Research article

Aberrant trafficking of NSCLC-associated EGFR mutants through the endocytic recycling pathway promotes interaction with Src@

Byung Min Chung[1], Srikumar M Raja[1], Robert J Clubb[1], Chun Tu[1], Manju George[1], Vimla Band[1,3,4] and Hamid Band*[1,2,3,4]

Address: [1]Eppley Institute for Cancer and Allied Diseases, University of Nebraska Medical Center, 985950 Nebraska Medical Center, Omaha, NE 68198-5950, USA, [2]Department of Biochemistry & Molecular Biology, University of Nebraska Medical Center, 985870 Nebraska Medical Center, Omaha, NE 68198-5870, USA, [3]Department of Genetics, Cell Biology & Anatomy, College of Medicine, University of Nebraska Medical Center, 985805 Nebraska Medical Center, Omaha, NE 68198-5805, USA and [4]UNMC-Eppley Cancer Center, University of Nebraska Medical Center, 985950 Nebraska Medical Center, Omaha, NE 68198-5950, USA

Email: Byung Min Chung - chungj@unmc.edu; Srikumar M Raja - sraja@unmc.edu; Robert J Clubb - rclubb@unmc.edu; Chun Tu - ctu@unmc.edu; Manju George - mgeorge@unmc.edu; Vimla Band - vband@unmc.edu; Hamid Band* - hband@unmc.edu

* Corresponding author

Abstract

Background: Epidermal growth factor receptor (EGFR) controls a wide range of cellular processes, and altered EGFR signaling contributes to human cancer. EGFR kinase domain mutants found in non-small cell lung cancer (NSCLC) are constitutively active, a trait critical for cell transformation through activation of downstream pathways. Endocytic trafficking of EGFR is a major regulatory mechanism as ligand-induced lysosomal degradation results in termination of signaling. While numerous studies have examined mutant EGFR signaling, the endocytic traffic of mutant EGFR within the NSCLC milieu remains less clear.

Results: This study shows that mutant EGFRs in NSCLC cell lines are constitutively endocytosed as shown by their colocalization with the early/recycling endosomal marker transferrin and the late endosomal/lysosomal marker LAMP1. Notably, mutant EGFRs, but not the wild-type EGFR, show a perinuclear accumulation and colocalization with recycling endosomal markers such as Rab11 and EHD1 upon treatment of cells with endocytic recycling inhibitor monensin, suggesting that mutant EGFRs preferentially traffic through the endocytic recycling compartments. Importantly, monensin treatment enhanced the mutant EGFR association and colocalization with Src, indicating that aberrant transit through the endocytic recycling compartment promotes mutant EGFR-Src association.

Conclusion: The findings presented in this study show that mutant EGFRs undergo aberrant traffic into the endocytic recycling compartment which allows mutant EGFRs to engage in a preferential interaction with Src, a critical partner for EGFR-mediated oncogenesis.

Background

Epidermal growth factor receptor (EGFR) is a prototype of receptor tyrosine kinases (RTKs) which control critical cellular responses to extra-cellular growth factors during development and tissue homeostasis [1,2]. Importantly, overexpression of EGFR and/or its ligands is frequently

observed in human cancers, and recent studies have identified activating mutations in EGFR as direct determinants of oncogenic transformation in human cancers [3]. For example, missense mutations or small in-frame deletions within the kinase domain, which render EGFR constitutively active, are observed in a subset of patients with non-small cell lung cancer (NSCLC) [4-6]. As mutational activation of EGFR imparts a higher sensitivity to inhibition by EGFR-selective tyrosine kinase inhibitors (TKIs), there is considerable interest in understanding biological mechanisms whereby mutant EGFRs mediate aberrant oncogenic signaling in cancer cells.

While the normal EGFR signaling cascade is initiated by ligand-dependent dimerization and subsequent trans-phosphorylation of tyrosine residues within the cytoplasmic tail of the receptor, constitutively active mutant EGFRs associated with human cancer are thought to engage downstream signaling pathways in a constitutive fashion. Indeed, biochemical analyses have demonstrated that NSCLC-associated EGFR mutants activate signaling through the Erk, Akt, Src and STAT pathways [4,7,8]. A notable finding from these studies has been that certain signaling pathways may be preferentially altered by mutationally-activated EGFRs. For example, phosphoinositide 3-kinase pathway activation by mutant EGFR was found to be highly sensitive to gefitinib, an EGFR tyrosine kinase inhibitor [4,8]. Other studies have indicated a relatively selective activation of Src downstream of mutant EGFRs [7-9].

In the context of Src, use of Src inhibitors [9,10] and mutation of Src-dependent phosphorylation sites within EGFR (Y845) [7,11] have demonstrated a critical role for Src activity in linking mutant EGFRs to activation of several signaling pathways, to cell survival and to mutant EGFR-mediated oncogenic transformation. However, the reasons why certain signaling pathways, such as Src activity-dependent events, might be particularly activated by oncogenic EGFR mutants have not been addressed.

A crucial determinant of events downstream of RTKs such as EGFR is their endocytic traffic [12]. Ligand-dependent internalization of EGFR with subsequent sorting of the internalized receptors for lysosomal degradation has emerged as a major mechanism for termination of signaling. While EGFR endocytosis is a pre-requisite for lysosomal targeting, the latter is not an invariant fate. It has become clear that endocytosed receptors undergo a sorting process whereby internalized receptors can either proceed to the lysosome through a series of vesicular fusion/maturation events or can be recycled back to the plasma membrane [13].

Recent studies have demonstrated that activation-dependent recruitment of the Cbl family of ubiquitin ligases is a major determinant of lysosomal targeting of EGFR [14,15]. Cbl-dependent mono-ubiquitinylation of the cytoplasmic tail of EGFR serves as a signal for receptor sorting to the inner vesicles of the multi-vesicular bodies, a key step in lysosomal targeting of RTKs [16]. Indeed, perturbation of Cbl protein expression or function alters the lysosomal degradation of EGFR and impacts the magnitude and/or duration of downstream signals [15,17]. Additional mechanisms that function either in concert with Cbl-dependent ubiquitin modification, such as sprouty2, Sts-1/Sts-2 and cortactin [18-20], or independently (e.g. Sorting nexins) [21] further contribute to EGFR downregulation through lysosomal targeting.

In contrast to EGF-induced lysosomal targeting of EGFR, TGFα binding appears to promote the recycling of EGFR rather than its lysosomal degradation, correlating with a more potent signaling response [22-24]. Notably, TGFα stimulation is associated with a more transient EGFR-Cbl association and EGFR ubiquitinylation [22]. EGFR heterodimerization with ErbB2, as is often observed in tumor cells, has also been shown to impair lysosomal degradation of EGFR apparently due to increased recycling and/or reduced internalization [25-27].

Given the importance of endocytic trafficking in dictating the lifespan of active EGFR and possibly the quality of downstream signaling events, it is of considerable interest to explore how oncogenic EGFRs traffic. In addition, the ability of mutant EGFRs to hyper-activate certain signaling pathways may be related to altered endocytic trafficking. Consistent with such a possibility, NSCLC-associated EGFR mutants appear to be impaired in their interaction with Cbl [28,29]. More recent studies suggest that specific endocytic routes may dictate the type of biological responses to EGFR stimulation. For example, clathrin-dependent endocytosis appears to be critical for proliferative responses to EGF, whereas clathrin-independent endocytosis appears to primarily promote EGFR degradation [30]. Furthermore, NSCLC-associated EGFR mutants have been shown to undergo EGF-independent internalization when expressed in a murine pro-B cell line [31]. Intracellularly distributed EGFR was also observed in NSCLC cell lines [32]. Here, we examined the subcellular localization of wild-type (wt) EGFR and oncogenic EGFR mutants in normal bronchial epithelial cells and NSCLC cell lines. Findings reported here demonstrate that mutant EGFRs undergo enhanced endocytic recycling and suggests a role of altered endocytic trafficking in mutant EGFR interaction with Src.

Results

NSCLC-associated oncogenic EGFR mutants are constitutively endocytosed

To examine the cell surface versus intracellular (endocytic vesicular) localization of EGFR, we carried out confocal immunofluorescence imaging analyses of a normal bronchial epithelial cell line HBE135 expressing wtEGFR and NSCLC cell lines expressing wtEGFR (H1666) or mutant EGFRs (H1650 and HCC827 expressing EGFR Δ746-750, HCC4006 expressing EGFR Δ747-749/A750P, and H1975 expressing EGFR L858R/T790 M) [4,33]. As anticipated for unstimulated wtEGFR [34], EGFR was essentially exclusively localized at the cell periphery in growth factor-deprived, unstimulated HBE135 and H1666 cell lines, and EGFR was only observed in punctate intracellular vesicles after EGF stimulation of these cells (Figure 1A). In contrast, all of the cell lines expressing mutant EGFRs showed predominantly punctate EGFR staining with a much lower level of peripheral cell surface staining when examined without EGF stimulation; the intracellular punctate staining increased further upon EGF stimulation (Figure 1A). Mutant EGFRs ectopically expressed in HBEC cell lines as EGFR-GFP chimeras showed similar results, indicating that the constitutive intracellular localization of mutant EGF receptors observed in Figure 1 was not due to cell type specificity (Additional File 1A). Correlating with the immunofluorescence results, mutant EGFRs displayed high levels of basal phosphorylation (with further increase upon EGF stimulation) whereas the phosphorylation of wtEGFR was observed only upon EGF stimulation (Figure 1B and Additional File 1B), as expected from previous biochemical analyses [4,35].
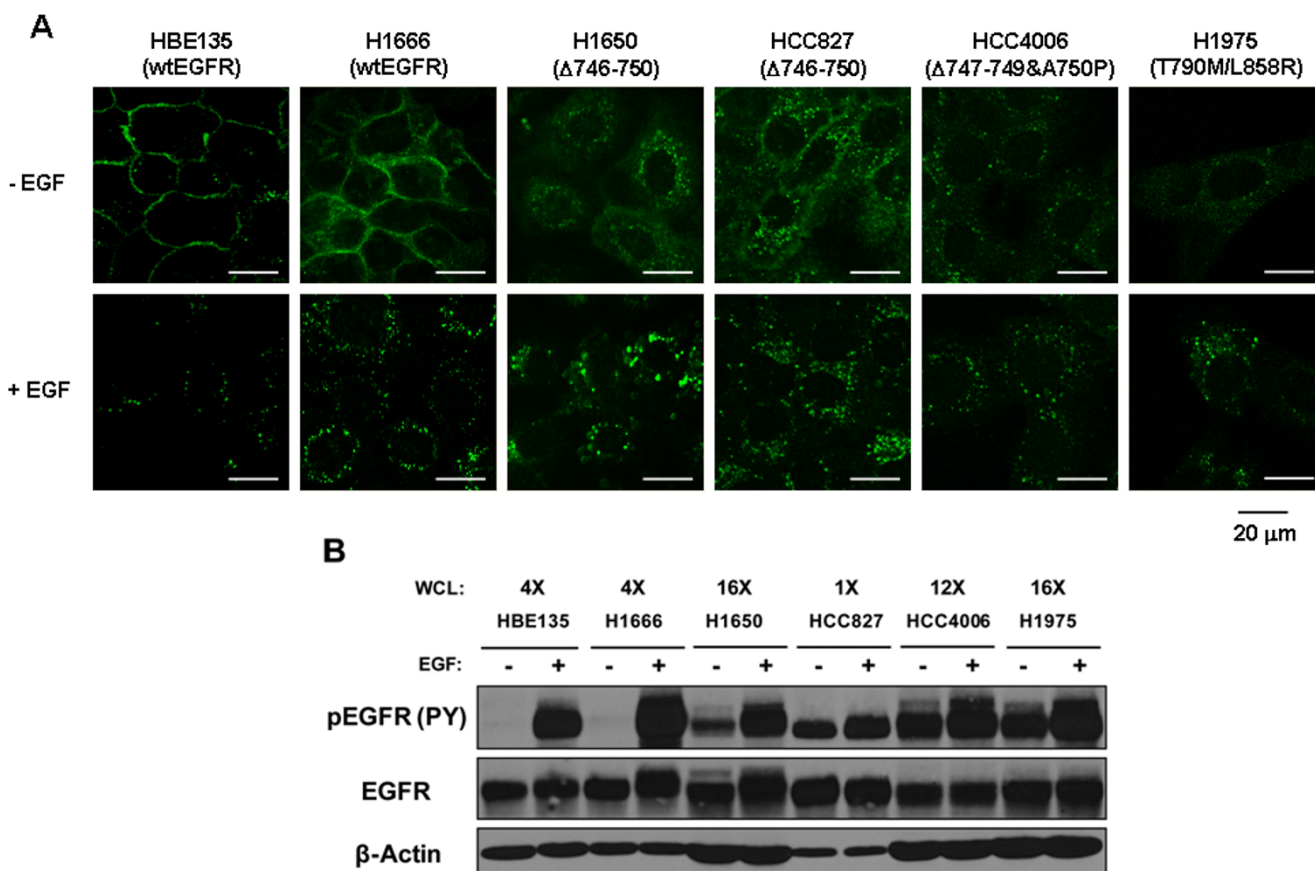

Figure 1
Constitutive intracellular vesicular localization and phosphorylation of mutant EGF receptors in NSCLC cell lines. (A) Cells were growth factor-deprived by culturing in D3 medium (HBE135) or 0.1% FBS-containing growth medium (all other cell lines) for 48 hr and then either left unstimulated (- EGF) or stimulated (+ EGF) with 100 ng/ml of EGF for 30 min. Cells were fixed, permeabilized, and immunostained with anti-EGFR antibody 528. Images were acquired under a confocal microscope at the medial plane. Bar represents 20 μm. (B) Cells were growth factor-deprived as in (A) for 48 hr and either left unstimulated (-) or stimulated (+) with 10 ng/ml EGF for 10 min. The indicated amounts of whole cell lysate protein were used for immunoblotting with antibodies against the indicated proteins.

To determine the identity of intracellular vesicles in which the mutant EGFRs reside, we loaded cells with fluorescently-labeled transferrin, an early and recycling endo-

somal marker, followed by immunostaining for EGFR and LAMP1, the latter as a late endosomal/lysosomal marker (Figure 2A-D). Labeled transferrin loading for 30 min
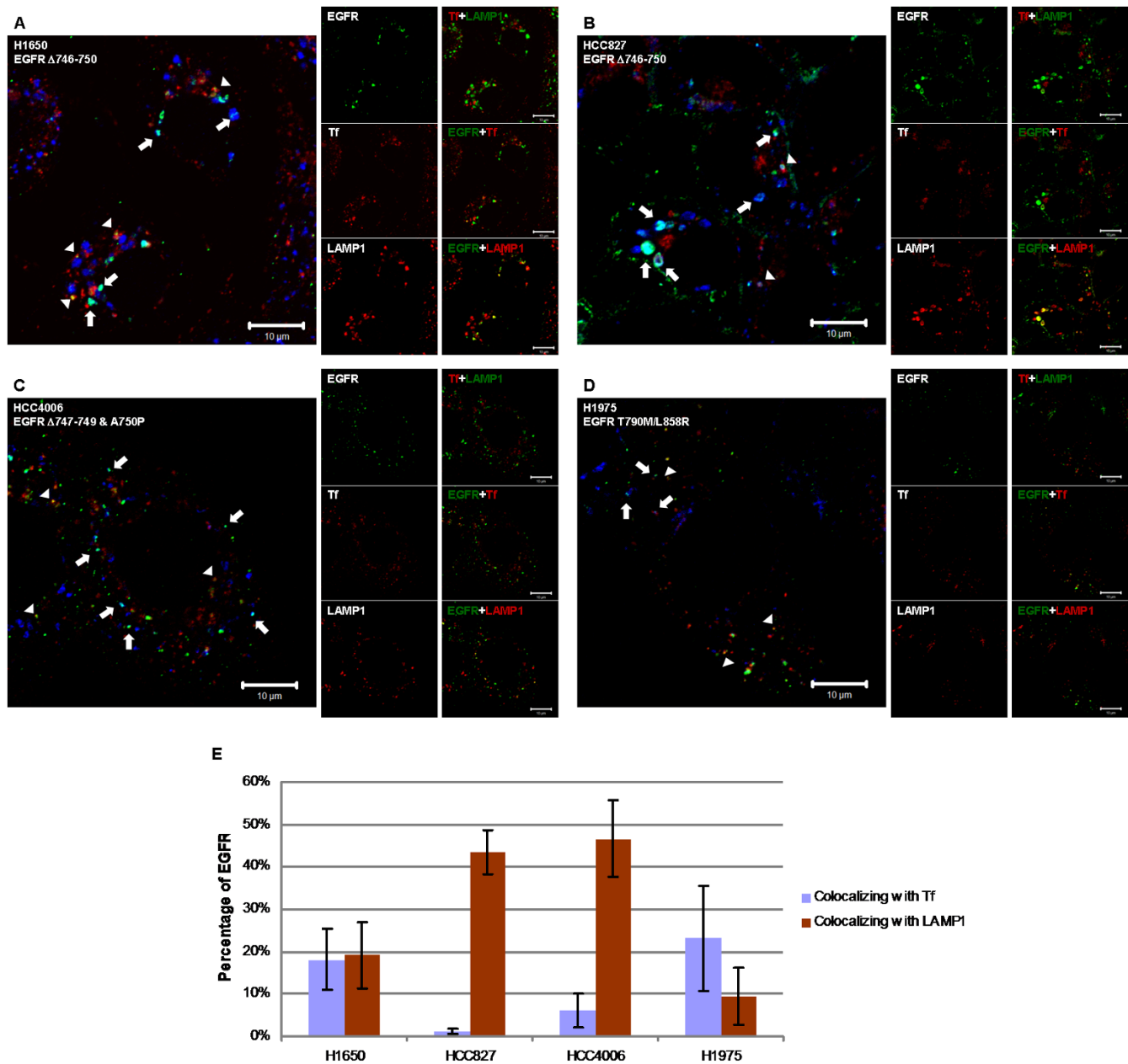

Figure 2
Constitutively endocytosed mutant EGF receptors colocalize with early/recycling endosomal marker transferrin and late endosomal/lysosomal marker LAMP1. NSCLC cell lines H1650 (A), HCC827 (B), HCC4006 (C) or H1975 (D) were serum-starved for 48 hr and incubated with 10 μg/ml of AF546-transferrin (Tf) (red) for 30 min at 37°C. Cells were fixed, permeabilized and immunostained with anti-EGFR antibody 528 (green), followed by anti-LAMP1 antibody H4A3 (blue). Images were acquired under a confocal microscope at the medial plane. Arrows indicate EGFR and LAMP1 colocalization, and arrowheads indicate EGFR and labeled-transferrin colocalization. Bars represent 10 μm. (E) Colocalization coefficients for the green channel (EGFR) from (A), (B), (C), and (D) were obtained using LSM510 Image Examiner software, and depicted as percentages of EGFR colocalizing with Tf or LAMP1.

allowed for an examination of both early and recycling endosomes [13]. Mutant EGFRs in all of the cell lines examined (H1650, HCC827, HCC4006 and H1975) colocalized both with transferrin and LAMP1, albeit at different levels in the steady-state conditions (Figure 2E). This data indicates that under steady-state conditions the mutant EGFRs are located in multiple endosomal compartments within the cell.

Incubation of cells at 16°C allows for continued internalization from the cell surface but blocks further progression of endocytosed receptors and cargo along the endocytic pathway and into the endocytic recycling compartments, resulting in enhanced accumulation in sorting endosomes [36]. Indeed, when NSCLC cell lines were incubated at 16°C, mutant EGFRs showed enhanced colocalization with labeled transferrin (Additional File 2). This result further suggests that mutant EGFRs transit along a transferrin-positive sorting compartment.

Treatment with monensin results in the accumulation of mutant EGFR, but not wtEGFR, in a perinuclear endocytic compartment

While our colocalization analyses demonstrated that constitutively endocytosed mutant EGFRs do transit to the lysosome, recent reports indicate that mutant EGFRs show reduced ligand-induced ubiquitinylation and degradation [28,37] which could promote their entry into the endocytic recycling pathway; colocalization of mutant EGFRs with transferrin (Figure 2) is consistent with this idea. To further test this possibility, we examined the localization of mutant EGFRs after treating cells with monensin, an agent that has been shown to inhibit exit of internalized receptors and other endocytic cargo from sorting endosomes and the endocytic recycling compartment [34,38,39]. To demonstrate the ability of monensin to inhibit the cargo exit from the endocytic recycling compartment, we first assessed its effects on transferrin recycling in the NSCLC cell line H1666. As expected, labeled transferrin exit out of the perinuclear endocytic recycling compartment was essentially complete within the 60 min chase period; however, monensin treatment markedly delayed this process (Additional File 3A).

To assess the impact of recycling inhibition on mutant versus wild-type EGFR, we carried out concurrent EGF stimulation and labeled transferrin chase in HBE135 and NSCLC cell lines with or without pre-incubation in monensin (Figure 3). While the relatively low uptake of transferrin in the HBE135 cell line did not permit a clear assessment of transferrin accumulation upon monensin treatment, all of the NSCLC cell lines, including the wtEGFR-expressing cell line H1666, showed a marked increase in perinuclear labeled transferrin staining in the presence of monensin, indicating an effective inhibition

of cargo exit from the endocytic recycling compartment. Importantly, monensin treatment induced the perinuclear accumulation of EGFR in H1650, HCC827, HCC4006 and H1975 cell lines bearing mutant EGFRs, but not detectably in HBE135 and H1666 cell lines bearing the wtEGFR, either in the presence or absence of EGF stimulation. Similar perinuclear mutant EGFR accumulation was observed upon monensin treatment of cells grown in regular growth media without any growth factor deprivation or EGF stimulation (Additional File 3B), and also in HBEC cell lines stably expressing ectopic mutant EGF receptors (Additional File 3C).

NSCLC-associated mutant EGFRs have been shown to attain sensitivity to Hsp90 inhibitor 17-(allylamino)-17-demethoxygeldanamycin (17-AAG) which targets the related receptor ErbB2 to degradation by enhancing its lysosomal targeting [37,40,41]. Notably, the presence of monensin prevented the lysosomal targeting of mutant EGFR and its degradation induced by 17-AAG (Additional File 4). 17-AAG treatment resulted in a decrease in mutant EGFR staining, indicating that mutant EGFR was targeted for degradation in the lysosomes. The 17-AAG-induced mutant EGFR downregulation was inhibited in monensin-treated cells and intracellular punctate staining of EGFR could still be observed. This is consistent with the concurrent effect of monensin to block traffic towards the lysosome [42].

To rule out the possibility that the perinuclear accumulation of mutant EGFRs may reflect an overall increase in the level of EGFR, we compared the EGFR expression levels in cells treated with DMSO versus monensin (Additional File 5A). Neither the overall EGFR levels nor the overall level of EGFR phosphorylation, as determined using anti-phosphotyrosine (PY) and anti-phospho-EGFR antibodies specific to pY845 and pY1173, showed a gross change upon monensin treatment (Additional File 5A).

Mutant EGFR colocalizes with markers of endocytic recycling compartment

Enhanced colocalization of mutant EGFRs with transferrin at 16°C together with perinuclear accumulation upon monensin treatment suggested that mutant EGFRs preferentially transit through the endocytic recycling compartment. Therefore, we carried out confocal imaging studies to assess if the constitutively endocytosed mutant EGFRs show colocalization with endocytic recycling compartment markers. Rab proteins are known to regulate various steps in endocytic traffic: Rab4 regulates fast/direct recycling from the early endosomes to the plasma membrane, while Rab11 regulates recycling from the deeper perinuclear recycling compartments [13,36]. The newly identified EHD protein family also controls endocytic recycling, with EHD1 functioning in the endocytic recycling com-
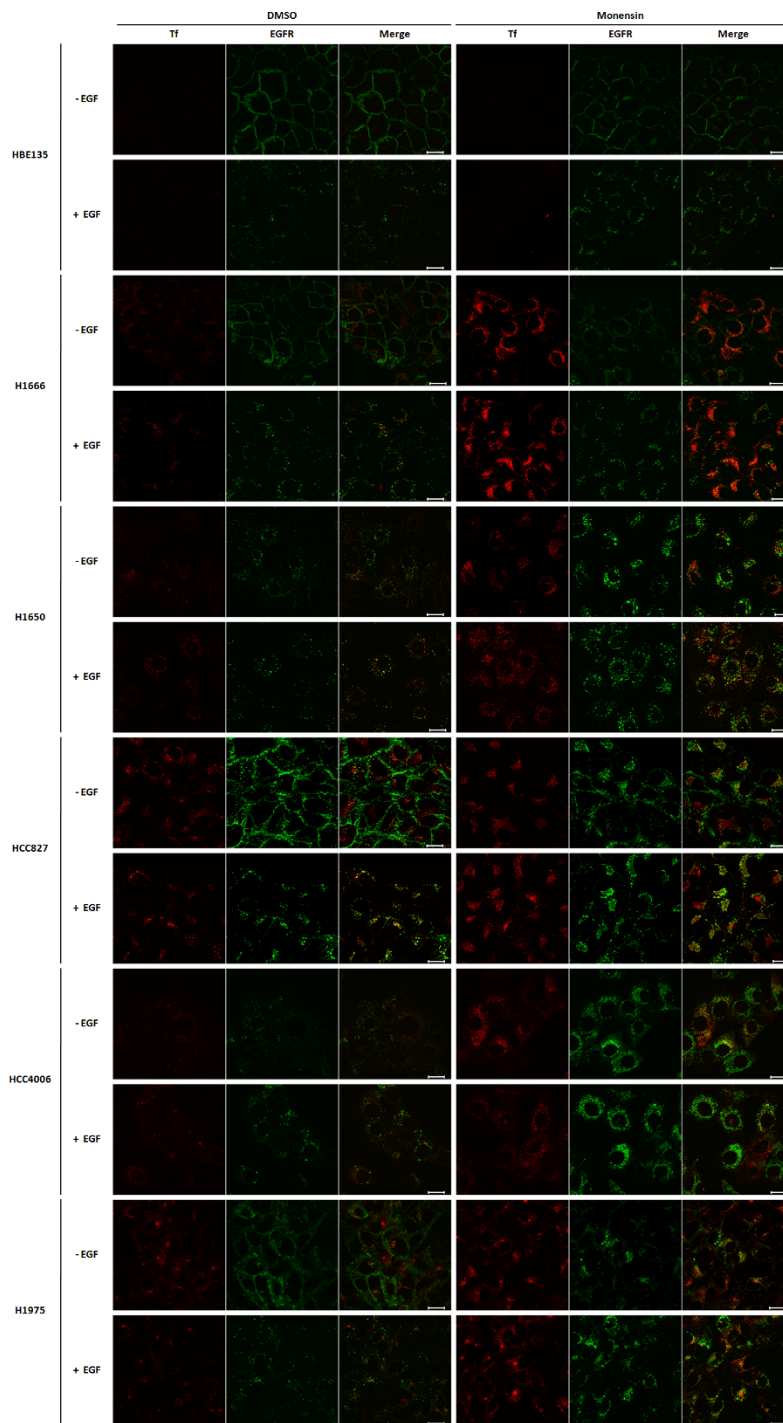
Figure 3
Monensin-treatment increases the accumulation of mutant EGF receptors in perinuclear endocytic vesicles.
Cells were growth factor-deprived in D3 medium (HBE135) or 0.1% FBS-containing growth medium (all other cell lines) for 48 hr and then preincubated with DMSO or 10 μM monensin for 3 hr. Cells were loaded with 10 μg/ml AF546-Tf (Tf) (red) for 45 min at 37°C (with continuation of DMSO or monensin treatment). This was followed by incubation with (+ EGF) or without (- EGF) 10 ng/ml EGF for 30 min with continued DMSO or monensin treatment. Cells were fixed, permeabilized, and immunos- tained with anti-EGFR antibody 528 (green). Images were acquired under a confocal microscope at the medial plane. Bars rep- resent 20 μm.

partment and EHD3 in the early endosomes [43,44]. The mutant EGFR-expressing cell line HCC827 was transiently transfected with expression vectors coding for GFP-tagged Rab11, Rab4, EHD1 or EHD3; after 48 hr, the cells were fixed and immunostained with an anti-EGFR antibody (Figure 4A). Partial colocalization of mutant EGFR with markers of early and recycling endosomes was observed (Figure 4A), and notably, enlarged GFP-positive vesicles were observed surrounding the EGFR-positive punctate structures (arrows), especially in cells transfected with Rab4-GFP. Monensin treatment further increased the appearance of these enlarged vesicles for all of the early and recycling endosomal markers as well as colocalization between these markers and mutant EGFR (Figure 4B and 4C), consistent with the monensin-induced block of exit from the endocytic recycling compartments. The confocal colocalization studies therefore further support the conclusion that mutant EGFRs traffic through the endocytic recycling compartments.

Src association with mutant EGF receptors in the endocytic recycling compartment
Constitutive localization of mutant EGFRs in the endocytic recycling compartments (Figure 4) could allow preferential interaction of mutant EGFRs with certain signaling pathways. A particular EGFR-relevant signaling partner in this regard is Src, which is known to localize on endocytic vesicles including the endocytic recycling compartment [45,46]. Furthermore, mutant EGFRs show increased constitutive association with Src, and Src-EGFR interaction plays an important role in mutant EGFR-induced oncogenic transformation [7,9,47]. Therefore, we examined the relative subcellular localizations of EGFR and Src in NSCLC cell lines that were serum-starved and then left untreated or treated with EGF for 10 min (Figure 5A). As observed above (Figure 1A), EGF-deprived H1666 cells (wtEGFR) showed predominantly surface-localized EGFR staining, whereas HCC827 cell line showed constitutive localization of mutant EGFR in intracellular vesicles. Anti-Src staining showed a predominantly perinuclear vesicular staining, consistent with previous reports [45,46]. While wtEGFR was internalized upon EGF stimulation of H1666 cells as expected, we observed very little, if any, colocalization between EGFR and Src under these conditions. In contrast, constitutively internalized mutant EGFR in the HCC827 cell line exhibited enhanced colocalization with Src when compared to wtEGFR in the H1666 cell line (arrows, Figure 5A and 5C). Enhanced colocalization between phospho-EGFR and phospho-Src was also observed in the HCC827 cell line, indicating that constitutive active mutant EGFR interacts with activated Src in endosomal compartments (Figure 5B and 5D). Similar results were seen with the H1650, HCC4006 and H1975 cell lines (data not shown).

As monensin treatment increased the mutant EGFR accumulation in the perinuclear endocytic vesicles (Figure 3), we examined the extent of EGFR and Src colocalization in cells treated with monensin. Treatments were carried out as in Figure 3, and cells were then immunostained for EGFR and Src (Figure 6A). Monensin treatment increased the perinuclear accumulation of mutant but not wild-type EGFR, similar to results in Figure 3. Notably, Src showed an enhanced colocalization with mutant EGFRs that accumulated in perinuclear vesicles; quantification of the relative colocalization (as a colocalization coefficient of Src) confirmed the enhancement upon monensin treatment (Figure 6B). Thus, mutant but not wild-type EGFR displayed enhanced colocalization with Src in endocytic vesicles, and such colocalization was further enhanced by inhibiting the exit of EGFR from the endocytic recycling compartment with monensin.

Monensin treatment enhances the mutant EGFR-Src association
In view of the increased colocalization of mutant EGFR and Src in monensin-treated cells, and recent findings that mutant EGFRs constitutively complex with Src [7,48], we asked if monensin-induced block of EGFR exit from the endocytic recycling compartment influences mutant EGFRs and Src association. Cells processed essentially as for confocal imaging in Figure 6 were used to carry out co-immunoprecipitation analyses to assess EGFR and Src association (Figure 7). In parallel with the increased mutant EGFR and Src colocalization seen in Figure 6, the amounts of Src that co-immunoprecipitated with mutant EGFRs, but not with wtEGFR, were enhanced in the presence of monensin. Similar results were seen when cells were grown and treated with monensin in regular growth media (data not shown).

Discussion
The outcome of RTK signaling involves a balance between various stimulatory and inhibitory mechanisms which in turn determine both the strength and duration of signals that are transmitted through networks of signaling cascades [49]. In this respect, endosomal sorting plays a key role in the regulation of EGFR signaling [12].

NSCLC-associated kinase domain mutations in EGFR promote its constitutive activation, and a number of studies have focused on delineating the signaling pathways whose activation contributes to oncogenesis [3]. The outcome of EGFR signaling is intimately linked to its endocytic traffic, which is normally triggered by ligand-induced dimerization [50] and phosphorylation-dependent as well as phosphorylation-independent recruitment of endocytic machinery components [51,52]. The nature of endocytic trafficking of NSCLC-associated EGFR mutants and any relationship of altered traffic with oncogenic sig-
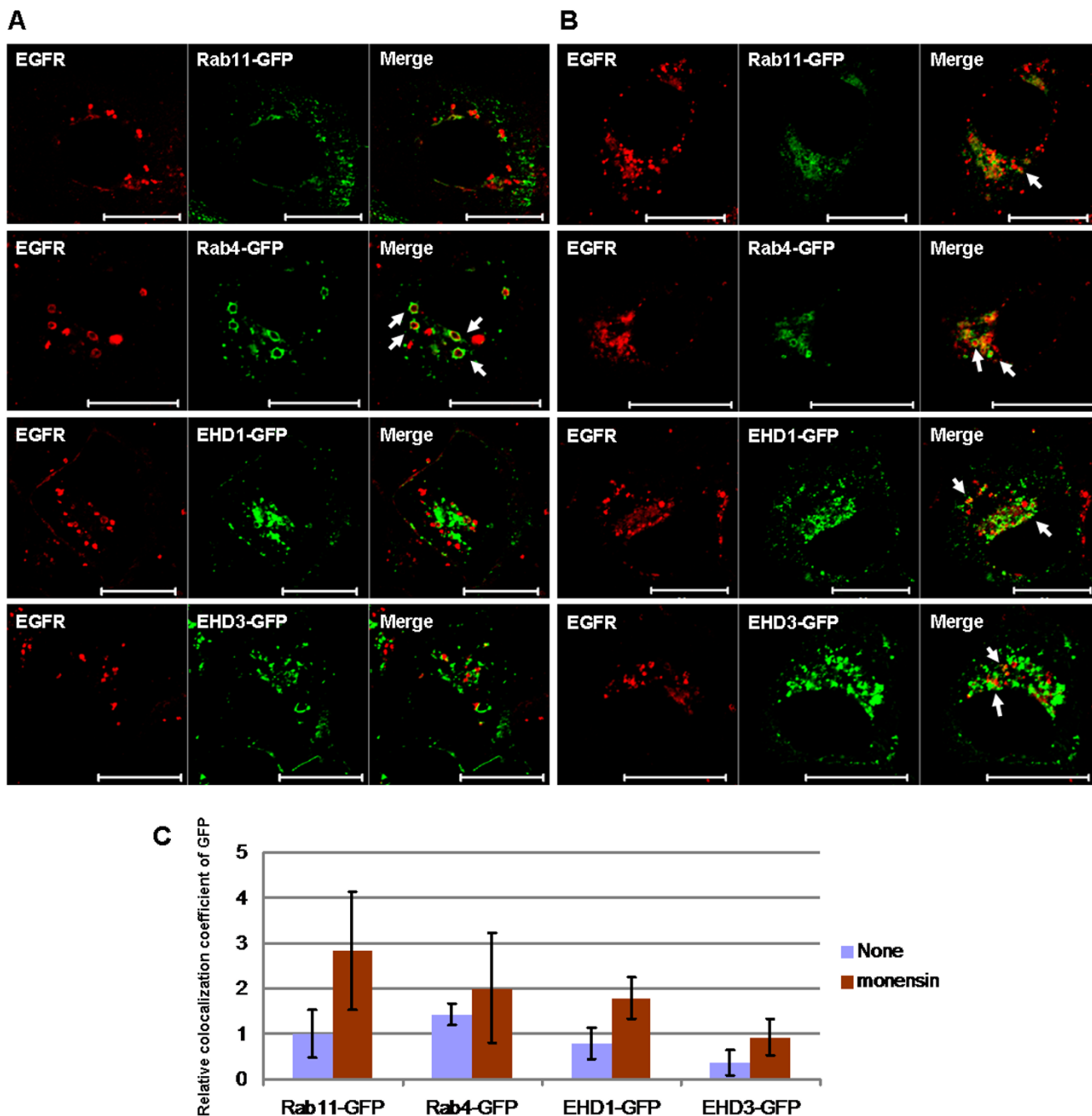
Figure 4
Mutant EGF receptor colocalization with endocytic recycling compartment markers. HCC827 NSCLC cell line was transiently transfected with Rab11-GFP, Rab4-GFP, EHD1-GFP or EHD3-GFP constructs. After 48 hr, cells were either left untreated (A) or treated with 10 μM monensin for 2 hr (B). Cells were fixed, permeabilized, and immunostained with anti-EGFR antibody (528) (red). Images were acquired under a confocal microscope at the medial plane. Arrows indicate mutant EGFR colocalization with GFP-tagged proteins (green). Bars represent 20 μm. (C) Colocalization coefficients for the green channel (GFP) were obtained using LSM510 Image Examiner software and normalized to the colocalization coefficient from Rab11-GFP without any treatment (none).
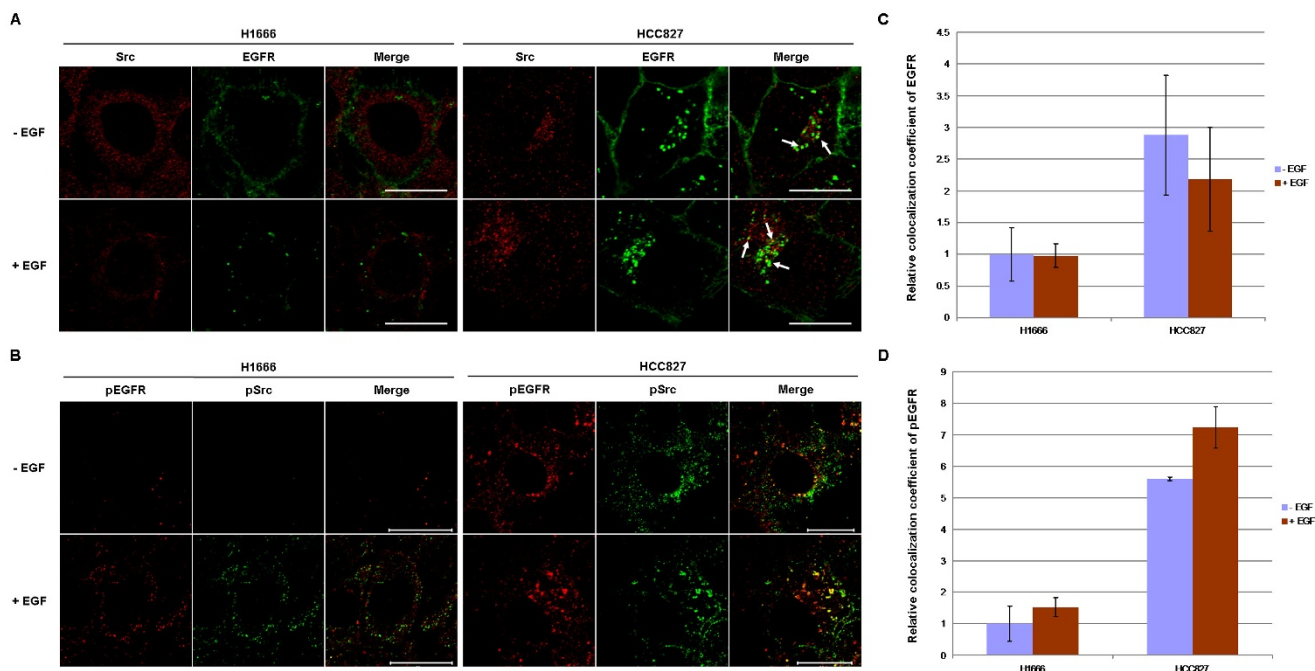
Figure 5

Mutant EGF receptors colocalize with NSCLCcell lines. Cells were growth factor-deprived in 0.1% FBS-containing growth medium for 48 hr and then either left unstimulated (- EGF) or stimulated (+ EGF) with 100 ng/ml of EGF for 10 min. Cells were fixed, permeabilized, and immunostained with (A) anti-EGFR antibody 528 (green) followed by anti-Src antibody SRC2 (red) staining or (B) anti-phospho-EGFR antibody (red) followed by anti-phospho-Src antibody (green). Images were acquired under a confocal microscope at the medial plane. Arrows indicate EGFR and Src colocalization. Bars represent 20 μm. (C) Colocalization coefficients for the green channel (EGFR) from (A), and (D) colocalization coefficients for the red channel (pEGFR) from (B) were obtained using LSM510 Image Examiner software and normalized to the colocalization coefficient from unstimulated (- EGF) H1666 cells.

naling remain poorly understood. Here, we have used NSCLC cell lines to demonstrate that oncogenic mutant EGFRs, but not wtEGFR, are constitutively endocytosed (Figure 1). Mutant EGFRs were found to localize in early and recycling endosomes based on colocalization with labeled transferrin (Figure 2) and GFP-tagged Rab4, Rab11, EHD1 and EHD3 proteins (Figure 4). Notably, blocking the exit of endocytosed receptors from endocytic recycling compartments with monensin led to a marked accumulation of mutant EGFR in a perinuclear endocytic compartment (Figure 3) and increased its colocalization with markers of sorting and endocytic recycling compartments (Figure 4B). Thus, these findings strongly suggest that mutant EGFRs transit through the endocytic recycling compartment.

Importantly, enhanced EGFR-Src as well as activated EGFR/phospho-Src colocalization was observed in endocytic vesicles of a mutant EGFR-expressing cell line (Figure 5). Furthermore, monensin treatment increased the colocalization of mutant EGFRs with Src in the perinuclear endosomal compartment (Figure 6) and enhanced the

biochemical association between mutant EGFRs and Src (Figure 7). Given the emerging evidence for a critical role of the constitutive engagement of Src-mediated signaling pathways in mutant EGFR-dependent oncogenesis [7,9,10], our results suggest a potentially important role of altered endocytic trafficking in the oncogenic behavior of mutant EGFRs.

In view of the critical role of ligand-induced internalization and lysosomal targeting in limiting EGFR signaling, the constitutive activation of downstream signaling pathways by NSCLC-associated mutant EGFRs has generated interest into potential alterations of endocytic trafficking. For example, given the critical role for the Cbl-family of ubiquitin ligases in orchestrating EGFR ubiquitinylation and subsequent lysosomal sorting, it is notable that a recent analysis of NSCLC-associated mutant EGFRs showed reduced Cbl-dependent lysosomal downregulation [28,29,53]. However, another study in an NSCLC cell line reported that mutant EGFR traffics into lysosomes upon EGF stimulation [54]. The present study extends beyond these observations by demonstrating that mutant
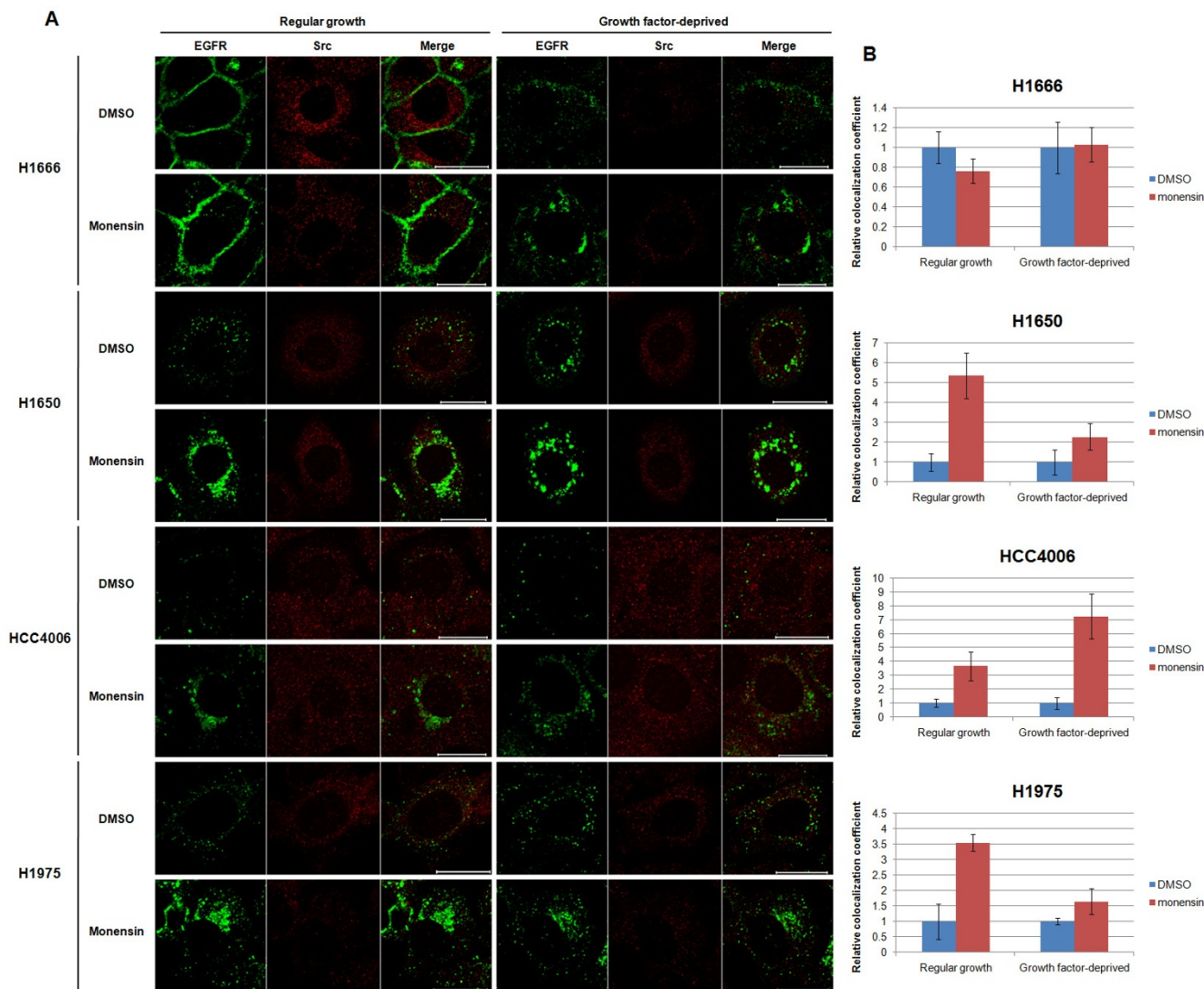
Figure 6
Monensin treatment enhances mutant EGFR-Src colocalization in endocytic vesicles. (A) Cells were either growth factor-deprived or left in regular growth medium for 48 hr and incubated with DMSO or 10 μM monensin for 3 hr. Cells were fixed, permeabilized, and immunostained with anti-EGFR antibody 528 (green) followed by anti-Src antibody SRC2 (red) staining. Images were acquired under a confocal microscope at the medial plane. Bars represent 20 μm. (B) Colocalization coefficients for the red channel (anti-Src staining) were obtained using LSM510 Image Examiner software and normalized to the colocalization coefficient from DMSO controls.

EGFRs traffic through the endocytic recycling compartment. Our observations, that mutant EGFRs localize to the lysosomes (Figure 2) and block of their endocytic transit by low temperature incubation (Additional File 2) or monensin treatment led to reduced degradation (Additional File 4), are consistent with the idea of mutant EGFRs trafficking into lysosomes. However, our observations do not contradict the defective ubiquitin-dependent trafficking of mutant EGFRs reported by Shtiegman et al., and others [28,29,37], as our studies did not address this issue.

Whether the increased transit through the endocytic recycling compartment is an intrinsic property of mutant EGFRs or is a secondary consequence of their reportedly reduced interaction with Cbl and ubiquitin-mediated lysosomal sorting machinery are important questions that will need to be addressed through appropriate manipulations in NSCLC cells as well as the use of ectopic gene expression approaches. In this regard, it is noteworthy that conditions that prevent EGFR interaction with Cbl or its Cbl-dependent ubiquitinylation lead to a more prolonged stay of EGFR in early/recycling endosomal com-
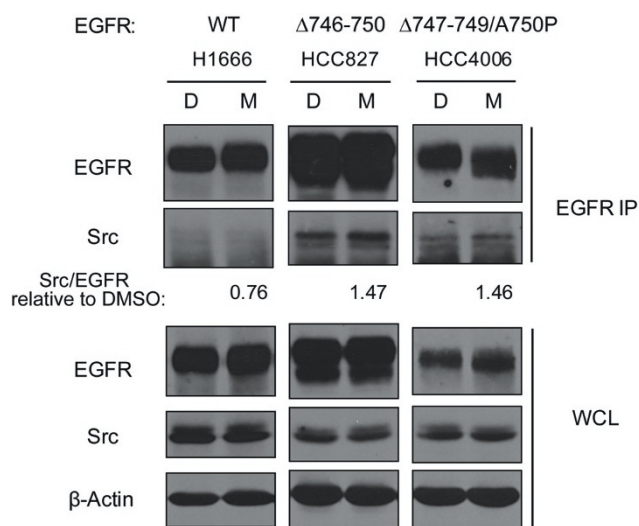
Figure 7
Monensin treatment enhances the mutant EGFR-Src association. Cells were growth factor-deprived for 48 hr and incubated with DMSO (D) or 10 μM monensin (M) for 3 hr. 1 mg aliquots of cell lysate proteins were immunoprecipitated with anti-EGFR antibody 528 and resolved together with 50 μg aliquots of whole cell lysates followed by immunoblotting with antibodies against the indicated proteins. The levels of EGFR and Src bands on blots were quantified using densitometry and immunoprecipitated Src relative to immunoprecipitated EGFR levels (Src/EGFR) were determined using the ImageJ software. Src/EGFR ratios in monensin-treated cells versus DMSO-treated cells are noted.

partments [15,55,56]. Physiologically, ligands such as TGFα that promote EGFR recycling rather than lysosomal degradation appear to engage the Cbl and ubiquitin machinery more transiently [22,57]. In addition to altered ubiquitinylation of mutant EGFRs, other defects in their signaling or protein-protein interactions could contribute to their propensity to enter the endocytic recycling compartment. For example, deubiquitinylating enzymes [58,59] as well as other factors (e.g. secretory membrane carrier protein SCAMP3) can regulate EGFR recycling versus lysosomal degradation [13,60]. Future studies to elucidate whether or not mutant EGFRs might aberrantly interact with such proteins will therefore be of considerable interest.

NSCLC-associated mutant EGFRs (both gefitinib-sensitive deletion mutants and gefitinib-resistant L858R/T790 M mutant) are constitutively active and constitutively endocytosed (Figure 1 and Additional File 1). Recent studies have demonstrated that NSCLC-associated kinase domain mutations promote constitutive dimerization of EGFR [61]. As dimerization is critical to EGFR endocytosis and may promote internalization in a kinase-dependent [2] or

kinase-independent [50,62] manner, constitutive dimerization may play an important role in the transit of mutant EGFRs into the endocytic recycling compartment. In this context, our observations using kinase inhibitors indicate that the kinase activity of EGFR is not essential for the constitutive endocytic localization of mutant EGFR (Additional File 6A). The intracellular localization of mutant EGFR was also unaffected by Src inhibitor PP2, indicating that there may be another determinant of constitutive endosomal localization of mutant EGFRs.

Transit of the constitutively-active mutant EGFR through the endocytic recycling compartment is likely to be biologically relevant. Analyses of EGFR as well as other RTKs have demonstrated that endocytic recycling, in addition to returning the internalized receptors for additional rounds of ligand-binding and signaling, can directly participate in signaling events [12]. For example, inhibition of EGFR internalization reduced the level of activation of Akt and MAPK downstream of the receptor [30,63]. Notably, initiation of EGFR activation directly at the level of endosomes has been shown to be sufficient to activate Erk and Akt, as well as promote cell survival and proliferation [34,64]. However, monensin treatment did not enhance Erk, Akt and STAT3 phosphorylation levels (Additional File 5B). The lack of monensin effect on downstream signaling is likely to reflect its ability to affect multiple endocytic compartments and/or its effects on other cellular processes [38,42,65]. Nevertheless, our observations of EGFR and Src colocalization and association are consistent with a role of signaling at the level of the endocytic recycling compartment in the biology of mutant EGFR.

Our analyses of mutant EGFR recycling in the context of Src were based on prior evidence that Src-dependent signaling is critical for EGFR-mediated oncogenesis; this has been established *in vitro* using Src inhibitors as well as mutational approaches [7], and Src is overexpressed or hyperactive in NSCLC as well as other cancers where EGFR mutations or overexpression have been implicated in oncogenesis [9,10]. Importantly, Src has been shown to localize to endosomes [46], and recent studies have shown that Src specifically localizes on recycling endosomes [45,66]. Thus, it appears plausible that mutant EGFRs, by virtue of their transit through the endocytic recycling compartment, may gain enhanced access to Src, providing a potential explanation for the higher level of constitutive Src-mutant EGFR association [7,48]. Confocal image analyses indeed support this possibility, as Src and mutant EGFRs show a detectable colocalization (versus essentially little detectable colocalization of Src with wtEGFR) (Figure 5); moreover, this colocalization was further increased by inhibiting the exit of EGFR from the endocytic recycling compartment using monensin (Figure 6). Also, a predominant pool of activated EGFR colocal-

ized with activated Src (Figure 5), and Src inhibitor slightly decreased the mutant EGFR-Src association (additional File 6), which suggest that Src activity might be important for colocalization and association with mutant EGFR. In a different study, a Src inhibitor did not inhibit mutant EGFR-Src association [48]. The difference between the two studies may be due to different types of inhibitor and/or cell lines tested.

Rather interestingly, monensin treatment led to a higher level of biochemically detectable EGFR-Src complexes (Figure 7). This, together with higher constitutive Src-mutant EGFR association, suggests the likelihood that Src-mutant EGFR complexes are either formed or more stable in the endocytic recycling compartment. As Src-dependent signaling is critical for mutant EGFR-mediated oncogenic transformation [7], these findings suggest that altered trafficking of mutant EGFRs into the endocytic recycling compartment may contribute to their oncogenic behavior. Further studies to perturb the endocytic recycling of oncogenic EGFR mutants should help address the biological role of the altered endocytic trafficking identified here.

It has been reported that a gefitinib-resistant version of H1650 NSCLC cell line showed increased internalization of EGFR upon ligand stimulation when compared to the parental gefitinib-sensitive cell line [67]. Notably, the wtEGFR in the gefitinib-resistant cell line did not undergo ligand-induced lysosomal sorting, even though the receptor was found in endocytic vesicles [54]. In our analyses, we observed a comparable pattern of subcellular localization and endocytic trafficking of gefitinib-sensitive (deletion) and gefitinib-resistant (L858R/T790 M) EGFR mutants (Figures 1, 2, 3 and Additional Files 2 and 3). Similarly, both gefitinib-resistant H1975 and gefitinib-sensitive H1650 cell lines showed delayed internalization of labeled EGF in comparison to the wtEGFR-expressing cell line H358 [28]. However, there were subtle differences among different cell lines harboring mutant EGFRs in the perinuclear accumulation of the mutant EGFR induced by monensin in the regular growth condition (Additional File 3B); the perinuclear accumulation of EGFR was dramatic in HCC827 and HCC4006, intermediate in H1650, and not readily apparent in H1975. Similarly, quantitative assessments of EGFR localization under steady-state conditions (Figure 2E) suggested differences between different NSCLC lines: the mutant EGFR is evenly divided between Tf-positive and LAMP1-positive vesicles in H1650, HCC827 and HCC4006 showed much more mutant EGFR in LAMP1-positive than in Tf-positive vesicles; and gefitinib-resistant mutant EGFR in H1975 colocalized more with Tf than with LAMP1. In addition, H1650 cell line displayed more sensitivity to EGF than other mutant EGFR-expressing cell lines (Figure 1 and Additional File 5A). Whether EGFR expression levels, the

nature of EGFR mutations, and/or activities of EGFR regulatory factors such as Src, Cbl or PTEN, which has been shown to be absent in the H1650 cell line [68], might contribute to the differences in the localization of mutant EGFR and their endocytic trafficking remain open questions. While it is possible that altered endocytic trafficking of EGFR relates to gefitinib resistance, extensive future studies are needed to determine if this is the case.

Conclusion
In summary, the results presented here show that mutant EGFRs in NSCLC cell lines constitutively transit through the sorting and endocytic recycling compartments. Impairment of EGFR exit from the endocytic recycling compartment enhances the mutant EGFR colocalization with Src in the endocytic recycling compartments and increases the Src-mutant EGFR association. Given the critical role of Src-mediated signaling in mutant EGFR-mediated oncogenic transformation, our findings suggest a potentially important role for altered endocytic trafficking in the biology of NSCLC-associated EGFR mutants.

Methods
Constructs
The EHD1-GFP and EHD3-GFP expression constructs in the pcDNA-DEST47 vector were described previously [43]. The Rab4-GFP and Rab11-GFP expression constructs in the EGFPN1 vector [69] were provided by Dr. Victor Hsu (Brigham and Women's Hospital, Harvard Medical School, Boston, MA). The lentiviral expression vectors pLenti6-V5-UbC GFP, wtEGFR-GFP, EGFR L858R-GFP, and EGFR Δ746-750-GFP were generated using the Gateway cloning technology (Invitrogen, Carlsbad, CA). EGFR was PCR amplified from pcDNA 3.1 EGFR using primers CACCATGCGACCCTCCGGGACGG and TGCTC-CAATAAATTCACTGCTTTG, and the amplified fragment was inserted into pENTR/SD/D-TOPO vector using the pENTR/SD/D-TOPO cloning kit (Invitrogen). LR recombination reaction was performed to insert EGFR into the pDEST47 vector to generate an EGFR-GFP chimera. EGFR-GFP was PCR amplified using primers CACCATGCGAC-CCTCCGGGACGG and TTATTTGTAGAGCTCATCCAT-GCC, inserted into the pENTR vector, and finally into the pLenti6-V5-UbC vector. PLenti6-V5-UbC EGFR L858R-GFP, and EGFR Δ746-750-GFP were generated using the QuikChange II XL Site-Directed Mutagenesis Kit (Strategene, La Jolla, CA) as previously described [7]. All PCR reactions were performed using the QuikChange II XL Site-Directed Mutagenesis Kit following the manufacturer's instructions.

Human bronchial epithelial cell line immortalization and lentiviral transfection
Primary normal bronchial epithelial cells (HBEC) obtained from a bronchoscopic biopsy sample were pro-

vided by Dr. Ravi Salgia (University of Chicago). Cells were transduced with retroviral supernatants of human papilloma virus (HPV) E6 and E7 and selected for several weeks to generate an immortalized human bronchial epithelial cell (HBEC) cell line. Lentiviral supernatants generated as per Gateway cloning technology protocol were used to make the HBEC cell line stably expressing pLenti6-V5-UbC vector, GFP, wtEGFR-GFP, EGFR L858R-GFP, or EGFR Δ746-750-GFP.

Cell culture and transient transfection

Immortalized bronchial epithelial cell line HBE135 (ATCC, Manassas, VA) and HBEC were grown in the DFCI-1 medium described in Band et al. [70]. NSCLC tumor cell lines H1666, H1650, HCC827, HCC4006 and H1975 (ATCC) were grown in RPMI-1640 medium (Invitrogen, Carlsbad, CA) containing 5% fetal bovine serum (FBS, Hyclone Inc., Logan, UT), 20 mM HEPES (pH 7.35), 1 mM sodium pyruvate, 1 mM each of nonessential amino acids, 100 units/ml penicillin, 100 μg/ml streptomycin, 2 mM L-Glutamine and 55 μM 2-Mercaptoethanol (all supplements were from Invitrogen) at 37°C in 5% CO_2. Cells were transiently transfected with the indicated plasmids using the FuGene6 Transfection Reagent (Roche, Indianapolis, IN) following the manufacturer's protocol.

Antibodies and other reagents

The following antibodies were obtained from commercial sources: rabbit polyclonal (pAb) anti-EGFR (1005), pAb anti-phospho-AKT (pAKT1/2/3) (Ser 473), pAb anti-phospho-Erk 1/2 (Thr 202/Tyr 204), pAb anti-Erk1 (K-23), and pAb anti-Src (SRC 2) were from Santa Cruz Biotechnology (Santa Cruz, CA); mouse monoclonal (mAb) anti-phospho-EGFR (activated form) was from BD Biosciences (San Jose, CA); pAb anti-phospho-Src (Tyr416), pAb anti-phospho-EGFR (Tyr1173), pAb anti-STAT3, Rabbit monoclonal anti-phospho-STAT3 (Tyr705), and pAb anti-phospho-EGFR (Tyr845) were from Cell Signaling Technology (Danvers, MA); mAb anti-β actin (Clone AC-15) was from Sigma-Aldrich (St Louis, MO); mAb anti-LAMP1 (H4A3) was from Developmental Studies Hybridoma Bank (Iowa City, IA); mAb anti-EGFR (clone 528; ATCC) was Protein G purified from hybridoma supernatants. Purified anti-phosphotyrosine mAb 4G10 [71] was provided by Dr. Brian Druker (Oregon Health Science University, Portland, OR). Purified mouse EGF, human holo-Transferrin, and monensin were from Sigma-Aldrich. EGFR inhibitor, Erlotinib (Tarceva), was obtained from the Hospital Pharmacy. Src inhibitor PP2 was from Calbiochem (San Diego, CA). Hsp90 inhibitor 17-AAG was from Biomol International (Plymouth, PA, U.S.A.).

Preparation of cell lysates, SDS-PAGE and immunoblotting

Cells at 50-60% confluence were incubated in normal growth medium, growth factor-deprived D3 medium (HBE135) [72] or 0.1% FBS-containing medium (H1666, H1650, HCC827, HCC4006 and H1975) for 48 hr. For EGF stimulation, cells preincubated in growth factor-deficient medium were either left as such or EGF was added at 10 ng/ml 10 min before cell lysis. Cell lysates were prepared in cold Triton X-100-based lysis buffer [7], and SDS-PAGE and immunoblotting were performed as previously described [7].

Immunoprecipitation

Cells were grown, EGF stimulation performed, and cell lysates prepared as above with the exception that the lysis buffer contained 0.25% NP-40 (instead of 0.5% Triton X-100), 50 mM Tris (pH 8.0), and 100 mM sodium chloride. Cell lysate aliquots were incubated with anti-EGFR (528) antibody, and immune complexes were captured using Protein A-Sepharose beads (GE Healthcare, Piscataway, NJ). Subsequent SDS-PAGE and immunoblotting were performed as described above.

Immunofluorescence microscopy

Cells were plated on glass coverslips (VWR, Batavia, IL) at 50-60% confluence and incubated in normal growth medium or growth factor-deficient medium for 48 hr. Cells were either left unstimulated or stimulated with EGF (10 ng/ml) for 30 min, washed in phosphate buffered saline (PBS, Cellgro, Manassas, VA), fixed in 3.7% formaldehyde (Sigma) in PBS for 20 min at RT, blocked in 2% FBS/PBS/0.02% sodium azide at 4°C for 24 hr, and permeabilized in immunostaining buffer with 0.05% Saponin (Sigma) and 0.2% BSA (Sigma) in PBS for 15 min. Cells were stained with primary antibodies diluted in immunostaining buffer for 1 hr and with Alexa 488- or Alexa 647-conjugated goat anti-mouse or goat anti-rabbit secondary antibodies (Invitrogen) for 1 hr. Coverslips were mounted on microscope slides with VECTASHIELD® Hard Set™ Mounting Medium with DAPI (Vector Laboratories, Burlingame, CA). Confocal fluorescence images were obtained with a LSM510 fluorescence confocal microscope (Carl Zeiss, Thornwood, NY) under a 63× oil immersion lens. Colocalization coefficients for each channel were calculated using the LSM510 Image Examiner software. Colocalization parameters were either set automatically by the software or thresholds were set using the scattergrams. Colocalization coefficients from at least three images were obtained, and averages were either represented as percentages or normalized and plotted with standard deviation as error bars.

Monensin Treatment

For analyses involving immunoblotting or immunoprecipitation, cells were starved in D3 or 0.1% FBS-contain-

ing media and preincubated in DMSO (0.1%) or 10 μM monensin for 4 hr. Cells were then continued as such or EGF (10 ng/ml) was added for 30 min followed by cell lysis. For immunofluorescence analyses, starved cells were preincubated in DMSO or monensin as above and loaded with 10 ug/ml of Alexa Fluor 546-conjugated transferrin (Invitrogen) for 45 min. Cells were then washed twice in PBS and either left unstimulated or stimulated with EGF (10 ng/ml) for 30 min. Cells were immunostained as described above.

Abbreviations
EGF: Epidermal growth factor; EGFR: Epidermal growth factor receptor; LAMP1: Lysosomal-associated membrane protein 1; NSCLC: Non small cell lung cancer; RTK: Receptor tyrosine kinase; Tf: transferrin; TKI: Tyrosine kinase inhibitor; WT: Wild-type.

Competing interests
The authors declare that they have no competing interests.

Authors' contributions
HB and VB conceived the study and established the initial design of studies. BMC, and SMR carried out the experimental work including design alterations and data analysis in consultation with HB. RJC, CT and MG participated in data analysis and provided critical comments on the study design and manuscript. BMC prepared the draft of the manuscript and HB edited and finalized it. All authors read and approved the final manuscript.

Note
@The work presented here was initiated while the investigators were in the Department of Medicine, Evanston Northwestern Healthcare (now Northshore University HealthSystem) Research Institute, Feinberg School of Medicine, Northwestern University, Evanston, IL, USA.

Additional material

Additional file 1
Constitutive intracellular vesicular localization of mutant EGF receptors stably expressed in HBEC cell line. (A) Cells were growth factor-deprived by culturing in D3 medium for 48 hr and then treated with 100 ng/ml EGF for indicated time periods. Cells were fixed, and GFP images were acquired under a confocal microscope at the medial plane. Bar represents 20 μm. (B) Cells were growth factor-deprived as in (A) for 48 hr and either left unstimulated (-) or stimulated (+) with 100 ng/ml EGF for 10 min. The indicated amounts of whole cell lysate protein were used for immunoblotting with antibodies against the indicated proteins. Exogenous EGFR-GFPs (EGFR-GFP) and endogenous EGFR (endogenous EGFR) are indicated with arrows.
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Additional file 2
Enhanced colocalization of mutant EGF receptors with labeled transferrin upon incubation of cells at 16°C. Cells were growth factor-deprived in 0.1% FBS-containing growth medium for 48 hr and preincubated at 37 or 16°C for 2 hr. Cells were loaded with 10 μg/ml AF546-Tf (Tf) (red) for 45 min. This was followed by incubation in growth factor-deprived medium with (+ EGF) or without (- EGF) 10 ng/ml EGF for 30 min. Cells were fixed, permeabilized, and immunostained with anti-EGFR antibody 528 (green). Images were acquired under a confocal microscope at the medial plane. Bars represent 10 μm.
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Additional file 3
Monensin treatment inhibits the exit of labeled transferrin and mutant EGFR from the perinuclear endocytic recycling compartment. (A) H1666 cells were growth factor deprived for 48 hr and preincubated with DMSO or 10 μM monensin for 3 hr. Cells were loaded with 10 μg/ml AF546-Tf (Tf) (red) for 30 min at 37°C and then chased in the presence of 2 mg/ml holo-transferrin for 0, 30 or 60 min in the continued presence of DMSO or 10 μM monensin. Cells were fixed, permeabilized and immunostained with anti-LAMP1 antibody (green). Images were acquired under a confocal microscope at the medial plane. (B) Cells were grown in regular growth medium and preincubated with DMSO or 10 μM monensin for 3 hr. Cells were loaded with 10 ug/ml AF546-Tf (Tf) (red) for 45 min at 37°C (with continuation of DMSO or monensin treatment). This was followed by incubation with regular growth medium for 30 min with continued DMSO or monensin treatment. Cells were fixed, permeabilized, and immunostained with anti-EGFR antibody 528 (green). Images were acquired under a confocal microscope at the medial plane. (C) HBEC cells stably expressing mutant EGF receptors were growth factor-deprived in D3 medium for 48 hr and then preincubated with DMSO or 10 μM monensin for 3 hr. Cells were incubated with (+EGF) or without (-EGF) 10 ng/ml EGF for 30 min with continued DMSO or monensin treatment. Cells were fixed, and GFP images were acquired under a confocal microscope at the medial plane. Bars represent 20 μm.
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Additional file 4
Monensin treatment prevents lysosomal targeting and degradation of mutant EGFR by 17-AAG. H1650 cells were grown in regular growth medium and preincubated with DMSO or 10 μM monensin for 4 hr. Cells were either left untreated or treated with 1 μM 17-AAG for 3 hr. Cells were fixed, permeabilized, and immunostained with anti-EGFR antibody 528 (green) followed by anti-LAMP1 antibody (red). Images were acquired under a confocal microscope at the medial plane. Bars represent 20 μm.
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Additional file 5

Monensin treatment does not alter the overall levels of phosphorylation or expression of EGFR and its downstream factors. Cells were growth factor-deprived in D3 medium (HBE135) or 0.1% FBS-containing growth medium (all other cell lines) for 48 hr and preincubated with DMSO or 10 μM monensin for 3 hr. Cells were then either left unstimulated (-) or stimulated (+) with 10 ng/ml EGF for 30 min. 50 μg aliquots of WCL were used for immunoblotting with antibodies against the indicated proteins.

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Additional file 6

Src inhibitor does not alter intracellular localization of mutant EGFR but reduces mutant EGFR-Src association. (A) HCC827 cells were growth factor deprived for 48 hr and preincubated with DMSO, 1 μM Erlotinib (ER) or 3 μM PP2 for 3 hr. Cells were fixed, permeabilized, and immunostained with anti-EGFR antibody 528 (green). Images were acquired under a confocal microscope at the medial plane. Bars represent 20 μm. (B) HCC827 cells were grown in regular growth medium and preincubated with DMSO, 1 μM Erlotinib (ER) or 3 μM PP2 for 3 hr. 1 mg aliquots of cell lysate proteins were immunoprecipitated with anti-EGFR antibody 528 (EGFR IP) and resolved together with 50 μg aliquots of whole cell lysates (WCL) followed by immunoblotting with antibodies against the indicated proteins.

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