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ORIGINAL ARTICLE

The role of cooperativity with Src in oncogenic transformation mediated by non-small cell lung cancer-associated EGF receptor mutants

BM Chung[1,5,9], M Dimri[6,9], M George[1,9], AL Reddi[7,9], G Chen[8,9], V Band[3,4,9] and H Band[1,2,3,4,9]

[1]*Eppley Institute for Cancer and Allied Diseases, University of Nebraska Medical Center, 986805 Nebraska Medical Center, Omaha, NE, USA;* [2]*Department of Biochemistry and Molecular Biology, University of Nebraska Medical Center, 986805 Nebraska Medical Center, Omaha, NE, USA;* [3]*Department of Genetics, Cell Biology and Anatomy, College of Medicine, University of Nebraska Medical Center, 986805 Nebraska Medical Center, Omaha, NE, USA and* [4]*UNMC-Eppley Cancer Center, University of Nebraska Medical Center, 986805 Nebraska Medical Center, Omaha, NE, USA*

Non-small cell lung cancer (NSCLC)-associated epidermal growth factor receptor (EGFR) mutants are constitutively active and induce ligand-independent transformation in non-malignant cell lines. We investigated the possibility that the ability of mutant EGFRs to transform cells reflects a constitutive cooperativity with Src using a system in which the overexpression of mutant, but not wild-type, EGFR induced anchorage-independent cell growth. Src was constitutively activated and showed enhanced interaction with mutant EGFRs, suggesting that constitutive EGFR–Src cooperativity may contribute to mutant EGFR-mediated oncogenesis. Indeed, the mutant EGFR-mediated cell transformation was inhibited by Src- as well as EGFR-directed inhibitors. Importantly, a tyrosine to phenylalanine mutation of the major Src phosphorylation site on EGFR, Y845, reduced the constitutive phosphorylation of NSCLC-EGFR mutants, as well as that of STAT3, Akt, Erk and Src, and reduced the mutant EGFR–Src association as well as proliferation, migration and anchorage-independent growth. Reduced anchorage-independent growth and migration were also observed when dominant-negative-Src was expressed in mutant EGFR-expressing cells. Overall, our findings show that mutant EGFR–Src interaction and cooperativity play critical roles in constitutive engagement of the downstream signaling pathways that allow NSCLC-associated EGFR mutants to mediate oncogenesis, and support the rationale to target Src-dependent signaling pathways in mutant EGFR-mediated malignancies.
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Introduction

Epidermal growth factor receptor (EGFR) is a member of the ErbB family (ErbB1–4) of receptor tyrosine kinases that plays important physiological functions by controlling critical processes, including proliferation, survival, migration and differentiation (Wiley and Burke, 2001; Herbst, 2004). Aberrant EGFR signaling through overexpression and/or mutation occurs in many cancers, including breast cancer, head-and-neck cancer, non-small cell lung cancer (NSCLC), renal cancer, ovarian cancer and colon cancer (Herbst, 2004). Notably, specific subtypes of NSCLC patients exhibit somatic EGFR mutations that impart a higher sensitivity to EGFR-directed tyrosine kinase inhibitors (TKIs) such as gefitinib (Iressa) or erlotinib (Tarceva) (Gschwind *et al*., 2004; Lynch *et al*., 2004). Consistent with *in vivo* findings, NSCLC cell lines with mutant EGFRs are hypersensitive to growth inhibition on gefitinib treatment (Sordella *et al*., 2004).

Gefitinib-sensitizing NSCLC-associated EGFR mutations are located in the catalytic domain of EGFR near the ATP-binding pocket, which is also the binding site for TKIs (Paez *et al*., 2004; Sordella *et al*., 2004). The most common NSCLC-associated mutations are in-frame deletions of codons 746–750 (EGFR Δ746–750) and a leucine-to-arginine point mutation at codon 858 (EGFR L858R) of the full-length EGFR (Mitsudomi *et al*., 2005).

Earlier studies of cells expressing mutant EGFRs indicate that mutant receptors are constitutively active (Lynch *et al*., 2004; Zhang *et al*., 2006; Yun *et al*., 2007) and transform non-malignant cell lines in a ligand-independent manner (Greulich *et al*., 2005).

Correspondence: Dr H Band, Eppley Institute for Cancer and Allied Diseases, University of Nebraska Medical Center, 986805 Nebraska Medical Center, Omaha, NE 68198-6805, USA.
E-mail: hband@unmc.edu
[5]BMC is a Ph.D. degree candidate of the Interdepartmental Biological Sciences Program of Weinberg College of Arts and Sciences, Northwestern University, Evanston, IL, USA.
[6]Current address: Evanston Northwestern Healthcare Research Institute, Evanston, IL, USA.
[7]Current address: Department of Medicine, Northwestern University Feinberg School of Medicine, Chicago, IL, USA.
[8]Current address: Coskata Energy, Warrenville, IL, USA.
The work presented here was initiated while the principal investigators were at: [9]Evanston Northwestern Healthcare Research Institute, Department of Medicine, Feinberg School of Medicine, Department of Biochemistry, Molecular Biology and Cell Biology, Weinberg College of Arts and Robert H. Lurie Comprehensive Cancer Center, Northwestern University, Evanston, IL, USA.
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Furthermore, cells harboring mutant EGFRs undergo 'oncogene addiction' and require the mutant receptor activity for survival (Sharma *et al*., 2007). In transgenic mouse models, reduced expression of mutant EGFR or inhibition of kinase activity caused rapid tumor regression, showing that mutant EGFR is required for tumor maintenance (Politi *et al*., 2006). These analyses support the overall rationale provided by clinical observations for EGFR-directed targeted therapy of mutant EGFR-expressing cancers. However, resistance to these drugs can develop rapidly, in part, because of secondary mutations that prevent TKI binding (Pao *et al*., 2005). Therefore, understanding the mechanisms that allow mutant EGFRs to engage in constitutive oncogenic signaling are likely to point to alternate strategies for treatment of mutant EGFR-driven cancers.

In contrast to mutant EGFRs, overexpression of wild-type EGFR (wtEGFR) in primary cells is not oncogenic. High levels of exogenous ligands have been reported to promote oncogenesis in certain cell systems (Di Fiore *et al*., 1987). Several studies have shown cooperative signaling between EGFR and Src to be an important determinant of EGFR-mediated oncogenesis (Bromann *et al*., 2004). Overexpression of both EGFR and Src in a mouse fibroblast model system led to synergistic increase in EGF-induced DNA synthesis, soft agar colony formation and tumor formation in nude mice (Bromann *et al*., 2004). This cooperativity has also been shown in the context of epithelial cell transformation: loss of polarity in three-dimensional cultures of non-malignant human mammary epithelial cells as well as their anchorage-independent growth were seen only when both EGFR and Src were co-overexpressed (Dimri *et al*., 2007). Consistent with these initial studies, EGFR and Src are often overexpressed in human cancers (Silva, 2004). In NSCLC, Src expression and its activity are elevated in tumor compared with normal tissue (Masaki *et al*., 2003).

The mechanism of EGFR and Src synergy involves reciprocal trans-activation. Src becomes activated on EGF stimulation and mediates phosphorylation of EGFR, including that on tyrosine 845 (Y845, Y869 in the full-length EGFR) (Biscardi *et al*., 1999). Mutational analysis has shown that phosphorylation of EGFR Y845 is critical for EGF-induced DNA synthesis (Biscardi *et al*., 1999).

Altogether, these findings suggest that one likely mechanism of oncogenic signaling by mutant EGFRs may be a constitutive engagement of the Src pathway. Consistent with this idea, Src was hyperphosphorylated and associated with EGFR in NSCLC cell lines harboring mutant EGFRs, and these cells are more sensitive to Src inhibitors for cell death than cells expressing wtEGFR (Song *et al*., 2006; Zhang *et al*., 2007; Yang *et al*., 2008). To systematically define the role of Src in mutant EGFR-mediated oncogenesis, we used an NIH 3T3 cell overexpression system in which the mutant, but not the wild-type, EGFR induces oncogenic transformation. Our findings suggest that a constitutive cooperativity with Src is critical for mutant EGFR-mediated oncogenic transformation, supporting the rationale for concurrent or alternative targeting of Src-dependent signaling for targeted therapy of mutant EGFR-driven cancers.

Results

Constitutive activity of mutant EGF receptors and cellular transformation on stable expression in NIH 3T3 cells

We first established retroviral transductants of NIH 3T3 cells stably expressing the vector (V), wtEGFR (WT), EGFR L858R (LR) or EGFR Δ746–750 (DEL) to confirm the mutant EGFR-induced transformation. Earlier studies have established that introduction of NSCLC-associated EGFR mutants into NIH 3T3 cells, which expresses very low levels of endogenous EGFR, leads to transformation (Greulich *et al*., 2005). We used this model system to examine the role of EGFR–Src interaction in oncogenesis. Consistent with earlier studies (Sordella *et al*., 2004), mutant EGFRs showed constitutive phosphorylation (Supplementary Figure S1A), whereas wtEGFR showed phosphorylation only on EGF stimulation.

We next used the soft agar colony assay to assess the transforming ability of mutant EGFRs (Supplementary Figure S1B). Cells expressing mutant EGFRs, but not the vector control or wtEGFR-expressing cells, showed colony formation in soft agar. It is interesting that, colony growth in low serum medium (0.1% fetal bovine serum (FBS)) was comparable with that in complete growth medium (10% FBS) (Supplementary Figure S1C). Thus, consistent with the earlier published data, mutant EGFRs expressed in NIH 3T3 cells are constitutively active and induce cellular transformation, providing a validated set of reagents to test the role of mutant EGFR cooperation with Src.

Constitutive Src signaling downstream of mutant EGF receptors

In an effort to identify molecular pathways through which mutant EGFRs trigger cell transformation, we examined the activation and expression levels of STAT3, Akt and Erk, major downstream targets of EGFR that control a wide array of cellular processes. Similar to EGFR phosphorylation (Supplementary Figure S1A), the phosphorylation levels of STAT3, Akt and Erk on EGF stimulation did not differ greatly between wtEGFR- and mutant EGFR-expressing cells (Figure 1a). In contrast, cells expressing mutant EGFRs showed enhanced phosphorylation of each of the analysed signaling proteins in the absence of EGF stimulation when compared with the vector control or wtEGFR cells.

As STAT activation mediates EGFR and Src synergism (Kloth *et al*., 2003) and Akt and Erk can also be activated by Src (Bromann *et al*., 2004), these findings suggest that mutant EGFRs induce a constitutive activation of the Src signaling pathway. Consistent with this suggestion, Src is hyperphosphorylated in NSCLC cell lines expressing mutant receptors, and these cell

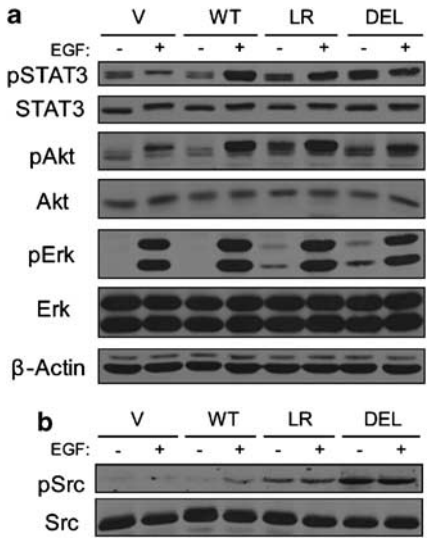

Figure 1 Constitutive activation of epidermal growth factor receptor (EGFR) downstream targets and Src by mutant EGFRs. NIH 3T3 cells stably expressing the vector (V), wild-type (wt)EGFR (WT), EGFR L858R (LR) or EGFR Δ746–750 (DEL) were serum deprived for 3 h and then either left unstimulated (−) or stimulated (+) with 10 ng/ml EGF for 10 min. (**a**) 50 μg aliquots of whole cell lysates were used for immunoblotting with antibodies against the indicated proteins. (**b**) 50 μg aliquots of whole cell lysates were used for immunoblotting with antibodies against phospho-Src (pSrc, Y416) and total Src (SRC 2).

lines are sensitive to Src inhibition (Song *et al*., 2006; Zhang *et al*., 2007). Indeed, levels of active Src were elevated in cells expressing mutant EGFRs (Figure 1b). Although Src phosphorylation was dependent on EGF stimulation in wtEGFR-expressing cells, Src was hyperphosphorylated and its phosphorylation was constitutive in mutant EGFR-expressing cells.

Next, we examined the complex formation between EGFR and Src. Earlier studies have shown that, unlike ErbB2, interaction of EGFR with Src is not stable (Muthuswamy and Muller, 1995; Kim *et al*., 2005). The differential association of Src with ErbB2 versus EGFR is mediated through differences in the kinase domain (Kim *et al*., 2005). As mutant EGFRs also share structural differences with wtEGFR in the kinase domain (Zhang *et al*., 2006), we surmized that mutant EGFRs may exhibit more stable association with Src. Therefore, we carried out co-immunoprecipitation experiments in unstimulated as well as EGF-stimulated cells to evaluate EGFR–Src association (Figure 2a). Consistent with an earlier report (Yang *et al*., 2008), when EGFR was immunoprecipitated and probed for Src, mutant EGFR-expressing cells showed increased levels of co-immunoprecipitated Src in the presence and absence of EGF stimulation compared with wtEGFR. Co-immunoprecipitation experiments on NSCLC cell lines expressing wtEGFR (H1666) versus mutant EGFR (HCC4006) confirmed these findings (Figure 2b). Thus, mutant EGFRs interact more stably with Src compared with wtEGFR. Pre-incubating cells with erlotinib abrogated mutant EGFR phosphorylation and association with Src, indicating that mutant EGFR kinase activity is critical for the enhanced association with Src (Figure 2c).

Src is required for mutant EGFR-mediated cell transformation

We then asked whether mutant EGFR-driven oncogenic transformation required the Src signaling pathway. We assessed the anchorage-independent growth of mutant EGFR-expressing cells in the presence or absence of the Src inhibitor PP2 (Figure 3a). Similar to Supplementary Figure S1B, only cells expressing mutant EGFRs showed evidence of colony growth in soft agar. As expected, on the basis of mutant EGFR-driven transformation, EGFR inhibition with erlotinib abolished the colony formation in mutant EGFR-expressing cells. It is interesting that increasing concentrations of PP2 also resulted in dose-related decreases in colony formation (Figure 3b). Treating mutant EGFR-expressing cells with erlotinib or PP2 also decreased phosphorylation of downstream targets (Supplementary Figure S2). These results support the integral role of Src activity in mutant EGFR-driven oncogenic transformation.

Earlier studies have shown that a key mechanism of EGFR–Src cooperative signaling in cell transformation is the Src-dependent phosphorylation on EGFR Y845 (Biscardi *et al*., 1999). Therefore, to genetically define the involvement of Src in the mutant EGFR-mediated transformation, we examined the importance of EGFR Y845 phosphorylation. For this purpose, we engineered Y845F mutations in wtEGFR, EGFR L858R and EGFR Δ746–750, and expressed these mutants in NIH 3T3 cells; the corresponding cell lines are designated as EGFR Y845F (YF), YF/LR (EGFR Y845F/L858R) and YF/DEL (EGFR Y845F/Δ746–750).

Each cell line had a comparable level of introduced EGFRs (Figure 4a). It is interesting that although overall levels of EGF-induced tyrosine phosphorylation of EGFRs bearing Y845F mutations were comparable, we observed that there was a notable decrease in levels of ligand-independent phosphorylation of YF/LR compared with LR, and to lesser degree of YF/DEL versus DEL (Figure 4a). Surprisingly, the use of phosphotyrosine 845-specific commercial antibody (pY845) for immunoblotting showed very little difference, if any, in EGF-mediated phosphorylation of Y845F mutants (data not shown). This was not because of the reversion of Y845F mutation, as sequencing of genomic PCR products from stable NIH 3T3 transductants confirmed the presence of appropriate mutants in each cell line (data not shown). The lack of significant decreases in the pY845 level among Y845F mutants may be because of a relative lack of specificity of the antibody at higher EGFR levels, as was noted earlier (Song *et al*., 2006).

In contrast to relatively similar levels of EGF-induced phosphorylation, mutant EGFRs with Y845F mutation showed a substantial reduction in basal phosphorylation. Cells were cultured in normal growth (10% FBS) or in serum-starvation (0.1% FBS) medium for 3 h, harvested and analysed for EGFR phosphorylation

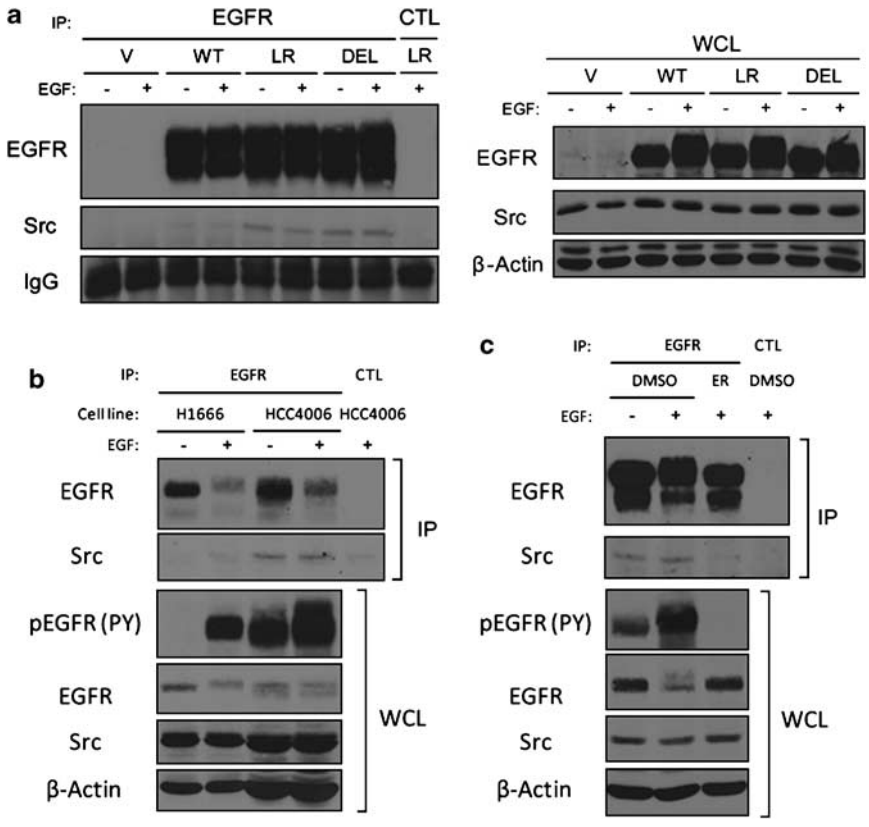

Figure 2 Constitutive interaction of mutant epidermal growth factor receptors (EGFRs) with Src. (**a**) NIH 3T3 cells were treated as described in Figure 1. 1 mg aliquots of cell lysates immunoprecipitated (IP) with anti-EGFR (528) or control (CTL) antibody or 50 μg aliquots of whole cell lysates (WCL) were resolved directly followed by immunoblotting with antibodies against the indicated proteins. (**b**) H1666 or HCC4006 cells were serum deprived for 48 h and then either left unstimulated (−) or stimulated (+) with 10 ng/ml EGF for 30 min. 667 μg (H1666) or 2 mg (HCC4006) aliquots of cell lysates immunoprecipitated with anti-EGFR (528) or control (CTL) antibody or 66.7 (H1666) or 200 (HCC4006) μg aliquots of whole cell lysates (WCL) were resolved directly followed by immunoblotting with antibodies against the indicated proteins. Different amounts of lysates were used to compensate for the difference in EGFR and Src expression levels between two cell lines. (**c**) NIH 3T3 EGFR L858R (LR) cells were serum deprived for 3 h, pre-incubated in dimethylsulphoxide (DMSO) or 1 μM erlotinib (ER) for 1 h, and either left unstimulated (−) or stimulated (+) with 10 ng/ml EGF for 30 min. 1 mg aliquots of cell lysates immunoprecipitated with anti-EGFR (528) or CTL antibody or 50 μg aliquots of WCL were resolved directly followed by immunoblotting with antibodies against the indicated proteins. V, vector; WT, wild-type (wt)EGFR; EGFR Δ746–750 (DEL).

(Figure 4b). In both cases, YF/LR and YF/DEL showed lower levels of phosphorylation when compared with LR and DEL, respectively. YF/LR and YF/DEL still showed high levels of phosphorylation over wtEGFR. These data suggest that although Y845F mutation does not significantly alter the overall phosphorylation of the receptor in the presence of EGF stimulation, it reduces the constitutive phosphorylation of mutant EGFRs.

Src phosphorylation on Y845 is critical for the mutant EGFR-mediated cell transformation, proliferation and migration

To assess the biological effect of Y845F mutation on mutant EGFR-driven oncogenic transformation, we compared the anchorage-independent growth of cell lines expressing NSCLC-associated EGFR mutants versus their Y845F mutants (Figure 5a). Cells expressing NSCLC mutants showed colony formation, whereas vector, wtEGFR- or EGFR Y845F-expressing cells did not. However, Y845F mutations decreased the abilities of EGFR mutants to promote colony growth. Although the decrease is more profound with YF/LR than with YF/DEL (Figure 5b), reduction in both cases was statistically significant.

We also examined the effect of Y845F mutations of mutant EGFRs on the rate of proliferation of transduced cells. As expected, similar rates of cell growth were observed between vector, wtEGFR- and EGFR Y845F-expressing cells (Figure 5c). As anticipated, cells expressing NSCLC mutants showed a dramatic increase in cell proliferation over those expressing vector, wtEGFR and EGFR Y845F. However, the advantage in cell growth was partially reduced by Y845F mutation of mutant EGFRs (Figure 5c).

To further confirm the requirement of Src and its activity in mutant EGFR-mediated transformation, vector or dominant-negative Src (DN-Src) was stably expressed in mutant EGFR-expressing cells (Supplementary Figure S3), and soft agar colony formation was assessed (Figures 5d–h). Anchorage-independent growth of both LR (Figure 5d) and DEL (Figure 5g) cells was

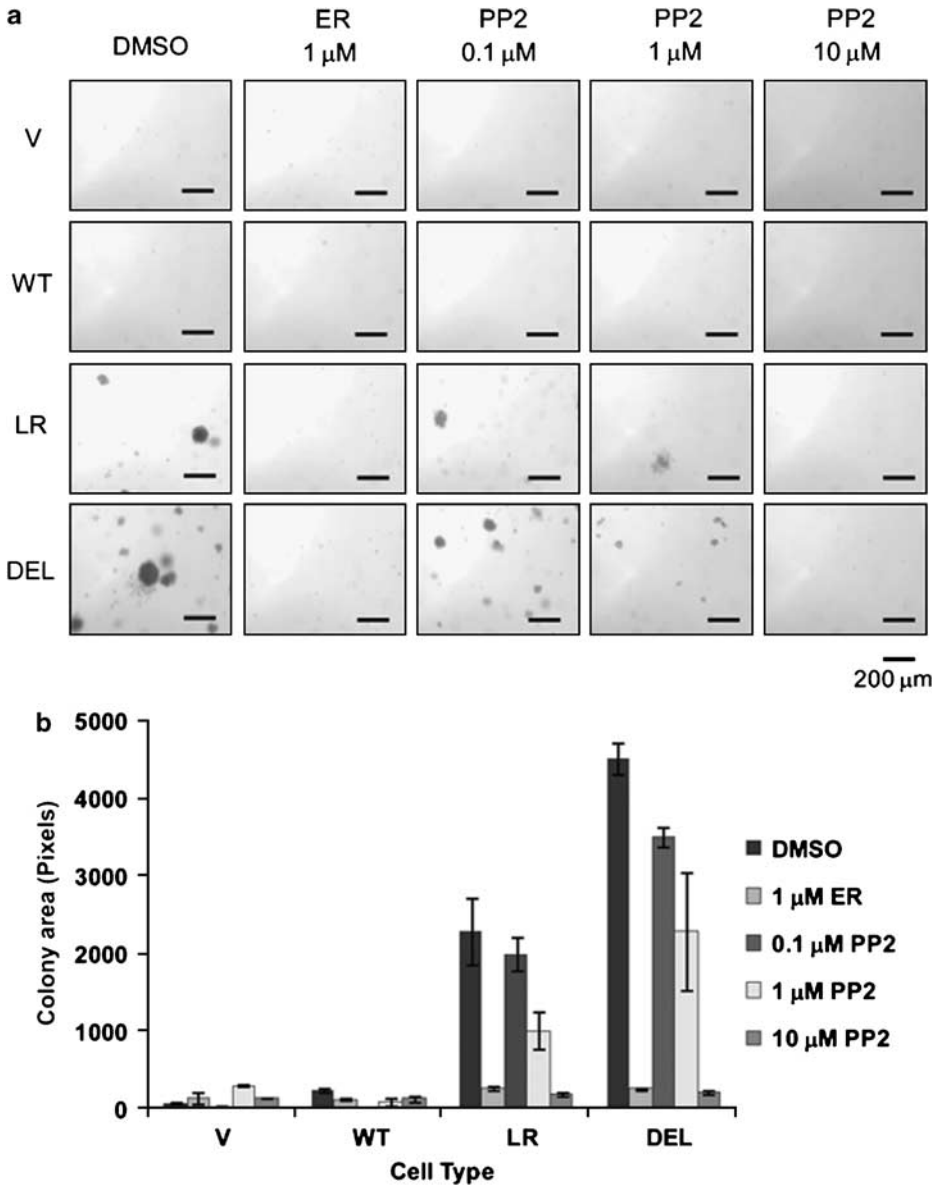

Figure 3 Src activity is required for mutant epidermal growth factor receptor (EGFR)-mediated transformation. (**a**) Cells expressing the indicated EGFRs were grown on soft agar with media containing dimethylsulphoxide (DMSO), 1 μM erlotinib (ER) or indicated concentration of Src inhibitor PP2 (PP2). After 3 weeks, pictures were taken with a phase-contrast microscope. (**b**) Images from (**a**) were quantified using the ImageJ software. Each condition was prepared in triplicate with four high power fields per replicate. Data points represent combined colony areas of four high power fields. Data were expressed as colony area (mean ± s.d.) of cells expressing indicated EGFRs. Analysis of Variance performed on the colony area yielded a statistically significant difference between conditions. V, vector; WT, wild-type (wt)EGFR; EGFR L858R (LR); EGFR Δ746–750 (DEL).

reduced by DN-Src expression as compared with vector controls; the reduction was statistically significant (Figures 5e and h, respectively).

We further examined the effect of Y845F mutation on the ability of NSCLC mutants to drive increased cell migration, another critical oncogenesis-related biological outcome (Figure 6a). Unstimulated vector control or wtEGFR-expressing cells showed minimal migration, which was substantially enhanced in the presence of EGF and a higher level of migration was seen in cells overexpressing wtEGFR (Figure 6a). Notably, EGFR Y845F-expressing cells showed a level of EGF-induced cell migration that was comparable to the vector control. Importantly, the basal migration of cells expressing NSCLC mutants was substantially higher compared with those of wtEGFR, and higher levels of migration were seen on EGF stimulation. Both the basal and EGF-stimulated cell migration was reduced in cells expressing NSCLC mutants with the Y845F mutation. Furthermore, the expression of DN-Src reduced the EGF-mediated cell migration of mutant EGFR-expressing cells (Figure 6c). Use of erlotinib, PP2 and wortmannin also confirmed the requirement of EGFR, Src and Akt in the mutant EGFR-mediated cell migration (Supplementary Figure S4). Collectively, the analyses of cells expressing Y845F mutations or DN-Src

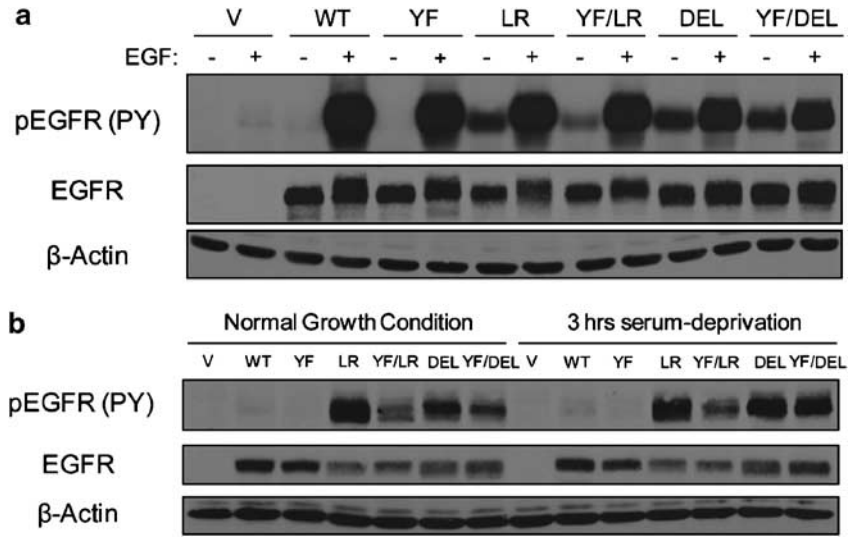

Figure 4 The Src phosphorylation site on epidermal growth factor receptor (EGFR), Y845, is important for constitutive activation of mutant EGF receptors. (**a**) Cells expressing the indicated EGFRs were serum deprived for 3 h and then either left unstimulated (−) or stimulated (+) with 10 ng/ml EGF for 10 min, or (**b**) Cells were placed in media with 10% fetal bovine serum (FBS) (Normal Growth Condition) or 0.1% FBS for 3 h (3 hrs serum-deprivation). In all, 50 μg aliquots of whole cell lysates were used for immunoblotting with antibodies against the indicated proteins. V, vector; WT, wild-type (wt)EGFR; EGFR L858R (LR); YF, EGFR Y845F; EGFR Δ746–750 (DEL).

provide strong support for the conclusion that constitutive activation of mutant EGFRs is transduced into oncogenic signaling through cooperative signaling with Src and involves an obligatory role of the major Src phosphorylation site on EGFR.

Y845 phosphorylation is important in constitutive downstream signaling by EGFR mutants

Given the substantial effects of the Y845F mutation and DN-Src on the biological behavior of NSCLC-associated EGFR mutants, it was of considerable interest to investigate whether the phosphorylation of Y845 was important to link mutant EGFRs to downstream signaling pathways that are constitutively activated by these mutants (Figures 1a and b). In view of the apparent lack of requirement for exogenous ligand for oncogenicity of mutant EGFRs, we focused on constitutive phosphorylation of the downstream effectors. For this purpose, we compared the phosphorylated and total protein levels of STAT3, Akt and Erk in NIH 3T3 cells expressing wtEGFR, EGFR L858R or Δ746–750 versus their Y845F mutants under normal growth conditions (Supplementary Figure S5) or after 3-h serum deprivation (Figure 7a). Although cells grown under normal growth conditions or those stimulated with EGF (Supplementary Figure S6) showed little to modest difference in signaling pathways, difference in signaling became evident when cells were serum deprived. Similar to Figure 1b, STAT3, Akt and Erk phosphorylation in EGFR L858R- and, more significantly, Δ746–750-expressing cells remained relatively high when compared with vector and wtEGFR-expressing cells on serum deprivation (Figure 7a). Notably, cells expressing Y845F mutants showed reduced phosphorylation levels of STAT3, Akt and Erk compared with cells expressing mutant EGFRs without Y845F mutations (quantified in Supplementary Table S2). Src phosphorylation between normally grown and serum-deprived cells harboring particular EGFRs was comparable (Supplementary Figure S5A and Figure 7b). However, Src phosphorylation levels decreased substantially as a result of Y845F mutation under both growth conditions.

As mutant EGFRs showed enhanced interactions with Src compared with wtEGFR (Figure 2), the effect of Y845F mutations on EGFR–Src association was also examined. For this purpose, anti-EGFR immunoprecipitation from cells grown in complete growth medium followed by anti-Src immunoblotting was carried out (Figure 8). In both wtEGFR and mutant EGFRs, Y845F mutation led to a weaker co-immunoprecipitation by Src (quantified in Supplementary Table S2). Similar analysis carried out on serum-deprived cell lysates yielded identical results (data not shown). Thus, Y845F mutation decreases the interaction of mutant EGFRs with Src.

Discussion

Somatic mutations in the kinase domain of EGFR impart increased binding to TKIs. Coupled with evidence that cells expressing mutant EGFRs undergo 'oncogene addiction', these findings have led to selective targeting of NSCLC patients with EGFR mutations with EGFR TKIs (Sharma *et al*., 2007). However, modulation of TKI sensitivity by acquired secondary mutations in EGFR or because of other genetic or epigenetic alteration in cancer cells necessitates the development of alternate strategies against mutant EGFR-driven NSCLC.

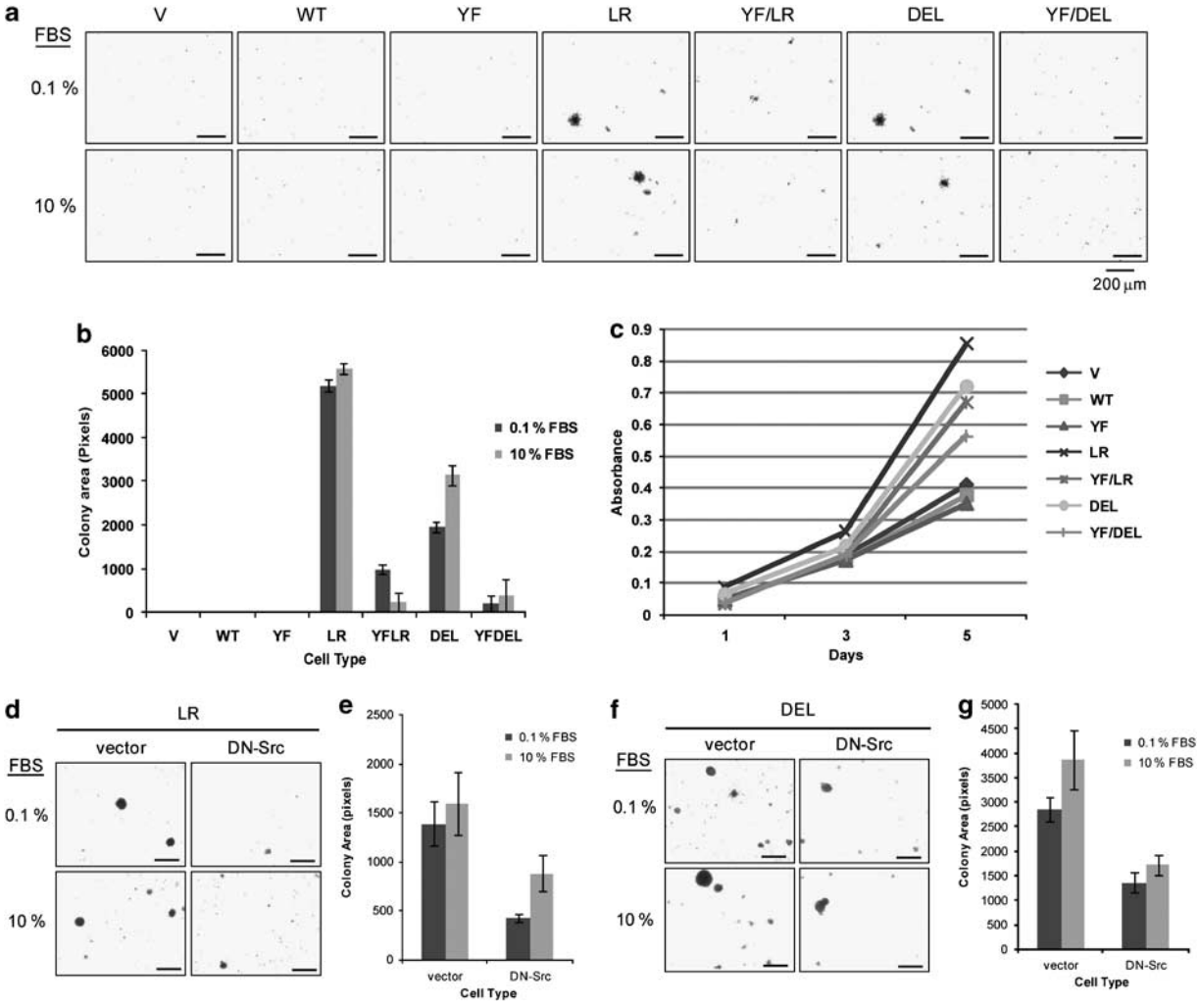

Figure 5 Src and its phosphorylation on epidermal growth factor receptor (EGFR), Y845, are critical for mutant EGFR-mediated cell transformation and proliferation. (**a**) Cells expressing the indicated EGFRs were grown in soft agar with media containing 0.1 or 10% fetal bovine serum (FBS). After 3 weeks, pictures were taken with a phase-contrast microscope. (**b**) Images from (**a**) were quantified using the ImageJ software. Each condition was prepared in triplicate with four high power fields per replicate. Data points represent combined colony areas of four high power fields. Data were expressed as colony area (mean ± s.d.) of cells expressing indicated EGFRs. Analysis of Variance (ANOVA) performed on the colony area yielded a statistically significant difference between cell types. (**c**) Cells were plated at day 0 and grown in media with 10% FBS. MTT dye incorporation was measured as an index of cell proliferation at day 1, 3 and 5. For each data point, 6 replicates were carried. Data were expressed as absorbance at 570 nm (mean ± s.d.) on days 1, 3 and 5 of cells expressing indicated EGFRs. EGFR L858R (LR) (**d**) or EGFR Δ746–750 (DEL) (**f**) cells expressing vector control or dominant-negative Src (DN-Src) were grown as described in (**a**) and quantified and analysed as described above in (**e** and **g**), respectively. Bar represents 200 μm. ANOVA performed on the colony area yielded a statistically significant difference between cell types. V, vector; WT, wild-type (wt)EGFR; YF, EGFR Y845F.

Recent studies have shown that in the process of oncogene addiction, cells become dependent not only on the oncogene itself, but also on the activity of signaling pathways that mediate the oncogenic activity (Sharma *et al*., 2007). A better understanding of how downstream signaling pathways are coupled to mutant EGFRs is therefore likely to identify critical therapeutic targets in NSCLC. Here, we used a NIH 3T3 cell transformation system to underscore the critical involvement of signaling through Src, orchestrated by the Src phosphorylation site on EGFR (Y845), which seems crucial in linking mutant EGFRs to oncogenesis and constitutive activation of specific signaling pathways.

Src dependence of EGFR Y845 phosphorylation is well established (Biscardi *et al*., 1999; Bromann *et al*., 2004; Ishizawar and Parsons, 2004). However, it has been suggested that phosphorylation of Y845 on one of the NSCLC-associated EGFR mutants, L861Q, represents an autophosphorylation site and is Src activity-independent on the basis of the use of EGFR and Src inhibitors (Yang *et al*., 2008). However, Y845 phosphorylation in EGFR L858R-expressing cells was still sensitive to Src inhibitors (Yang *et al*., 2008). Although it is reasonable that activated mutant EGFRs might acquire the ability to autophosphorylate Y845, constitutive activity of Src and the enhanced EGFR–Src interaction in mutant EGFR-expressing cells suggest

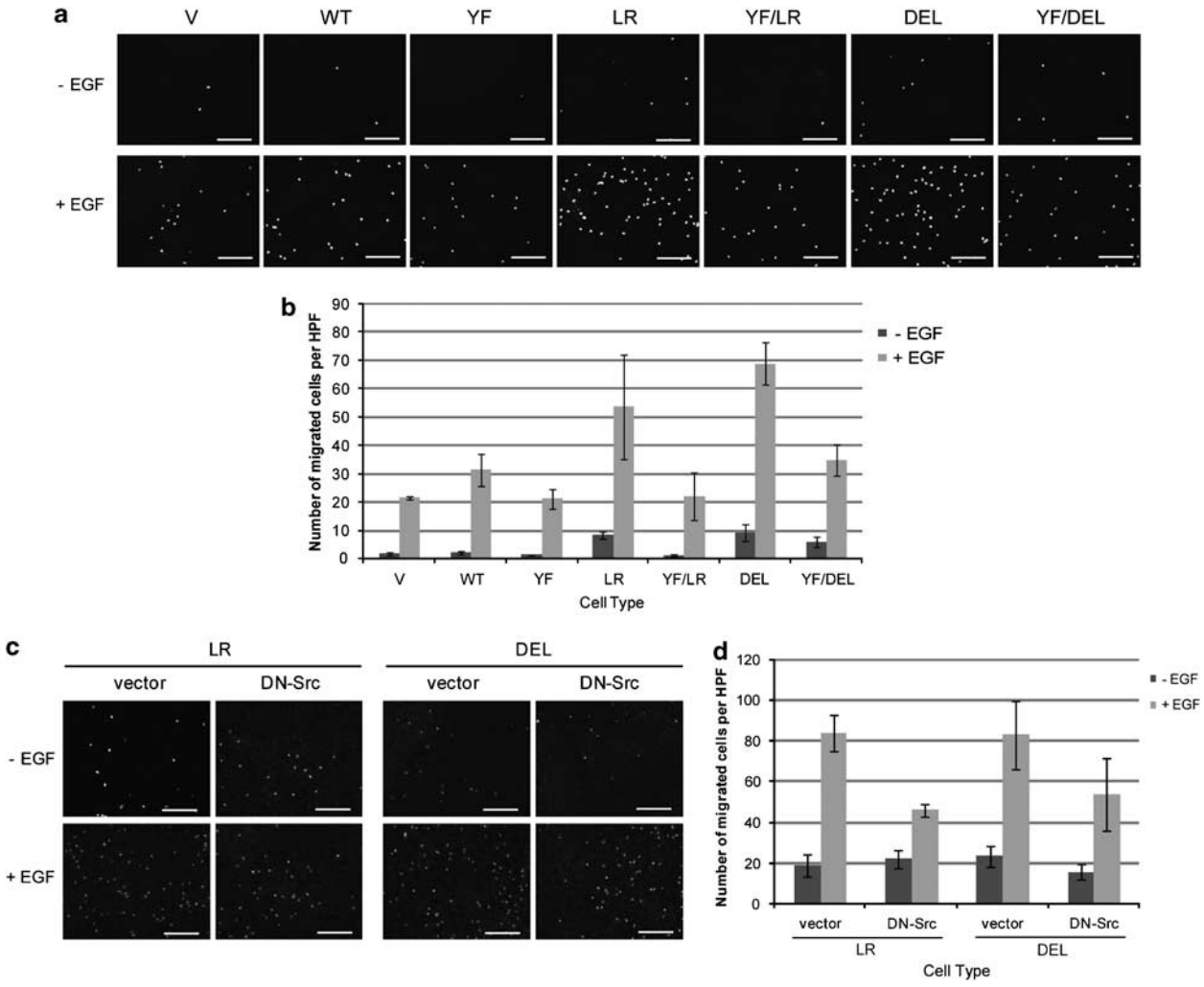

Figure 6 Src and its phosphorylation on epidermal growth factor receptor (EGFR) Y845 are critical for mutant EGFR-mediated cell migration. Transwell migration was carried out on cells expressing the indicated EGFRs (**a**) or indicated Src (**c**) with or without 10 ng/ml EGF on the bottom chamber. After 16 h, cells were fixed, their nuclei stained with propidium iodide and pictures were taken with a fluorescence microscope. Each condition was prepared in triplicate with four high power fields per replicate. Bar represents 200 μm. (**b**) The number of cells migrated from (**a**) or (**d**) number of cells migrated from (**c**) were quantified using the ImageJ software. Data points represent the average number of migrated cells in four high power fields (HPF). Data were expressed as number of migrated cells per HPF (mean ± s.d.). Analysis of Variance performed on the number of migrated cells yielded a statistically significant difference between cell types. V, vector; WT, wild-type (wt)EGFR; EGFR L858R (LR); YF, EGFR Y845F; EGFR Δ746–750 (DEL).

that phosphorylation of Y845 is quite likely to be predominantly Src-dependent. Different EGFR mutations may use these mechanisms (autophosphorylation versus Src-dependent phosphorylation) to different extents. It is also important to note that in addition to questions about available pY845 antibody, as mentioned above, the specificity of Src inhibitors has been questioned, as they seem to also inhibit other kinases, such as Abl (Golas *et al*., 2003; Tatton *et al*., 2003; Lombardo *et al*., 2004) and transforming growth factor-β receptors (Maeda *et al*., 2006). Therefore, our studies of the potential role of Src in the oncogenic transforming ability of NSCLC-associated EGFR mutants used genetic ablation of this potential interaction rather than the use of Src-inhibitors alone.

Earlier studies have established the cooperativity between EGFR and Src signaling pathways using overexpression of both tyrosine kinases in fibroblasts (Maa *et al*., 1995) and more recently in epithelial cells (Dimri *et al*., 2007). However, a specific role of Src in mediating oncogenic transformation by NSCLC-associated EGFR mutants has not been clearly established nor have the specific signaling pathways linked to mutant EGFR-Src interaction been delineated. In this regard, our studies provide new insights that clarify the importance of Src interaction with mutant EGFR in oncogenic transformation.

Our studies show that not only does the chemical inhibition of Src kinase activity inhibit oncogenic transformation driven by mutant EGFRs (Figure 3), but that specific mutation of a Src tyrosine kinase phosphorylation site on mutant EGFRs or the expression of DN-Src substantially reduced their transforming ability (Figure 5). Concomitant with the overall decrease in transformation, the Y845F mutation also reduced the ability of mutant

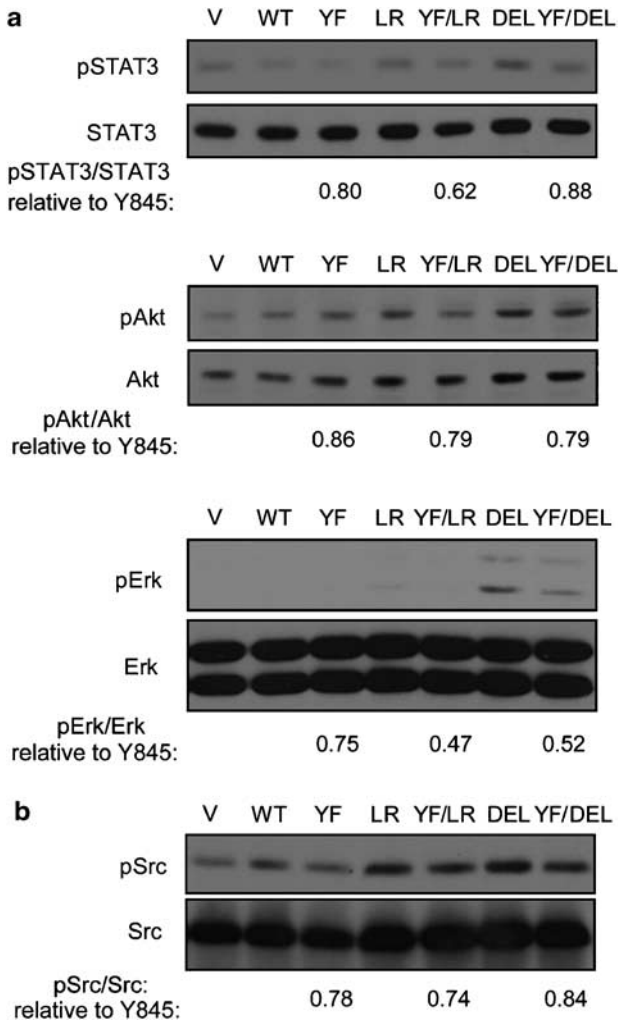

Figure 7 Y845 phosphorylation is key to constitutive downstream signaling by epidermal growth factor receptor mutants. NIH 3T3 cells were placed in media with 0.1% fetal bovine serum for 3 h. (**a**) 50 μg of whole cell lysates were used for immunoblotting with antibodies against the indicated proteins; and (**b**) 1 mg aliquots of cell lysates were immunoprecipitated with anti-Src (B-12) antibody followed by immunoblotting with antibodies against the indicated proteins. The levels of phosphorylated and total proteins were quantified by densitometry using the ImageJ software. Mean phosphorylated protein levels to the total levels of cells with Y845F mutation relative to that of corresponding cells without Y845F mutation from at least three experiments are noted. V, vector; WT, wild-type (wt)EGFR; YF, EGFR Y845F; EGFR L858R (LR); EGFR Δ746–750 (DEL).

EGFRs to drive cell proliferation and migration, important determinants of a transformed phenotype. Consistent with these findings, a recent study showed that an intact Y845 is important to maintain the overall phosphotyrosine level of mutant EGFRs (Fu *et al*., 2008).

The model system in our studies provides important insights into altered EGFR–Src interactions as a result of NSCLC-associated EGFR kinase domain mutations and the role of a Src phosphorylation site in EGFR as a critical determinant of mutant EGFR–Src interactions. Src was constitutively activated (Figure 1b) and constitutively enhanced, yet EGFR kinase activity-dependent, EGFR–Src interactions (Figure 2) were observed in mutant EGFR- but not wtEGFR-expressing cells. Although the precise mechanism of the enhanced interaction is not clear, several factors could account for this. As noted above, Src associates more stably with ErbB2 than with EGFR. It has been suggested that mutant EGFRs may dimerize with ErbB2 (Shtiegman *et al*., 2007). Mutant EGFRs also seem to acquire ErbB2-like properties, such as an association with molecular chaperone Hsp90 and sensitivity to Hsp90 inhibitors (Kim *et al*., 2005; Shimamura *et al*., 2005; Yang *et al*., 2006); this may account for a higher ErbB2-like affinity for Src.

Use of mutant EGFRs with Y845F mutation for parallel biological analyses and examination of downstream signaling targets also allowed us to determine the importance of the EGFR–Src interaction in activating key pathways important in cellular transformation and oncogene addiction. NSCLC-associated mutants enhance the activation of Erk, Akt and STAT3, and Akt and STAT pathways are critical for cell survival (Lynch *et al*., 2004; Sordella *et al*., 2004; Haura *et al*., 2005; Alvarez *et al*., 2006). Our results show that similar to mutant EGFR-expressing NSCLC cell lines, NIH 3T3 cells expressing mutant EGFRs exhibit a constitutive activation of major downstream targets of EGFR, including STAT3, Akt and Erk (Figure 1a).

Based on the effects of Y845F mutations in mutant EGFRs, together with EGFR and Src inhibitors on STAT3, Akt and Erk activity (Figure 7a and Supplementary Figure S2), it is reasonable to suggest that increased EGFR–Src interaction contributes to oncogenic signaling by linking mutant EGFRs to downstream signaling pathways. Consistent with this suggestion, activation of STATs has been reported as a key mediator of wtEGFR and Src synergism, and STATs are required for oncogenic transformation by Src (Turkson *et al*., 1998; Kazansky and Rosen, 2001; Kloth *et al*., 2003). EGFR–Src interaction has also been linked to an enhanced Akt activation (Dimri *et al*., 2007).

Also rather unexpectedly, we observed that Y845F mutation of NSCLC-associated EGFR mutants decreased the pool of activated Src (Figure 7b) and reduced the level of EGFR–Src association (Figure 8). Unlike the requirement of Y845 in EGFR signaling and mitogenesis (Bromann *et al*., 2004), the role of this phosphorylation site in EGFR–Src association is not known. It is likely that reduced kinase activity of Y845F mutants is in part responsible for reduced Src phosphorylation and EGFR–Src association. It is likely that the cooperative activation of these two tyrosine kinases involves a complex mechanism that requires further investigation.

Overall, our data suggests that Src interaction and phosphorylation of mutant EGFRs at Y845 play a significant role in mutant EGFR-driven and Src-dependent activation of downstream signaling pathways and in mutant EGFR-driven oncogenesis. Further studies to elucidate the mechanism of interplay between mutant EGFRs and Src should help design more effective ways to treat mutant EGFR-dependent NSCLC.

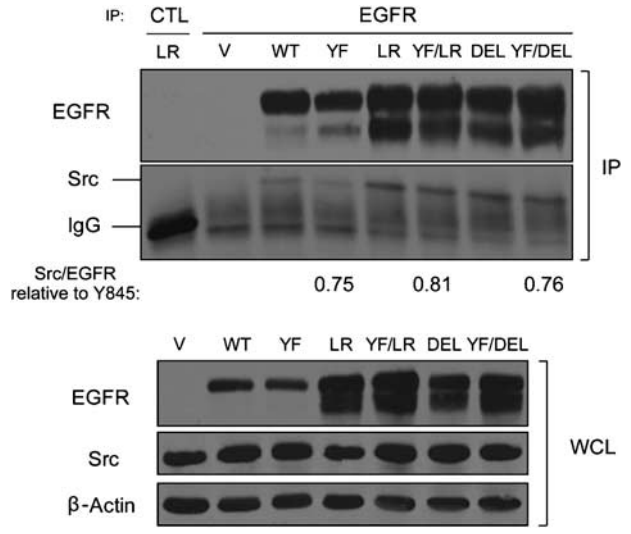

Figure 8 Y845F mutation reduces Src interaction with non-small cell lung cancer-associated epidermal growth factor receptor (EGFR) mutants. NIH 3T3 cells expressing the indicated EGFRs were grown in complete growth medium. 1 mg aliquots of cell lysates were immunoprecipitated (IP) with anti-EGFR (528) antibody or control (CTL) antibody or 50 μg of whole cell lysates (WCL) were resolved directly followed by immunoblotting with antibodies against the indicated proteins. Co-immunoprecipitated levels of Src relative to immunoprecipitated EGFRs (Src/EGFR) were determined by densitometry using the ImageJ software. Mean Src/EGFRs of cells with Y845F mutation relative to that of corresponding cells without Y845F mutation from at least three experiments are noted. V, vector; WT, wild-type (wt)EGFR; YF, EGFR Y845F; EGFR L858R (LR); EGFR Δ746–750 (DEL); IgG, immunoglobulin G.

Materials and methods

Retroviral constructs
The retroviral expression vectors pMSCV-puro-EGFR and pMSCV-hygro-Src K297R have been described earlier (Dimri *et al*., 2007). EGFR mutants and dominant-negative Src (K297R/Y529F) were generated using the QuikChange II XL Site-Directed Mutagenesis Kit (Strategene, La Jolla, CA, USA) following the manufacturer's instructions; the primers used are shown in Supplementary Table S1. All constructs were verified by DNA sequencing.

Cell culture and retroviral infections
NIH 3T3 cells (ATCC, Manassas, VA, USA) were grown in Alpha Modified Eagle Medium (Invitrogen, Carlsbad, CA, USA) containing 10% fetal bovine serum (FBS, Hyclone Inc., Logan, UT, USA), 20 mM HEPES (4-(2-hydroxyethyl)piperazine-1-ethanesulfonic acid), pH 7.35, 1 mM sodium pyruvate, 1 mM each of non-essential 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. Retroviral supernatants were generated and infected as described earlier (Reddi *et al*., 2007).

Antibodies and other reagents
The following antibodies were obtained from commercial sources: rabbit polyclonal (pAb) anti-EGFR (1005), pAb anti-Src (SRC 2), pAb anti-phosphorylated AKT (pAKT1/2/3) (Ser 473), pAb anti-pErk 1/2 (Thr 202/Tyr 204), pAb anti-Erk1 (K-23), mouse monoclonal (mAb) anti-AKT (B-1), mAb anti-Syk (4D10) and mAb anti-Src (B-12) were from Santa Cruz Biotechnology (Santa Cruz, CA, USA); pAb anti-phospho-EGFR (Tyr1068), pAb anti-phospho-EGFR (Tyr845), pAb anti-phospho-Src (Tyr416), pAb anti-STAT3 and Rabbit monoclonal anti-phospho-STAT3 (Tyr705) were from Cell Signaling Technology (Danvers, MA, USA); mAb anti-β actin (Clone AC-15) was from Sigma-Aldrich (St Louis, MO, USA); mAb anti-EGFR (clone 528; ATCC) was protein G purified from hybridoma supernatants. Purified anti-phosphotyrosine (PY) mAb 4G10 was provided by Dr Brian Druker (Oregon Health Science University, Portland, OR, USA). Purified mouse EGF was from Sigma-Aldrich. EGFR inhibitor, erlotinib (Tarceva), was obtained from the Hospital Pharmacy. Src inhibitor PP2 was from Calbiochem (San Diego, CA, USA).

Preparation of cell lysates, SDS–polyacrylamide gel electrophoresis and immunoblotting
Cells at 80–90% confluence were incubated in the 10 or 0.1% FBS-containing medium for 3 h. For EGF stimulation, cells earlier incubated for 3 h in 0.1% FBS-containing medium were either left unstimulated or stimulated with EGF (10 ng/ml) for 10 min. Cell lysates were prepared in cold lysis buffer as described earlier (Dimri *et al*., 2007). SDS–polyacrylamide gel electrophoresis and immunoblotting was carried out as described earlier (Reddi *et al*., 2007).

Immunoprecipitation
Cells were grown, EGF stimulation carried out and cell lysates prepared as above with the exception that the lysis buffer contained 0.25% NP-40 (instead of Triton X-100), 50 mM Tris (pH 8.0) and 100 mM NaCl. Cell lysate aliquots were incubated with anti-EGFR (528) or anti-Src (B-12) antibody and anti-Syk antibody as a control, and immune complexes captured using the Protein A-Sepharose beads (GE Healthcare, Piscataway, NJ, USA). Subsequent SDS–polyacrylamide gel electrophoresis and immunoblotting were carried out as above.

Anchorage-independent growth in soft agar
Soft agar colony growth assay was carried out as described earlier (Dimri *et al*., 2007). Phase-contrast images were processed using the ImageJ software (http://rsb.info.nih.gov/ij) to quantify colony areas. All conditions were performed in triplicates and four high power fields were imaged per replicate.

MTT assay
In all, 1000 cells were plated per well of 96-well plate at day 0. Absorbance at 570 nm after adding the MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) dye was measured at day 1, 3 and 5 using the Vybrant MTT Cell Proliferation Assay Kit (Molecular Probes, Eugene, OR, USA).

Transwell cell migration assay
The Transwell chambers with 8 μm pores (VWR, West Chester, PA, USA) were coated overnight at 4 °C with 10 μg/ml fibronectin (Sigma-Aldrich) followed by blocking with 0.1% FBS-containing starvation medium for 1 h at 4 °C. The cells (2.5×10^4) were serum deprived for 4 h in starvation medium, and plated in the top chamber for 3 h to allow attachment. Starvation medium, with or without 10 ng/ml EGF, was then added in the bottom chamber. After 16 h, cells were washed, fixed in methanol at −20 °C and stained with propidium iodide. Cotton swab was used to remove cells from the top chamber. Fluorescence images of migrated cells were processed using the ImageJ software to quantify the number of cells

migrated. All conditions were carried out in triplicates and four high power fields were imaged per replicate.

Conflict of interest

The authors declare no conflict of interest.

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Supplementary Information accompanies the paper on the Oncogene website (http://www.nature.com/onc)