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Yes and Lyn play a role in nuclear translocation of the Epidermal Growth Factor Receptor

Mari Iida*, Toni M. Brand*, David A Campbell, Chunrong Li, and Deric L. Wheeler¹

Department of Human Oncology, University of Wisconsin School of Medicine and Public Health, 1111 Highland Ave., WIMR 3159, Madison, Wisconsin, 53705 USA

Abstract

The epidermal growth factor receptor (EGFR) is a central regulator of tumor progression in human cancers. Cetuximab is an anti-EGFR antibody that has been approved for use in oncology. Previously we investigated mechanisms of resistance to cetuximab using a model derived from the non-small cell lung cancer line NCI-H226. We demonstrated that cetuximab-resistant clones (Ctx^R) had increased nuclear localization of the EGFR. This process was mediated by Src family kinases (SFK), and nuclear EGFR played a role in resistance to cetuximab. To better understand SFK mediated nuclear translocation of EGFR, we investigated which SFK member(s) controlled this process as well as the EGFR tyrosine residues that are involved. Analyses of mRNA and protein expression indicated up-regulation of the SFK members Yes and Lyn in all Ctx^R clones. Further, immunoprecipitation analysis revealed that EGFR interacts with Yes and Lyn in Ctx^R clones, but not in cetuximab-sensitive (Ctx^S) parental cells. Using RNAi interference, we found that knockdown of either Yes or Lyn led to loss of EGFR translocation to the nucleus. Conversely, overexpression of Yes or Lyn in low nuclear EGFR expressing Ctx^S parental cells led to increased nuclear EGFR. Chromatin immunoprecipitation (ChIP) assays confirmed nuclear EGFR complexes associated with the promoter of the known EGFR target genes B-Myb and iNOS. Further, all Ctx^R clones exhibited up-regulation of B-Myb and iNOS at the mRNA and protein levels. siRNAs directed at Yes or Lyn led to decreased binding of EGFR complexes to the B-Myb and iNOS promoters based on ChIP analyses. SFKs have been shown to phosphorylate EGFR on tyrosines 845 and 1101 (Y845 and Y1101) and mutation of Y1101, but not Y845, impaired nuclear entry of the EGFR. Taken together, our findings demonstrate that Yes and Lyn phosphorylate EGFR at Y1101 which influences EGFR nuclear translocation in this model of cetuximab resistance.

Keywords

nuclear EGFR; SFK; Yes; Lyn

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¹To whom requests for reprints should be addressed: Deric L. Wheeler PhD, Department of Human Oncology, University of Wisconsin Comprehensive Cancer Center, 1111 Highland Avenue, WIMR 3159, Madison, Wisconsin 53705. Phone: (608) 262-7837; fax: (608) 263-9947; dlwheeler@wisc.edu.

*These authors contributed equally to this work.

Conflict of interest

The authors declare no conflict of interest.

INTRODUCTION

Activation of the epidermal growth factor receptor (EGFR), a receptor tyrosine kinase (RTK), provides cells with potent growth and survival signals that enable tumors to manifest (1–3). Aberrant expression or activity of the EGFR is identified as a major etiological factor in many human epithelial cancers including colorectal cancer (CRC), head and neck squamous cell carcinoma (HNSCC), non-small cell lung cancer (NSCLC) and brain cancer (2, 4, 5). In the classical EGFR signaling pathway ligand binding to the EGFR allows for receptor homo- or hetero-dimerization at the plasma membrane. This interaction activates each receptor's tyrosine kinase domain and induces autophosphorylation of each dimer's cytoplasmic tail. The phosphorylated cytoplasmic tail of the EGFR serves as docking sites for numerous proteins that initiates key oncogenic pathways including the RAS/RAF/MEK/ERK and phosphatidylinositol 3-kinase (PI3K)-Akt pathways; however, the activation of src family tyrosine kinases (SFKs), Phospholipase C-gamma (PLC), Protein kinase C (PKC) and Signal Transducers and Activators of Transcription (STAT) proteins have also been documented (1, 6).

In addition to the classical signaling pathways initiated by the EGFR at the cell surface, there is now an emerging novel signaling pathway influenced by EGFR located in the nucleus. The full-length EGFR can be shuttled from the plasma membrane to the nucleus in a series of well-defined steps (7–9). These events include receptor internalization to the early endosome and interaction with importin β 1 via its tripartite nuclear localization sequence (NLS), followed by COPI-mediated retrograde trafficking to the Golgi apparatus and the endoplasmic reticulum (ER) (10, 11). Once in the ER the EGFR-importin β 1 complex moves to the outer nuclear membrane where importin β 1 interacts with nucleoporin 62 lining the nuclear pore channel to shuttle the EGFR-importin β 1 complex to the inner nuclear membrane. Here the complex interacts with the Sec61 β translocon to be released from the membrane into the nucleus (12, 13).

Within the nucleus, EGFR serves as a transcriptional co-activator for a series of tumor promoting genes including cyclin D1, inducible nitric oxide synthase (iNOS), Aurora Kinase A, B-Myb, COX2, c-Myc, Breast Cancer Related Protein (BCRP) and GRP78 (14–21). Additionally, nuclear EGFR can phosphorylate and stabilize the proliferating cell nuclear antigen (PCNA) at the replication fork of the dividing cell (22), and activate DNA-PK to enhance DNA repair (23).

High levels of nuclear EGFR correlate with poor clinical outcome in breast cancer, oropharyngeal squamous cell cancer, ovarian cancer, and gallbladder cancer (24–28). Nuclear EGFR also contributes to cancer cells resistance to cetuximab (29), gefitinib (20), cisplatin and radiation therapy (30–33). Taken together these pieces of evidence suggest that nuclear EGFR plays a role in the promotion of cancer and provides a rationale for studying the mechanisms of EGFR nuclear translocation in order to target the nuclear functions of the EGFR.

It is well established that SFKs are necessary for full activation of the EGFR (34, 35). Src kinase is the prototype member of this family of non-RTKs that include Yes, Fyn, Lyn, Lck, Hck Fgr, Blk and Yrk (36, 37). These SFKs mediate mitogenic signals from a variety of RTKs (38, 39). It has been observed that SFKs can phosphorylate EGFR at both tyrosine 845 (Y845) and tyrosine 1101 (Y1101). EGFR Y845 is located in the activation loop of the kinase domain that is highly conserved among other RTKs. Phosphorylation of EGFR Y845 appears to be critical for EGFR-mediated mitogenesis, and is critical for the phosphorylation and activation of the STAT5b transcription factor (34, 40, 41). The second known Src-mediated phosphorylation site is Y1101, which lies within the carboxyl-terminal region of the EGFR; however the function of Y1101 has not been fully elucidated (34). Oncogenic cooperation between Src and EGFR has been well established in breast cancer (34, 42), glioblastoma (43), HNSCC and NSCLC (44–47).

We established six clonal Ctx^R variants of the NCI-H226 NSCLC line (29, 48, 49). In previous reports we found that Ctx^R clones had a dramatic increase in nuclear EGFR localization, in addition to having increased SFK activity (29, 44). Further, we reported that the SFK inhibitor dasatinib (BMS-354825, SpryzelTM) could 1) block SFK activation, 2) decrease nuclear EGFR translocation, 3) increase plasma membrane levels of the EGFR, and 4) re-sensitize Ctx^R cell lines to cetuximab. Collectively these findings suggest that SFKs play a crucial role in nuclear translocation of the EGFR in this model of cetuximab-resistance. However, the specific SFKs involved in the mediation of EGFR nuclear translocation and how they mediate this process are unknown.

In the current study we demonstrate that Ctx^R clones had increased expression of the SFKs Yes and Lyn. Both Yes and Lyn were strongly associated with EGFR in Ctx^R clones as compared to the Ctx^S parental cell line. Depletion of either Yes or Lyn kinase decreased EGFR nuclear translocation, and reduced phosphorylation at Y845 and Y1101 of the EGFR. Reciprocally, overexpression of Yes or Lyn increased EGFR nuclear translocation in Ctx^S parental cell line. Furthermore, mutation of Y1101 of the EGFR impaired its nuclear translocation. Collectively these data suggest that Yes, Lyn and Y1101 of the EGFR are involved in EGFR nuclear translocation in this model of acquired resistance to cetuximab.

RESULTS

The SFK inhibitor Dasatinib blocks nuclear translocation of the EGFR

We have previously reported that Ctx^R clones have increased nuclear EGFR and activation of SFKs (Figure 1A, 1B) (29, 48). Using this model, we determined the effects of the SFK inhibitor dasatinib on the phosphorylation status of the EGFR in three Ctx^R clones (HC1, HC4 and HC8) and the Ctx^S parental clone (HP) after treatment with 100 nM of dasatinib for 24 hours. Dasatinib inhibited the full activation of SFKs as indicated by the loss of phospho-Y419 and decreased the phosphorylation of EGFR at the known SFK phosphorylation sites Y845 and Y1101 (Figure 1B). Also, treatment with dasatinib led to modest increases in steady state expression of total SFKs in all Ctx^R clones.

To determine the effects of dasatinib treatment on nuclear translocation of EGFR, we treated the Ctx^R clones and the Ctx^S parental clone with 100 nM of dasatinib for 24 hours followed

by nuclear fractionation. As illustrated in Figure 1C, dasatinib treatment reduced EGFR nuclear translocation in Ctx^R clones. The Ctx^S parental clone has very low levels of nuclear EGFR and dasatinib treatment had no effect. Thus, the inhibition of SFK activity decreased the phosphorylation of EGFR at Y845 and Y1101 as well as impaired nuclear entry of the EGFR. These results suggested that SFK phosphorylation of EGFR may play a role in inducing its nuclear translocation.

Yes and Lyn are overexpressed and associate with the EGFR in Ctx^R clones

Based on our previous findings with clonal Ctx^R variants of the NCI-H226 NSCLC line we hypothesized that SFK member(s) may regulate EGFR nuclear translocation. To identify the specific SFKs that are necessary for EGFR nuclear translocation, we performed microarray analysis comparing Ctx^S HP parental cells to the three Ctx^R clones (HC1, HC4 and HC8). Microarray analysis demonstrated a ~4-fold up-regulation of Yes and Lyn kinases and ~3-fold down-regulation of Src kinase in all of three Ctx^R cells (data not shown). Other SFK family members did not exhibit significant expression level changes in the three Ctx^R clones (HC1, HC4 and HC8) compared to sensitive parental line (HP).

To validate the microarray findings we performed qPCR analysis. These results confirmed the microarray data indicating up-regulation of Lyn (~ 3-fold) and Yes (~1.5 fold), and down-regulation of Src kinase (~2-fold) in all three Ctx^R clones (Figure 2A). Next, we examined if these increased mRNA levels reflected total protein levels in Ctx^R when compared to Ctx^S cells. We found that total protein levels of Yes and Lyn were increased 1.3- to 2.1-fold in Ctx^R clones compared to parental cells (Figure 2B). Finally, we investigated whether EGFR associated with Yes and Lyn. Immunoprecipitation analysis of EGFR binding partners indicated that EGFR displayed increased association to Yes and Lyn in all three Ctx^R clones as compared to the Ctx^S HP cell line (Figure 2C). Collectively, these results indicate that Yes and Lyn are up-regulated in Ctx^R clones and have increased association with the EGFR.

Yes and Lyn are necessary for nuclear translocation of EGFR in Ctx^R cells

To further investigate if Yes and/or Lyn expression altered EGFR activation and nuclear translocation we performed gene-silencing experiments using siRNA directed against Yes or Lyn in Ctx^R clones (HC1, HC4 and HC8). Ctx^S cells were not included in these siRNA studies because the cells have negligible levels of nuclear EGFR. After treatment with siYES or siLYN in Ctx^R clones for 72 hours we observed decreased phosphorylation of EGFR Y845 (70–99%) and EGFR Y1101 (40–85%) relative to control non-targeting siRNA (NT) (Figure 3A). Moreover, siYES and siLYN decreased the nuclear localization of EGFR in Ctx^R clones (Figure 3B).

Knockdown studies of Yes or Lyn in cells with high nuclear EGFR expression led to decreased nuclear EGFR levels. Therefore, we hypothesized that the overexpression of Yes or Lyn could increase the level of nuclear EGFR in a cell line with low levels of EGFR in the nucleus. To test this hypothesis we overexpressed Yes or Lyn in Ctx^S cells, which express low-levels of nuclear EGFR. First, Yes and Lyn were cloned into mammalian expression vectors, expressed in CHO-K1 cells and characterized for increased total Yes and

Lyn protein expression and activity (Figure 4A). Comparable increases in Yes and Lyn expression and activation were observed in Ctx^S cells after transfection compared to vector only (Figure 4B). Consistent with siRNA observations, transient transfection of Yes or Lyn into Ctx^S cells significantly increased (3~3.5 fold) nuclear EGFR translocation (Figure 4C). Interestingly, Yes and Lyn were also detected in nucleus of HP cells after transfection. To determine if Ctx^R cells with increased of nuclear EGFR also express more Yes and Lyn in the nucleus compared to Ctx^S cells, we determined nuclear Yes and Lyn levels in Ctx^R clones and Ctx^S cells. As seen with EGFR, increased levels of both Yes and Lyn were found in the nucleus of Ctx^R clones compared to Ctx^S cells (Figure 4D). These siRNA and overexpression results suggest that Yes and Lyn play a role in EGFR nuclear translocation.

Depletion of Yes or Lyn decreases binding of nuclear EGFR complexes to the B-Myb and iNOS promoter regions

Nuclear EGFR and various transcription factor complexes have been shown to bind promoter regions and regulate the transcription of multiple genes including cyclin D1, iNOS, B-Myb, Aurora Kinase A, COX2, c-Myc, BCRP and GRP78 (14–21). To confirm that nuclear EGFR complexes in Ctx^R clones bound to known EGFR target gene promoters we performed Chromatin immunoprecipitation-quantitative PCR (ChIP-qPCR) analysis of the B-Myb and iNOS promoters. We demonstrated that Ctx^R clones have increased EGFR association with the B-Myb (3–9-fold increase in EGFR binding) and iNOS (6–12-fold increase in EGFR binding) promoter regions as compared to Ctx^S parental cell line (Figure 5A). These results indicate that nuclear EGFR complexes bind known EGFR target promoters in Ctx^R clones more strongly than in Ctx^S HP cells.

Given the high binding of nuclear EGFR complexes to the B-Myb and iNOS promoters we performed qPCR to determine whether this binding resulted in increased expression of B-Myb and iNOS genes as previously reported (15, 16). Results in Figure 5B indicated that B-Myb mRNA expression was increased approximately 4-fold in all Ctx^R clones as compared to Ctx^S parental cells. Whereas, iNOS mRNA expression was increased 4–11-fold in Ctx^R clones when compared to Ctx^S parental cells. Furthermore, B-Myb protein expression was up-regulated approximately 3-fold and iNOS protein expression was up-regulated 2–14-fold in Ctx^R clones (Figure 5C). Using the Ctx^R HC4 clone we demonstrate that silencing of Yes or Lyn using siRNA reduced nuclear EGFR complex formation with the B-Myb and iNOS promoters as detected by ChIP-qPCR (Figure 5D). Additionally, the protein expression of both B-Myb and iNOS were decreased (60–70 %) after siYES or siLYN transfection compared to control non-targeting siRNA in HC4 (Figure 5D). Collectively, these data demonstrate that Ctx^R clones with high levels of nuclear EGFR associate more strongly with known EGFR regulated promoter regions, and that these association (demonstrated with B-Myb and iNOS) can be prevented upon depletion of Yes or Lyn.

EGFR Y1101 is involved in nuclear translocation

Analysis of known SFK phosphorylation sites on the EGFR showed increased phosphorylation of Y845 and Y1101 in Ctx^R clones (Figure 1B). However, the relevance of these two tyrosine sites for EGFR nuclear translocation is unknown. To determine if phosphorylation of one or both of these tyrosine residues is involved in EGFR nuclear

translocation, MCF-7 breast cancer cells (which express very low EGFR levels) were transiently transfected with cDNAs encoding wild-type EGFR (WT) or the following EGFR mutants: EGFR-Y845F or EGFR-Y1101F. Immunoprecipitation of WT and mutant EGFR followed by immunoblotting with antibodies directed against pEGFR^{Y845} or pEGFR^{Y1101} showed reduced phosphorylation of Y845 and Y1101. (Figure 6A). To induce nuclear translocation of the EGFR we treated the transfected cells with EGF for 45 minutes prior to cell lysis and nuclear fractionation. The results of this experiment indicated that EGF was able to induce nuclear translocation of EGFR in both EGFR-WT and EGFR-Y845F mutant cells (Figure 6B). However, EGF-induced EGFR nuclear translocation was diminished ~70% in EGFR-Y1101F mutant cells as compared to EGFR-WT expressing cells (Figure 6B). Furthermore qPCR analysis revealed that B-Myb and iNOS mRNA expression were downregulated in cells transfected with EGFR-Y1101F mutant compared to EGFR-WT transfected cells (Figure 6C). Collectively, these data suggest that the phosphorylation of Y1101 is important for the nuclear translocation of EGFR while the phosphorylation of Y845 does not appear to be essential for this process.

Discussion

The nuclear localization of receptor tyrosine kinases (RTKs) have been observed for over 20 years, however only in the last 10 years has research begun to focus on how they translocate from the cell surface to the nucleus and what functions they perform there. All four HER family members have been identified in the nucleus of various types of human cancer cells and tumor specimens (9, 50–53). Currently, eight target genes of nuclear EGFR have been identified (14–21), and nuclear EGFR has been correlated with resistance to cetuximab, radiation, cisplatin and gefitinib therapies (20, 23, 29–33). Collectively, these results suggest an emerging role of the nuclear EGFR signaling network in cancer progression and response to therapeutic modalities.

Several studies have examined how EGFR moves from the plasma membrane to the nucleus of the cell. It has been shown that the full-length EGFR can be shuttled from the plasma membrane to the nucleus through associations with importin β 1, the nuclear pore complex, and the Sec61 β translocon (7, 10, 13). Despite this mechanism of EGFR nuclear translocation, the early events at the plasma membrane that may serve as critical initiating signals for the movement of the EGFR to the nucleus have yet to be defined and form the basis of the current study.

To further elucidate the molecular requirements for EGFR nuclear transport we utilized a previously established model of acquired resistance to cetuximab in the NCI-H226 NSCLC cell line (48). In this model, cetuximab-resistant cells were observed to have increased levels of nuclear EGFR as compared to their cetuximab-sensitive parental cells, making it an ideal model for investigating events involved in nuclear translocation of the EGFR (29). Additionally, cetuximab-resistant cell lines were shown to have increased expression and activity of SFKs (44). Further investigation using dasatinib, an inhibitor of SFKs, demonstrated that SFK activity was necessary for the nuclear transport of EGFR in this model of cetuximab-resistance (29). In the current study, we identified Yes and Lyn to have increased expression and association with the EGFR (Figure 2). This result is consistent

with other reports identifying Yes and Lyn interaction and activation of the EGFR (54–57). In addition, loss of Yes and Lyn expression using siRNA technology, led to reduced phosphorylation of Y845 and Y1101 of the EGFR and more importantly impaired nuclear EGFR accumulation (Figure 3). Consistent with this observation, overexpression of Yes and Lyn in Ctx^S cells that express low-levels of nuclear EGFR significantly increased (3~3.5 fold) nuclear EGFR translocation (Figure 4B). ChIP assays demonstrated that nuclear EGFR complexes bind to B-Myb and iNOS promoter regions and siYES and siLYN decreased binding to these promoters (Figure 5). Mutagenesis studies of Y845 and Y1101 indicated that Y1101, not Y845, might be necessary for nuclear translocation of the EGFR from the membrane to the nucleus (Figure 6). Recently, Jaganathan et al. reported that Src and EGFR associate in the nucleus with the transcription factor STAT3 to regulate the expression of the c-Myc gene in pancreatic cancer (19). Consistent with this report, we found that Yes or Lyn not only increased the levels of nuclear EGFR but also had increased nuclear localization themselves, suggesting that they may have been imported into the nucleus with the EGFR. Collectively, these studies provide evidence for the role of SFKs in mediating nuclear translocation of the EGFR. However, it remains to be investigated whether Yes or Lyn are solely responsible for this nuclear translocation, or if SFKs exhibit a functional redundancy where the overexpression of one or more SFK may result in the induction of nuclear EGFR in various cancers.

Y845 and Y1101 of the EGFR are phosphorylated by SFKs (34). Biscardi et al. utilized a GST bound SH2 domain of the c-Src protein to demonstrate its specific binding to the EGFR via affinity chromatography (34). Subsequently, these investigators identified and validated that EGFR was indeed phosphorylated by c-Src at Y845 and Y1101. Breast cancer cell lines with high levels of Src activity also had increased levels of phospho Y845 and Y1101 of the EGFR. Researchers further showed that phospho-Y845 was necessary for full EGFR activation and EGF-induced DNA synthesis (34). This study represented a landmark finding by identifying novel Src phosphorylation sites on the EGFR and the role of tyrosine 845 in the complete activation of the EGFR. Further studies looking at the function of EGFR Y845 demonstrated that Y845 mediated EGFR binding to the mitochondrial protein cytochrome c oxidase subunit II at the mitochondria; however, EGFR Y845 was not necessary for its movement to the mitochondria (58). These findings support our data that EGFR Y845 may not be required for the intracellular trafficking of the EGFR.

In the current study, we corroborate findings of Biscardi et al. by showing that Y845 and Y1101 are Src specific phosphorylation sites through the use of the SFK inhibitor dasatinib (Figure 1B). In addition, siRNA directed towards Yes and Lyn decreased the phosphorylation of EGFR Y845 and Y1101 (Figure 3A). Our data further suggests that Y1101, not Y845, may be a critical molecular determinant in the localization of nuclear EGFR as indicated by site-directed mutagenesis (Figure 6B). It should be noted, however, that mutation of Y1101 did not completely block translocation of the EGFR to the nucleus, suggesting that other post-translational modifications of the EGFR may be necessary. Recent evidence has identified another key phosphorylation site on the EGFR, serine 229 (S229), as being necessary for EGFR translocation to the nucleus (20). It was reported that the serine/threonine kinase AKT can influence the nuclear translocation of the EGFR by phosphorylating S229 on the EGFR in a model of gefitinib resistance. In this model,

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gefinitinib-resistant A431 cells have both increased AKT activity and increased nuclear EGFR as compared to gefinitinib-sensitive A431 cells. Using an antibody that recognizes the phosphorylated consensus motif of AKT substrates and subsequent mass spectrometry, Huang et al. revealed that EGFR was phosphorylated by AKT at S229. Inhibition of AKT kinase activity prevented this phosphorylation event, and decreased the nuclear transport of EGFR providing evidence for the role of alternative kinases and post-translational modifications of the EGFR that indeed affect its nuclear translocation. Collectively, these findings suggest that the phosphorylation of the EGFR on Y1101 by Yes and Lyn together with AKT phosphorylation of S229 may be critical molecular determinants that influence the nuclear localization of the EGFR.

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EGFR is tightly linked to the etiology of HNSCC, NSCLC, CRC, breast and brain cancers. Accordingly, five EGFR inhibitors, three tyrosine kinase inhibitors, and two monoclonal antibodies have been developed for clinical use to inhibit EGFR activation and downstream signaling. Despite the successes of these agents, many tumors do not respond to EGFR inhibition, or eventually become resistant to this therapeutic strategy. Accumulating evidence suggests that nuclear EGFR plays a role in resistance to radiation, cetuximab, cisplatin and gefinitinib therapies (20, 23, 29–33). The mechanisms for how nuclear EGFR leads to this resistance are not clear. However work from our laboratory suggests that nuclear translocation can protect EGFR from the inhibitory effects of cetuximab causing resistance to this therapy (29). The results presented in this study provide a potential mechanism for the key molecules involved in nuclear localization of the EGFR providing rational targets to prevent nuclear translocation and thus nuclear function of the EGFR.

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In summary, the data presented in the current study has identified the SFKs Yes and Lyn to play a crucial role in nuclear translocation of the EGFR in a model of cetuximab-resistance. In addition, the SFK phosphorylation site Y1101 of the EGFR appears to be involved in translocation of the EGFR from the plasma membrane to the nucleus. These findings are of instrumental value in understanding the molecular requirements for nuclear EGFR transport, and for potentially targeting nuclear EGFR in the future.

MATERIALS AND METHODS

Cell lines

The human NSCLC line NCI-H226, the human breast cancer line MCF-7 and Chinese hamster ovary K1 (CHO-K1) cells were purchased from ATCC (Manassas, VA, USA). The cells were maintained in 10% fetal bovine serum (FBS) in RPMI-1640 for H226, DMEM/F12K for MCF-7 and F12K for CHO-K1 (Mediatech Inc., Manassas, VA, USA) with 1% penicillin and streptomycin. The development of cells with acquired resistance to cetuximab has been previously described (48).

Plasmid constructs and transfection

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EGFR wild-type (WT), Y845F and Y1101F mutants, were kindly provided from Dr. Julie Boerner (Wayne State University School of Medicine, Karmanos Cancer Institute, MI). The presence of 845F and 1101F mutations were confirmed by DNA sequencing. For transient

transfections, MCF-7 cells were transfected with plasmid DNA for each construct or pcDNA3.1 vector using Lipofectamine LTX and Opti-MEM I (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's recommendations. Either 24 hr (for RNA) or 48 hr (for protein) after transfection, EGF (100 ng/ml, Millipore, Billerica, MA, USA) was added to the plates for 45 min. Cells were collected, isolated RNA or fractionated and screened for their EGFR expression levels by quantitative PCR (qPCR) or immunoblotting as described below. For siRNAs, Ctx^R cells (HC1, HC4 and HC8) were transiently transfected with siYES (ON-TARGETplus SMARTpool YES1: L-003184-00, Dharmacon, Lafayette, CO, USA) or siLYN (ON-TARGETplus SMARTpool LYN: L-003153-00) using Lipofectamine RNAiMAX according to the manufacturer's instructions (Invitrogen). The non-targeting siRNA (ON-TARGETplus Non-targeting Pool, D-001810-10) was obtained from Dharmacon as a control. Cells were then lysed for analysis of protein knockdown by immunoblotting 72hr after siRNA transfection. Wild-type human YES (source ID: 5260751) and LYN (source ID: 8992174) cDNAs were purchased from Open Biosystems (Lafayette, CO, USA) and cloned into the NOTI/PACI restriction sites of the pQCXIP expression vector (Clontech, Mountain View, CA, USA). YES-PQCXIP, LYN-PQCXIP, or PQCXIP vector were transiently transfected into CHO-K1 cells with using Lipofectamine 2000 (Invitrogen) according to the manufacturer's recommendations. Forty-eight hours after transfection, cells were collected and lysed. HP parental cells were transiently transfected with the same constructs using Lipofectamine LTX (Invitrogen) according to the manufacturer's recommendations. Forty-eight hours after transfection, cells were collected and fractionated for nuclear protein. Nuclear EGFR expression levels were then detected via immunoblot analysis.

Compounds

Dasatinib (BMS-354825, SprycelTM) was generously provided by Bristol-Myers Squibb (New York, NY, USA).

Antibodies

All antibodies were purchased from commercial sources as indicated below: EGFR, B-Myb, Actin, Histone H3, HRP-conjugated goat-anti-rabbit IgG, goat-anti-mouse IgG and donkey-anti-goat IgG were obtained from Santa Cruz Biotechnology Inc., (Santa Cruz, CA, USA). SFK, YES, LYN, pSFK (Y419) and normal mouse IgG were obtained from Cell Signaling Technology (Beverly, MA, USA). pEGFR (Y1101) was purchased by Abcam (Cambridge, MA, USA). Anti-mouse EGFR and pEGFR (Y845) were purchased from Invitrogen. Polyclonal iNOS was obtained from BD Biosciences (San Jose, CA, USA). α -tubulin was purchased from Calbiochem (San Diego, CA, USA).

Cellular fractionation and Immunoblotting analysis

Cells were swelled in cytoplasmic lysis buffer (20 mM HEPES, pH 7.0, 10 mM KCl, 2 mM MgCl₂, 0.5% NP40, 1 mM Na₃VO₄, 1 mM PMSF, 1mM BGP, 10 μ g/ml of leupeptin and aprotinin) for 10 min on ice and homogenized by 20–30 strokes in a tightly fitting Dounce homogenizer. The homogenate was centrifuged at 1,500 g for 5 min at 4°C to sediment the nuclei. The supernatant was then centrifuged at 15,000 g for 10 min at 4°C, and the resulting supernatant formed the non-nuclear fraction. The nuclear pellet was washed three times in

cytoplasmic lysis buffer and re-suspended in the same buffer containing 0.5 M NaCl to extract nuclear proteins. After sonication and vortex, the extracted sample was centrifuged at 15,000 g for 10 min at 4°C. Whole cell protein lysate was obtained by tween-20 lysis buffer (50 mM HEPES, pH 7.4, 150 mM NaCl, 0.1% Tween-20, 10% glycerol, 2.5 mM EGTA, 1 mM EDTA, 1 mM DTT, 1mM Na₃VO₄, 1 mM PMSF, 1mM BGP and 10 µg/ml of leupeptin and aprotinin). Samples were sonicated and then centrifuged at 15,000 g for 10 min at 4°C. Protein concentrations were determined by Bradford assay (Bio-Rad Laboratories, Hercules, CA). Equal amounts of protein were fractionated by SDS-PAGE, transferred to a PVDF membrane (Millipore), and analyzed by incubation with the appropriate primary antibody. Proteins were detected via incubation with HRP-conjugated secondary antibodies and ECL chemiluminescence detection system (GE Healthcare Life Sciences, Piscataway, NJ, USA), ECL Western Blotting Substrate (Promega Cooperation, Madison, WI, USA), SuperSignal* West Dura Extended Duration Chemiluminescent Substrate or SuperSignal* West Femto Maximum Sensitivity Chemiluminescent Substrate (Thermo Fisher Scientific, Waltham, MA, USA).

Immunoprecipitation

Cells were lysed with NP-40 lysis buffer (50 mM HEPES, pH 7.4, 150 mM NaCl, 1% NP-40, 0.5% Deoxycholic acid, 10% glycerol, 2.5 mM EGTA, 1 mM EDTA, 1 mM DTT, 1 mM PMSF, 1mM BGP and 10 µg/ml of leupeptin and aprotinin). Cell lysates containing 0.5 mg of protein were incubated overnight at 4°C with 1 µg of anti-mouse EGFR antibody (Invitrogen) or normal mouse IgG (Cell Signaling Technology). After adding 25 µl of protein A/G agarose beads (Santa Cruz), cell lysates were incubated for another 2 hours at 4°C. The immunoprecipitates were pelleted by centrifugation and washed several times with NP-40 lysis buffer. The captured immunocomplexes were then eluted by boiling the beads in 2x SDS sample buffer for 5 minutes and subjected to immunoblot analysis as described above.

Microarray analysis

Total RNAs extracted from HP, HC1, HC4 and HC8 using an RNeasy kit (Qiagen Inc., Valencia, CA, USA). Gene expression profiling using the HT-HG-U133 Human Genome Array (Affymetrix, Santa Clara, CA, USA) containing over 22,000 well characterized genes. After RMA normalization, data were analyzed using Partek Discovery Suite (St. Louis, MO, USA) and signature genes were genes increased or decreased > 2-fold expression levels in three Ctx^R clones (HC1, HC4 and HC8) compared to sensitive parental line (HP) with p value <0.05.

cDNA synthesis and quantitative PCR

cDNA from total RNA of HP, HC1, HC4 and HC8 were synthesized using SuperScript III First-Strand Synthesis System (Invitrogen). Quantitative PCR (qPCR) analysis was performed using a Bio-Rad iQ5 Real-Time PCR Detection System (Bio-Rad Laboratories) using the iQ Supermix as recommended by manufacturer. All reactions were performed in triplicate. The sequences of primer sets used for this analysis are as follows: Lyn-F: 5'-GGCTCCAGA AGCAATCAACT-3', Lyn-R: 5'-TCACGTGGCATTAGTTCTC-3'; Yes-F: 5'-CTAGTAACA AAGGGCCGAGTG-3', Yes-R: 5'-

ATCCTGTATCCTCGCTCCAC-3'; Src-F: 5'-GAGGAG CCCATTTACATCGT-3', Src-R: 5'-TGAGAAAGTCCAGCAAACCTCC-3', B-Myb-F: 5'-ATG TCCAGTGCCTGGAAGAC-3', B-Myb-R: 5'-AGATGAGGGTCCGAGATGTG-3'. iNOS-F: 5'-CCATAAGGCCAAAGGGATT-3', iNOS-R: 5'-ATCTGGAGGGGTAGGCTTGT-3'. Fold increases or decreases in gene expression were determined by quantitation of cDNA from target samples (HC1, HC4 and HC8) relative to a calibrator sample (HP). Human β -actin gene (F: 5'-CAGCCATGTACGTTGCTATCCAGG-3', R: 5'-AGGTCCAGA CGCAGGATGGCATG-3') was used as the endogenous control for normalization of initial RNA levels. To determine this normalized value, $2^{-\Delta\Delta C_t}$ values were compared between target and calibrator samples, where the change in crossing threshold (ΔC_t) = $C_{t_{\text{target gene}}} - C_{t_{\beta\text{-actin}}}$ and $\Delta\Delta C_t = C_{t_{\text{HC1, HC4 or HC8}}} - C_{t_{\text{HP}}}$.

Chromatin immunoprecipitation (ChIP) assay

Cells were fixed with formaldehyde at a final concentration of 1 % for 15 min at room temperature, stopped fixation by 1.25 M glycine for 5 min. Subsequently, cells were washed with ice-cold PBS and collected in the tube and centrifuge at 4°C for 5 min. The cell pellets were lysed in cell lysis buffer (5 mM HEPES, pH 8.0, 85 mM KCl, 0.5% NP-40 and 10 mM sodium pyrophosphate) by a Dounce homogenizer. After centrifuge, supernatant was removed, and the nuclei pellets were lysed in nuclei lysis buffer (Tris-HCl 50 mM, pH 8.1, 10 mM EDTA, 1% SDS and 10 mM sodium pyrophosphate). The lysate was sonicated on ice to shear DNA, and the supernatant was pre-cleared with protein A/G agarose beads (Santa Cruz) in dilution buffer (16.7 mM Tris-HCl, pH 8.1, 1.2 mM EDTA, 167 mM NaCl, 1.1% Triton X-100, 0.01% SDS and 10 mM sodium pyrophosphate) for 1h at 4°C. The pre-cleared lysates were immunoprecipitated by incubating with protein A/G beads containing 1 μ g of anti-EGFR antibody or IgG and rotated at 4°C for overnight. The beads were washed with wash buffer I (25 mM Tris-HCl, pH 8.0, 2 mM EDTA, 150 mM NaCl, 1% Triton X-100, 0.1% SDS and 10 mM sodium pyrophosphate), wash buffer II (25 mM Tris-HCl, pH 8.0, 2 mM EDTA, 500 mM NaCl, 1% Triton X-100, 0.1% SDS and 10 mM sodium pyrophosphate), wash buffer III (10 mM Tris-HCl, pH 8.0, 1 mM EDTA, 250 mM LiCl, 1% NP-40, 1% deoxycholic acid and 10 mM sodium pyrophosphate) and TE buffer (10 mM Tris-HCl, pH 8.0, 1 mM EDTA, 10 mM sodium pyrophosphate). The bound protein was eluted twice with elute buffer (100 mM NaHCO₃ and 1% SDS). Then, 5 M NaCl was added to the pooled eluent and incubated at 68°C overnight. The DNA was recovered and purified using DNA purification kit (Qiagen). The purified chromatin immunoprecipitated DNA was used as a template for the qPCR of the promoter regions using the following primer pairs: B-Myb-F: 5'-CTGGTCTTAGCTACCCGTGAG TTGA-3' and B-Myb-R: 5'-CAGGAGTATCCCACATAGCGAACAC-3' (15), iNOS-F: 5'-TGATGAACTGCCACCTTGGAC-3' and iNOS-R: 5'-TTCACCCAACCC ACCTCTTTC-3' (16). The qPCR program was: 95°C for 3min, followed by 40 cycles of 95°C for 15 s and 60°C for 30 s for B-Myb or 55°C for 30 s for iNOS. The qPCR was performed using the iQ5 Real-time PCR Detection system (Bio-Rad).

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The abbreviations used are

BCRP	breast cancer related protein
CRC	colorectal cancer
Ctx^R	cetuximab-resistant
Ctx^S	cetuximab-sensitive
DMSO	dimethyl sulfoxide
EGF	epidermal growth factor
EGFR	epidermal growth factor receptor
ER	endoplasmic reticulum
FBS	fetal bovine serum
FGFR	fibroblast growth factor receptor
HNSCC	head and neck squamous cell carcinoma
iNOS	inducible nitric oxide synthase
Lyn	v-yes-1 Yamaguchi sarcoma viral related oncogene homolog
mAb	monoclonal antibody
NSCLC	non-small cell lung cancer
PI3K	phosphatidylinositol 3-kinase
PKC	protein kinase C
PLCγ	phospholipase C-gamma
qPCR	quantitative PCR
PGFR	platelet derived growth factor
RTK	receptor tyrosine kinase
SFK	Src-family kinases
STAT	signal transducers and activators of transcription
Yes	v-Yes-1 yamaguchi sarcoma viral oncogene

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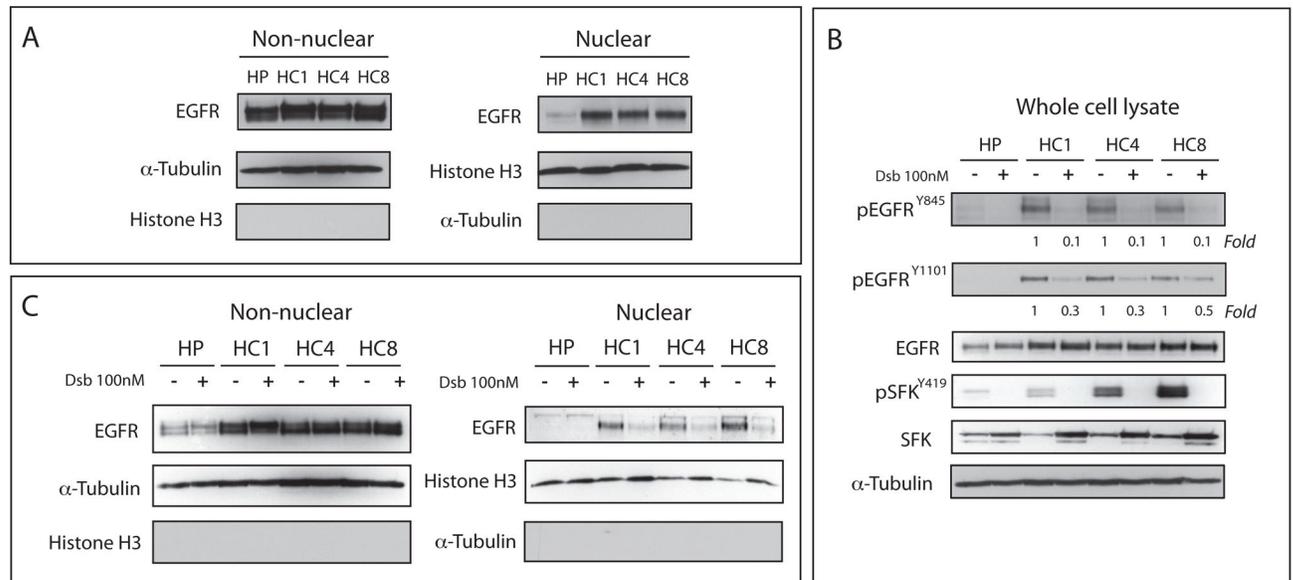


Figure 1. SFKs are essential for EGFR translocation to the nucleus

(A) Ctx^R clones (HC1, HC4 and HC8) have increased nuclear EGFR localization as compared to Ctx^S cell line (HP). Cells were harvested for non-nuclear and nuclear protein, and fractionated on SDS-PAGE followed by immunoblotting for indicated proteins. Histone H3 and α -tubulin were used as loading and purity controls for the nuclear and non-nuclear fractions, respectively. (B) Dasatinib decreased EGFR activity in Ctx^R cells. Ctx^R clones (HC1, HC4 and HC8) and Ctx^S HP cell line were treated with 100 nM dasatinib for 24 hr. Cells were harvested and protein lysates were fractionated on SDS-PAGE followed by immunoblotting for the indicated proteins. α -tubulin was used as a loading control. Expression was quantitated using ImageJ software. (C) Dasatinib can inhibit EGFR nuclear translocation in Ctx^R clones. Ctx^R clones (HC1, HC4, and HC8) and Ctx^S cell line were treated with 100 nM dasatinib for 24 hr. Cells were harvested for non-nuclear and nuclear protein, and fractionated on SDS-PAGE followed by immunoblotting for indicated proteins. Histone H3 and α -tubulin were used as loading and purity controls for the nuclear and non-nuclear fractions, respectively.

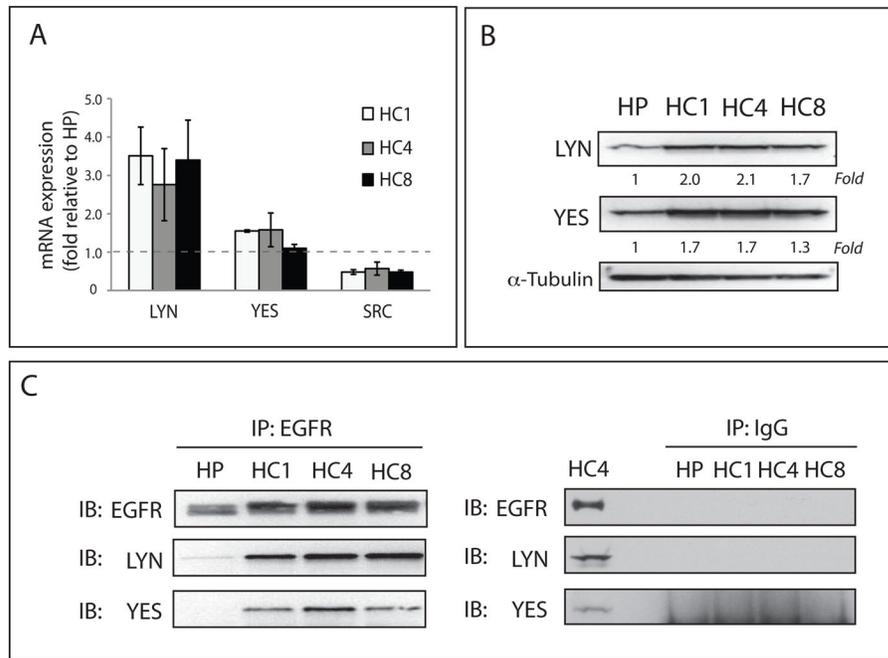


Figure 2. Yes and Lyn SFK family members are overexpressed in Ctx^R cells

(A) Yes and Lyn mRNA is up-regulated in Ctx^R clones (HC1, HC4, and HC8). Lyn (3.6~4.5-fold), Yes (~1.5-fold) and Src (~2.3~3.5-fold) mRNA expression levels were compared to that of the Ctx^S cell line (HP) by qPCR. (B) Total protein levels of Yes and Lyn were increased (1.3~2.1-fold) in Ctx^R clones (HC1, HC4, and HC8) as compared to the Ctx^S cell line (HP). Cells were harvested and protein lysates were fractionated on SDS-PAGE followed by immunoblotting for the indicated proteins. α -tubulin was used as a loading control. Protein expression was quantitated using ImageJ software. (C) Analysis of EGFR binding partners in Ctx^R cells using immunoprecipitation assay indicated that EGFR displayed increased binding with Yes and Lyn as compared to the Ctx^S parental cell line. Cells were harvested and EGFR or IgG were immunoprecipitated with anti-mouse EGFR antibody or normal mouse IgG. The immunoprecipitate complexes were fractionated on SDS-PAGE followed by immunoblotting for indicated proteins.

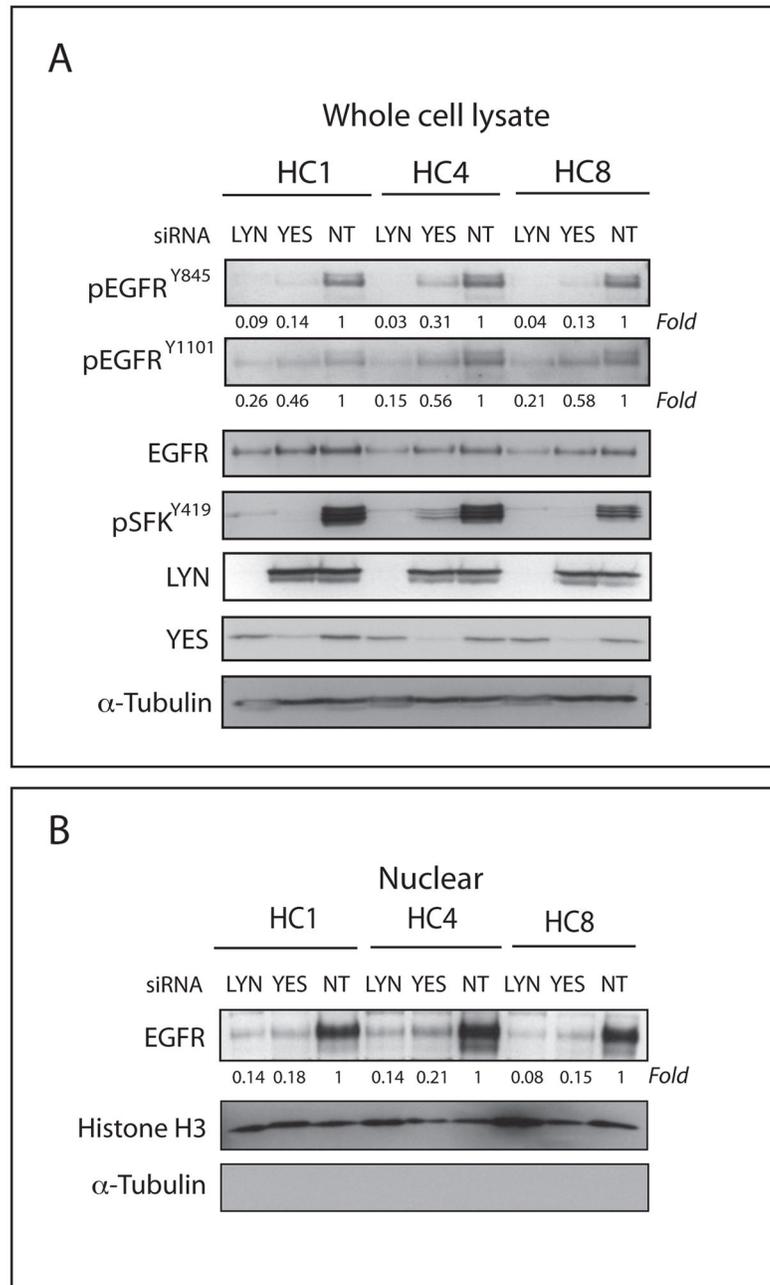


Figure 3. siYES and siLYN can reduce the nuclear localization of the EGFR

(A) siYES and siLYN decreased phosphorylation of EGFR tyrosine 845 (Y845), tyrosine 1101 (Y1101) and SFK tyrosine 419. Cells were harvested 72 hr after treatment with either siYES or siLYN, and protein lysates were fractionated on SDS-PAGE followed by immunoblotting for the indicated proteins. The non-targeting siRNA (NT) was used as a control. α -tubulin was used as a loading control. Protein expression was quantitated using ImageJ software. (B) siYES and siLYN can reduce the nuclear localization of the EGFR. Cells were harvested for non-nuclear and nuclear protein, and fractionated on SDS-PAGE followed by immunoblotting for indicated proteins after 72hr treatment with either siYES or

siLYN. The non-targeting siRNA (NT) was used as a control. Histone H3 and α -tubulin were used as loading and purity controls for the nuclear and non-nuclear fractions, respectively. Protein expression was quantitated using ImageJ software.

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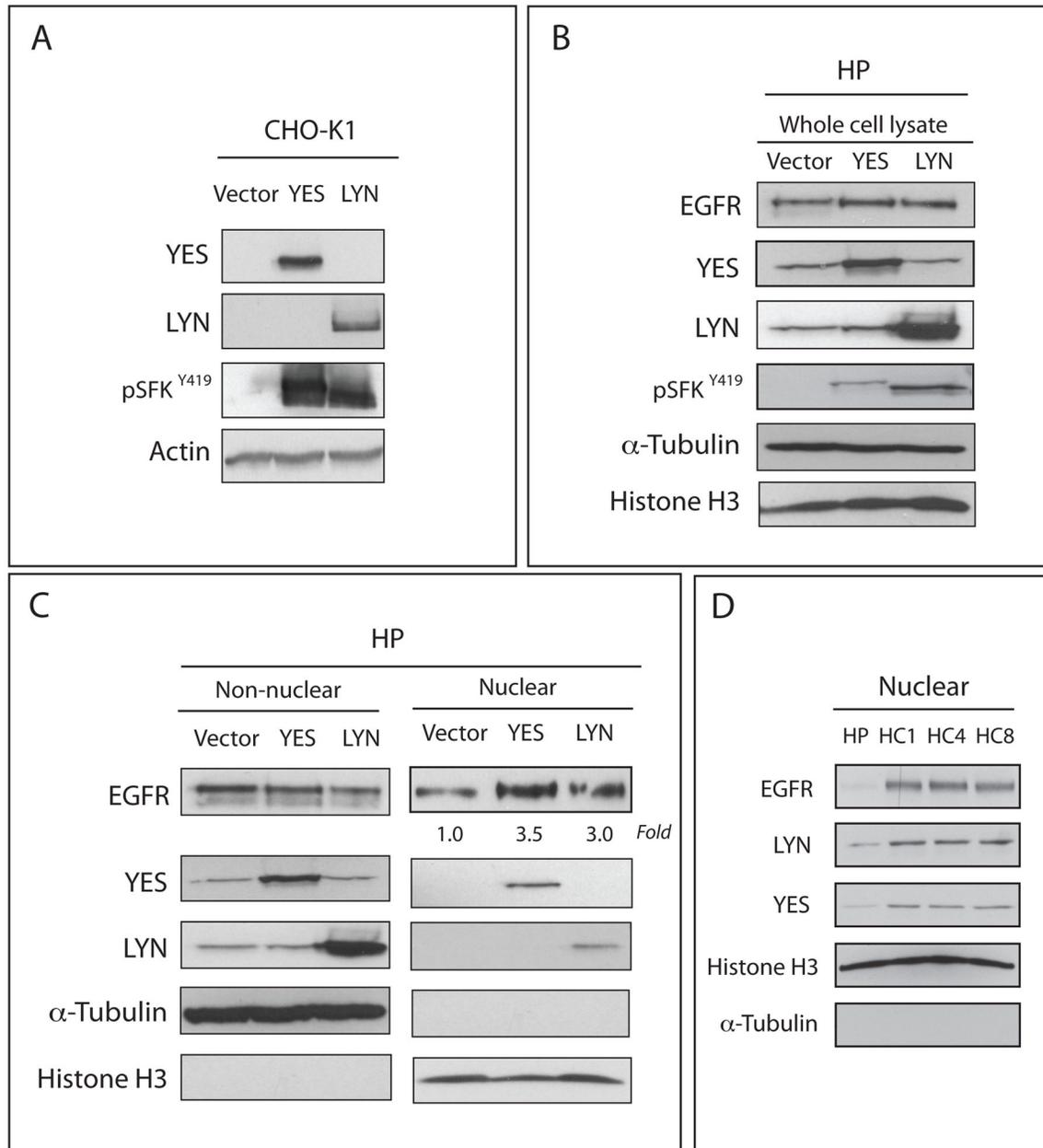


Figure 4. YES and LYN can induce the nuclear localization of the EGFR

(A) Increased levels of total protein expression of Yes and Lyn were detected in Chinese hamster ovary (CHO-K1) cells post 48hr transfection with either a Yes or Lyn mammalian expression vector. (B) Increased Yes and Lyn expression as well as increased levels of pSFK Y419 were observed in Ctx^S cells after 48hr transfection compared to vector only. (C) Nuclear EGFR translocation was increased by overexpression of Yes and Lyn. Cells were transfected with a Yes or Lyn mammalian expression vector and harvested for non-nuclear and nuclear protein. Each protein was fractionated on SDS-PAGE followed by immunoblotting. Histone H3 and α -tubulin were used as loading and purity controls for the nuclear and non-nuclear fractions, respectively. (D) Ctx^R clones (HC1, HC4 and HC8) have

increased nuclear Yes and Lyn localization as compared to Ctx^s cell line (HP). Cells were harvested for non-nuclear and nuclear protein, and fractionated on SDS-PAGE followed by immunoblotting for indicated proteins. Histone H3 and α -tubulin were used as loading and purity controls for the nuclear and non-nuclear fractions, respectively.

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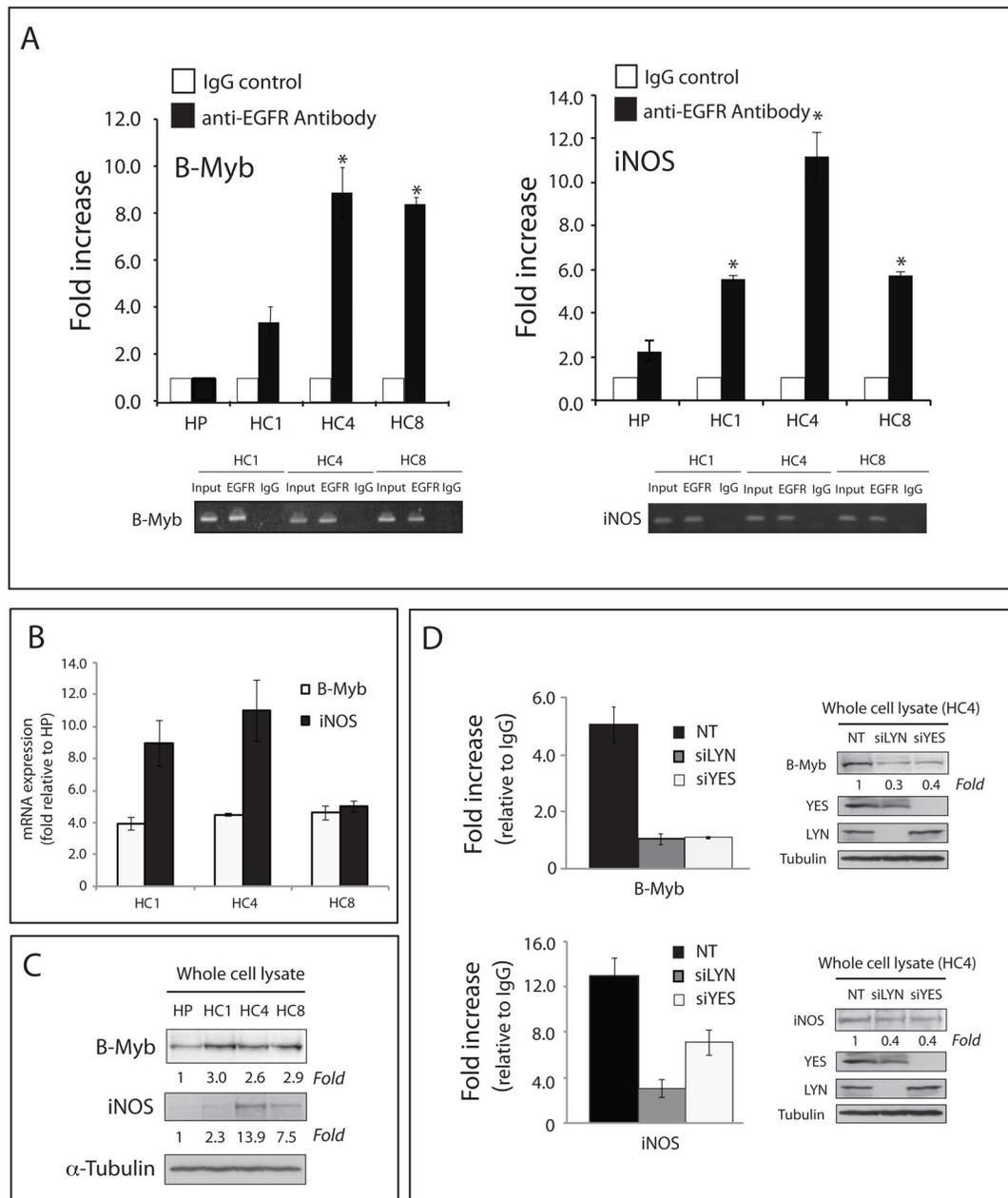


Figure 5. Yes and Lyn influence the binding of EGFR complexes to the B-Myb and iNOS promoter regions

(A) EGFR regulated gene promoter regions are more strongly associated with EGFR in Ctx^R clones (HC1, HC4, and HC8) as compared to Ctx^S cells (HP). EGFR-ChIP and subsequent qPCR from the ChIP sample for the presence of B-Myb and iNOS promoter sequences. Data points are represented as mean \pm SEM (n = 3). *p < 0.05. qPCR specificity for the B-Myb and iNOS promoter regions was also confirmed by agarose gel electrophoresis of semi-quantitative PCR products. (B) B-Myb and iNOS mRNA levels were significantly up-regulated in Ctx^R cells (HC1, HC4, and HC8) as compared to the Ctx^S cell line (HP) by qPCR. The mRNA expression of B-Myb and iNOS in HP, HC1, HC4 and HC8 were

determined by qPCR. Data points are represented as mean \pm SEM (n = 3). (C) B-Myb and iNOS protein levels were increased in Ctx^R cells (HC1, HC4, and HC8) as compared to the Ctx^S cell line (HP) by immunoblot analysis. Cells were harvested and protein lysates were fractionated on SDS-PAGE followed by immunoblotting for the indicated proteins. α -tubulin was used as a loading control. (D) Loss of Yes or Lyn prevents EGFR association with B-Myb and iNOS promoters, and corresponds with a decrease in protein expression. EGFR-ChIP and subsequent qPCR from the ChIP sample for the presence of B-Myb and iNOS promoter sequences. The non-targeting siRNA (NT) was used as a control. B-Myb and iNOS protein levels were decreased in HC4 after siYES or siLYN treatment by immunoblot analysis. Cells were harvested after treatment with siLYN or siYES for 72 hr and protein lysates were fractionated on SDS-PAGE followed by immunoblotting for the indicated proteins. α -tubulin was used as a loading control.

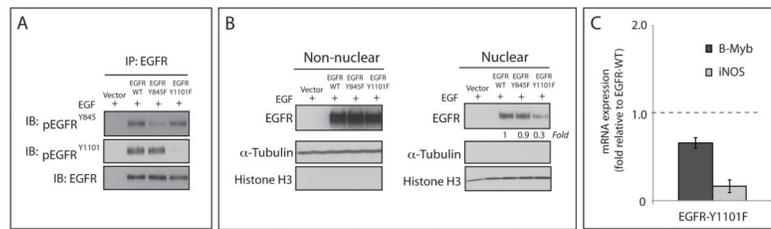


Figure 6. Phosphorylation of EGFR at Y1101 is involved in the nuclear localization of EGFR (A) Immunoprecipitation of total EGFR followed by immunoblotting with anti-pEGFR Y845, anti-pEGFR Y1101 or pan-EGFR antibodies. MCF7 Cells overexpressing EGFR-WT, EGFR-Y845F or EGFR-Y1101F were harvested after stimulation with 100 ng/mL of EGF for 45 min. A total of 500 ug of cell lysate was immunoprecipitated with pan-EGFR antibody. The immunoprecipitates were fractionated on SDS-PAGE followed by immunoblotting for the indicated proteins. (B) Mutation of Y1101, but not Y845, reduces the nuclear localization of EGFR in MCF-7 breast cancer cells. Cells were transiently transfected with plasmids encoding the EGFR wild-type (WT), EGFR-Y845F, EGFR-Y1101F or vector only. 48hr post-transfection the cells were incubated with EGF (100 ng/ml) for 45min, harvested for whole cell lysate, non-nuclear, and nuclear protein, and fractionated on SDS-PAGE followed by immunoblotting for indicated proteins. Histone H3 and α -tubulin were used as loading and purity controls for the nuclear and non-nuclear fractions, respectively. (C) B-Myb and iNOS mRNA levels were down-regulated in HC4 cells transfected with EGFR-Y1101F mutant compared to EGFR-WT transfected cells by qPCR. Cells were transiently transfected with plasmids encoding the EGFR wild-type (WT) or EGFR-Y1101F. 24hr post-transfection the cells were treated with EGF (100 ng/ml) for 45min, and harvested for RNA. The mRNA expression of B-Myb and iNOS was determined by qPCR.