in Figure 3D. An increase in the correlation function at short distances (< 50 nm) was seen for both mutants, providing further evidence for increased self-association of EGFR mutants in the absence of ligand (Figure 3D). Cross-correlation values for EGFR-L858R within the dimerization-permissive distance were higher than for EGFR-ΔL747-P753insS, consistent with modeling predictions reported in Figure 1C for a lower relative K_D (see Tables S3 and S4).

Activating kinase mutations alter the ectodomain structure. Prior studies using recombinant EGFR kinase domain has shown that NSCLC-associated EGFR mutant kinases exhibit increased catalytic activity (Zhang et al., 2006), possibly by adopting an active conformation (Shan et al., 2012). Our studies of full-length receptors in intact cells have indicated an additional property of both EGFR-L858R and EGFR-ΔL747-P753insS mutations: they promote the formation of stable homodimers within the plasma membrane of live cells in the absence of ligand. Thus, our observations imply that the EGFR kinase domain mutations induce structural changes that facilitate receptor dimerization.
To test this hypothesis, we monitored the conformational states of the EGFR ectodomain via FRET measurements in living cells using fluorescence lifetime imaging microscopy (FLIM) (Ziomkiewicz et al., 2013), shown schematically in Figure 4A. EGFR-WT and EGFR-L858R were generated with N-terminal acyl carrier protein (ACP) tags and expressed in CHO cells. The ACP-tag was coupled to a small FRET donor, Oregon Green, through the covalent attachment of labeled-CoA. Cells were subsequently labeled with the FRET acceptor, NR12S, a lipid probe that is retained in the outer leaflet of the plasma membrane (Kucherak et al., 2010) (Figure S5).

The extent of energy transfer between the EGFR donor tag and lipid acceptor, was determined by monitoring the donor fluorescence lifetime under various conditions (Figure 4B-D, Table S2). As shown previously (Ziomkiewicz et al., 2013), EGFR-WT exhibited a shorter donor lifetime (corresponding to high energy transfer) in the absence of ligand (3.14 ± 0.05 ns), which significantly increased upon EGF addition (3.37 ± 0.05 ns) (Figure 4D, blue). This reduction in FRET with EGF addition is consistent with ligand-induced extension of the extracellular domain away from the plasma membrane (Burgess et al., 2003; Ziomkiewicz et al., 2013) (Figure S6), in contrast to previously proposed models (Webb et al., 2008; Kästner et al., 2009; Tynan et al., 2011). Interestingly, we observed a longer donor lifetime (lower FRET efficiency) for EGFR-L858R in the absence of ligand (3.34 ± 0.03 ns) relative to unliganded EGFR-WT (Figure 4D, red), suggesting a bias in ectodomain structure in the kinase mutant favoring the extended, dimer-competent conformation. We note that the further increase in lifetime for EGFR-L858R upon EGF addition (3.53 ± 0.04 ns) is consistent with ligand-induced activity (Figure 1A) and an increased ligand-binding affinity (Choi et al., 2007), however the relationship between structure and affinity is not fully understood (Mattoon et al., 2004).

**Ectodomain engagement is required for robust, ligand-independent signaling of EGFR-L858R.** To understand whether ectodomain interactions were indeed required for ligand-independent activity of EGFR-L858R, we introduced a series of mutations to amino acids within the dimerization arm (246-253*) that are critical for receptor dimerization and activity (Dawson et al., 2005) and expressed these mutants in HeLa cells. As predicted based on these biochemical studies, mutation of the dimerization arm resulted in no change in EGFR-WT mobility upon the addition of saturating EGF (Figure S7) indicating that ectodomain dimerization is indispensable for ligand-induced activity. Consistent with results in CHO cells (Figure 1-2), EGFR-L858R
shows ligand-independent phosphorylation and exhibits decreased mobility relative to EGFR-WT (Figure 5). We found that EGFR-L858R/246-253*, containing both an activating kinase mutation and mutation of the ectodomain dimerization arm, had significantly diminished ligand-independent phosphorylation (Figure 5A-B, Figure S8) and also increased mobility relative to EGFR-L858R kinase mutant alone (Figure 5C-D). The doubly mutated EGFR-L858R/246-253* retained measurable levels of ligand-independent activity compared to the EGFR-WT (Figure 5A, B). These results confirm a critical role for ectodomain engagement via interactions in the dimerization arm in the ligand-independent signaling of intracellular kinase mutants associated with NSCLC.

DISCUSSION

In this study, we have applied complimentary high-resolution imaging techniques to better understand the molecular mechanisms underlying the aberrant activity of EGFR NSCLC mutants. We show that NSCLC mutations facilitate ligand-independent receptor dimerization. Our results additionally reveal that the mechanism of signal initiation for the kinase domain L858R mutant involves ectodomain interactions via the dimerization arm, stabilizing the receptor in the extended, dimer-competent conformation. Therefore, our data support the hypothesis that oncogenic signaling from these NSCLC-associated EGFR mutants occurs as a result of enhanced, productive dimerization between unliganded receptors.

Several studies have demonstrated that asymmetric kinase dimerization is required for the ligand-independent activity of NSCLC-associated EGFR kinase mutants (Cho et al., 2013; Red-Brewer et al., 2013). Crystal structures showed that such mutations shift the equilibrium of the kinase domain toward an active conformation (Yun et al., 2007), and recent studies demonstrated that this occurs by suppressing intrinsic disorder within the kinase domain N-lobe (Shan et al., 2012), allowing the mutant kinase domains to act as “super-acceptors” within an asymmetric kinase dimer (Red-Brewer et al., 2013). Interestingly, other mutations, such as exon 19 deletion and exon 20 insertions, are active as either activator or receiver (Cho et al., 2013). Given this result, an important consideration is the interaction between NSCLC EGFR variants and either
the wild type EGFR or erbB2. It has been reported that patients with L858R mutation or exon 19 deletions are heterozygous (Lynch et al., 2004), suggesting that oncogenic, pro-survival is a result of signaling via one mutant and one wild type EGFR. Our results in CHO cells, which lack endogenous EGFR-WT, and in HeLa cells, which have endogenous EGFR-WT, are consistent, indicating that EGFR-WT:EGFR-L858R kinase interactions do not overcome the need for ectodomain engagement. Further work is needed to determine whether the EGFR-WT:EGFR-L858R or erbB2:EGFR-L858R heterodimers form stable interactions as do to the L858R and exon 19 deletion receptor homodimer species studied here. Nevertheless, in the context of our results, it is clear that the function of NSCLC kinase mutants is dependent on dimerization via coordinated interactions both in the kinase domain and ectodomain.

The influence of the kinase domain mutations on extracellular domain behavior would suggest a structural coupling between the two domains. The relationship between the structure of the intracellular and extracellular domains of EGFR remains largely controversial (Bessman and Lemmon, 2012). There is experimental evidence for a well-defined structural linkage between intracellular and extracellular domains, where alterations to intracellular regions, including the juxtamembrane and kinase domains, influence extracellular ligand binding properties of the receptor (Choi et al., 2007; Macdonald and Pike, 2008; Alvarado et al., 2010). If these domains are rigidly linked, activating mutations in the kinase domain may drive extension of the ectodomain independent of kinase interactions. Alternatively, recent results show remarkable flexibility between intracellular and extracellular domains. Using disulfide cross-linking in cells as well as negative-stain EM of purified, near full-length EGFR (tEGFR), Lu et al. provided evidence that receptor dimerization can be driven through the kinase domain when it is stabilized in the active conformation. These results showed a high degree of structural variability in the ectodomain and no contacts in the dimerization arm domain compared to that of EGF-mediated dimers (Lu et al., 2012). In an additional study, simultaneous visualization of extracellular and intracellular domains of tEGFR in the presence of Gefitinib, an EGFR-specific tyrosine kinase inhibitor which stabilizes the active and dimeric kinase domain, suggests a range of ectodomain conformations and little conformational coupling between these domains (Mi et al., 2011). In the case that the intracellular and extracellular domains are flexibly linked, ligand-independent activity of NSCLC mutants would conceivably be initiated via kinase domain dimerization and stabilized by subsequent interactions within the ectodomain.
While our results cannot distinguish between these mechanisms of dimerization, we have shown that ectodomain engagement through the dimerization arm is required for optimal ligand-independent signaling of EGFR kinase mutants. It is conceivable that such a structural linkage exists in the wild type receptor, and, considering that the ensemble of structural states exist in equilibrium, constitutive activity of overexpressed EGFR-WT may be explained by an increased probability for productive receptor encounters, leading to ligand-independent dimerization (Martin-Fernandez et al., 2002; Lidke et al., 2003; Nagy et al., 2010; Endres et al., 2013).

Interestingly, mutation of the dimerization arm does not completely abolish phosphorylation of EGFR-L858R (Figure 5A-B), indicating that kinase domain dimerization alone, while perhaps less stable in the absence of ectodomain engagement, can still produce some level of ligand-independent signaling. This dominating influence of the ectodomain in dimer stabilization is consistent with the ability of ligand-bound EGFR to form dimers when the kinase domain is either inhibited or removed (Chung et al., 2010; Low-Nam et al., 2011).

We have discussed EGFR signal initiation primarily in the context of dimerization as a result of extended ectodomain conformation (Burgess et al., 2003), which is stabilized by ligand binding or mutation. However, we acknowledge the existence of alternative models for EGFR activation. For example, some studies have suggested that the presence of preformed dimers in the absence of EGF that transition to tetramers upon EGF binding (Clayton et al., 2005). Other studies have indicated alternative ectodomain structures, including evidence that the EGFR ectodomain exists in an extended conformation in the cell membrane independent of ligand status (Kozer et al., 2011) or that liganded receptor or preformed dimer collapses on the plasma membrane (Tynan et al., 2011; Arkhipov et al., 2013). Here, we have confirmed that while wild type receptor interactions in the absence of ligand can occur, they are short-lived (Figure 2B). Furthermore, quantitative analysis of super-resolution data showed a nearly random distribution of unliganded EGFR-WT (Figure 3), indicating predominantly monomeric receptor in the absence of ligand. These results are not consistent with preformed dimers as the predominant species in the absence of ligand. Our results are consistent with a monomer-dimer transition driving signaling. Receptor interactions are longer-lived in the presence of EGF (Figure 2C, Figure 3C) or by mutation to an active kinase domain (Figure 2D and Figure 3D). Furthermore, on the basis of live cell FRET data, the relatively long lived interactions of liganded or mutated receptors are associated with greater occupancy of the ectodomain extended conformation.
Recent MD simulations of the full-length, glycosolated receptor in a complex lipid environment lend support for our live cell FRET by demonstrating that glycosolation prevents such a collapsed receptor conformation (Kaszuba et al., 2015). We note that many of the apparent inconsistencies between studies may be explained by experimental variations such as use of fixed cells, non-physiological temperatures, pharmacological inhibitors, or steric hindrance due to labeling. While our live cell data supports the model of dimers as the fundamental signaling unit, we cannot rule out a potential role for higher-order oligomers.

Our result that dimerization arm interactions are critical for robust ligand-independent signaling of the NSCLC-associated L858R mutant (Figure 5) suggests that targeting the ectodomain for EGFR-L858R, may inhibit the ligand-independent activity of these mutants. Such targeting molecules could include antibodies against the EGFR ectodomain (Li et al., 2008; Rivera et al., 2008; Schmiedel et al., 2008; Talavera et al., 2009) or peptides which disrupt EGFR dimerization arm-mediated interactions (Hanold et al., 2015a, 2015b). Cetuximab is a potent inhibitor of EGFR activity and functions by both blocking ligand binding and by preventing the receptor from adopting the extended conformation (Li et al., 2005). Several studies have used Cetuximab to block activity of NSCLC EGFR mutants with varying results (Wang et al., 2011; Cho et al., 2013).

The results presented here provide a biophysical basis for signal initiation in specific mutations associated with NSCLC. However, it remains unknown whether this mechanism of activation—including ectodomain engagement—is required in other variants of EGFR. The EGFRvIII variant, commonly found in glioblastomas (Gan et al., 2009), lacks key residues in the ectodomain—including the dimerization arm—and therefore circumvents the need for dimerization arm engagement. The acquisition of a secondary, “gatekeeper” mutation within the EGFR kinase domain (T790M) renders patients insensitive to clinical TKIs (Kobayashi et al., 2005; Pao et al., 2005; Godin-Heymann et al., 2008). Previous results suggested ligand-independent activity EGFR-L858R/T790M expressed in Ba/F3 cells was dimerization-independent based on resistance to Cetuximab (Cho et al., 2013). The increased affinity for ATP of EGFR-L858R/T790M (Yun et al., 2008) may accelerate receptor phosphorylation and
effectively circumvent the requirement for stable dimer formation, however this remains unknown.

In summary, this work provides critical new insight into the structural and molecular mechanisms of signaling by the gain-of-function EGFR mutations common in NSCLC. Our results in live cells support a model in which these mutations mediate signaling through enhanced dimerization in the absence of ligand via coordinated interactions that include ectodomain dimerization arm engagement. The altered ectodomain behavior induced by kinase domain mutations provides evidence for inside-out structural modulation, previously termed “inside-out signaling” (Macdonald-Obermann and Pike, 2009; Lu et al., 2012), as a mechanism underlying aberrant activity by NSCLC-associated kinase mutants of EGFR. Our findings have implications for future therapeutic strategies, since it is clear that the constitutively active receptors studied here still require dimerization for oncogenic signaling, including engagement of the dimerization arm. Combinations of dimer-disrupting agents, which target the ectodomain, and kinase-selective inhibitors may offer a critical therapeutic advantage.

MATERIALS AND METHODS

Cell lines and reagents

CHO cells and HeLa were cultured in Dulbecco’s Modified Eagle Medium (Life Technologies # 10313-021) supplemented with 10% fetal bovine serum (HyClone), penicillin-streptomycin, and 2 mM L-glutamine. Antibodies against phosphorylated EGFR (clone 1H12 #2236S) and total EGFR (clone 15F8, #4405S) used for immunoblotting and immunofluorescence were from Cell Signaling Technology. HRP conjugated goat anti-rabbit and anti-mouse secondary antibodies (sc-2004, sc-2005), and EGFR antibody used for super-resolution imaging (anti-EGFR, R-1, sc-101) were from Santa Cruz Biotechnology. Anti-HA-biotin (12158167001) and anti-HA-FITC (11988506001), high Affinity rat monoclonal antibody Fab fragments (clone 3F10) were purchased from Roche. EGF-biotin conjugate (E-3477), Qdot 585 streptavidin conjugate (Q10111MP), Qdot 655 streptavidin conjugate (Q10121MP), and Alexa Fluor 647 carboxylic acid, succinimidyl ester (A-20006) were purchased from Life Technologies. Cy3B NHS-ester (PA63100) was purchased from GE Healthcare Life Sciences.
Cloning and generation of stable cell lines

Cloning of HA-tagged and ACP-tagged EGFR constructs and mutations was performed as previously described (Ziomkiewicz et al., 2013; Steinkamp et al., 2014), inserting the HA-tag immediately downstream of the receptor signal peptide.

CHO cells were transfected with cDNA encoding either HA-tagged EGFR or ACP-tagged EGFR (WT, L858R, or ΔL747-P753insS) by Amaxa nucleofection. Briefly, 3x10^6 cells were transfected with 4 μg DNA using Amaxa Nucleofection Kit V optimized for CHO cells per manufacturer’s instructions. Cells were selected using 1 mg/ml G418 and sorted using either EGF-AlexaFluor488 (LifeTechnologies #E13345) or EGF-AlexaFluor647 (LifeTechnologies #E35351). After sorting, cells were maintained in G418 and routinely sorted, yielding a heterogeneous non-clonal population. Membrane localization of the HA- or ACP-tagged receptors was confirmed by live labeling, and activity and ligand-sensitivity of HA-EGFR or ACP-EGFR was confirmed by immunofluorescence and immunoblotting.

Transient transfection of HeLa cells

Before transfection, HeLa cells were grown to ~75% confluence. For transfection, 1.5x10^6 cells were transfected with 2.5 μg of the indicated plasmid (pcDNA3.1+ HA-EGFR-WT, HA-EGFR-L858R, or HA-EGFR-L858R+246-253*) using Amaxa Nucleofection Kit R (Lonza) following manufacturer’s instructions. Cells were immediately transferred to complete culture media and seeded onto 8-well chamber slides (Lab-Tec, Thermo Scientific #155411) for single particle tracking or immunofluorescence labeling. The remaining cells were plated onto a 100 mm dish for lysis and western blot analysis. Cells were incubated for ~24 hours at 37°C before single particle tracking (see below), fixation and immunolabeling, or lysis and western blot analysis. Separate transfections were done for independent experiments.

Immunoblotting

CHO cells expressing HA-EGFR (wild type or mutants) were seeded on 60 mm or 100 mm dishes and grown to ~80% confluency. Cells were pre-treated with tyrosine kinase inhibitor
as indicated followed by treatment with EGF (50 nM) for 8 minutes. After washing with cold PBS, cells were lysed in RIPA buffer on ice for 1 hour. Protein concentration of cleared lysates was measured by BCA assay (Pierce, Rockford IL), and equal amounts of total protein were separated on a 4-15% polyacrylamide gel (BioRad), transferred to nitrocellulose (iBlot transfer system, Life Technologies), probed with the indicated antibody, and imaged using enhanced chemiluminescence on a BioRad ChemiDoc.

Immunochemistry

CHO cells expressing HA-EGFR (wild type or mutants) were seeded on 18 mm coverslips overnight and labeled live using an anti-HA-FITC (Fab) at 2 μg/ml for 1 hour at 37 °C in serum free media followed by four washes in serum free media. Cells were treated without or with dark (i.e. non-fluorescently labeled or unconjugated) EGF (50 nM) for 5 minutes and immediately fixed in 4% paraformaldehyde for 15 minutes. Cells were washed extensively with 10 mM Tris (pH 7.2) and PBS, and permeabilized with 0.1% Triton X-100 (w/v) in 5% BSA for 20 min. Cells were labeled with anti-pY1068 and an anti-mouse Alexa647 secondary antibody. Cells were mounted on slides using Prolong Gold with DAPI and imaged using a Zeiss LSM510. Immunofluorescence labeling of transiently transfected HeLa cells was carried out in 8-well chamber slides (Lab-Tec, Thermo Scientific #155411). All image processing was performed using Matlab (The MathWorks, Inc., Natick, MA) in conjunction with the image processing library DIPImage (Delft University of Technology).

Immunochemistry assays of EGFR abundance and phosphorylation

For each confocal fluorescence image, total receptor abundance was characterized in terms of the per-pixel intensity of the receptor label (anti-HA FITC), and the level of receptor phosphorylation was similarly characterized using a site-specific antibody (α-pY1068 EGFR). After thresholding on total receptor intensity to mask the membrane of expressing cells, the total receptor intensity and corresponding phosphorylated EGFR level was averaged for each pixel to generate a plot showing the relationship between receptor expression and receptor phosphorylation (Figure 1C).
Model-based analysis of immunofluorescence data

See Supplementary Note.

Single particle tracking, image registration, and processing

Single- and dual-color SPT of unliganded and ligand-bound EGFR, diffusion analysis, and HMM analysis, were carried out using anti-HA- and EGF-conjugated QDs as previously described (Low-Nam et al., 2011; Steinkamp et al., 2014). Briefly, equal molar ratios of anti-HA-biotin or EGF-biotin and QD585 or QD655 streptavidin were incubated in PBS + 1% BSA at 4 °C for 2 hours with agitation before imaging. CHO cells expressing EGFR-WT, EGFR-L858R, or EGFR-ΔL747-P753insS were seeded in 8-well chamber slides (Lab-Tec, Thermo Scientific #155411) and allowed to adhere to the glass overnight. Cell culture media was exchanged for Tyrodes imaging buffer (135 mM NaCl, 10 mM KCl, 0.4 mM MgCl2, 1 mM CaCl2, 10 mM HEPES, 20 mM glucose, 0.1% BSA, pH 7.2), and cells were incubated with anti-HA-QD585/655 or EGF-QD585/655 to obtain single molecule density on the apical cell surface. After extensive washing with imaging buffer, cells were imaged for up to 8 minutes to avoid imaging of internalized receptors.

Wide field imaging for SPT was performed using an Olympus IX71 inverted microscope equipped with a 60× 1.2 N.A. water objective; an objective heater (Bioptechs, Butler, PA) maintained physiological temperature at 34-36 °C. Wide field excitation was provided by a mercury lamp with a 436/10 nm bandpass excitation filter and a 50/50 neutral density filter. Emission was collected by an EMCCD camera (Andor iXon 887) and pixel size was 166.67 nm. QD emission was collected using the OptoSplit image splitter (Cairn Research) to simultaneously image two spectrally distinct QDs, using a 600 nm dichroic and the appropriate filters, 655/40 nm and 585/20 nm bandpass filters (Chroma, Rockingham, VT). Fiducial channel registration datasets were acquired periodically using 0.2 μm Tetraspeck fluorescent beads (Invitrogen).

Single molecule localization and trajectory connection were carried out using GPU computing as previously described (Smith et al., 2010). For details of SPT analysis, see Low-
Diffusion analysis of trajectories was conducted by square displacement analysis and two-component fitting, as previously described (de Keijzer et al., 2008; Low-Nam et al., 2011). All image processing was performed using Matlab (The MathWorks, Inc., Natick, MA) in conjunction with the image processing library DIPImage (Delft University of Technology). Dimer events were identified using a three-state hidden Markov model (HMM), as previously described (Low-Nam et al., 2011; Steinkamp et al., 2014). The Viterbi algorithm (Forney, 1973) is used to identify the most likely state within individual QD interactions.

**Super-resolution imaging and analysis**

CHO cells expressing EGFR and mutants were washed with PBS and fixed in 4% paraformaldehyde + 0.2% glutaraldehyde for ~2 hours to minimize receptor mobility after fixation (Tanaka et al., 2010). Cells were washed extensively with PBS, once with 0.1% NaBH₄, and once with 10 mM Tris-HCl (pH 7.2) to reduce background fluorescence and quench cross-linkers. After blocking with PBS + 2% BSA, cells were labeled with Alexa647- or Cy3B-conjugated monoclonal, primary antibodies against EGFR. Identical, monoclonal antibody against EGFR (anti-EGFR, R-1, Santa Cruz Biotechnology #sc-101) was used for Alexa647- and Cy3B-labeling. Alexa647-conjugated anti-EGFR was purchased commercially (Santa Cruz Biotechnology #sc-101 AF647), and Cy3B-conjugated antibody was made using the carrier-free version of anti-EGFR R-1 along with reactive Cy3B NHS-ester (GE Healthcare Life Sciences #PA63100). Cells were labeled with Alexa647- and Cy3B-conjugated anti-EGFR in a 1:1 ratio in PBS + 2% BSA either overnight at 4 °C or 2 hours at room temperature followed by extensive washing with PBS + 2% BSA. For activation of EGFR-WT, cells were exposed to 50 nM EGF for 8 minutes at room temperature prior to fixation; a sufficient concentration and duration to ensure dimerization of EGFR on the basal surface of the cell.

Two-color super-resolution imaging via direct stochastic optical reconstruction microscopy (dSTORM) was performed as described (van de Linde et al., 2011) and analyzed similar to previously used techniques (Semrau et al., 2011; Veatch et al., 2012). Imaging was performed using an inverted microscope (IX71; Olympus America, Center Valley, PA) equipped with an oil-immersion objective 1.45-NA total internal reflection fluorescence objective (U-APO
150×/NA 1.45; Olympus America). A 637-nm laser (collimated from a laser diode, HL63133DG, Thorlabs) was used for Alexa647 excitation (~0.5 kW/cm²), and a 561-nm laser (Sapphire 561, Coherent, Inc.) was used for Cy3B excitation (~0.5 kW/cm²). Additional 405 excitation (up to ~25 W/cm²) was used for activation of both Alexa647 and Cy3B dyes as necessary. All laser light was configured in total internal reflection geometry. Cells were imaged in standard dSTORM imaging buffer (Dempsey et al., 2011) with enzymatic oxygen scavenging system and primary thiol: 50mM Tris, 10mM NaCl, 10% w/v glucose, 168.8 U/ml glucose oxidase (Sigma #G2133) and 1404 U/ml catalase (Sigma #C9332), 10 mM 2-aminoethanethiol (MEA), pH 8.5.

A quad-band dichroic and emission filter set (LF405/488/561/635-A; Semrock, Rochester, NY) set was used for sample illumination and emission. Emission light was separated into two channels using a custom built emission filter setup using a 655 dichroic and the appropriate filters for Alexa647 (692/40 nm) and Cy3B (600/37 nm), directing emission light for Alexa647 and Cy3B onto different halves of an iXon 897 electron-multiplying charge-coupled device (EM CCD) camera (Andor Technologies, South Windsor, CT). The EMCCD gain was set to 200, and frames were 256 × 256 pixels (for each channel) with a pixel size of 106.7 nm. Images were acquired at maximum camera speed (~15 ms exposure for 256 × 256 pixel region) and a total of 10,000-20,000 frames were collected. The sample chamber was mounted in a three-dimensional piezostage (Nano-LPS; Mad City Labs, Madison, WI) with a resolution along the xyz-axes of 0.2 nm. Sample drift was corrected for throughout the imaging procedure using a custom-built stage stabilization routine.

Imaging was carried out sequentially starting with Alexa647 followed by Cy3B, and two-color super-resolution data was overlayed using an affine transformation matrix generated from the localization of fiducial beads with fluorescence emission in both channels from 0.2 μm Tetraspeck fluorescent beads (Invitrogen). Fiducial bead datasets were acquired periodically throughout imaging, and the overlay accuracy was calculated by applying the affine transformation matrix from one channel registration to other channel registration sets within a single day (Figure S9). The overlay error is given as a mean ± standard deviation of the root mean squared error.
Two-color super-resolution datasets were analyzed by localization-based cross-correlation analysis similar to previously described methods (Semrau et al., 2011; Sengupta et al., 2011; Veatch et al., 2012; Shelby et al., 2013). Briefly, a subregion within each cell (typically 25-100 μm², excluding the lateral membrane region) was selected, and opposite color localizations were binned radially in 20 nm bins. Data was normalized to total center counts and to the average number of localizations per unit area. Shown is the normalized radial distribution function (RDF) with 20 nm bin size of 10-20 cells imaged over several days, treating the RDF of each cell as independent (mean ± standard error).

Measurement and analysis of FLIM data

FRET measurements between EGFR and the plasma membrane were performed as previously described (Ziomkiewicz et al., 2013).

Donor and acceptor cell labeling, spectra and R₀. The ACP-tagged EGFR expressing CHO cells were plated and grown in 4-well Lab-Tek chambered coverglass slides (Thermo Fisher) in DMEM with 10% FCS. Prior to imaging, cells were starved for 1 hour before labeling with 4 µM Oregon Green CoA in Tyrodes buffer containing 0.5% BSA and 10 mM MgCl₂ for 20 min at room temperature by addition of Sfp PPTase from Bacillus subtilis and washed 3 times over 15 minutes with RAB buffer before measuring. Acceptor NR12S (Kucherak et al., 2010) (the kind gift of A. Klymchenko) was diluted from a DMSO stock with vigorous vortexing to 4 µM in Tyrodes buffer and immediately added to an equal volume of buffer on the cells with rapid mixing. The excitation and emission spectra of the donor and acceptor are shown in Figure S5. An R₀ of 5.8 nm was calculated from the Förster overlap integral. Figure S6 shows possible distances between the ACP tag and the cell membrane if the EGFR is in the inhibited, unliganded or the extended, liganded conformations.

TCSPC Hardware. The excitation light source was a Fianium SC400-4-2 white-light laser system pulsed at 20 MHz that was coupled to an AOTFnC-400.650-TN, AA Optic set at 467 nm in the case of one system and the excitation wavelength isolated from a Fianium Whitelase SC450 by a 467/10 nm Semrock filter in the case of the other system. Excitation was directed into an IX71 Olympus microscope equipped with a 60x or 100x Olympus objective, 1.49 NA and a stage scanning system. Data acquisition was recorded with a 510/10nm Semrock
BP emission filter in front of a Micron Photon Devices (Bolzano, Italy) PDM Series SPAD detector coupled to a PicoQuant PicoHarp 300 TCSPC correlator. The data were collected at 32 ps/ch with 5 ms/pixel dwell time and a 0.5 µm/pixel resolution.

**TCSPC Analysis Software.** Multicomponent lifetime analysis routines were written in Mathematica (Wolfram Research). The IRF was fit to an analytical function (*pulse*) composed of a combination of a Gaussian and an integrated Gaussian-exponential functions. The entire course of the fluorescence response (*signal*) from cells exposed to excitation pulses were then fit to the analytical convolution of the IRF and 1, 2, or 3 additional components according to the equations described in Ziomkiewicz et al. (Ziomkiewicz et al., 2013). The fit parameters in *signal* are the lifetime (*τ*), and a corresponding amplitude, both of which can be determined for one or more components. Single pixel decays could only be fit by a single component. More precise two-component analyses were possible after ordering and binning of the pixel data into groups (generally 2-10, each with 10-1000 members) in order of decreasing peak signal magnitude. The first component was assigned to background and the second component assigned to the donor. In the case of unlabeled cells and cells labeled only with acceptor, fitting required only a corresponding amplitude factor. One- or two-sided masks were generated on the image data to restrict the analysis primarily to foreground pixels. Donor lifetimes were back-mapped onto the 2-D cell images. Derived amplitudes and lifetimes can be subjected to further analysis based on the corresponding images and two-dimensional histograms. Examples of the back-mapped data for ACP-EGFR-WT and ACP-EGFR-L858R are shown in Figure 4B and 4C (bottom row of images), respectively.

**ACKNOWLEDGEMENTS**

This study was supported by NSF MCB-0845062 (DSL), the Oxnard Foundation (DSL), NIH P50GM085273 (BSW) and NSF 0954836 (KAL). DJA-J received financial support from the Max-Planck Society. Financial support by the Deutsche Forschungsgemeinschaft is gratefully acknowledged (SFB 937, project A5). N.K. is grateful to the Niedersächsisches Ministerium für Wissenschaft and Kultur for a MWK stipend. Use of the UNM Cancer Center Microscopy and Flow Cytometry facilities, and NIH support for these cores, is acknowledged (http://hsc.unm.edu/crtc/microscopy/acknowledgement.shtml). We gratefully thank Thomas
Jovin for analysis of FRET/FLIM data, input regarding the equilibrium modeling (Supplementary Note), and insightful discussions. We thank Shalini T. Low-Nam, Samantha L. Schwartz, and Patrick J. Cutler for assistance with single particle tracking development and analysis. Additionally, we thank Jörg Enderlein for providing the TCSPC facilities for FLIM measurements, Andrey Klymchenko for the gift of the NR12S membrane probe, and Reinhard Klement for MD calculations.

REFERENCES


Talavera, A. et al. (2009). Nimotuzumab, an antitumor antibody that targets the epidermal growth factor receptor, blocks ligand binding while permitting the active receptor conformation. Cancer Res. 69, 5851–5859.


Figure 1. Non-small cell lung carcinoma-associated EGFR kinase domain mutants are constitutively active and show increased phosphorylation with increasing receptor expression. (A) CHO cells expressing HA-tagged EGFR (HA-EGFR): EGFR-WT, EGFR-L858R, or EGFR-ΔL747-P753insS were serum starved followed by treatment without and with EGF. Cells were pre-treated with tyrosine kinase inhibitor PD153035 (1 μM) as indicated. Lysates were probed for phosphorylated EGFR (top) as well as total EGFR (bottom). (B) CHO cells expressing the indicated EGFR construct were labeled with a FITC-labeled α-HA Fab (green), then fixed, permeabilized, and labeled using α-pY1068 (red). (C) Cells imaged as in (B) were quantified for EGFR expression and phosphorylation. Each data point represents the mean pY1068 fluorescence intensity for a given EGFR intensity value per pixel across multiple images after thresholding (arrowhead and dashed line) to eliminate contribution from cells with no detectable EGFR expression; error bars illustrate the standard deviation of the measurement. Solid curves were obtained from Model 1 (Eqs. (5) and (6) with the constraints on parameter values indicated in Supplemental Methods); these curves reflect differences in the kinase activities of mutant and wild type forms of EGFR. Dashed curves were obtained from Model 2 (Eqs. (5) and (6) with the constraints on parameter values indicated in Supplemental Methods); these curves reflect differences in dimerization affinities of mutant and wild type forms of EGFR. Best-fit values for Model 1 and Model 2 and confidence limits from bootstrapping are given in Table S3. Individual plots for EGFR-WT, -L858R, and -ΔL747-P753insS are shown in Figure S2. A complete description each model is provided in Supplementary Note.
Figure 2. Detection of dimerization events by EGFR-WT and mutants on the surface of living cells using two-color SPT. (A) Schematic of EGFR dimers species identified using two-color QD tracking, including unliganded receptor dimers (0-EGF:2-EGFR, left), EGF-bound receptor dimers (2-EGF:2-EGFR, center), and singly-liganded EGFR dimers (1-EGF:2-EGFR, right). (B-D) Top: Raw, diffraction-limited data (pixelated, Gaussian-filtered green/magenta image) and corresponding localizations (green/magenta circles) for two-color SPT of EGFR dimerization. Bottom: Plot showing the changes in separation distance for the corresponding pair over time, overlaid with the resulting state assignments from the Viterbi analysis (Free, Domain Confined, or Dimer). Shown are EGFR-WT in the absence of ligand, tracking with QD-HA (B), EGFR-WT bound to EGF, tracking with EGF-QD (C), and EGFR-L858R in the absence of ligand, tracking with QD-HA (D). Note the difference in dimer duration between conditions. (E) The dimer off-rates calculated from the HMM analysis confirm that mutants form stable dimers in the absence of ligand. (F) Raw data (pixelated images) and corresponding trajectories (red lines) for unliganded EGFR-WT, ligand-bound EGFR-WT, unliganded EGFR-L858R, and unliganded EGFR-ΔL747-P753insS illustrating differences in receptor mobility. The trajectory in each figure represents 20 seconds of tracked data. Scale bar is 1μm. (G) Receptor mobility was quantified using a cumulative probability plot of squared displacements. Solid lines represent unliganded EGFR tracked with QD-HA, and dashed lines represent ligand-bound receptor tracked with EGF-QD (see schematic inset). Shown are EGFR-WT (blue), EGFR-L858R (red) and EGFR-ΔL747-P753insS (green). (H) Diffusion coefficients determined from fitting the distribution of squared displacements for EGFR and mutants, both unliganded (black bars) and ligand-bound (gray bars). Shown is the mean ± standard error for a minimum of three independent experiments (* p < 0.001 relative to the unliganded EGFR-WT, and ** p < 0.05 comparing unliganded and ligand-bound EGFR-L858R or EGFR-ΔL747-P753insS). Dimer off-rates and diffusion values are summarized in Table S1.
Figure 3. Two-color dSTORM super-resolution imaging shows ligand induced EGFR dimerization and ligand-independent dimerization of EGFR mutants. (A) Representative two-color dSTORM reconstructed image of a CHO cell expressing EGFR-WT in the presence of EGF, fixed and labeled with a monoclonal anti-EGFR antibody conjugated with either Alexa647 (magenta) or Cy3B (green). Shown is a reconstructed superresolution image, where each localization is represented as a 2D Gaussian with σ proportional to its localization precision. The mean localization precision for Alexa647 and Cy3B is approximately 10 and 12 nm, respectively. (B) Zoomed regions of (A) highlighting potential EGFR dimers (white boxes) separated by < 50 nm. (C) Cross-correlation analysis of Alexa647 and Cy3B channels clearly shows EGFR-WT clustering after stimulation with EGF (red), indicated by the peak in the radial distribution function at short distances (< 50 nm) relative to control cells (black). The cross-correlation for control cells (black) remains flat, indicating no spatial correlation in the absence of ligand. (D) Similar two-color super-resolution and cross-correlation analysis in resting cells expressing EGFR mutants shows ligand-independent aggregation of EGFR-L858R (red) and EGFR-ΔL747-P753insS (blue), indicated by the increase in the radial distribution function at short distances (< 50 nm). EGFR-WT remains flat (black), indicating little aggregation in the absence of ligand.
Figure 4. FRET-FLIM and structural mutations reveal that unliganded EGFR-L858R adopts the extended conformation and requires ectodomain interactions for signaling. (A) Schematic of the EGFR ectodomain in the tethered, autoinhibited conformation (left) and the extended conformation (right). The donor fluorophore (Oregon Green 488, green) is covalently linked at the EGFR N-terminus via a small acyl carrier protein (ACP) tag, and the acceptor fluorophore (NR12S, a derivate of Nile Red, red) is embedded in the outer leaflet of the plasma membrane. By measuring FRET between donor and acceptor, the relative apparent separation between the EGFR N-terminus and the plasma membrane was determined. (B-C) Images of EGFR-WT (B) or EGFR-L858R (C) cells labeled with donor only (D only, left), donor with acceptor (D + A, middle), and donor with acceptor in the presence of EGF (D + A + EGF, right). Shown are the masked intensity images restricted primarily to the plasma membrane (top) and donor fluorescence lifetime values, $\tau_2$, corresponding to the masked pixels (bottom). (D) Donor fluorescence lifetime values were averaged over many cells from 5-6 independent experiments for EGFR-WT and EGFR-L858R ± 2 µM NR12S acceptor and ± 30 nM EGF. Fluorescence lifetime results are summarized in Table S2.
Figure 5. Ectodomain engagement is required for robust ligand-independent activity of EGFR-L858R. (A-B) Mutation of the EGFR dimerization arm within the context of the L858R kinase mutant causes a decrease in receptor phosphorylation at Y1068. HeLa cells were transfected with HA-EGFR-WT, -L858R, or -L858R/246-253* followed by lysis and analysis via western blot (A) and quantification (B). Shown is the mean ± s.e.m of four independent experiments (* p < 0.01). (C-D) Single color QD tracking of HA-EGFR-WT, -L858R, and -L858R/246-253* in HeLa cells demonstrates a decrease in mobility of EGFR-L858R relative to EGFR-WT, and that addition of the 246-253* mutation significantly increases mobility of the EGFR-L858R mutant. Shown are the cumulative probability plot (C) and extracted diffusion coefficients (D) * p < 0.05, n.s. not significant.