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ORIGINAL ARTICLE

Defective ubiquitinylation of EGFR mutants of lung cancer confers prolonged signaling

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Several distinct mutations within the kinase domain of the epidermal growth factor receptor (EGFR) are associated with non-small cell lung cancer, but mechanisms underlying their oncogenic potential are incompletely understood. Although normally ligand-induced kinase activation targets EGFR to Cbl-mediated receptor ubiquitinylation and subsequent degradation in lysosomes, we report that certain EGFR mutants escape this regulation. Defective endocytosis characterizes a deletion mutant of EGFR, as well as a point mutant (L858R-EGFR), whose association with c-Cbl and ubiquitinylation are impaired. Our data raise the possibility that refractoriness of L858R-EGFR to downregulation is due to enhanced heterodimerization with the oncogene product HER2, which leads to persistent stimulation.
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Introduction

Excessive epidermal growth factor (EGF) signaling due to genetic aberrations is richly implicated in human cancer (Hynes *et al*., 2001; Yarden and Sliwkowski, 2001). To generate intracellular signals, EGF receptor (EGFR) must homodimerize or heterodimerize with members of its family, primarily a ligand-less oncogenic receptor called HER2/ErbB-2. These receptors promote cellular activation by recruiting several pathways, including the mitogen-activated protein kinase (MAPK) and the phosphoinositide 3-kinase – protein kinase B (PI3K-PKB/Akt) pathway. Following stimulation, EGFR undergoes ubiquitinylation by an E3 ubiquitin ligase, c-Cbl, which sorts hyper-phosphorylated receptors to degradation in lysosomes (reviewed in Dikic and Giordano, 2003).

Several groups reported in 2004 that certain heterozygous, somatically acquired EGFR mutations in lung cancer predict significant clinical responses to kinase inhibitors specific to EGFR (Lynch *et al*., 2004; Paez *et al*., 2004; Pao *et al*., 2004). All mutations are restricted to the tyrosine kinase domain of EGFR. The most frequent point mutation is a substitution of an arginine for leucine at position 858 (L858R). Yet other mutants carry in-frame short deletions. A second mutation, T790M, has been associated with acquisition of secondary resistance of patients to kinase inhibitors (Kobayashi *et al*., 2005; Pao *et al*., 2005). Differential patterns of autophosphorylation, resulting in superior Akt and Stat signaling, have been associated with EGFR mutants, which explains how kinase inhibitors selectively induce apoptosis of mutant-expressing cells (Sordella *et al*., 2004; Tracy *et al*., 2004). Along with pathway-selective activation by EGFR mutants, their ligand-induced autophosphorylation decays relatively slowly (Lynch *et al*., 2004), suggesting that persistent stimulation contributes to cell transformation. To examine this model and resolve the underlying mechanism, we tested mutant EGFRs in lung cancer cells and in ectopic systems. Our data indicate that ligand-induced inactivation of L858R, Δ746-50 and the double-mutant L858R/T790M, is defective. The defect may be due to a propensity of the mutants to heterodimerize with HER2, thereby evading c-Cbl-mediated ubiquitinylation and subsequent sorting to degradation in lysosomes. Hence, our studies raise the possibility that HER2 collaborates with EGFR mutants of lung cancer.

Results

Downregulation of EGFR mutants is impaired when examined in transfected cells

We constructed six of the previously identified EGFR mutations (Lynch *et al*., 2004; Paez *et al*., 2004) in a

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mammalian expression vector. These include G719C and G719S, L858R, L861Q, Δ746-50 and Δ747-53S. Chinese hamster ovary (CHO) cells that express no endogenous EGFR were transfected with plasmids encoding either WT-EGFR or the respective mutant. By using a surface biotinylation assay, which labels surface-exposed, rather than intracellular proteins, we determined that all mutants reached the cell surface (Figure 1a). Furthermore, all mutants retained activation by EGF when assayed with anti-phosphotyrosine antibodies (data not shown). It has been reported that L858R, Δ747-53S and Δ746-50 exhibit enhanced activation of downstream effectors such as signal transduction and activators of transcription (Stat proteins) (Sordella *et al*., 2004). Indeed, Stat3 activation was significantly increased, both basally and in response to EGF, in CHO cells transfected with plasmids encoding for L858R and L861Q (Figure 1b).

It has been previously reported that ligand-induced autophosphorylation of EGFR mutants decays relatively slowly (Lynch *et al*., 2004). Hence, we raised the possibility that EGF-induced downregulation of the mutants, namely their removal from the plasma membrane through an endocytic process, is impaired. To examine this model, we first stably knocked down the expression of the endogenous EGFR of HeLa cells, by transfection with either a control vector (pSuper) or a vector encoding an EGFR-specific siRNA. Immunoblotting analysis confirmed loss of EGFR expression in cells transfected with the pSUPER-EGFR plasmid (Figure 1c, upper left panel). Two stable clones were drug-selected, and immunoblotting of whole-cell extracts confirmed loss of expression in one clone (Clone 1; Figure 1c, upper right panel). Using clone 1 cells for adoptive expression of L858R-EGFR, Δ746-50-EGFR or WT-EGFR (all forms of EGFR were made resistant to the siRNA by introducing mutations at the respective target sites), enabled us to compare the rates of receptor downregulation under the same experimental conditions. The results presented in Figure 1c (lower panel) indicate that L858R-EGFR undergoes relatively slow endocytosis following treatment with EGF, in support of defective desensitization of lung cancer mutants of EGFR.

Ligand-induced downregulation of L858R/T790M-EGFR is defective in lung cancer cells

To confirm the defect in receptor downregulation and also address the biochemical mechanisms underlying

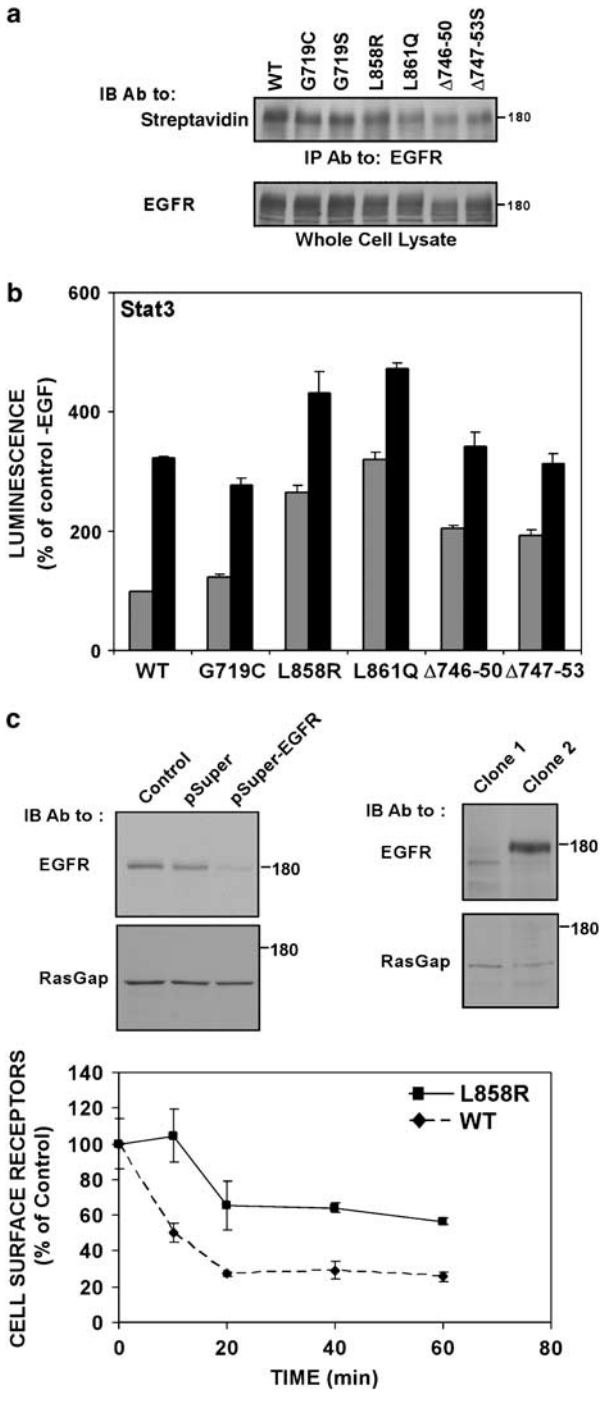

Figure 1 Downregulation of epidermal growth factor receptor (EGFR) mutants is impaired when examined in transfected cells. (**a**) The surface of Chinese hamster ovary (CHO) cells transiently expressing the indicated EGFR mutants was labeled with biotin and EGFR immunoprecipitated (IP). After electrophoresis, proteins were transferred to a nitrocellulose filter and immunoblotted (IB) with streptavidin horseradish peroxidase (HRP). Whole-cell lysates were also resolved and IB with anti-EGFR antibodies (lower panel). (**b**) CHO cells were cotransfected with plasmids encoding a Stat3-luciferase reporter, green fluorescent protein (GFP) and the indicated EGFR mutant. Following serum starvation, cells were treated with (black columns) or without (grey columns) EGF for 8 h, before determination of the relative luminescence signal. The results are the means ± s.d. of triplicates, normalized to the GFP signal. (**c**) Upper panels: forty-eight hours post transfection with a plasmid containing siRNA directed against EGFR (*pSuper-EGFR*), or a control vector (*pSuper*), HeLa monolayers were lysed and analysed with the indicated antibodies (left panel). The right panel presents immunoblotting analysis of EGFR expression in two HeLa stable clones transfected with a plasmid containing siRNA directed against EGFR. Lower panel: a stable clone (Clone 1) of HeLa cells transiently expressing siRNA-resistant L858R-EGFR or WT-EGFR, was incubated with EGF (5 ng/ml) at 37°C for the indicated time intervals. Cell-bound ligand was removed, and the level of surface receptors was determined by binding of a radiolabeled EGF at 4°C. The averages of triplicates and s.d. values (bars) are shown.

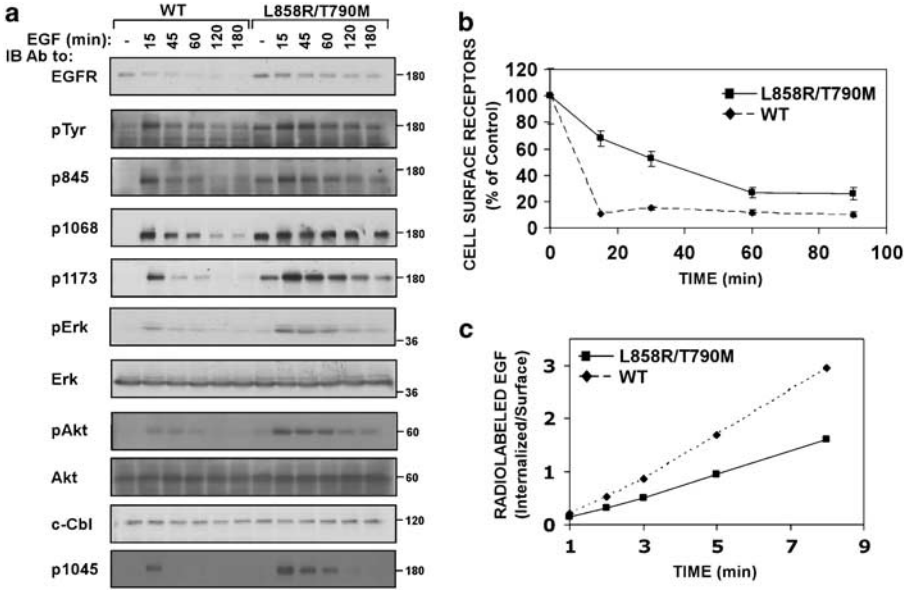

Figure 2 Ligand-induced downregulation of L858R/T790M-epidermal growth factor receptor (EGFR) is defective. (**a**) H358 (WT-EGFR) and H1975 (WT and L858R/T790M-EGFR) lung cancer cells were stimulated with EGF for the indicated time intervals and cell lysates analysed with the indicated antibodies. The experiment was repeated thrice. (**b**) H358 and H1975 NSCLC cells were incubated with EGF at 37°C for the indicated time intervals. Cell-bound ligand was removed, and the level of surface receptors was determined by binding of a radiolabeled EGF at 4°C. The average of triplicates and the respective s.d. (bars) are shown. H358 cells – dashed line, and H1975 cells – solid line. (**c**) H358 and H1975 cell monolayers were incubated at 37°C with a radiolabled EGF (10 ng/ml). At the indicated times, cell monolayers were acid – washed to remove surface-bound EGF. Radioactivity present in the acidic fraction was quantified in triplicates and the variation did not exceed 10% of the signal. The remaining cell-associated radioactivity was quantified similarly. The ratio obtained at each time point is presented.

enhanced signaling of L858R-EGFR, we used lung cancer cell lines naturally expressing EGFR. The cells we used were H358, which endogenously express WT-EGFR, and H1975 cells expressing a combination of WT and L858R-EGFR. According to several reports, the aberrant form of EGFR expressed in H1975 cells carries two mutations within the kinase domain (L858R/T790M); the T790M alteration prevents inhibition by Gefitinib (Kobayashi *et al*., 2005; Pao *et al*., 2005). When comparing the two lung cancer cell lines, we observed relatively high basal phosphorylation of the double mutant, as well as prolonged activation by EGF (Figure 2a). Consistent with these observations, L858R/T790M displayed enhanced activation of Akt and MAPK (Figure 2a).

Concentrating on ligand-induced downregulation of EGFR, we noted that ligand-activated WT-EGFR of H358 cells almost completely disappeared 15 min after stimulation with EGF, but EGFR of H1975 cells was detectable 2 h poststimulation (Figure 2a). This difference was confirmed by quantification of the immunoblots (data not shown). Hence, persistent autophosphorylation of L858R/T790M on several tyrosine residues, as well as prolonged activation of Akt and MAPK, might be attributed to a defect in ligand-induced degradation. Direct examination of receptor downregulation confirmed that L858R/T790M-EGFR is defective in ligand-induced endocytosis (Figure 2b), in similarity to the observations made in a reconstituted cell system (Figure 1c). Further, the rate of EGF internalization of a radioactive EGF into H1975 cells coexpressing WT-EGFR and L858R/T790M, was slower compared to H358 cells expressing WT-EGFR alone (Figure 2c). In contrast, internalization of a fluorescent derivative of transferrin displayed similar rates in H1975 and H358 cells (data not shown), suggesting intactness of the endocytic machinery of H1975 cells. The differences in receptor downregulation rates were highly reproducible in lung cancer cells, as well as in transfected cells (Figure 1c and data not shown), leading us to conclude that the ability of L858R/T790M-EGFR to generate enhanced and prolonged intracellular signals is attributable to a major defect in receptor desensitization.

An internal deletion mutant of EGFR evades ligand-induced downregulation

We utilized the H1650 lung cancer cell line that expresses Δ746-50-EGFR to determine whether additional non-small cell lung cancer (NSCLC) mutants, as revealed with L858R/T790M-EGFR, utilize defective receptor downregulation to prolong their signaling. Similar to the L858R/T790M mutant, Δ746-50-EGFR not only enhances, but also prolongs intracellular signals such as phosphorylation of Erk/MAPK and Akt (Figure 3a). Persistent activation of downstream effectors is likely due to a reduction in both the rate of EGFR internalization (Figure 3b), and the rate of ligand-induced receptor degradation (Figure 3a). Because the rate of receptor endocytosis depends on the extent of saturation by EGF (Sigismund *et al*., 2005),

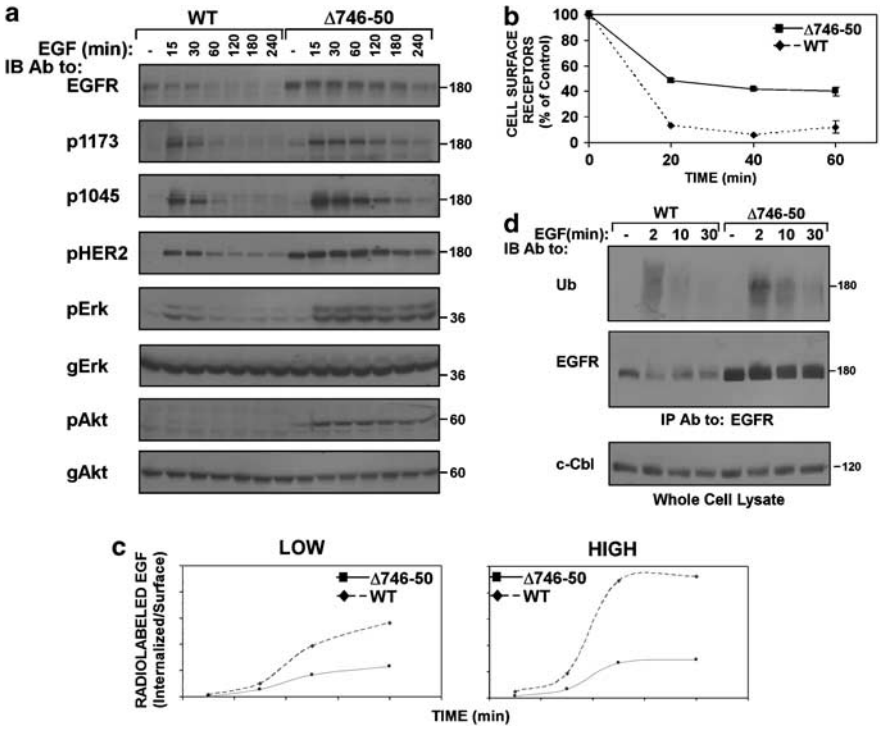

Figure 3 A deletion mutant of epidermal growth factor receptor (EGFR) evades EGF-induced downregulation. (**a**) The H358 (WT-EGFR) and the H1650 cell line (Δ746-50-EGFR) were stimulated with EGF for the indicated time intervals. Whole-cell lysates were analysed with the indicated antibodies. (**b**) EGFR downregulation was analysed, in triplicates, with H358 and H1650 cell monolayers as described in (**b**) of Figure 2. S.d. are shown (bars). (**c**) H358 (WT-EGFR) and H1650 (Δ746-50-EGFR) cell monolayers were incubated at 37°C with a radio-labled EGF (2 or 50 ng/ml). At the indicated time intervals, the medium was removed and cell monolayers were acid – washed to remove surface-bound EGF. Radioactivity present in the acidic fraction ('Surface') was quantified in triplicates. The remaining cell-associated radioactivity ('Internalized') was similarly quantified. The ratio obtained at each time point is presented. (**d**) Monolayers of H358 and H1650 cell lines were stimulated with EGF for the indicated time intervals and cell lysates were analysed with the indicated antibodies. The difference in receptor ubiquitinylation was observed in three other experiments.

we tested the rate of EGF internalization at low and high ligand concentrations and observed a consistent defect in the case of Δ746-50-EGFR (Figure 3c). Because ubiquitinylation by an E3 ubiquitin ligase, c-Cbl, is an essential step preceding ligand-induced degradation of EGFR (reviewed in Dikic and Giordano, 2003; Marmor and Yarden, 2004), we compared the patterns of EGF-induced modification of Δ746-50-EGFR and WT-EGFR (Figure 3d). This analysis revealed relatively weak ligand-induced ubiquitinylation and degradation of Δ746-50-EGFR (note limited band up-smearing indicative of inefficient ubiquitinylation). A similar defect in ligand-induced ubiquitinylation was observed with the partly overlapping deletion mutant, Δ747-53S-EGFR, expressed in an ectopic cellular system (data not shown). In conclusion, evasion from desensitization is a shared mechanism exploited by several NSCLC mutants of EGFR.

L858R-EGFR displays abnormal ligand-induced ubiquitinylation and coupling to c-Cbl

Comparative analysis of ligand-induced receptor ubiquitinylation revealed a similar defect in L858R/T790M-EGFR modification in H1975 cells (Figure 4a). The defective ubiquitinylation of L858R/T790M-EGFR was despite similar levels of c-Cbl and could be further distinguished by the lack of high molecular weight smearing when analysed with an anti-phosphotyrosine antibody (Figure 4a; lowermost panel). It is important noting that H1975 cells coexpress WT and mutant EGFR molecules, which partly masks the true characteristics of the mutant receptors. In contrast, ectopic expression in CHO cells enables direct analysis of mutant forms. Indeed, transfection of single mutants (L858R and T790M), the respective double mutant, or Δ746-50, into CHO and into HEK-293T cells confirmed the difference in ubiquitinylation (data not shown).

In order for c-Cbl to ubiquitinylate EGFR, it must form a physical complex with and undergo phosphorylation by EGFR (Levkowitz *et al*., 1999). Although the L858R/T790M-EGFR mutant is expressed at higher levels, the interaction between this form of EGFR and c-Cbl is impaired as revealed by testing EGF-induced coprecipitation of c-Cbl with either WT-EGFR or L858R/T790M-EGFR (Figure 4b). Unlike EGF-induced physical association of WT-EGFR with c-Cbl, association of the L858R/T790M mutant with c-Cbl was only weakly affected upon stimulation with EGF. Lack of c-Cbl-EGFR complex formation was confirmed in HEK-293T cells transfected with L858R-EGFR and stimulated with EGF (Figure 4c). In line with this

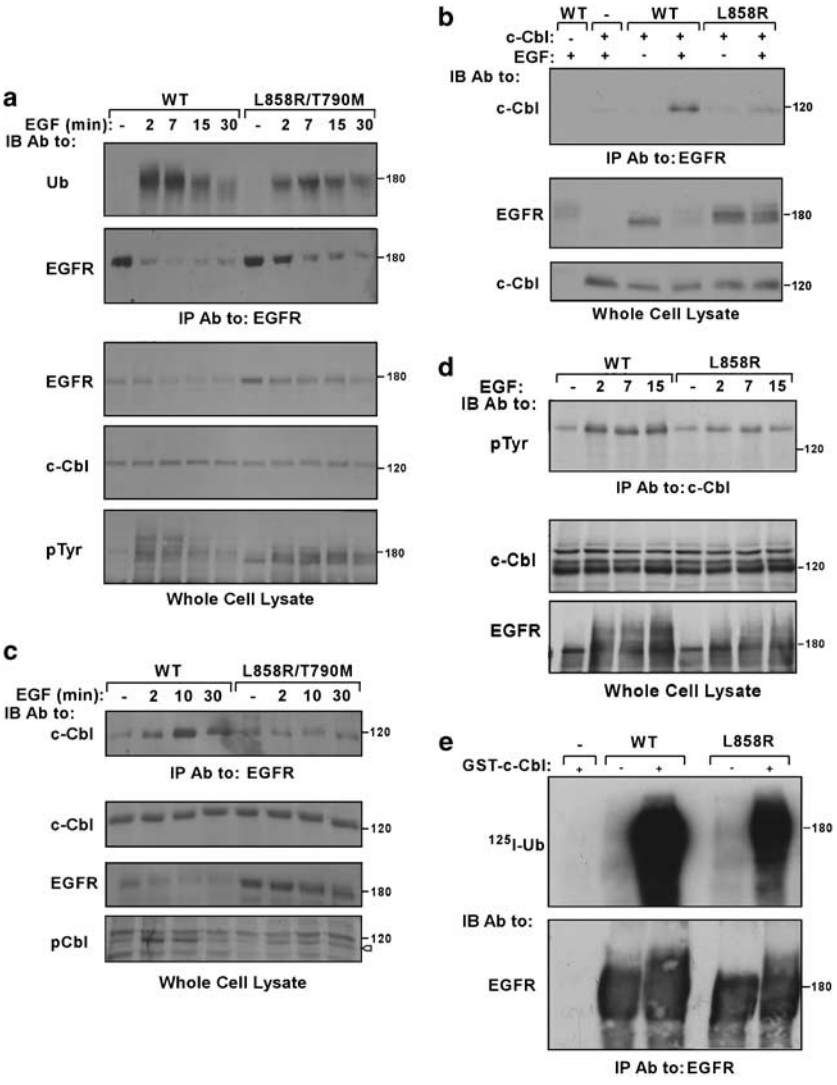

Figure 4 L858R-epidermal growth factor receptor (EGFR) displays abnormal ligand-induced ubiquitinylation and coupling to c-Cbl. (**a**) The H358 (WT-EGFR) and the H1975 cell lines (L858R/T790M-EGFR) were treated with EGF for the indicated time intervals. Cell lysates and EGFR immunoprecipitates were analysed with anti-ubiquitin (Ub) and other antibodies. (**b**) Coimmunoprecipitation of EGFR with c-Cbl was analysed in H358 and in H1975 cells expressing the indicated forms of EGFR. Cells were stimulated with EGF and the presence of c-Cbl in EGFR immunoprecipitates was analysed. Tyrosine-phosphorylated Cbl proteins (arrow) were detected with an antibody specific to phosphorylated Cbl (*pCbl*; at tyrosine 731). (**c**) HEK-293T cells were transfected with vectors encoding for the indicated forms of EGFR, along with a c-Cbl plasmid. Following 48 h, cell monolayers were starved and thereafter stimulated with EGF (20 ng/ml) for 8 min. EGFR was IP and after electrophoresis, immunoprecipitates and whole-cell lysates were IB with the indicated antibodies. (**d**) Chinese hamster ovary (CHO) cells were transiently transfected with vectors encoding for the indicated forms of EGFR, along with a c-Cbl plasmid. Following 48 h, cell monolayers were incubated with EGF for the indicated time intervals. c-Cbl was IP and after electrophoresis, immunoprecipitates and whole-cell lysates were IB with the indicated antibodies. (**e**) HEK-293T cells were transfected with plasmids encoding for the indicated forms of EGFR. Forty-eight hours post transfection, WT- and L858R-EGFR were IP and subjected to an *in vitro* ubiquitinylation assay with a radiolabeled ubiquitin and with or without GST-c-Cbl. Receptor immunoprecipitates were resolved by electrophoresis and proteins transferred to nitrocellulose filters, which were first autoradiographed (upper panels) and then IB (lower panels).

finding, analysis of the status of phosphorylation of c-Cbl, by using either antibodies specific to phosphorylated c-Cbl (pCbl; Figure 4c), or antibodies to phosphotyrosine (pTyr; Figure 4d), revealed relatively weak modification upon stimulation of the mutant receptor in both NSCLC cells and in transfected CHO cells, respectively. To assess whether L858R/T790M-EGFR is inherently refractory to ubiquitinylation, we employed an *in vitro* assay. This assay used a radiolabeled ubiquitin and a recombinant c-Cbl protein, along with reticulocyte lysate. The reaction was performed with EGFR molecules isolated from either transfected cells (Figure 4e), or from lung cancer cells (data not shown). When removed from the context of living cells, L858R-EGFR and L858R/T790M-EGFR underwent an apparently normal, c-Cbl-dependent ubiquitinylation (Figure 4e and data not shown). Conceivably, the altered structure of the mutant kinase domain does

not abrogate ubiquitinylation. Instead, we explore below the possibility that cellular factors extrinsic to EGFR and c-Cbl help sequester the mutant receptor from ubiquitinylation and downregulation.

Enhanced interaction of L858R/T790M-EGFR with HER2

The most direct mechanism to abrogate c-Cbl-mediated ubiquitinylation of L858R/T790M would be differential phosphorylation at tyrosine 1045 of EGFR, the only direct docking site for c-Cbl (Levkowitz *et al*., 1999). However, tyrosine 1045 of both L858R/T790M-EGFR and Δ746-50-EGFR underwent robust phosphorylation (Figures 2a, 3a and 5a). An alternative mechanism involves heterodimerization with HER2, an internalization-defective member of the HER/ErbB family (Baulida *et al*., 1996), whose interaction with c-Cbl is impaired, probably because HER2 cannot *trans*-phosphorylate tyrosine 1045 of EGFR (Levkowitz *et al*., 1996; Graus Porta *et al*., 1997; Muthuswamy *et al*., 1999; Worthylake *et al*., 1999). Consistent with this scenario, we observed high basal phosphorylation of HER2 in both H1975 and H1650 cells relative to H358

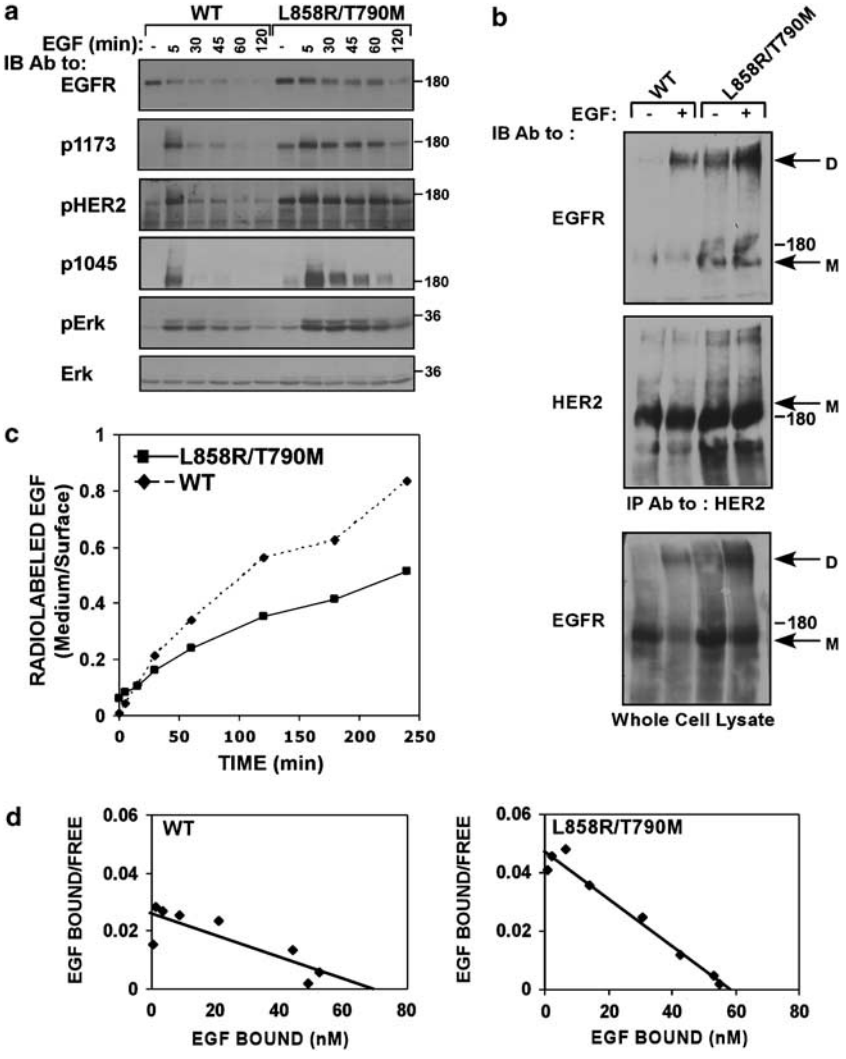

Figure 5 Enhanced functional and physical interactions of L858R/T790M-epidermal growth factor receptor (EGFR) with HER2. (**a**) The H358 (WT-EGFR) and H1975 (L858R/T790M-EGFR) cell lines were treated with EGF for the indicated time intervals. Whole-cell lysates were analysed with the indicated antibodies. (**b**) H358 and H1975 cell monolayers were incubated with or without EGF at 4°C for 60 min. Cells were lysed in the presence of a chemical cross-linking reagent (BS^3). Thereafter, cell lysates were cleared and analysed with the indicated antibodies. D: dimers; M: monomers. (**c**) Triplicate monolayers of H358 and H1975 cells were incubated with a radiolabeled EGF (2 ng/ml) for 2 h at 4°C. Cell monolayers were washed and fresh binding buffer was replaced. At the indicated times, the medium was collected and radioactivity present was quantified. The remaining cell-associated radioactivity was similarly quantified and the ratio obtained at each time point is presented. The experiment was repeated thrice and the variation within triplicates did not exceed 10%. (**d**) H358 (WT-EGFR) and H1975 (WT and L858R/T790M-EGFR) cell monolayers were incubated with sequential dilutions of a radiolabeled EGF for 2 h at 4°C. Duplicate monolayers were incubated with the same dilutions except in the presence of 100-fold-unlabeled EGF. After incubation, cell monolayers were washed and the remaining cell-associated radioactivity was quantified. Specific binding of EGF was calculated and presented as Scatchard plots. The K_d values calculated as 1.25 ± 0.24 nM ((L858R/T790M-EGFR) and 2.5 ± 0.9 nM (WT-EGFR).

cells, as well as prolonged ligand-induced tyrosine phosphorylation of HER2 (Figures 3a and 5a).

To test the prediction that L858R/T790M-EGFR interacts with HER2 before stimulation with EGF, we applied a covalent crosslinking reagent capable of stabilizing EGFR–HER2 complexes. Lung cancer cells expressing WT-EGFR were either stimulated with EGF or left untreated, and the crosslinking reagent added to whole-cell extracts, before immunoprecipitation of HER2. Blotting HER2 immunoprecipitates with anti-EGFR antibodies detected a covalently linked heterodimeric form of WT-EGFR only after stimulation with EGF (Figure 5b). In contrast and in line with our prediction, significant EGF-independent heterodimerization of L858R/T790M-EGFR was detectable when analysing HER2 immunoprecipitates prepared from H1975 cells (compare the dimer bands of untreated cells in the uppermost panel of Figure 5b).

Heterodimerization with HER2 decelerates ligand dissociation rates, thereby increasing binding affinity (Sliwkowski *et al*., 1994; Karunagaran *et al*., 1996). Hence, as an independent test for the enhanced presence of HER2-L858R/T790M heterodimers, we examined the prediction that L858R/T790M-expressing cells bind EGF with higher affinity and release the ligand more slowly than WT-EGFR-expressing cells. Lung cancer cells were loaded at 4°C with a radiolabeled EGF and the rates of release of radioactivity were monitored. The results of this assay showed that L858R/T790M-expressing cells are characterized by a slower ligand dissociation rate relative to the rapid release from WT-EGFR expressing cells (Figure 5c). In addition, L858R/T790M-expressing cells displayed higher EGF binding affinity than H358 cells ($K_d = 1.25$ vs 2.5 nM; Figure 5d). Taken together, these observations are consistent with a significant propensity of the L858R/T790M-EGFR to recruit HER2 into heterodimers.

Enhanced interaction with HER2 suggests a mechanism for defective downregulation of L858R-EGFR

To further examine the inferred propensity of EGFR mutants to recruit HER2, we reconstituted the interaction between L858R-EGFR and HER2 in CHO cells. As expected, when coexpressed with WT-EGFR, HER2 underwent no tyrosine phosphorylation, unless the cells were prestimulated with EGF (Figure 6a; panel labeled pHER2). In contrast, when coexpressed with the mutant form of EGFR, HER2 displayed high basal phosphorylation similar to the extent of phosphorylation observed in EGF-stimulated cells coexpressing WT-EGFR. These observations are consistent with stronger interactions between HER2 and the L858R mutant, relative to the interaction of HER2 with WT-EGFR. Indeed, an independent assay of downstream signaling to Stat3 demonstrated that relative to WT-EGFR, coexpression of L858R and HER2 significantly elevated the basal STAT3 activity, almost to the level achieved upon stimulation with EGF (Figure 6b).

To establish the involvement of HER2 in the resistance of L858R to ligand-induced degradation, we attempted downregulation of HER2 using a combination of two monoclonal antibodies to HER2, which is known to effectively target the receptor for degradation in lysosomes (Friedman *et al*., 2005). The results, we obtained, (Figure 6c) indicate that antibody-induced downregulation of HER2 transforms the relatively ligand-refractory mutant receptor (compare the decline from lanes 1 to 4) into a ligand-sensitive state, which displays significant downregulation (compare the decline from lanes 5 to 8). This conclusion relates not only to EGFR's protein level, but also to the relative decrease in receptor autophosphorylation (lower panel in Figure 6c). These observations are in line with the notion that HER2 protects WT-EGFR from EGF-induced desensitization (Muthuswamy *et al*., 1999), and our contention that mutant receptors are better coupled to HER2. To firmly test this model, we gradually overexpressed HER2 in EGFR-expressing CHO cells and stimulated them with EGF. Along with stabilization of ligand-activated WT-EGFR and L858R-EGFR, HER2 overexpression remarkably reduced ubiquitinylation of L858R-EGFR, but only moderately affected ubiquitinylation WT-EGFR (Figure 6d). In conclusion, the ability of HER2 to regulate ubiquitinylation (Figure 6d) and degradation of L858R-EGFR (Figure 6c) is consistent with the observed physical pre-association (Figure 5b) and *trans*-phosphorylation of HER2 in lung cancer cells (Figure 5b), which explain how aberrant receptors evade physiological desensitization.

Discussion

The major process of homeostatic regulation of EGFR and other receptor tyrosine kinases involves clustering of ligand-activated receptors in clathrin-coated areas of the plasma membrane, ubiquitinylation and sorting for degradation in lysosomes (reviewed in Wiley and Burke, 2001; Marmor and Yarden, 2004). Several growth factor receptors escape this inactivation pathway by means of overexpression, which saturates the clathrin-mediated route of endocytosis. The overexpression mechanism may be relevant to head and neck and other types of cancer, which overexpress EGFR. However, although a few mutant forms of EGFR are moderately overexpressed in NSCLC (Tracy *et al*., 2004; Amann *et al*., 2005), additional modes of escape are needed to maintain the active state of EGFR mutants at the plasma membrane, the site from which EGFR generates mitogenic (Waterman *et al*., 2002) and oncogenic signals (Wells *et al*., 1990). By concentrating on a frequently observed mutant, L858R, we raise the possibility that this mutant receptor evades homeostatic regulation by collaborating with HER2.

Unlike the transient EGF-induced signals that characterize WT-EGFR, the Akt and to some extent also the Erk pathway, are persistently activated in NSCLC cells expressing mutant forms of EGFR (Sordella *et al*., 2004; Amann *et al*., 2005). In contrast with WT-EGFR, which undergoes rapid clearance from the plasma membrane

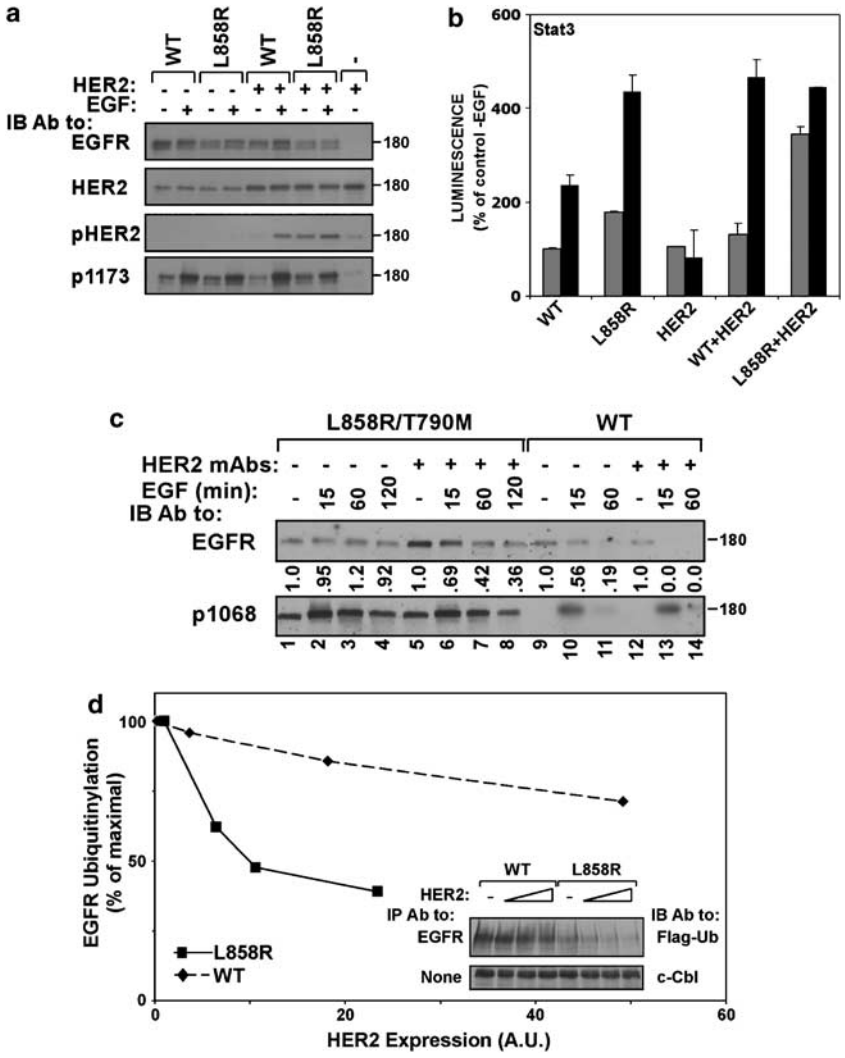

Figure 6 Enhanced interactions with HER2 may explain the defective downregulation of L858R-epidermal growth factor receptor (EGFR). (**a**) Chinese hamster ovary (CHO) cells were cotransfected with vectors encoding for either WT-EGFR or L858R-EGFR, with or without an HER2 expression plasmid. Forty-eight hours post transfection, cell monolayers were treated with EGF for 15 min. Cell lysates were analysed with the indicated antibodies. (**b**) CHO cells cotransfected with the indicated vectors (EGFR and HER2), along with plasmids encoding for a Stat3-luciferase reporter gene and GFP, were treated with (black columns) or without (grey columns) EGF and the relative luminescence signal determined and normalized as described in (**b**) of Figure 1. S.d. within triplicates are presented (bars). (**c**) H358 (WT-EGFR) and H1975 (L858R/T790M-EGFR) cells were treated with a mixture of anti-HER2 monoclonal antibodies (Herceptin and L26; 10 μg/ml each). Twenty hours after antibody treatment, cell monolayers were treated with EGF for the indicated time intervals and lysates electrophoresed and IB with the indicated antibodies. (**d**) CHO cells were cotransfected with vectors encoding for WT-EGFR or L858R-EGFR, Flag-Ub, c-Cbl and an increasing dose of a HER2-encoding plasmid. Cells monolayers were stimulated for 8 min with EGF before immunoblotting analyses. Receptor ubiquitinylation signals, obtained from the major ubiquitinylated species, were quantified and presented as a function of HER2 expression. AU, arbitrary units.

followed by lysosomal degradation, the mutants we studied (primarily L858R, Δ746-50 and L858R/T790M) undergo relatively slow endocytosis and they internalize bound EGF molecules significantly slower than WT-EGFR (Figures 2 and 3). As a result, EGFR mutants resist ligand-induced degradation, such that their phosphorylated forms are detectable for several hours after stimulation with EGF (Figures 2a and 3a). Conceivably, the relatively slow clearance of mutant EGFR molecules enables persistent signaling and leads to stronger mitogenic and antiapoptotic signals.

Why is the EGF-induced downregulation of mutant forms of EGFR defective? Ligand-induced receptor autophosphorylation facilitates Cbl-mediated receptor ubiquitinylation. This generates multiple docking sites for endocytotic adaptor proteins possessing ubiquitin-binding domains (Polo *et al*., 2002). Our studies revealed a major failure in the initial steps of receptor ubiquitinylation and endocytosis. The most prominent aspect is a relatively weak ligand-induced ubiquitinylation of L858R, L858R/T790M and Δ746-50, along with an inability of L858R to physically recruit and phosphorylate c-Cbl upon stimulation with EGF (Figure 4). The mechanism underlying this defect is incompletely understood, but our data suggest involvement of a third component, namely HER2. Heterodimers comprising

EGFR and HER2 are endowed with relatively high transforming activity (Kokai *et al*., 1989). The ability of HER2 to enhance EGFR signaling has been attributed to several unique aspects of the heterodimers, which allow them to signal persistently. These include a slow rate of EGF dissociation (Karunagaran *et al*., 1996) and a relatively slow rate of EGF-induced receptor degradation (Worthylake *et al*., 1999). Presumably, the endocytosis defect of HER2 is due to a reduced ability to recruit c-Cbl (Levkowitz *et al*., 1996), and a defect in the ability of EGFR to interact with c-Cbl when present in HER2-containing heterodimers (Muthuswamy *et al*., 1999). According to an alternative model, HER2 inhibits downregulation of EGFR in a mechanism that entails serine phosphorylation at residue 1113, which flanks a c-Cbl-docking site at tyrosine 1112 (Ouyang *et al*., 2001).

For several reasons, we propose that a propensity to engage into heterodimers with HER2 underlies the ability of mutant EGFRs like L858R to escape ubiquitinylation by c-Cbl. First, a mutant form of EGFR displays ligand-independent heterodimerization with HER2, unlike the WT receptor (Figure 5b). Second, HER2 is constitutively hyperphosphorylated in mutant-expressing lung cancer cells and CHO cells (Figures 3a, 5a, and 6a). Third, EGF binds more avidly to mutant-expressing cells, and its rate of dissociation is retarded (Figure 5), consistent with abundance of EGFR-HER2 heterodimers. In addition, forced overexpression of HER2 enhances the basal transcriptional signaling of L858R (Figure 6b) and reduces ubiquitinylation of the mutant receptor (Figure 6d). On the other hand, reducing HER2 expression using a combination of monoclonal antibodies enhanced ligand-mediated receptor desensitization (Figure 6c). These observations imply that mutants like L858R evolve a propensity to heterodimerize with HER2 even in the absence of a stimulating ligand. Within the heterodimer, EGFR *trans*-phosphorylates HER2 and undergoes enhanced phosphorylation. This leads to recruitment of several signaling molecules, but c-Cbl is excluded from the complex (Levkowitz *et al*., 1996; Graus Porta *et al*., 1997; Muthuswamy *et al*., 1999; Worthylake *et al*., 1999), probably because HER2 cannot *trans*-phosphorylate tyrosine 1113 of EGFR (Muthuswamy *et al*., 1999), and therefore ubiquitinylation and downregulation of mutant forms of EGFR are defective. According to a recently proposed alternative model, heat shock protein 90 uncouples mutant forms of EGFR from c-Cbl (Yang *et al*., 2006). Also relevant to our model is the ability of EGFR mutants to recruit another family member, namely ErbB-3 (Engelman *et al*., 2005), a kinase-defective receptor whose downregulation is defective (Waterman *et al*., 1999).

In summary, our studies identified a defect in ligand-induced ubiquitinylation and degradation of mutant forms of EGFR. In addition, based on several indirect lines of evidence, we propose that a partnership with HER2 underlies the ability of mutant receptors to evade desensitization and prolong signaling. If this model is confirmed by additional studies, it will be interesting to examine the prediction that intercepting HER2 in lung cancer will reduce oncogenicity of mutated forms of EGFR.

Materials and methods

Reagents and antibodies

Unless indicated, materials were purchased from Sigma (St Louis, MO, USA). $Na^{125}I$ and chemiluminescence kit were from Amersham Pharmacia Biotech (Buckinghamshire, UK) and IODOGEN from Pierce (Rockford, IL, USA). Lipofectamine and oligofectamine were supplied by Invitrogen (Carlsbad, CA, USA). Monoclonal antibodies to EGFR and HER2 were from Alexis Biotechnology (Lausen, Switzerland) or generated in our laboratory. Antibodies to p845-, p1045-, p1068-, p1173-EGFR, p1221-HER2, p731-c-Cbl and phospho-Akt were obtained from Cell Signaling (Beverly, MA, USA). Antibodies to ubiquitin were obtained from Babco (Berkley, CA, USA). NSCLC cell lines were purchased from the American Type Tissue Culture collection (Manassas, VA, USA).

Buffers

The following buffers were used: Binding buffer: Dulbecco's modified Eagle's medium supplemented with 0.5% bovine serum albumin and 20 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) (pH 7.5). Lysis buffer: 0.1 N NaOH and 0.1% sodium dodecyl sulfate. HNTG: 20 mM HEPES (pH 7.5), 150 mM NaCl, 0.1% Triton X-100 and 10% glycerol. Tris-buffered saline Tween-20 (TBST): 20 mM Tris–HCL (pH 7.5), 0.15 M NaCl and 0.05% Tween-20. Solubilization buffer: 50 mM HEPES (pH 7.5), 150 mM NaCl, 10% glycerol, 1% Triton X-100, 1 mM ethylenediaminetetraacetic acid, 1 mM ethylene glycol bis(α-aminoethylether)-*N*,*N*,*N*′,*N*′, -tetraacetic acid, 0.2 mM Na_3VO_4 and a protease inhibitor cocktail.

Construction and transfection of expression vectors

EGFR mutants were constructed by site-directed mutagenesis using the QuikChange mutagenesis kit (Stratagene, La-Jolla, CA, USA). All other vectors have been described (Levkowitz *et al*., 1999). For transfection, cells were grown to 30–50% confluence and transfection was preformed using Oligofectamine (NSCLC cells) or Lipofectamine (CHO cells). The total amount of DNA in each transfection was normalized with the pcDNA3 plasmid.

Immunoprecipitation and immunoblotting analyses

In experiments, where EGF was introduced, the cells were starved overnight in serum-free medium, and incubated for the indicated times at 37°C with EGF (20 ng/ml). For immunoprecipitation, lysates were incubated for 1–2 h at 4°C with antibodies precoupled to anti-mouse immunoglobulin G-agarose beads. The immunoprecipitates were washed thrice with HNTG solution, resolved by gel electrophoresis and electrophoretically transferred to a nitrocellulose membrane. Membranes were blocked in TBST buffer containing 10% low-fat milk, blotted with a primary antibody for 1 h, washed with TBST and incubated for 30 min with a secondary antibody linked to horseradish peroxidase (HRP). Immunoreactive bands were detected using chemiluminescence.

Ligand binding and dissociation studies

Monolayers of cells were incubated with different concentrations of ^{125}I-EGF for 2 h at 4°C. Simultaneously, identical cell

monolayers were coincubated with different concentrations of ^{125}I-EGF and excess (100-fold) unlabeled ligand. The cells were washed three times with ice-cold binding buffer. Labeled cells were lysed in lysis buffer and their radioactivity was determined. For ligand dissociation analysis, cells were first incubated with ^{125}I-EGF (2 ng/ml) for 2 h at 4°C and then washed three times. Ligand dissociation was monitored by incubating the cells in binding buffer for various periods of time at 4°C, and the amounts of released and cell-associated radioactive ligand were determined.

Stat3 transcription assay
Cells were transfected with plasmids encoding green fluorescent protein (GFP), WT-EGFR, EGFR mutants or HER2, along with a Stat3-luciferase plasmid containing a Stat3 response element fused to a luciferase reporter gene. Twenty-four hours later, the cells were split into 24-well plates, and following 12 more hours, the cells were serum starved for 12 h and then treated with EGF (20 ng/ml) for 8 h or left untreated. The luciferase reporter assay was performed using a kit (Promega; Madison, WI, USA). Light intensity was normalized to GFP expression levels.

Cell surface biotinylation
For biotinylation, cells were washed with ice-cold saline and then incubated for 60 min at 4°C with *N*-hydroxysuccinimide-biotin (biotin-X-NHS, 0.5 mg/ml; Calbiochem, San Diego, CA, USA) dissolved in borate buffer (10 mM boric acid, 150 mM NaCl; pH 8.0). Coupling of biotin was blocked by cell rinsing with a solution of 15 mM glycine in saline.

Ligand internalization assay
EGF was labeled with ^{125}I and IODOGEN as recommended by the manufacturer. Ligand-induced internalization was measured as follows. Cells were incubated with ^{125}I-EGF (10 ng/ml) at 37°C for 2–10 min. At the indicated times, the monolayers were rapidly washed and surface-bound ^{125}I-EGF was removed using acetic acid wash (surface-bound ligand). The cells were then solubilized (internalized ligand). Non-specific binding, measured in the presence of molar excess (200-fold) of unlabeled ligand, represented less than 10% of the total cell-associated radioactivity.

Receptor downregulation assay
Cells grown in 24-well plates were serum-starved for at least 12 h. Thereafter, cell monolayers were incubated at 37°C for the indicated time intervals with EGF (20 ng/ml) in binding buffer and then rinsed. Surface-bound EGF was removed by using acid wash followed by neutralization with cold binding buffer. The number of surface-exposed ligand binding sites was determined by incubating cells for 90 min at 4°C with a radiolabeled EGF. Nonspecific binding was measured in the presence of excess of unlabeled ligand.

Covalent crosslinking assay
Cell monolayers were incubated with EGF on ice for 1 h. Thereafter, cells were washed twice and scraped in cold saline. Then, cells were lysed in the presence of BS^3 (2 mM). Following incubation for 20 min on ice, the reaction was quenched with glycine (50 mM), aggregates and nuclei precipitated (20 min at 14 000 r.p.m.) and covalent complexes analysed using immunoprecipitation and gel electrophoresis.

In vitro *ubiquitinylation assay*
Ubiquitin (10 μg) was incubated with Na^{125}I (1mCurie) and the IODOGEN reagent (Pierce, Rockford, IL, USA). Following incubation at 22°C (5 min), free iodine was separated from ubiquitin using D-Salt Excellulose Desalting Column (Pierce). The reaction mixture contained rabbit reticulocyte lysate supplemented with 40 mM Tris, pH 7.5, 5 mM $MgCl_2$, 2 mM dithiothreitol, 2 mM ATP-γ-S and ^{125}I-labeled ubiquitin. Bacterially purified recombinant GST-Cbl (50 μg) was added as indicated. EGFR was immunopurified from lung cancer cells and receptor-bound beads were incubated with the reaction mixture for 1 h at 30°C. Thereafter, the beads were washed extensively and analysed by electrophoresis and autoradiography.

Statistical analyses
All analyses presented were repeated at least thrice, and all quantitative analyses were performed in triplicates. S.d. were calculated and shown (as bars), but since variation within triplicates rarely exceeded 10% of the signal, the bars do not show in all figures.

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