Enhanced dimerization drives ligand-independent activity of mutant epidermal growth factor receptor in lung cancer

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ABSTRACT **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 subdiffraction-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 superresolution localization microscopy confirmed ligand-independent aggregation of EGFR mutants. Live-cell Förster resonance energy transfer 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.**

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Abbreviations used: ACP, acyl carrier protein; BSA, bovine serum albumin; CoA, coenzyme A; dSTORM, direct stochastic optical reconstruction microscopy; EGF, epidermal growth factor; EGFR, epidermal growth factor receptor; EMCCD, electron-multiplying charge-coupled device; FITC, fluorescein isothiocyanate; FLIM, fluorescence-lifetime imaging microscopy; FRET, Förster resonance energy transfer; HA, hemagglutinin tag; HMM, hidden Markov model; NSCLC, non–small cell lung carcinoma; PBS, phosphate-buffered saline; QD, quantum dot; RDF, radial distribution function; SPT, single-particle tracking; TKI, tyrosine kinase inhibitor.

INTRODUCTION

The epidermal growth factor receptor (EGFR/erbB1/HER1) is a member of the erbB family of receptor tyrosine kinases 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 a number of 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 on 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 interactions 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 in 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.* (2013) 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. 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 Leu-858 to arginine (EGFR-L858R or EGFR-L834R in the mature protein numbering) and 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, nearly 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 subdiffraction-limit imaging modalities, we reveal the altered behavior of NSCLC-associated EGFR mutants in living cells. We directly show that NSCLC-mutant EGFRs 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 hemagglutinin 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 Tyr-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 with 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 Tyr-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 (Supplemental Figure S1). Quantification of these images confirmed that the NSCLC mutants

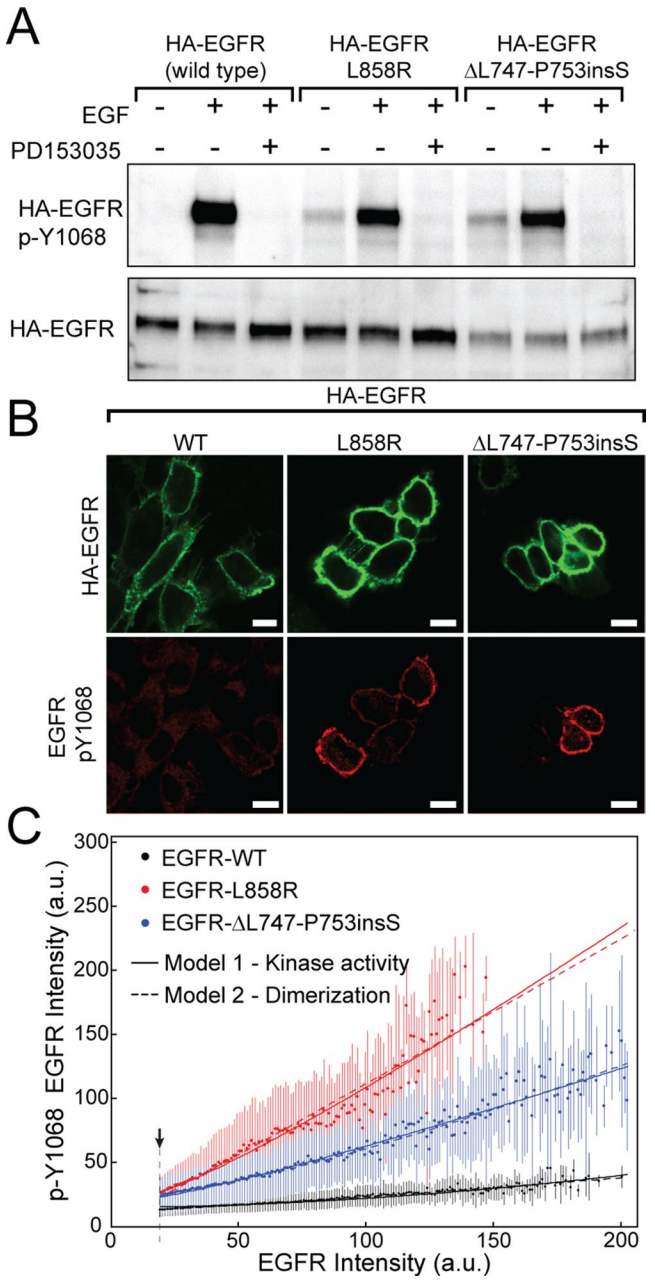

FIGURE 1: NSCLC-associated EGFR kinase domain mutants are constitutively active and show increased phosphorylation with increasing receptor expression. (A) CHO cells expressing HA-tagged EGFR (HA-EGFR): EGFR-WT, EGFR-L858R, or EGFR-ΔL747-P753insS were serum starved; this was followed by treatment without and with EGF. Cells were pretreated with TKI 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 an 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 SD of the measurement. Solid curves were obtained from model 1 (Eqs. 5 and 6 with the constraints on parameter values indicated in the Supplemental Methods); these curves reflect differences in the kinase activities of mutant and WT forms of EGFR. Dashed curves were obtained from model 2 (Eqs. 5 and 6 with the constraints on parameter values indicated in the Supplemental Methods); these curves reflect differences in dimerization affinities of mutant and WT forms of EGFR. Best-fit values for models 1 and 2 and confidence limits from bootstrapping are given in Supplemental Table S3. Individual plots for EGFR-WT, EGFR-L858R, and EGFR- ΔL747-P753insS are shown in Supplemental Figure S2. A complete description each model is provided in the Supplementary Note.

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 whether one mechanism might dominate, we fitted the data in Figure 1C to a monomer–dimer equilibrium model assuming either 1) higher intrinsic kinase activity (Zhang *et al.*, 2006; Yun *et al.*, 2007; see Supplementary Note and Supplemental Table S3, Model 1) or 2) increased dimerization affinity (Shan *et al.*, 2012; see Supplementary Note and Supplemental Table S3, Model 2). As shown in Figure 1C, however, both models yielded equal goodness of fit (compare the solid and dashed curves; see also Supplemental 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 the 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 single-particle tracking (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 (Supplemental Figure S3).

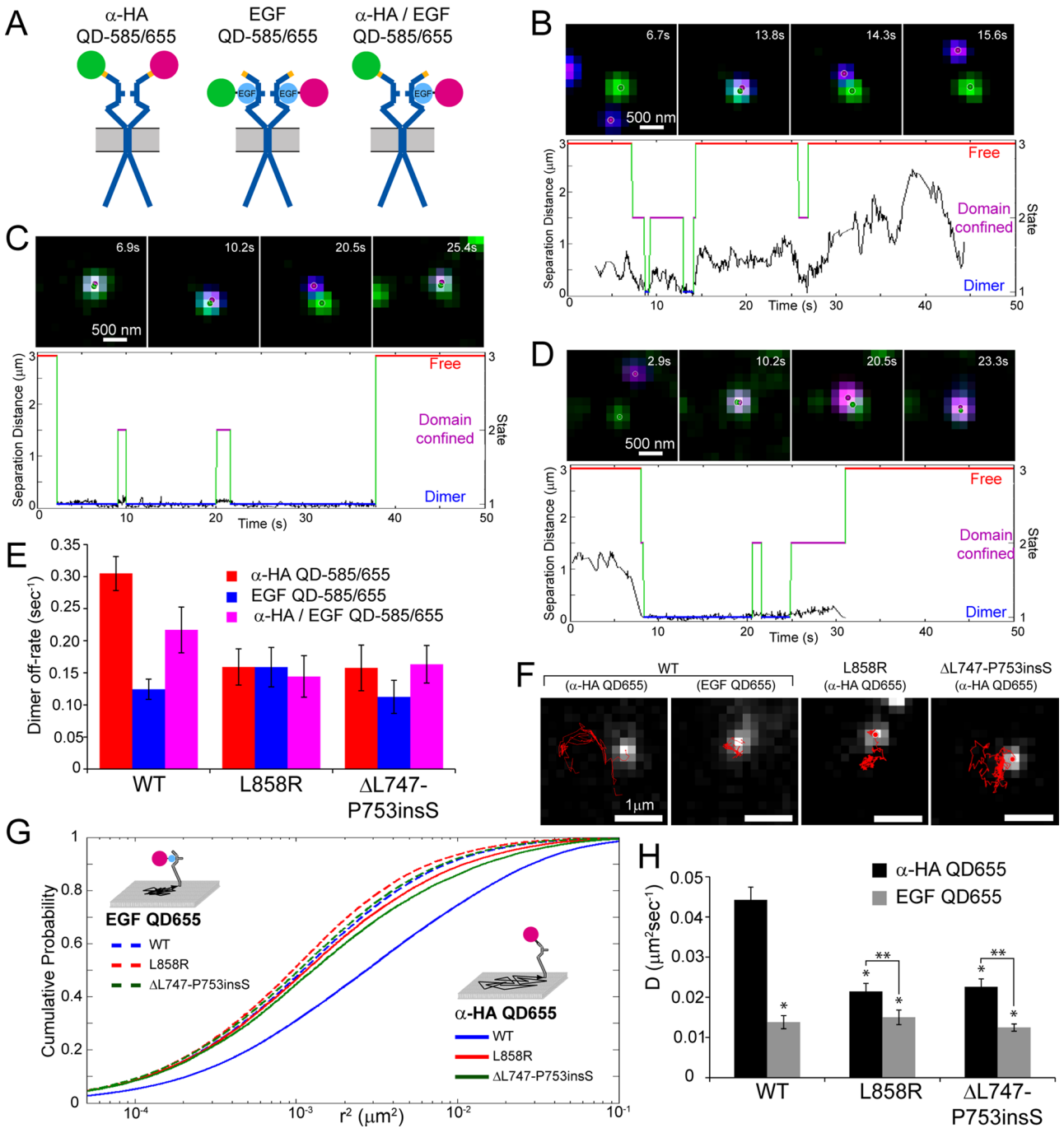

FIGURE 2: Detection of dimerization events by EGFR-WT and mutants on the surface of living cells using two-color SPT. (A) Schematic of EGFR dimers species identified using two-color QD tracking, including unliganded receptor dimers (0-EGF:2-EGFR, left), EGF-bound receptor dimers (2-EGF:2-EGFR, center), 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 s of tracked data. Scale bar: 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

Figure 2, B–D, shows examples of repeated dimerization events between receptor pairs with varying dimer stability. The unliganded EGFR-WT exhibited transient dimers (Figure 2B and Supplemental Movie S1), whereas the ligand-bound EGFR-WT formed longer-lived dimers (Figure 2C and Supplemental Movie S2). In contrast to EGFR-WT, both mutants demonstrated long-lived interactions in the absence of ligand (Figure 2D, Supplemental Figure S4, and Supplemental Movies 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 nondimer (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 2, B–D, lower panels). The resulting dimer off-rates calculated over many dimer interactions are shown in Figure 2E and summarized in Supplemental 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, $k_{off} = 0.31\ s^{-1}$) that shifted to longer-lived dimers with ligand bound ($k_{off} = 0.12\ s^{-1}$). The singly liganded 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 underestimation 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 Supplemental Table S1). On ligand binding, both mutants slowed further to a degree comparable with 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 superresolution 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 3, A and B). Receptor proximity across multiple samples was quantified using localization-based cross-correlation analysis of Alexa Fluor 647– 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 Supplemental Tables S3 and S4).

Activating kinase mutation alters the ectodomain structure

Prior studies using recombinant EGFR kinase domain have 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 coenzyme A (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 4, B–D, and Supplemental 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)

and mutants, both unliganded (black bars) and ligand bound (gray bars). Shown is the mean ± SE for a minimum of three independent experiments. *, $p < 0.05$ relative to the unliganded EGFR-WT; **, $p < 0.001$ comparing unliganded and ligand-bound EGFR-L858R or EGFR-ΔL747-P753insS. Dimer off-rates and diffusion values are summarized in Supplemental Table S1.

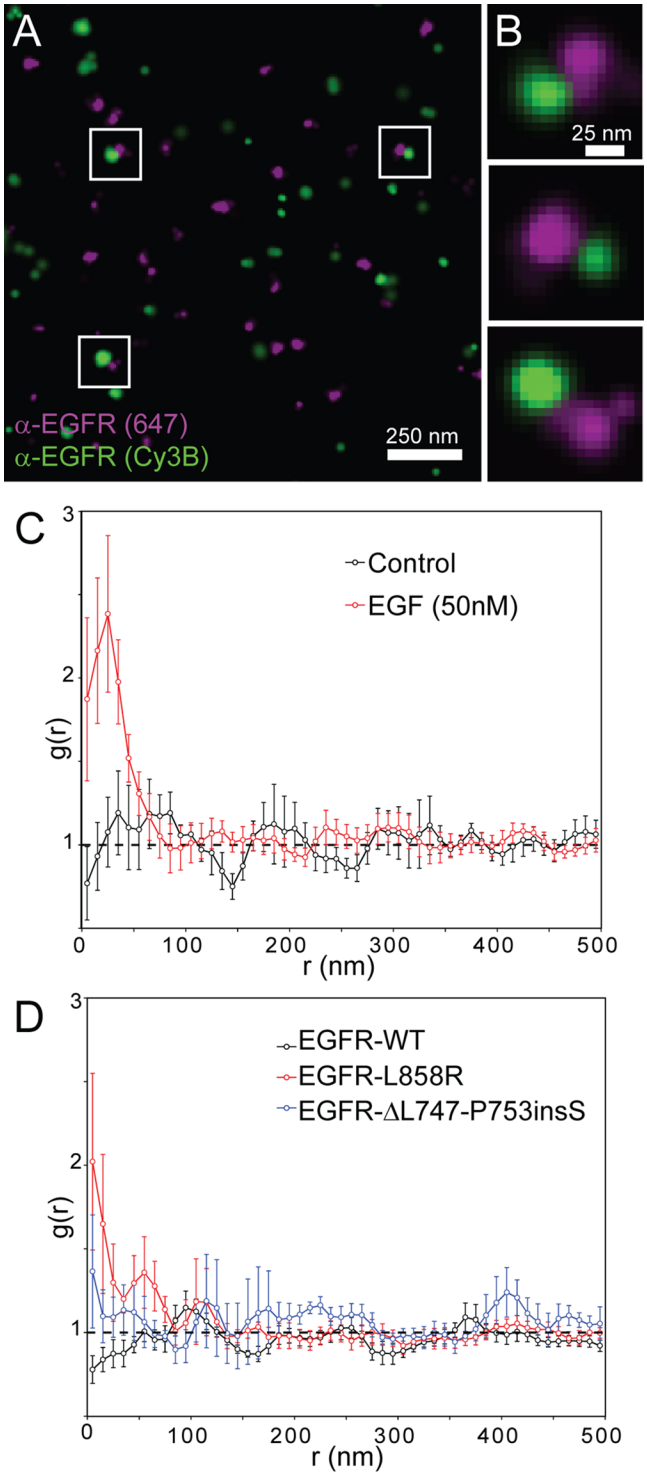

FIGURE 3: Two-color dSTORM superresolution imaging shows ligand-induced EGFR dimerization and ligand-independent dimerization of EGFR mutants. (A) Representative two-color dSTORM-reconstructed image of a CHO cell expressing EGFR-WT in the presence of EGF, fixed and labeled with a monoclonal anti-EGFR antibody conjugated with either Alexa Fluor 647 (magenta) or Cy3B (green). Shown is a reconstructed superresolution image, in which each localization is represented as a two-dimensional Gaussian with σ proportional to its localization precision. The mean localization precisions for Alexa Fluor 647 and Cy3B are ~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 Alexa Fluor 647 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 superresolution 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 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; Supplemental 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 is 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 (Supplemental Figure S7), indicating that ectodomain dimerization is indispensable for ligand-induced activity. Consistent with results in CHO cells (Figures 1 and 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 5, A and B, and Supplemental Figure S8) and also increased mobility relative to EGFR-L858R kinase mutant alone (Figure 5, C and D). The doubly mutated EGFR-L858R/246-253* retained measurable levels of ligand-independent activity compared with the EGFR-WT (Figure 5, A and 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 complementary 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

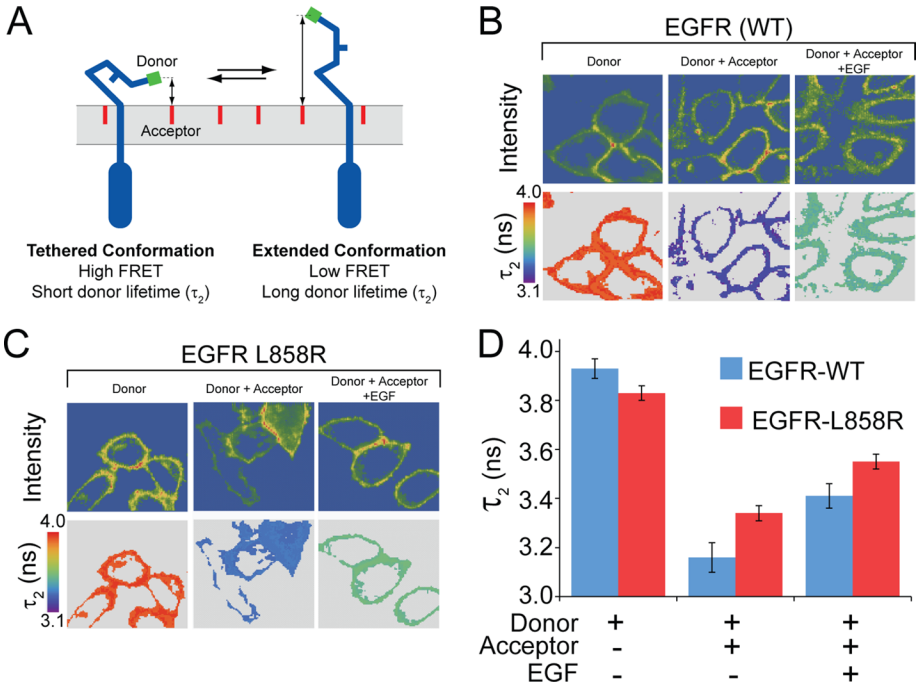

FIGURE 4: FRET-FLIM reveals that unliganded EGFR-L858R adopts the extended conformation and requires ectodomain interactions for signaling. (A) Schematic of the EGFR ectodomain in the tethered, autoinhibited conformation (left) and the extended conformation (right). The donor fluorophore (Oregon Green 488, green) is covalently linked at the EGFR N-terminus via a small ACP tag, and the acceptor fluorophore (NR12S, a derivate of Nile Red, red) is embedded in the outer leaflet of the plasma membrane. The relative apparent separation between the EGFR N-terminus and the plasma membrane was determined by measuring FRET between donor and acceptor. (B and C) Images of EGFR-WT (B) or EGFR-L858R (C) cells labeled with donor only (left), donor with acceptor (middle), and donor with acceptor in the presence of EGF (right). Shown are the masked intensity images restricted primarily to the plasma membrane (top) and donor fluorescence lifetime values, τ_2, corresponding to the masked pixels (bottom). (D) Donor fluorescence lifetime values were averaged over many cells from five to six independent experiments for EGFR-WT and EGFR-L858R ± 2 μM NR12S acceptor and ± 30 nM EGF. Fluorescence lifetime results are summarized in Supplemental Table S2.

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 "superacceptors" 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 activators or receivers (Cho *et al.*, 2013). Given this result, an important consideration is the interaction between NSCLC EGFR variants and either the WT EGFR or erbB2. It has been reported that patients with the L858R mutation or exon 19 deletions are heterozygous (Lynch *et al.*, 2004), suggesting that oncogenic prosurvival is a result of signaling via one mutant and one WT 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 in both the kinase domain and the 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 and negative-stain electron microscopy of purified, nearly full-length EGFR (tEGFR), Lu *et al.* (2012) 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 with those of EGF-mediated dimers. In an additional study, simultaneous visualization of extracellular and intracellular domains of tEGFR in the presence of Gefitinib, an EGFR-specific TKI that 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 WT 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

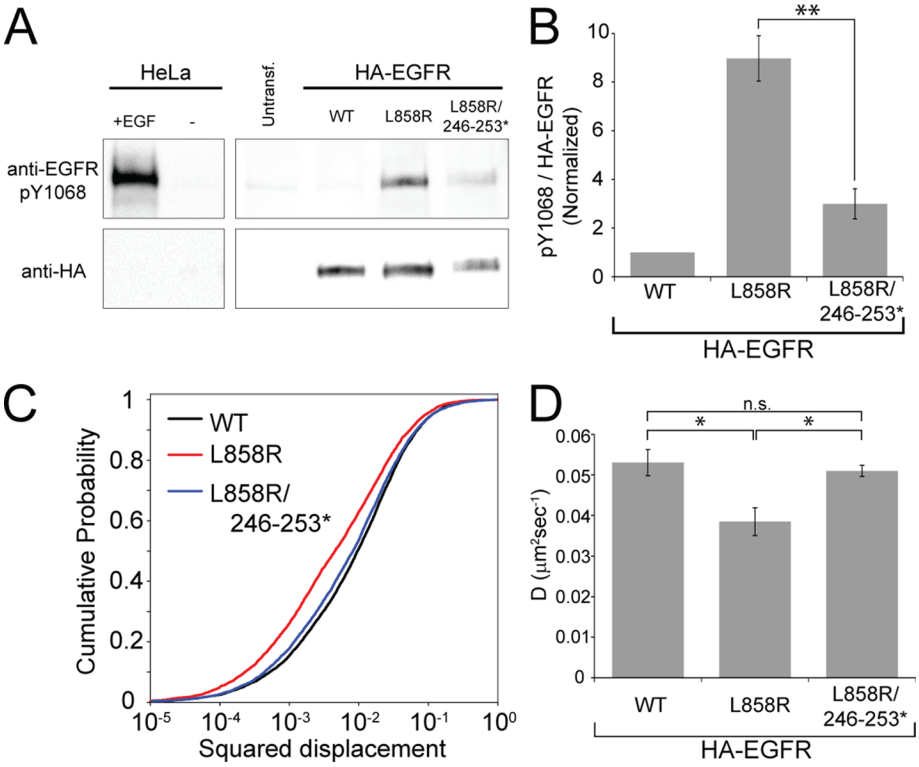

FIGURE 5: Ectodomain engagement is required for robust ligand-independent activity of EGFR-L858R. (A and B) Mutation of the EGFR dimerization arm within the context of the L858R kinase mutant causes a decrease in receptor phosphorylation at Y1068. HeLa cells were transfected with HA-EGFR-WT, HA-EGFR-L858R, or HA-EGFR-L858R/246-253* followed by lysis and analysis via Western blot (A) and quantification (B). Shown is the mean ± SEM of four independent experiments; **, $p < 0.01$. (C and D) Single-color QD tracking of HA-EGFR-WT, HA-EGFR-L858R, and HA-EGFR-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.

phosphorylation of EGFR-L858R (Figure 5, A and 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). In this study, we have confirmed that, while WT receptor interactions in the absence of ligand can occur, they are short lived (Figure 2B). Furthermore, quantitative analysis of superresolution 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 (Figures 2C and 3C) or by mutation to an active kinase domain (Figures 2D and 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 (Figure 4; Ziomkiewicz *et al.*, 2013). Recent molecular dynamics (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, nonphysiological temperatures, pharmacological inhibitors, or steric hindrance due to labeling. While our live-cell data support 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 that disrupt EGFR dimerization arm–mediated interactions (Hanold *et al.*, 2015a,b). Cetuximab is a potent inhibitor of EGFR activity and functions both by 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 of EGFR-L858R/T790M expressed in Ba/F3 cells was dimerization-independent based on resistance to Cetuximab (Cho *et al.*, 2013). Whether the increased affinity for ATP of EGFR-L858R/T790M (Yun *et al.*, 2008) accelerates receptor phosphorylation and effectively circumvents the requirement for stable dimer formation 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 DMEM (Life Technologies, Carlsbad, CA; #10313-021) supplemented with 10% fetal bovine serum (HyClone, Logan, UT), 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 (Danvers, MA). Horseradish peroxidase–conjugated goat anti-rabbit and anti-mouse secondary antibodies (sc-2004, sc-2005), and EGFR antibody used for superresolution imaging (anti-EGFR, R-1, sc-101) were from Santa Cruz Biotechnology (Dallas, TX). Anti HA-biotin (12158167001) and anti HA–fluorescein isothiocyanate (FITC; 11988506001) high-affinity rat monoclonal antibody Fab fragments (clone 3F10) were purchased from Roche (Basel, Switzerland). 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 (Pittsburgh, PA).

Cloning and generation of stable cell lines

Cloning of HA- 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- or ACP-tagged EGFR (WT, L858R, or ΔL747-P753insS) by Amaxa nucleofection. Briefly, 3×10^6 cells were transfected with 4 µg DNA using the Amaxa Nucleofection Kit V optimized for CHO cells per the manufacturer's instructions. Cells were selected using 1 mg/ml G418 and sorted using either EGF–Alexa Fluor 488 (LifeTechnologies, #E13345) or EGF–Alexa Fluor 647 (Life Technologies; #E35351). After sorting, cells were maintained in G418 and routinely sorted, yielding a heterogeneous nonclonal 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, 1.5×10^6 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 the Amaxa Nucleofection Kit R (Lonza, Basel, Switzerland) according to the manufacturer's instructions. Cells were immediately transferred to complete culture media and seeded onto eight-well chamber slides (Lab-Tec; Thermo Scientific, Waltham, MA; #155411) for SPT or immunofluorescence labeling. The remaining cells were plated onto a 100-mm dish for lysis and Western blot analysis. Cells were incubated for ~24 h at 37°C before SPT, fixation, and immunolabeling, or lysis and Western blot analysis. Separate transfections were done for independent experiments.

Immunoblotting

CHO cells expressing HA-EGFR (WT or mutants) were seeded on 60-or 100-mm dishes and grown to ~80% confluency. Cells were pretreated with TKI as indicated; this was followed by treatment with EGF (50 nM) for 8 min. After being washed with cold PBS, cells were lysed in RIPA buffer on ice for 1 h. Protein concentration of cleared lysates was measured by bicinchoninic acid (BCA) assay (Pierce, Rockford, IL), and equal amounts of total protein were separated on a 4–15% polyacrylamide gel (Bio-Rad, Hercules, CA), transferred to nitrocellulose (iBlot transfer system; Life Technologies), probed with the indicated antibody, and imaged using enhanced chemiluminescence on a Bio-Rad ChemiDoc.

Immunofluorescence

CHO cells expressing HA-EGFR (WT or mutants) were seeded on 18-mm coverslips overnight and labeled live using an anti HA-FITC (Fab) at 2 µg/ml for 1 h 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 min and immediately fixed in 4% paraformaldehyde for 15 min. Cells were washed extensively with 10 mM Tris (pH 7.2) and PBS, and permeabilized with 0.1% Triton X-100 (wt/vol) in 5% bovine serum albumin (BSA) for 20 min. Cells were labeled with anti-pY1068 and an anti-mouse Alexa Fluor 647 secondary antibody. Cells were mounted on slides using Prolong Gold with 4′,6-diamidino-2-phenylindole and imaged using a Zeiss LSM510. Immunofluorescence labeling of transiently transfected HeLa cells was carried out in eight-well chamber slides (Lab-Tec, #155411; Thermo Scientific). All image processing was performed using Matlab (MathWorks, Natick, MA) in conjunction with the image-processing library DIPImage (Delft University of Technology, The Netherlands).

Immunofluorescence 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 the Supplementary Note.

SPT, 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 h with agitation before imaging. CHO cells expressing EGFR-WT, EGFR-L858R, or EGFR-ΔL747-P753insS were seeded in eight-well chamber slides (Lab-Tec, #155411; Thermo Scientific) and allowed to adhere to the glass overnight. Cell culture medium was exchanged for Tyrodes imaging buffer (135 mM NaCl, 10 mM KCl, 0.4 mM $MgCl_2$, 1 mM $CaCl_2$, 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 min 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 NA 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 band-pass 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 band-pass filters (Chroma, Rockingham, VT). Fiducial channel registration data sets were acquired periodically using 0.2 µm Tetraspeck fluorescent beads (Invitrogen).

Single-molecule localization and trajectory connection were carried out using graphics processor unit (GPU) computing as previously described (Smith *et al.*, 2010). For details of SPT analysis, see Low-Nam *et al.* (2011). 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 (MathWorks) in conjunction with the image-processing library DIPImage (Delft University of Technology). Dimer events were identified using a three-state HMM, as previously described (Low-Nam *et al.*, 2011; Steinkamp *et al.*, 2014). The Viterbi algorithm (Forney, 1973) was used to identify the most likely state within individual QD interactions.

Superresolution imaging and analysis

CHO cells expressing EGFR and mutants were washed with PBS and fixed in 4% paraformaldehyde + 0.2% glutaraldehyde for ~2 h to minimize receptor mobility after fixation (Tanaka *et al.*, 2010). Cells were washed extensively with PBS, once with 0.1% $NaBH_4$, and once with 10 mM Tris-HCl (pH 7.2) to reduce background fluorescence and quench cross-linkers. After being blocked with PBS + 2% BSA, cells were labeled with Alexa Fluor 647– or Cy3B-conjugated monoclonal, primary antibodies against EGFR. Identical, monoclonal antibody against EGFR (anti-EGFR, R-1, #sc-101; Santa Cruz Biotechnology) was used for Alexa Fluor 647 and Cy3B labeling. Alexa Fluor 647–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 Alexa Fluor 647– and Cy3B-conjugated anti-EGFR in a 1:1 ratio in PBS + 2% BSA, either overnight at 4°C or for 2 h 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 min at room temperature before fixation; a sufficient concentration and duration to ensure dimerization of EGFR on the basal surface of the cell.

Two-color superresolution imaging via 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 Alexa Fluor 647 excitation (~0.5 kW/cm^2), and a 561-nm laser (Sapphire 561; Coherrent) was used for Cy3B excitation (~0.5 kW/cm^2). Additional 405 excitation (up to ~25 W/cm^2) was used for activation of both Alexa Fluor 647 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 an enzymatic oxygen-scavenging system and primary thiol: 50 mM Tris, 10 mM NaCl, 10% wt/vol 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) was used for sample illumination and emission. Emission light was separated into two channels using a custom-built emission filter setup with a 655 dichroic and the appropriate filters for Alexa Fluor 647 (692/40 nm) and Cy3B (600/37 nm), directing emission light for Alexa Fluor 647 and Cy3B onto different halves of an iXon 897 electron-multiplying charge-coupled device (EMCCD) 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 *x,y,z*-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 Alexa Fluor 647 followed by Cy3B, and two-color superresolution data were overlaid 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 data sets 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 (Supplemental Figure S9). The overlay error is given as a mean ± SD of the root-mean-square error.

Two-color superresolution data sets 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 µm^2, excluding the lateral membrane region) was selected, and opposite color localizations were binned radially in 20-nm bins. Data were 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 ± SE).

Measurement and analysis of FLIM data

FRET measurements between EGFR and the plasma membrane were performed using time-correlated single-photon counting as previously described (Ziomkiewicz *et al.*, 2013).

Donor and acceptor cell labeling, spectra and R_0. The ACP-tagged EGFR-expressing CHO cells were plated and grown in four-well Lab-Tek chambered coverglass slides (Thermo Fisher) in DMEM

with 10% fetal calf serum. Before being imaged, cells were starved for 1 h before being labeled with 4 μM Oregon Green CoA in Tyrodes buffer containing 0.5% BSA and 10 mM $MgCl_2$ for 20 min at room temperature by addition of Sfp PPTase from *Bacillus subtilis* and washed three times over 15 min with RAB buffer before being measured. Acceptor NR12S (the kind gift of A. Klymchenko; Kucherak *et al.*, 2010) was diluted from a dimethyl sulfoxide 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 Supplemental Figure S5. An R_0 of 5.8 nm was calculated from the Förster overlap integral. Supplemental 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.

Time-correlated single-photon counting (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 60× or 100× Olympus objective, 1.49 NA, and a stage scanning system. Data acquisition was recorded with a 510/10-nm 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/channel with a 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 instrument response function (IRF) was fitted to an analytical function (*pulse*) composed of a combination of a Gaussian and an integrated Gaussian-exponential function. The entire course of the fluorescence response (*signal*) from cells exposed to excitation pulses was then fitted to the analytical convolution of the IRF and one, two, or three additional components according to the equations described in 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 be fitted only by a single component. More precise two-component analyses were possible after ordering and binning of the pixel data into groups (generally two to 10, each with 10–1000 members) in order of decreasing peak signal magnitude. The first component was assigned to background, and the second component was 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 two-dimensional 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 4, B and C (bottom row of images), respectively.

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