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The Phosphoinositide Kinase PIKfyve Mediates EGF Receptor Trafficking to the Nucleus

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Abstract

ErbB receptor tyrosine kinases can transit to nuclei in tumor cells, where they have been shown to regulate gene expression as components of transcriptional complexes. Quantitative analysis of a human bladder cancer tissue microarray identified nuclear EGFR in tumor cells and also showed an increased frequency of this histologic feature in cancer relative to normal tissues. This observation suggests a potential role for nuclear EGFR in bladder cancer. We confirmed that EGFR could be induced to transit to nuclei in cultured human bladder cancer cells in response to the urothelial cell growth factor and EGFR ligand, HB-EGF. Mass spectrometric analysis of EGFR immune complexes from a transitional carcinoma cell line (TCCSUP) identified the phosphoinositide kinase, PIKfyve, as a potential component of the EGFR trafficking mechanism. RNA silencing indicated that PIKfyve is a mediator of HB-EGF-stimulated EGFR nuclear trafficking, EGFR binding to the cyclin D1 promoter, and cell cycle progression. These results identify a novel mediator of the EGFR transcription function and further suggest that nuclear EGFR and the lipid kinase PIKfyve may play a role in bladder oncogenesis.

Keywords

nuclear EGFR; bladder cancer; PIKfyve; trafficking

Introduction

The epidermal growth factor receptor tyrosine kinase (EGFR/ErbB1/HER1) is a signaling protein that plays a prominent role in the maintenance of epithelial tissues and in malignant transformation at many organ sites (1), with the result that the EGFR is now a major focus of targeted pharmacotherapy (2). Pharmacologic inhibitors of the EGFR are in clinical use for several malignancies, including non-small cell lung cancer and metastatic colon cancer, often in combination with other agents (3). Increased expression of the EGFR is associated with disease progression in transitional cell carcinoma (TCC) of the bladder and is an

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independent predictor of tumor stage and disease-specific mortality in this disease (4, 5). Activation of the EGFR occurs in response to binding of the extracellular domain by one or more soluble proteins containing an EGF-like motif. Expression of transforming growth factor-α (TGFα), an EGFR ligand, was shown to correlate with recurrence of superficial bladder cancer (6). Another EGFR ligand, heparin-binding EGF-like growth factor (HB-EGF), is an autocrine urothelial cell mitogen that is synthesized normally by bladder urothelium and smooth muscle cells (7, 8). HB-EGF is upregulated in bladder smooth muscle cells in vivo in response to hypertrophic stimuli (9), suggesting a physiologic role for HB-EGF/EGFR signaling in the urinary tract.

Several growth factors and growth factor receptors have been reported to localize to non-canonical subcellular compartments (10–12). FGF-2 accumulating in the nucleus was implicated in regulation of tumor cell survival and metastatic potential in vivo (13, 14). The EGF-like growth factors, HB-EGF (15, 16), TGF-α and amphiregulin, have all been reported to accumulate in nuclei in several cell types. The HB-EGF precursor was reported by our laboratory to localize to tumor cell nuclei in human TCC in a manner that positively correlated with disease progression (15). This result was recently confirmed independently by another group in a larger bladder cancer series (5). Nuclear-localized HB-EGF in TCC was also shown to be mobilized by oxidative stress into an autocrine loop involving transport of the growth factor from the nucleus to the cell surface, followed by regulated ligand shedding and EGFR activation (16).

EGFR and related receptor tyrosine kinases (RTKs), ErbB2, ErbB3, and ErbB4, have similarly been found in nuclei, where these proteins have been shown to perform roles distinct from their function as plasma membrane signal transducers (10, 17–20). This non-canonical signaling function of RTKs is still poorly understood. Nuclear EGFR has been detected in hepatocytes in regenerating liver (21) and nuclear ErbB4 was recently identified as a component of transcription complexes involved in astrocyte differentiation in the mouse (22). Expression levels of nuclear EGFR were recently reported to correlate with the cell proliferation markers, cyclin D1 and Ki-67, in a large breast cancer series (23), in which the extent of localization of EGFR to tumor cell nuclei was inversely correlated with patient survival. EGFR has been demonstrated to contain a functional nuclear localization sequence within the juxtamembrane region (24) and to act as a transcriptional co-activator by participating in the formation of a transcription complex at the cyclin D1 (17) and inducible nitric oxide synthase (iNOS) promoters (18), resulting in the activation of gene expression from these regulatory domains.

When ErbB receptors translocate to nuclei they likely associate with accessory proteins specialized for receptor transit, stability, or assembly into functional chromatin complexes. In its nuclear regulatory role, ErbB4 has been shown to complex with the MAP3K-interacting protein TAB2 and the corepressor N-CoR (22, 25). However, the precise regulatory mechanisms of EGFR/ErbB receptor trafficking to nuclei, and the manner in which RTKs are stabilized and turned over in nuclei, are still poorly understood. To date, a direct mediator of EGFR nuclear trafficking from the cytosol to the nucleus has not been identified.

In this study, we present evidence for a role for nuclear EGFR in human bladder cancer by providing the first identification of nuclear EGFR in patient-derived tumor tissues. In addition, we have applied a mass spectrometric approach toward analysis of EGFR translocation complexes in TCC cells. Here we report the identification of the lipid kinase PIKfyve as a novel regulator of EGFR transit to nuclei.

Materials and Methods

Immunostaining and automated quantitative immunohistochemical analysis (AQUA) of human tissues

A human tissue microarray (TMA) consisting of normal bladder tissues (n=3) and bladder cancer tissues (n=18) from Brigham and Women's Hospital, Boston, MA, was stained with an anti-mouse EGFR antibody (Biosource Inc., Camarillo, CA), at a dilution of 1:50. Four-μm sections from the TMA paraffin block were transferred to an adhesive coated slide system. Standard indirect immunoperoxidase procedures were used for immunohistochemistry after antigen retrieval in citrate buffer (pH=6.0), as described (26). Nuclear localization of EGFR was analyzed quantitatively using the AQUA system (27). TMA sections were processed for immunofluorescence using a rabbit anti-cytokeratin polyclonal antibody (1:500 dilution) (WWS, DAKO Corporation, Carpinteria) to localize epithelial cells and the cytosolic compartment. An anti-mouse EGFR (Biosource Inc., Camarillo, CA), used at a dilution of 1:50, was applied to the sections simultaneously. Cytokeratin was visualized using a secondary anti-rabbit FITC conjugated antibody. EGFR was detected using Cy5-tyramide (NEN, Life Science Products, Boston, MA), as described (27). 4′,6′-diamidino-2-phenylindole (DAPI) was used to identify the nuclear compartment. Using this approach, subcellular compartments were identified on the basis of molecular co-localization. The cytokeratin compartment corresponds to all epithelial cells in the 0.6mm TMA spot. The tumor mask was determined by gating the pixels in this image, in which an intensity threshold was set by visual inspection, and each pixel was recorded as "on" (tumor) or "off "(non-tumor) by the software on the basis of the threshold. The DAPI image, which was used to identify the nuclei, was subjected to a rapid exponential subtraction algorithm that improves signal to noise ratio by subtracting the out-of-focus image from the in-focus image. DAPI is the area defined as cell nuclei. Images of the TMA core sections were captured with an Olympus AX51 microscope and analyzed with the AQUA software (IPLabs, Scanalytics Inc., Fairfax, VA). A detailed description of this method has been described (28). Commercially available software SPSS 11.1 (SPSS Inc., Chicago, IL) was used for statistical analysis.

Indirect immunofluorescence microscopy of cultured cells

Cells seeded at low density in chamber slides were treated with HB-EGF, fixed with 3% paraformaldehyde and incubated with various antibodies in 1% BSA/PBS solution, followed by species-specific secondary antibodies conjugated to FITC or Texas-Red fluorophores. Slides were mounted in Vectashield mounting medium containing DAPI (Vector Laboratories, Inc., Burlingame, CA) and analyzed with oil immersion objectives using a LSM 510 META NLO laser scanning confocal microscope or an Axioplan 2 microscope (Carl Zeiss MicroImaging, Inc. Thornwood, NY).

Cell culture and transfection

TCCSUP or 253J bladder cancer cells (ATCC) were cultured in DMEM or MEM containing 10% FBS at 37°C and 5% CO_2. Cells were transiently transfected with siRNAs (siEGFR, siPIKfyve-1, siPIKfyve-2, siPIKfyve-3, or siPIKfyve-pool) or various constructs (vector only, wild type PIKfyve and dominant-negative PIKfyve, PIKfyve^{K1831E}) (29) using Lipofectamine 2000 according to the manufacturer's instructions (Invitrogen, Carlsbad, CA). The siEGFR was generated by partial enzyme digestion of a PCR product corresponding to positions 3118 to 3806 of NM_005228 (RH-0079, SuperArray Bioscience, Frcderick, MD). All PIKfyve siRNAs were purchased from Dharmacon, Inc. (Chicago, IL). The siPIKfyve-pool contains Sense Sequence, GUAGAAUGCUGGCCGAUUAUU and Antisense Sequence, 5′-P UAAUCGCCAGCAUUCUACUU (D-005058-03, Dharmacon). The siPIKfyve-l contains Sense Sequence, GAAUGGAAGUUUCAGGAUCAUU and

Antisense Sequence, 5′-P UGAUCCUGAAACUCCAUUCUU (D-005058-09-0005). The siPIKfyve-2 contains Sense Sequence, GGAAAUCUCCUGCUCGAAAUU and Antisense Sequence, 5′-P UUUCGAGCAGGAGAUUUCCUU (D-005058-10-0005). The siPIKfyve-3 contains Sense Sequence, UGAAGAAGGUGACGAUAAUUU and Antisense Sequence, 5′-P AUUAUCGUCACCUUCUUCAUU (D-005058-11-0005). The siCONTROL Non-targeting siRNA was obtained from Dharmacon (D-001210-14-05).

Chromatin immunoprecipitation assay (ChIP)

Cells were serum-starved for 16 h and stimulated with HB-EGF (100 ng/ml). At the indicated time points, formaldehyde (1% final concentration) was added for 10 min to cross-link proteins to DNA. Cells were washed with ice-cold PBS and resuspended in 500 μl of hypotonic buffer [10 mM Tris-HCl, pH 7.4, 10 mM KCl, 1 mM dithiothreitol], followed by passage through a 25-gauge needle (20 times). Nuclear pellets were resuspended in 200 μl SDS lysis buffer [1% SDS, 10 mM EDTA, 50 mM Tris-HCl, pH 8.0, and protease inhibitors]. After sonication, the supernatant was diluted 1:10 with IP buffer [50 mM Tris-HCl, pH 8.0, 150 mM NaCl, 5 mM EDTA, 0.5% NP40]. IP using anti-EGFR antibody was performed and immunoprecipitates were washed with RIPA buffer [150 mM NaCl, 50 mM Tris-HCl, pH 8.0, 0.1% SDS, 0.5% sodium deoxycholate, 1.0% NP40], with high-salt buffer [500 mM NaCl, 1.0% NP40, 0.1% SDS, 50 mM Tris-HCl, pH 8.0), with LiCl buffer [250 mM LiCl, 1.0% NP40, 0.5% sodium deoxycholate, 1 mM EDTA, 50 mM Tris-HCl, pH 8.0] and TE buffer [10 mM Tris-HCl, pH 8.0, 1 mM EDTA]. DNA was harvested by the following steps: incubation with RNase solution (50 mg/ml, 30 min, 37 °C) and Proteinase K/SDS solution (0.25% SDS, 250 mg/ml proteinase K, 37 °C, overnight), and heating (65 °C, 6 h), extraction with phenol/chloroform (1:1), and precipitation with ethanol. The cyclin D1 promoter in the immunoprecipitates was detected by PCR with primers S (sense) (5′-GAGGGGACTAATATTTCCAGCAA-3′) and AS (anti-sense) (5′-TAAAGGGATTTCAGCTTAGCA-3′)(17).

Cell cycle analysis by FACS

Cells were serum starved overnight and stimulated with 100ng/ml HB-EGF for the indicated times. After harvesting, cells were stained with propidium iodide, and assessed by flow cytometry (16).

Immunoprecipitation and western blot analysis

For whole lysates, proteins were extracted into lysis buffer [1% Nonidet P-40; 50 mM Tris pH 7.4; 10 mM NaCl; 1 mM NaF; 5 mM $MgCl_2$; 0.1 mM EDTA; 1 mM PMSF; and COMPLETE protease inhibitor cocktail tablet (Roche)] and centrifuged at 12,000× g for 15 min. Supernatants were applied to each immunoprecipitation after protein measurement. Each 1 μg Abs including control IgG was incubated (16 h, 4°C) with 500 μg protein samples, followed by incubation with protein A/G beads for 1h. Immunocomplexes were washed with modified lysis buffer three times, subjected to SDS-PAGE and transferred to nitrocellulose membranes. Target proteins were assessed by western blot using specific Abs. Cytoplasmic and nuclear fractionation and blotting were performed as described (16).

Protein identification by mass spectrometry

Mass spectrometric analysis with EGFR immunoprecipitates from TCCSUP cells was performed to search for binding partners of the translocating EGFR. The protein bands from native SDS-PAGE gels were reduced and alkylated by DTT and iodoacetamide, respectively. Gel bands were dehydrated in acetonitrile, followed by trypsin digestion (500 ng/band) in NH_4HCO_3 buffer (50 μl/band) for 16h at 37°C. MALDI-TOF analysis was performed using a Voyager-DE STR mass spectrometer (Applied Biosystems). α-Cyano-4-

hydroxy-cinnamic acid matrix (0.5 μl, saturated in 50% acetonitrile and 0.1% trifluoroacetic acid) was used for each sample (0.5 μl). In the reflector mode, 20,000V of accelerating voltage and 200 ns of extraction delay time were applied, under the acquisition mass range of 750–4500 Da with 600 Da low mass gate. The Expasy Proteomics Tool (http://www.expasy.org) and Protein Prospector (http://prospector.ucsf.edu/) were applied for peak analysis and protein identification using one trypsin peptide (MW=842.5099) as the internal standard.

Statistical Analysis

Data were compared using a paired Student's t-test. P values < 0.05 were considered significant.

Results

Nuclear localization of EGFR in bladder cancer cells and tissues

The discovery that HB-EGF localizes to nuclei in TCC, and that this subcellular feature can have functional consequences (5, 15, 16), led us to question whether the EGFR, the primary cognate receptor for HB-EGF, might also localize to nuclei in bladder cancer. To test this hypothesis, we analyzed EGFR expression and localization in a bladder cancer TMA consisting of 18 tumors and 3 normal tissues. Using standard immunohistochemistry, we identified sporadic nuclear localization of EGFR, which otherwise exhibited a predominantly membrane/cytosolic pattern (Figure 1A). Automated quantitative analysis (AQUA) (27) of this TMA after immunostaining with antibodies against EGFR and keratin demonstrated that nuclear EGFR was identifiable and quantifiable in this series of tissues (Figure 1A, B). Nuclear EGFR levels were increased in bladder cancer relative to benign tissues (p=0.003). Cytosolic EGFR was also detected and quantitated by the AQUA system, however, no correlation with disease status was observed (p=0.12) (Figure 1B).

We next attempted to test whether EGFR could be detected in nuclei in TCCSUP bladder cancer cells, a cell line that previously was shown to accumulate HB-EGF in nuclei (15, 16). EGFR localization was initially assessed by subcellular fractionation and western blot analysis. In these experiments we were able to show that the intact (170kDa) form of the EGFR was detectable in the nuclear fraction (Figure 1C). No smaller fragments were seen in the blots, consistent with previous findings that intact EGFR can transit to nuclei (30). The specificity of the antibody detection result was demonstrated by RNA interference. Liposome-mediated transfection of TCCSUP cells with control and anti-EGFR small interfering RNA (siRNA) duplexes demonstrated that the intensity of the EGFR immunoreactive signal in the nuclear subcellular fractions was reduced by the EGFR siRNA but not by the control siRNA (Figure 1C). Consistent with published data (17), we demonstrated association of the nuclear EGFR with the endogenous cyclin D1 promoter in a chromatin complex by ChIP using a specific anti-EGFR antibody (Figure 1D), strongly suggesting that the protein performs a similar role in cyclin D1 regulation in the bladder cancer cell background.

Translocation of EGFR to the nucleus is followed by association of the receptor with the cyclin D1 promoter

Indirect immunofluorescence cell staining indicated that cell surface EGFR moved to the nucleus within 30 min after TCCSUP and 253J bladder cancer cells were treated with HB-EGF (Figure 2A). A western blot time course showed that the EGFR accumulated and declined in the nuclear fraction with similar kinetics (Figure 2B). The specificity of the antibody-derived signal detecting the trafficking event was confirmed by RNA interference (Figure 2B). These results suggest that nuclear EGFR is degraded in TCCSUP cells upon

HB-EGF treatment, consistent with previous observations demonstrating proteasome- and ubiquitin-mediated EGFR degradation following receptor activation and clearance from the cell surface (31, 32). The cytoplasmic and nuclear fractions employed in this and subsequent experiments showed enrichment for the cytoplasmic marker, β-tubulin, in the cytoplasmic fraction and the nuclear marker, lamin, in the nuclear fraction, with no detectable cross-contamination.

To assess the potential function of nuclear EGFR in bladder cancer cells, we attempted to follow EGFR association with the cyclin D1 promoter kinetically following receptor activation with HB-EGF. The expression level of cyclin D1 has been positively correlated with high proliferation rate in tumor cells (33, 34). Treatment of TCCSUP cells with HB-EGF enhanced the association of the EGFR with the cyclin D1 promoter in a dose- and time-dependent manner, as assessed by ChIP (Figure 2C). The effect of HB-EGF on association of EGFR with the cyclin D1 promoter reached a maximum level at 100 ng/ml after 30 min incubation with the growth factor. Incorporation of EGFR into the chromatin complex was inhibited by EGFR siRNA but not by control siRNA, verifying the authenticity of the ChIP result (Figure 2C).

EGFR signaling pathways involved in EGFR transit to nuclei

As an independent confirmation of the effect of EGFR activation on EGFR nuclear trafficking and association of the receptor with the cyclin D1 promoter, we attempted to confirm dependence of these events on EGFR pathway activation. Immunofluorescence staining using a specific phospho-EGFR antibody demonstrated that nuclear EGFR is phosphorylated in response to HB-EGF (Figure 3A). Treatment of TCCSUP cells with either one of two different pharmacologic inhibitors of the EGFR kinase activity (AG1478 and PD153035) inhibited HB-EGF-stimulated nuclear translocation of EGFR (Figure 3B). In addition, AG1478 suppressed complex formation of the EGFR with the cyclin D1 promoter in a dose-dependent manner as assessed by ChIP (Figure 3B). A time course experiment demonstrated that HB-EGF induced increased expression of cyclin D1 (Figure 3C). We demonstrated in a published study that HB-EGF increases the rate of cell cycle transit in an EGFR kinase-dependent manner in TCCSUP cells (data not shown) (16). These data verify that EGFR activation is sufficient to induce EGFR transit to nuclei and association of the receptor with chromatin complexes, leading to enhancement of cell proliferation.

Consistent with this interpretation, when TCCSUP cells were pretreated with inhibitors of signal transduction pathways known to be downstream from the EGFR (LY294002, PI3-kinase inhibitor; PD98059, MEK inhibitor) prior to addition of HB-EGF, EGFR trafficking to the nucleus was substantially suppressed, based on both western blot analysis of nuclear fractions and ChIP (Figure 3D). Both the PI3-kinase/Akt and Erk/MAPK pathways were activated in TCCSUP cells in response to HB-EGF (Figure 3D).

TCCSUP cells engineered to stably express the membrane-anchored, precursor form of HB-EGF (proHB-EGF) were also analyzed (15, 16). ProHB-EGF transfectant cells contain EGFR on plasma membrane and nucleus under steady-state conditions (Figure 4A). Membrane EGFR was lost by treatment with H_2O_2, a source of reactive oxygen species that promotes cleavage and secretion of membrane-associated proHB-EGF. Western blot analysis confirmed that EGFR accumulated in nuclei in response to H_2O_2 (Figure 4B), suggesting that membrane EGFR trafficked to nucleus. Treatment of these cells with other known inducers of proteolytic cleavage of proHB-EGF and liberation of soluble HB-EGF (the phorbol ester, TPA; and ionomycin) also induced EGFR accumulation in the nucleus (Figure 4B). These data indicate that nuclear trafficking of the EGFR occurs following activation of the receptor by multiple routes.

Identification of PIKfyve as a binding partner of cytoplasmic EGFR

The mechanism that mediates transit of the EGFR across the complex vesicular trafficking network in the cytoplasmic space during translocation of the receptor to the nucleus is unknown. We sought to approach this question by identifying proteins that bind EGFR during the translocation process. Whole cell lysates from TCCSUP cells were subjected to IP with a mono-specific anti-EGFR antibody as described in Materials and Methods. Mass spectrometric analysis of immunoprecipitated proteins was then performed. These experiments identified a phosphoinositide kinase, PIKfyve, as a member of the cytoplasmic EGFR immune complex in 5 independent trials. PIKfyve synthesizes phosphatidylinositol(3,5)P_2 (35, 36) and has been linked to endosomal dynamics and intracellular trafficking (35, 37–41), suggesting the hypothesis that PIKfyve is a direct mediator of EGFR transit to nuclei.

To validate the association of EGFR with PIKfyve in TCCSUP cells, whole cell lysates were subjected to co-IP followed by western blot. The data demonstrated that endogenous EGFR associated with endogenous PIKfyve (Figure 5A). When TCCSUP cells were treated with HB-EGF, and EGFR complexes were isolated with anti-EGFR antibody, the binding of EGFR and PIKfyve increased approximately 10-fold, compared to the unstimulated condition. Consistent with previous reports about PIKfyve, the protein was detected exclusively in the cytoplasm, not nuclei, by immunofluorescent cell staining (Figure 5B).

Enforced expression of PIKfyve was performed to examine the effect on EGFR trafficking to nuclei. Transfection of wild-type PIKfyve increased the level of nuclear-resident EGFR (Figure 5C), while EGFR translocation to nuclei was significantly inhibited in transfectants with a dominant-interfering, kinase-dead (KD) PIKfyve mutant, PIKfyve^{K1831E} (29) (Figure 5D). These data suggest that PIKfyve plays a role specifically in the trafficking mechanism. Binding of EGFR and PIKfyve was not significantly affected in PIKfyve^{K1831E}-transfected cells (data not shown).

To further investigate the regulatory role of PIKfyve, gene silencing experiments were performed using three different PIKfyve siRNAs as well as pooled siRNA PIKfyve oligos (Figure 6A, B). Nuclear accumulation of EGFR was inhibited significantly when expression levels of PIKfyve were knocked down by siRNAs differentially targeting PIKfyve (Figure 6A). No effect on levels of PIKfyve and nuclear EGFR was observed using two sets of control oligos (Figure 6A). Cell cytometry analysis showed cell cycle transit was arrested at the G_0/G_1 phase when 70–80% of endogenous PIKfyve in TCCSUP cells was down-regulated by RNA interference (Figure 6B). As assessed by immunofluorescent cell staining, HB-EGF enhanced translocation of EGFR from the cell surface to the nucleus, and knockdown of PIKfyve protein expression significantly attenuated this effect (Figure 6C). ChIP analysis demonstrated that silencing PIKfyve RNA also reduced complex formation between EGFR and the cyclin D1 promoter under conditions of log-phase growth as well as under HB-EGF-stimulated conditions (Figure 6D). We conclude from these data that transient depletion of PIKfyve significantly reduced the effects elicited by HB-EGF on cell cycle transit, EGFR relocalization to nuclei, and complex formation between EGFR and chromatin.

Taken together, our findings indicate that nuclear EGFR plays a significant role in cell cycle progression in bladder cancer cells, and that PIKfyve is an essential mediator of EGFR transit to nuclei and association of the receptor with chromatin.

Discussion

In this study we provide the first evidence from human tissue that EGFR/HER1 accumulates in tumor cell nuclei in TCC and we provide evidence that nuclear EGFR plays a role in bladder cancer cell proliferation by identifying a novel mediator of the EGFR nuclear trafficking mechanism and showing that this protein is involved in cell cycle progression. Quantitative analysis of EGFR localization patterns in a human bladder cancer TMA verified the presence of EGFR in tumor cell nuclei and identified an apparently significant quantitative increase in nuclear EGFR associated with TCC in comparison to benign bladder tissue. Further analysis is necessary to assess the possibility that nuclear EGFR is an independent prognostic indicator capable of providing information of clinical value. At least one previous study that confirmed the presence of nuclear HB-EGF in TCC specimens, originally reported by our group (15, 16), did not find significant nuclear EGFR (5). Although in our study we did find nuclear EGFR in bladder cancer tissues using conventional immunohistochemical staining, our study benefited from the use of AQUA, a highly sensitive and quantitative system for localizing immunoreactive signals to subcellular compartments in tissue. Nuclear EGFR has been reported in human breast cancers and in normal tissues, including regenerating liver (21).

We also found that nuclear EGFR could be demonstrated in human bladder cancer cells in culture. We demonstrated that receptor activation by the cognate EGFR ligand, HB-EGF, stimulates translocation of full-length EGFR to the nucleus in TCCSUP bladder cancer cells, and that this results in binding of the receptor to chromatin within 30 min. The kinetics of nuclear transit we observed are consistent with the kinetics reported by other groups for nuclear translocation of the EGFR (17, 18). Notably, the rapid translocation of EGFR appears distinct from the slower translocation process seen with ErbB4, where proteolytic cleavage of the intracellular domain of the receptor precedes translocation of the cleaved fragment to active chromatin. In the present study we also identified the lipid kinase, PIKfyve, as a component of an EGFR cytosolic trafficking complex. Down-regulation of PIKfyve by RNA interference inhibited EGFR trafficking to nuclei, association of the receptor with the endogenous cyclin D1 promoter and cell cycle progression in TCCSUP cells. This is the first report to link nuclear EGFR with bladder cancer and the first to identify PIKfyve as a mediator of EGFR intracellular trafficking to the nucleus in any cell type.

Ligand activation of the EGFR promotes numerous downstream signal transduction processes, leading to cell proliferation, resistance to apoptosis, aspects of development, and migration (42, 43). Modulation of receptor activity through endocytosis, degradation and vesicular trafficking regulates the downstream targets of EGFR. The physiologic roles for nuclear trafficking of RTKs, including EGFR, FGFR, and other receptor families are currently under intense scrutiny (10, 13, 17, 44–46). Vesicular and other trafficking devices, such as involvement of the exportin CRM1, are likely to be important elements of the regulatory mechanics of nuclear-localized RTKs (47–49). EGFR has been shown to co-localize and interact with the nuclear pore protein Nup358 as well as the nuclear importing cofactors importins $\alpha 1/\beta 1$ (49). Nuclear EGFR also interacts with signal transducer and activator of transcription-3 (STAT-3), leading to transcriptional activation of iNOS (18). Current evidence suggests that RTKs from several protein families employ similar strategies for transit to nuclei. Despite some information about likely candidates for co-factors in the translocation mechanism, nothing has been published previously about the mechanism whereby the ligand-activated EGFR in transit to the nucleus navigates the cytosolic vesicular compartments.

Our finding that PIKfyve is a facilitator of EGFR nuclear trafficking has shed light on this question. PIKfyve is the mammalian ortholog of the yeast protein, Fab1p. The principal in vivo activity of both enzymes is the production of phosphatidylinositol (3,5) bisphosphate from phosphatidylinositol (3)-monophosphate (35, 36). PIKfyve has been implicated in vesicular trafficking in mammalian cells and direct comparisons between the yeast and mammalian forms of the protein indicate that their predominant role appears to be conserved across a great evolutionary distance (39–41, 50). Perturbations in the PIKfyve function by protein silencing or expression of a dominant-negative, kinase-deficient form in human cells did not significantly affect internalization, recycling or degradative sorting of the EGFR (40, 50). However, PIKfyve suppression did result in defective exit from early endosomes and trafficking between the early endosomal compartment and the trans-Golgi network (40, 41, 50). Our data are consistent with these findings and further implicate PIKfyve in the transport of EGFR from the cell surface through the cytoplasmic vesicular space to the nucleus. We generated multiple lines of evidence that support this conclusion. Endogenous PIKfyve co-precipitated with EGFR from TCCSUP cytosolic fractions, indicating that the two proteins are associated within a cytosolic multi-protein complex. siRNA knock-down of PIKfyve and/or enforced expression of the dominant-negative mutant, PIKfyve^{K1831E} inhibited multiple endpoints associated with EGFR nuclear trafficking, including progression through the cell cycle in response to EGFR activation. These data indicate that PIKfyve kinase activity is a requisite process for relocation of surface EGFR to the nucleus. In addition, our data demonstrate that intracellular signaling pathways including PI3-kinase and Erk/MAPK are also involved in the HB-EGF-stimulated nuclear localization of EGFR. It remains to be elucidated how PIKfyve lipid products are related to these pathways in the context of EGFR nuclear transit.

In summary, we have identified a novel mediator of nuclear shuttling of the EGFR and have provided a new pathologic context for this phenomenon. Because EGFR can be over-expressed and is believed to be functionally involved in some bladder cancers (4–6), our findings indicate that EGFR likely plays a role in both conventional cell signaling initiated from the plasma membrane and also by direct intervention, at the level of chromatin, in transcriptional regulatory mechanisms of tumor cell proliferation in this disease. Because nuclear HB-EGF can be mobilized into an autocrine loop leading to EGFR activation (16), our conclusions are consistent with those of Kramer et al. with respect to the point that disease progression in TCC may be related to the mode of HB-EGF signaling (5). We propose a model in which PIKfyve facilitates transit of EGFR from the cytosolic vesicular compartments to the nucleus, where EGFR rapidly and reversibly associates with chromatin to regulate gene expression. This study provides the first evidence of functional involvement of PIKfyve in mechanisms of TCC oncogenesis, suggesting that targeting this lipid kinase may be fruitful as a novel therapeutic strategy in circumstances where changes in EGFR or HB-EGF expression or subcellular localization are evident histologically.

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List of abbreviations used

EGFR/ErbB1/HER1	epidermal growth factor receptor tyrosine kinase
TCC	transitional cell carcinoma

TGF	transforming growth factor
HB-EGF	heparin-binding EGF-like growth factor
RTKs	receptor tyrosine kinases
iNOS	inducible nitric oxide synthase
AQUA	automated quantitative analysis
TMA	tissue microarray
DAPI	4′,6′-diamidino-2-phenylindole
ChIP	chromatin immunoprecipitation
siRNA	small interfering RNA
proHB-EGF	heparin-binding EGF-like growth factor precursor

References

1. Roskoski R Jr. The ErbB/HER receptor protein-tyrosine kinases and cancer. Biochem Biophys Res Commun. 2004; 319:1–11. [PubMed: 15158434]
2. Baselga J, Arteaga CL. Critical update and emerging trends in epidermal growth factor receptor targeting in cancer. J Clin Oncol. 2005; 23:2445–59. [PubMed: 15753456]
3. Ji H, Sharpless NE, Wong KK. EGFR targeted therapy: view from biological standpoint. Cell Cycle. 2006; 5:2072–6. [PubMed: 16969107]
4. Nguyen PL, Swanson PE, Jaszcz W, et al. Expression of epidermal growth factor receptor in invasive transitional cell carcinoma of the urinary bladder. A multivariate survival analysis. Am J Clin Pathol. 1994; 101:166–76.
5. Kramer C, Klasmeyer K, Bojar H, Schulz WA, Ackermann R, Grimm MO. Heparin-binding epidermal growth factor-like growth factor isoforms and epidermal growth factor receptor/ErbB1 expression in bladder cancer and their relation to clinical outcome. Cancer. 2007
6. Turkeri LN, Erton ML, Cevik I, Akdas A. Impact of the expression of epidermal growth factor, transforming growth factor alpha, and epidermal growth factor receptor on the prognosis of superficial bladder cancer. Urology. 1998; 51:645–9.
7. Freeman MR, Yoo JJ, Raab G, et al. Heparin-binding EGF-like growth factor is an autocrine growth factor for human urothelial cells and is synthesized by epithelial and smooth muscle cells in the human bladder. J Clin Invest. 1997; 99:1028–36.
8. Raab G, Klagsbrun M. Heparin-binding EGF-like growth factor. Biochim Biophys Acta. 1997; 1333:F179–99.
9. Adam RM, Eaton SH, Estrada C, et al. Mechanical stretch is a highly selective regulator of gene expression in human bladder smooth muscle cells. Physiol Genomics. 2004; 20:36–44.
10. Carpenter G. Nuclear localization and possible functions of receptor tyrosine kinases. Curr Opin Cell Biol. 2003; 15:143–8. [PubMed: 12648669]
11. Clevenger CV. Nuclear localization and function of polypeptide ligands and their receptors: a new paradigm for hormone specificity within the mammary gland? Breast Cancer Res. 2003; 5:181–7.
12. Keresztes M, Boonstra J. Import(ance) of growth factors in(to) the nucleus. J Cell Biol. 1999; 145:421–4.
13. Maher PA. Nuclear Translocation of fibroblast growth factor (FGF) receptors in response to FGF-2. J Cell Biol. 1996; 134:529–36. [PubMed: 8707835]
14. Reilly JF, Maher PA. Importin beta-mediated nuclear import of fibroblast growth factor receptor: role in cell proliferation. J Cell Biol. 2001; 152:1307–12. [PubMed: 11257130]
15. Adam RM, Danciu T, McLellan DL, et al. A nuclear form of the heparin-binding epidermal growth factor-like growth factor precursor is a feature of aggressive transitional cell carcinoma. Cancer Res. 2003; 63:484–90. [PubMed: 12543806]

16. Kim J, Adam RM, Freeman MR. Trafficking of nuclear heparin-binding epidermal growth factor-like growth factor into an epidermal growth factor receptor-dependent autocrine loop in response to oxidative stress. Cancer Res. 2005; 65:8242–9.

17. Lin SY, Makino K, Xia W, et al. Nuclear localization of EGF receptor and its potential new role as a transcription factor. Nat Cell Biol. 2001; 3:802–8. [PubMed: 11533659]

18. Lo HW, Hsu SC, Ali-Seyed M, et al. Nuclear interaction of EGFR and STAT3 in the activation of the iNOS/NO pathway. Cancer Cell. 2005; 7:575–89. [PubMed: 15950906]

19. Wang SC, Lien HC, Xia W, et al. Binding at and transactivation of the COX-2 promoter by nuclear tyrosine kinase receptor ErbB-2. Cancer Cell. 2004; 6:251–61.

20. Offterdinger M, Schofer C, Weipoltshammer K, Grunt TW. c-erbB-3: a nuclear protein in mammary epithelial cells. J Cell Biol. 2002; 157:929–39.

21. Marti U, Hug M. Acinar and cellular distribution and mRNA expression of the epidermal growth factor receptor are changed during liver regeneration. J Hepatol. 1995; 23:318–27. [PubMed: 8550996]

22. Sardi SP, Murtie J, Koirala S, Patten BA, Corfas G. Presenilin-dependent ErbB4 nuclear signaling regulates the timing of astrogenesis in the developing brain. Cell. 2006; 127:185–97. [PubMed: 17018285]

23. Lo HW, Xia W, Wei Y, Ali-Seyed M, Huang SF, Hung MC. Novel prognostic value of nuclear epidermal growth factor receptor in breast cancer. Cancer Res. 2005; 65:338–48. [PubMed: 15665312]

24. Hsu SC, Hung MC. Characterization of a novel tripartite nuclear localization sequence in the EGFR family. J Biol Chem. 2007

25. Schlessinger J, Lemmon MA. Nuclear signaling by receptor tyrosine kinases: the first robin of spring. Cell. 2006; 127:45–8. [PubMed: 17018275]

26. Tornillo L, Duchini G, Carafa V, et al. Patterns of gene amplification in gastrointestinal stromal tumors (GIST). Lab Invest. 2005; 85:921–31. [PubMed: 15864317]

27. Rubin MA, Zerkowski MP, Camp RL, et al. Quantitative determination of expression of the prostate cancer protein alpha-methylacyl-CoA racemase using automated quantitative analysis (AQUA): a novel paradigm for automated and continuous biomarker measurements. Am J Pathol. 2004; 164:831–40. [PubMed: 14982837]

28. Camp RL, Chung GG, Rimm DL. Automated subcellular localization and quantification of protein expression in tissue microarrays. Nat Med. 2002; 8:1323–7. [PubMed: 12389040]

29. Ikonomov OC, Sbrissa D, Shisheva A. Mammalian cell morphology and endocytic membrane homeostasis require enzymatically active phosphoinositide 5-kinase PIKfyve. J Biol Chem. 2001; 276:26141–7. [PubMed: 11285266]

30. Wang SC, Nakajima Y, Yu YL, et al. Tyrosine phosphorylation controls PCNA function through protein stability. Nat Cell Biol. 2006; 8:1359–68.

31. Alwan HA, van Zoelen EJ, van Leeuwen JE. Ligand-induced lysosomal epidermal growth factor receptor (EGFR) degradation is preceded by proteasome-dependent EGFR de-ubiquitination. J Biol Chem. 2003; 278:35781–90. [PubMed: 12829707]

32. Haglund K, Sigismund S, Polo S, Szymkiewicz I, Di Fiore PP, Dikic I. Multiple monoubiquitination of RTKs is sufficient for their endocytosis and degradation. Nat Cell Biol. 2003; 5:461–6. [PubMed: 12717448]

33. Yang CC, Chu KC, Chen HY, Chen WC. Expression of p16 and cyclin D1 in bladder cancer and correlation in cancer progression. Urol Int. 2002; 69:190–4. [PubMed: 12372886]

34. Ongusaha PP, Kwak JC, Zwible AJ, et al. HB-EGF is a potent inducer of tumor growth and angiogenesis. Cancer Res. 2004; 64:5283–90. [PubMed: 15289334]

35. Sbrissa D, Ikonomov OC, Shisheva A. PIKfyve, a mammalian ortholog of yeast Fab1p lipid kinase, synthesizes 5-phosphoinositides. Effect of insulin. J Biol Chem. 1999; 274:21589–97.

36. Shisheva A. PIKfyve: the road to PtdIns 5-P and PtdIns 3,5-P(2). Cell Biol Int. 2001; 25:1201–6. [PubMed: 11748912]

37. Berwick DC, Dell GC, Welsh GI, et al. Protein kinase B phosphorylation of PIKfyve regulates the trafficking of GLUT4 vesicles. J Cell Sci. 2004; 117:5985–93. [PubMed: 15546921]

38. Cabezas A, Pattni K, Stenmark H. Cloning and subcellular localization of a human phosphatidylinositol 3-phosphate 5-kinase, PIKfyve/Fab1. Gene. 2006; 371:34–41. [PubMed: 16448788]
39. Ikonomov OC, Sbrissa D, Mlak K, Kanzaki M, Pessin J, Shisheva A. Functional dissection of lipid and protein kinase signals of PIKfyve reveals the role of PtdIns 3,5-P2 production for endomembrane integrity. J Biol Chem. 2002; 277:9206–11. [PubMed: 11714711]
40. Ikonomov OC, Sbrissa D, Foti M, Carpentier JL, Shisheva A. PIKfyve controls fluid phase endocytosis but not recycling/degradation of endocytosed receptors or sorting of procathepsin D by regulating multivesicular body morphogenesis. Mol Biol Cell. 2003; 14:4581–91.
41. Ikonomov OC, Sbrissa D, Shisheva A. Localized PtdIns 3,5-P2 synthesis to regulate early endosome dynamics and fusion. Am J Physiol Cell Physiol. 2006; 291:C393–404. [PubMed: 16510848]
42. Scaltriti M, Baselga J. The epidermal growth factor receptor pathway: a model for targeted therapy. Clin Cancer Res. 2006; 12:5268–72.
43. Arslan MA, Kutuk O, Basaga H. Protein kinases as drug targets in cancer. Curr Cancer Drug Targets. 2006; 6:623–34.
44. Rakowicz-Szulczynska EM, Herlyn M, Koprowski H. Nerve growth factor receptors in chromatin of melanoma cells, proliferating melanocytes, and colorectal carcinoma cells in vitro. Cancer Res. 1988; 48:7200–6.
45. Planque N. Nuclear trafficking of secreted factors and cell-surface receptors: new pathways to regulate cell proliferation and differentiation, and involvement in cancers. Cell Commun Signal. 2006; 4:7.
46. Ni CY, Murphy MP, Golde TE, Carpenter G. gamma-Secretase cleavage and nuclear localization of ErbB-4 receptor tyrosine kinase. Science. 2001; 294:2179–81. [PubMed: 11679632]
47. Bryant DM, Stow JL. Nuclear translocation of cell-surface receptors: lessons from fibroblast growth factor. Traffic. 2005; 6:947–54. [PubMed: 16138907]
48. Giri DK, Ali-Seyed M, Li LY, et al. Endosomal transport of ErbB-2: mechanism for nuclear entry of the cell surface receptor. Mol Cell Biol. 2005; 25:11005–18.
49. Lo HW, Ali-Seyed M, Wu Y, Bartholomeusz G, Hsu SC, Hung MC. Nuclear-cytoplasmic transport of EGFR involves receptor endocytosis, importin beta1 and CRM1. J Cell Biochem. 2006; 98:1570–83. [PubMed: 16552725]
50. Rutherford AC, Traer C, Wassmer T, et al. The mammalian phosphatidylinositol 3-phosphate 5-kinase (PIKfyve) regulates endosome-to-TGN retrograde transport. J Cell Sci. 2006; 119:3944–57. [PubMed: 16954148]

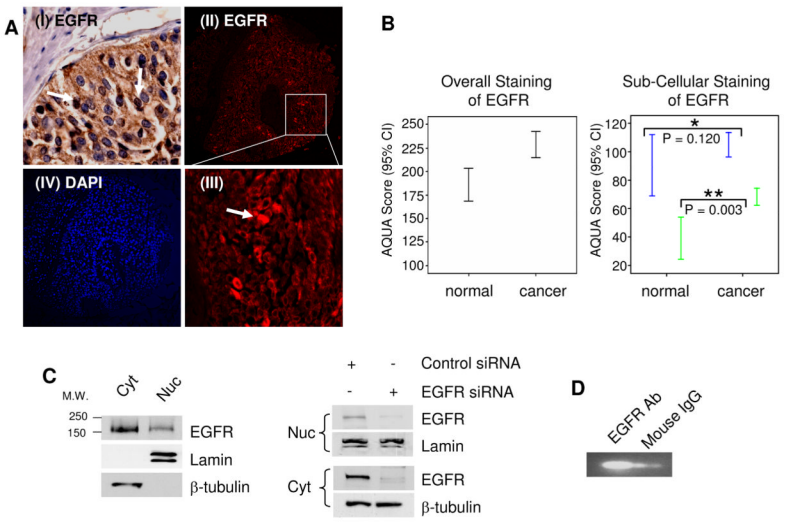


Figure 1.

Nuclear residency of EGFR in bladder cancer. A. (I) EGFR protein expression by standard immunohistochemistry in a human bladder cancer TMA containing human tumor tissues and normal samples. Arrows indicate nuclear localization. EGFR protein expression as determined by Automated quantitative analysis (AQUA). The Cy-5 expression (red), scored on a scale of 0–250, identifies the area of EGFR expression (II), magnified in (III), where nuclear localization of EGFR is indicated by the arrow. DAPI staining (IV) identifies nuclei both in the stroma and epithelial compartment. For each core, areas of tumor are distinguished from stromal elements by an epithelial tumor mask from the keratin signal (not shown). B. Expression of EGFR overlapping to cytokeratin and to DAPI quantified by the AQUA software. Overall and subcellular AQUA score for EGFR is shown (subcellular staining graph: blue, EGFR in cytoplasmic compartment; green, EGFR in nuclear compartment) as error bars of EGFR protein expression with 95% confidence intervals(27). The intensity is corrected for area for each tissue microarray spot and measured using arbitrary units. Expression results demonstrate measurable levels of EGFR in the nuclear compartment that are significantly higher in the tumor group than in normal samples, as demonstrated by non overlapping error bars with 95% confidence intervals (green). EGFR AQUA score in the cytosolic compartment is not significantly different between tumors and normal tissues. C. Localization of EGFR to the nuclear fraction in TCCSUP bladder cancer cells. Lamin A/C and β-tubulin were used for markers of nuclear and cytoplasmic fractions, respectively. Reduced EGFR expression elicited by EGFR siRNA was verified by western blot. D. Association of EGFR with the cyclin D1 promoter, as assayed by ChIP analysis.

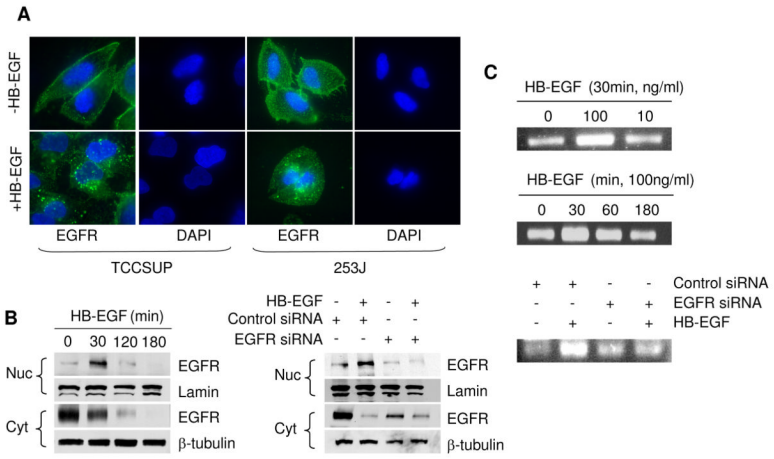


Figure 2.

EGFR nuclear trafficking stimulated by HB-EGF in bladder cancer cells. A. Immunofluorescent localization of EGFR in response to 100 ng/ml HB-EGF for 30 min (green, EGFR; blue, nucleus) in TCCSUP and 253J bladder cancer cells. B. Time-dependent localization of EGFR to nuclear fractions in response to HB-EGF (100 ng/ml, 30 min). EGFR siRNA reduced EGFR levels substantially. C. Time- and dose-ChIP experiments were performed using anti-EGFR antibody. Increased association of nuclear EGFR and the cyclin D1 promoter region following HB-EGF treatment is shown. Down-regulation of EGFR by gene silencing suppressed the association of EGFR with the cyclin D1 promoter.

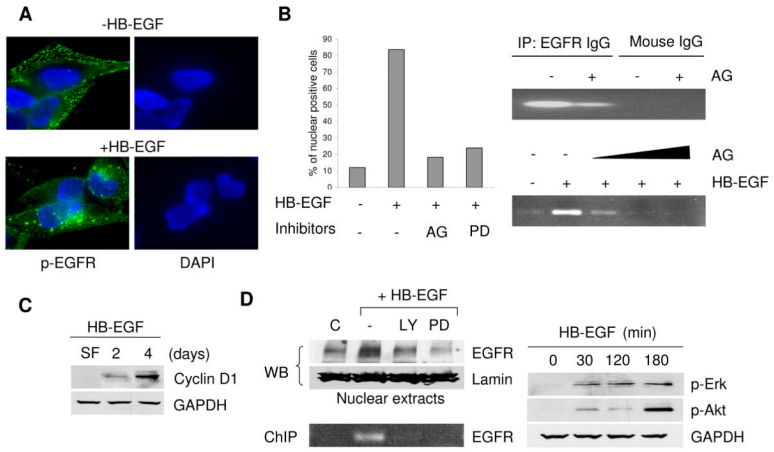


Figure 3.

Signaling pathways required for EGFR nuclear trafficking in TCCSUP cells treated with HB-EGF. A. Immunofluorescence micrographs demonstrating active (phosphorylated) EGFR in the nucleus (green, phosphorylated EGFR; blue, nucleus). B. Effect of EGFR kinase inhibitors (AG, AG1478; PD, PD153035) on EGFR trafficking under conditions where cells were treated with HB-EGF for 30 min (graph). Effect of the EGFR inhibitor, AG1478, on assembly of the nuclear EGFR/cyclin D1 promoter complex (right panels). C. Western blot analysis was performed and demonstrated that HB-EGF treatment increased expression of cyclin D1 (SF=serum-free control). D. Effect of PI3-kinase (LY, LY294002) and MEK (PD, PD98059) inhibitors on nuclear trafficking of EGFR. Nuclear extracts were subjected to Western blot analysis after 30 min of HB-EGF treatment in the absence or presence of inhibitors (upper panel). ChIP analysis was performed under the same experimental conditions as above (lower panel). Both PI3-kinase and Erk/MAPK signal pathways were activated by HB-EGF (right panel).

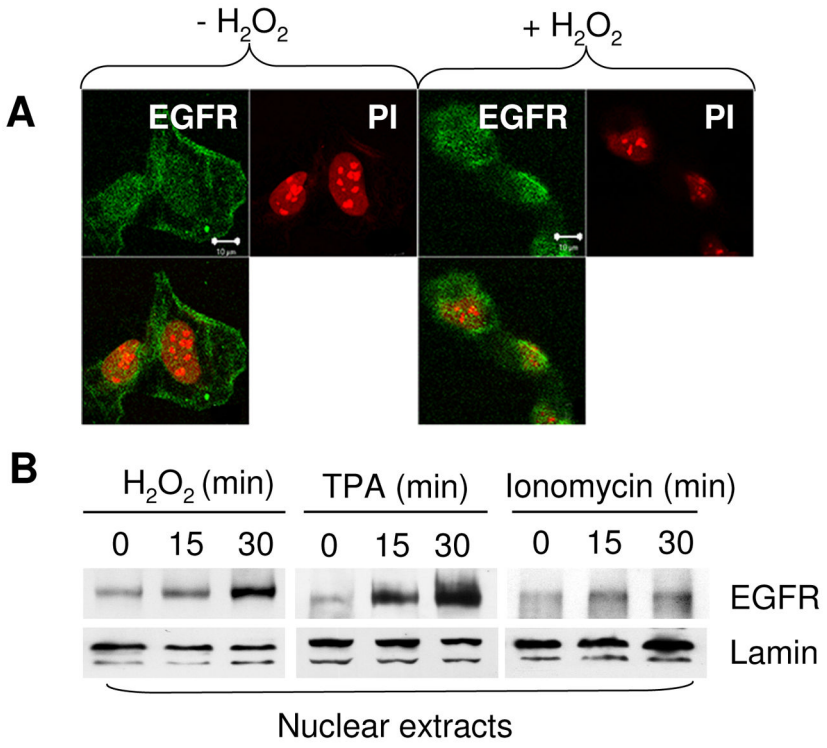


Figure 4.

EGFR nuclear localization induced by agents that stimulate cleavage of proHB-EGF from TCCSUP cells which stably overexpress proHB-EGF. A. Surface EGFR translocated to the nucleus in TCCSUP/proHB-EGF cells by treatment with the strong HB-EGF secretion inducer, H_2O_2 (green, EGFR; red, nuclei stained with propidium iodide). B. EGFR accumulated in nuclei upon treatment with HB-EGF secretion inducers H_2O_2 (0,01%), TPA (10 μM), and ionomycin (10 μM).

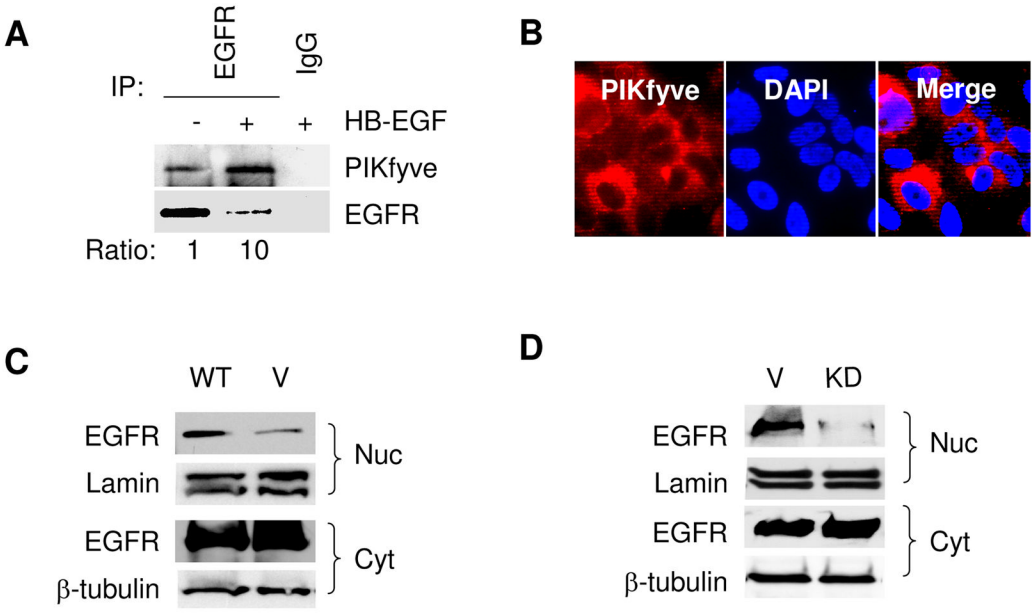
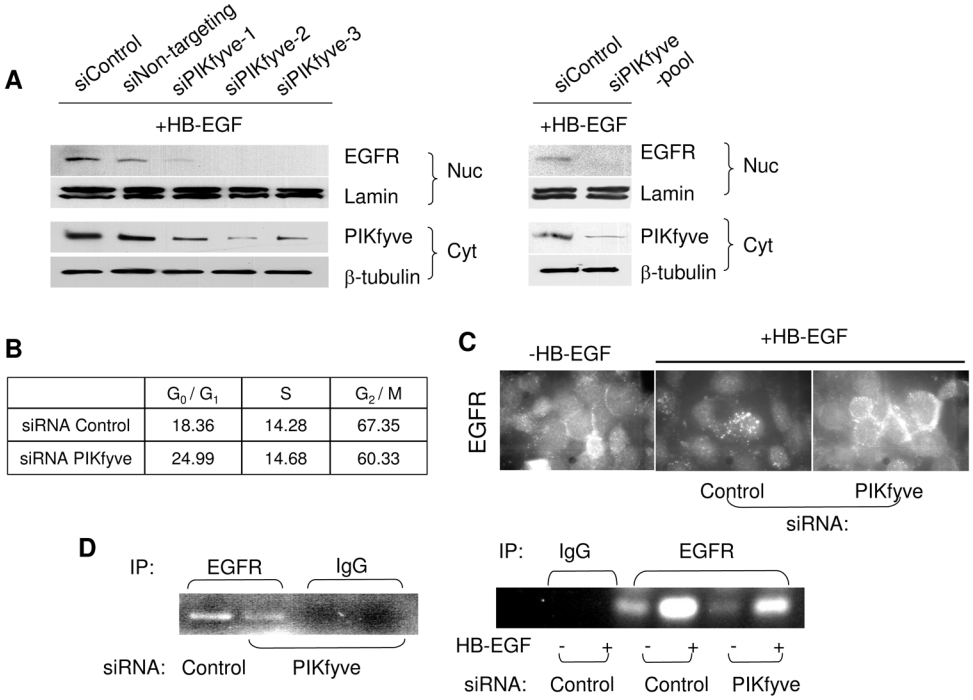


Figure 5.

Association of PIKfyve with the EGFR trafficking complex. A. Association of EGFR and PIKfyve were verified by co-IP and western blot analysis. TCCSUP whole cell lysates were subjected to IP with anti-EGFR antibody (Ratio: PIKfyve/EGFR). B. Immunofluorescence staining shows the cytoplasmic location of PIKfyve TCCSUP cells (red, PIKfyve; blue, nucleus). C–D. Effect of PIKfyve^{WT} or dominant-negative, kinase-dead PIKfyve^{K1831E} on nuclear EGFR level. After transient transfection with control (V, vector only), PIKfyve^{WT} (WT, wild-type) or PIKfyve^{K1831E} (KD, kinase dead), cells were harvested for nuclear extraction, followed by western blot. C. EGFR level in nuclear (Nuc) and cytosolic (Cyt) fractions from PIKfyve^{WT} transfectants after enforced expression. D. Blockade of EGFR localization to nuclei by PIKfyve^{K1831E}.



	G_0/G_1	S	G_2/M
siRNA Control	18.36	14.28	67.35
siRNA PIKfyve	24.99	14.68	60.33

Figure 6.

Cellular effects of PIKfyve loss of function demonstrated by RNA interference. A. EGFR localization after transient transfection with PIKfyve siRNA or control siRNA, +/− HB-EGF. Transfectants with siRNAs were serum starved and treated with HB-EGF for 30 min. Nuclear fractions were subjected to western blot analysis using antibodies against EGFR or Lamin. Cytoplasmic fractions were used to determine the level of PIKfyve relative to control under knockdown conditions. B. PIKfyve knockdown reduces cell cycle transit. Serum-starved TCCSUP cells were stimulated by FBS for 2 d in the presence of nocodazole, a G_2/M transition blocker. FACS analysis was performed using separate cell populations at different stages in the cell cycle. C. EGFR trafficking is blocked by PIKfyve knockdown. D. Reduction of complex formation between nuclear EGFR and cyclin D1 promoter demonstrated by ChIP (upper panel, log phase growth; lower panel, HB-EGF stimulated conditions).