Oncogene (2015), 1–9

www.nature.com/onc

ORIGINAL ARTICLE

Nuclear trafficking of EGFR by Vps34 represses *Arf* expression to promote lung tumor cell survival

D Dayde[1,2], M Guerard[1,2], P Perron[1,2], A-S Hatat[1,2], C Barrial[1,2], B Eymin[1,2] and S Gazzeri[1,2]

Epidermal growth factor receptor (EGFR) is a cell surface receptor that has an essential role in cell proliferation and survival, and overexpression of EGFR is a common feature of human cancers. In Non-small-cell lung cancer (NSCLC), activating mutations of EGFR have also been described. We recently showed that mutant EGFR-L858R inhibits the expression of the p14ARF tumor-suppressor protein to promote cell survival. In this study, we defined the molecular bases by which EGFR controls *Arf* expression. Using various lung tumor models, we showed that EGF stimulation inhibits *Arf* transcription by a mechanism involving the nuclear transport and recruitment of EGFR to the *Arf* promoter. We unraveled the vesicular trafficking protein Vps34 as a mediator of EGFR nuclear trafficking and showed that its neutralization prevents the accumulation of EGFR to the *Arf* promoter in response to ligand activation. Finally, in lung tumor cells that carry mutant EGFR-L858R, we demonstrated that inhibition of Vps34 using small interfering RNA restrains nuclear EGFR location and restores *Arf* expression leading to apoptosis. These findings identify the *Arf* tumor suppressor as a new transcriptional target of nuclear EGFR and highlight Vps34 as an important regulator of the nuclear EGFR/Arf survival pathway. As a whole, they provide a mechanistic explanation to the inverse correlation between nuclear expression of EGFR and overall survival in NSCLC patients.

Oncogene advance online publication, 21 December 2015; doi:10.1038/onc.2015.480

INTRODUCTION

Epidermal growth factor receptor (EGFR) belongs to a large family of membrane-bound receptor tyrosine kinases (RTKs) that serve as mediators of cell signalling by extracellular growth factors and have a pivotal role in physiological cell proliferation and behaviour. Increased levels of EGFR gene expression are observed in many cancers and frequently associated with an adverse prognosis.[1,2] In addition, in lung cancer, oncogenic mutations inside the tyrosine kinase domain of EGFR have been reported to confer tumor cell dependence on EGFR-mediated pro-survival signalling and high susceptibility to apoptosis induced by EGFR tyrosine kinase inhibitors (Gefitinib).[3–5] Binding of growth factor such as EGF induces EGFR homodimerization or heterodimerization and autophosphorylation of the intracellular domain. This promotes the recruitment of adaptor molecules that trigger the activation of signal transduction pathways, including the RAS/mitogen-activated protein kinase, the phosphoinositide-3-kinase (PI3K)/AKT and the signal transducer and activator of transcription (STAT) pathways.[6–10] These downstream signalling activities regulate proliferation, survival, mobility and differentiation in many different cell types. Results of several recent studies show that RTK such as EGFR can also transmit signal from subcellular localizations other than their classical plasma membrane location, including the nucleus.[11–13] Hence, nuclear EGFR has been shown to regulate a variety of cellular functions, such as cell proliferation, DNA replication and repair, and signal transduction both in normal tissues and in human diseases, including cancer.[14–17] The nuclear presence of EGFR is emerging as an important marker in many human cancers,[13,18] and some mechanisms underlying nuclear EGFR-mediated tumor progression have been described. As an example, in nasopharyngeal carcinoma induced by Epstein–Barr virus, the Epstein–Barr virus-encoded latent membrane protein 1 was found to enhance transactivation of *cyclin D1* and *cyclin E* by nuclear EGFR, thereby fostering cell proliferation.[19] In lung cancer, nuclear presence of EGFR was recently associated with poor clinical prognosis.[18] However, the molecular mechanisms by which a nuclear signalization of EGFR may contribute to lung cancer progression are not understood.

Arf tumor suppressor (p14ARF in humans, p19ARF in mice) is encoded by the *Ink4a/Arf* locus, which also houses the cyclin-dependent kinase inhibitor p16INK4a. Because of splicing events, separate promoters and unique first exons, the two proteins share no homology and have distinct functions.[20,21] P14ARF is not only a key activator of the p53 pathway but also displays inhibitory cell growth properties independently of p53.[22,23] Furthermore, p14ARF is involved in the maintenance of genomic stability in response to DNA damage and oncogenic stress, and its expression is a major break to cancer development, including lung cancer.[24] We recently identified a cross talk between EGFR and *Arf* signalling pathways in lung tumor cells and showed that activated EGFR inhibits the expression of the p14ARF protein.[25] Here we decipher the molecular mechanisms by which EGFR represses the p14ARF expression. We demonstrate that EGFR inhibits *Arf* transcription by a mechanism involving its nuclear translocation and accumulation to the *Arf* promoter. We also show that the Class III PI3K Vps34 facilitates nuclear trafficking and recruitment of EGFR to the *Arf* promoter and demonstrate that this Vps34/nuclear EGFR network counteracts *Arf* expression and pro-apoptotic signalling in lung tumor cells expressing mutant EGFR. These results provide the first

[1]Equipe Bases Moléculaires de la Progression des Cancers du Poumon, Centre de Recherche INSERM U823, Grenoble, France and [2]Université Joseph Fourier, Institut Albert Bonniot, Grenoble, France. Correspondence: Dr S Gazzeri, Equipe Bases Moléculaires de la Progression des Cancers du Poumon, Centre de Recherche INSERM U823, Institut Albert Bonniot, Grenoble 38042, France.
E-mail: Sylvie.Gazzeri@ujf-grenoble.fr
Received 25 March 2015; revised 2 October 2015; accepted 6 November 2015

evidence that a Vps34/nuclear EGFR trafficking contributes to the survival of lung tumor cells through inhibition of the *Arf* tumor suppressor.

RESULTS

EGFR decreases *Arf* transcript level

We previously reported a decreased expression of the p14ARF protein by activated EGFR in H1719 (ligand-stimulated wild-type (WT) EGFR) and H1975 (mutant EGFR-L858R) cells.[25] Here we investigated the molecular mechanisms involved. To address this, we first neutralized EGFR expression using specific small interfering RNA (siRNA) and studied the *Arf* expression by western blotting and reverse transcriptase/quantitative PCR (qPCR) analyses in both cell lines. Consistent with our previous data,[25] the expression level of the p14ARF protein was increased in EGFR-depleted cells (Figure 1a, bottom). Interestingly, we observed that accumulation of p14ARF protein correlated with a significant upregulation of its transcript expression level in both cellular models (Figure 1a, top). Same results were obtained in the HCC827 cell line, which express the active mutant EGFR-Del19, ensuring against potential cell-type-specific effects. To confirm the negative effect of EGFR on *Arf* transcripts, cells were serum starved for 24 h and *Arf* expression was studied after EGF stimulation. In all cellular models, the downregulation of p14ARF protein was associated with a significant diminution of its transcripts (Figure 1b and Supplementary Figure S1). Same results were obtained when H1719 cells were treated with Amphiregulin, another natural ligand for EGFR (Figure 1c). Importantly, expression of the *INK4a* transcript as well as the p16INK4a protein, which are also encoded by the *INK4a-Arf* locus, was not affected by ligand stimulation or when EGFR was neutralized using specific siRNA in all three cell lines (Figure 1d and Supplementary Figure S2). Taken together, these data demonstrated that WT and mutant EGFRs specifically downregulate *Arf* transcript level. Of note we observed that EGF does not decrease the stability of Arf mRNAs (Supplementary Figure S3), suggesting a role of EGFR in a negative control of *Arf* transcription.

Nuclear EGFR signalling pathway inhibits *Arf* transcription

Recent evidences indicate that RTKs such as EGFR act as transcriptional regulators when they are translocated to the nucleus.[14,26] Therefore, to investigate whether nuclear EGFR might control *Arf* transcription, we first checked whether EGFR accumulates in the nucleus of our cellular models in response to EGF stimulation. As shown by western blotting following subcellular fractionation, we found that EGF ligand increased nuclear EGFR expression in all three lung cancer cell lines (Figure 2a). These results were also supported by examination of EGFR by fluorescence pseudo-confocal (apotome) microscopy showing enrichment of a nuclear punctuated EGFR signal after EGF stimulation using three different antibodies (Figure 2b and Supplementary Figure S4). To go further, we used siRNA against *importin β1* or Brefeldin A treatment to block nuclear trafficking of EGFR. In both context, inhibiting the EGFR transport to the nucleus prevented the downregulation of both the mRNA and the p14ARF protein upon EGF stimulation (Supplementary Figure S5). As siRNA against *importin β1* and Brefeldin A do not target specifically EGFR trafficking, we took advantage of CHO cells stably expressing WT EGFR or an EGFR-pNLS mutant in which the nuclear localization signal (NLS) of EGFR is mutated.[27] In agreement with the inhibition of *Arf* by EGFR, EGF stimulation decreased the expression of both *Arf* transcript and protein in CHO-EGFR cells (data not shown). More importantly, we observed that the expression level of both *Arf* mRNA and protein was significantly increased in CHO-EGFR-pNLS, which do not express nuclear EGFR, compared with CHO-EGFR cells in which EGFR was present in the nucleus (Figures 2c and d). In agreement with our previous results, expression of *INK4a* did not vary significantly in CHO-EGFR-pNLS cells (Supplementary Figures S2E and F). As a whole, these data indicated nuclear EGFR is important for the repression of *Arf* expression.

As a transcription factor, the nuclear EGFR complex is known to target AT-rich minimal consensus sequences.[28] Very interestingly, '*in silico*' analysis of the *Arf* promoter highlighted several AT-rich sequence sites as putative EGFR-targeting sequences. Therefore, we used *in vivo* chromatin immunoprecipitation (ChIP)–qPCR assays to study whether nuclear EGFR is recruited to the *Arf* promoter. HCC827 cells were first used in this setting as they express high levels of nuclear EGFR (Figure 2a). Nuclear EGFR was precipitated from cells stimulated or not with EGF, and qPCR was performed using primers surrounding the three most proximal putative AT-rich sequence sites within the *Arf* promoter. We showed that nuclear EGFR accumulates on the *Arf* promoter in response to ligand stimulation (Figure 2e). In contrast, binding was not detected when ChIP was performed with control immunoglobulin G (IgG) and/or when irrelevant primers (Neg) were used for the qPCR, ensuring the specificity of the ChIP results. Same data were obtained in the H1975 model (Supplementary Figure S6). Altogether, these results demonstrated that nuclear EGFR is recruited to the *Arf* promoter in response to EGF stimulation in both EGFR mutant cell lines and inhibits Arf transcription. Unfortunately and despite several attempts, we could not obtain reliable ChIP results in H1719 cells. This could be due to the low level of expression of endogenous nuclear EGFR in these cells compared with H1975 and HCC827 cells in which the EGFR gene is strongly amplified.

PI3K but not AKT controls the nuclear translocation of EGFR

Next we wanted to characterize the molecular mechanisms controlling EGFR nuclear trafficking. Interestingly, we observed that pharmacological inhibition of EGFR prevents its nuclear accumulation following ligand activation and rescues the expression of both the *Arf* transcript and protein (Supplementary Figure S7). Therefore, we speculated that signal transduction pathways known to be downstream from the EGFR may promote its oncogenic nuclear translocation. In order to assess which pathways could be involved, we used specific pharmacological inhibitors. We found that pharmacological suppression of the RAS/mitogen-activated protein kinase or STAT pathways using U0126 and cucurbitacin, respectively, did not prevent the nuclear trafficking of EGFR in response to EGF nor the p14ARF downregulation (Supplementary Figures S8A and B). In contrast, the PI3K/AKT inhibitor wortmanin strongly inhibited EGFR nuclear transport in the same conditions whatever the EGFR status (WT or mutant) (Figures 3a and b and data not shown). This was accompanied by a diminished accumulation of EGFR on the *Arf* promoter (Figure 3c) and more importantly by the rescue of *Arf* mRNA expression (Figure 3d). To go further, we investigated the role of AKT by using Triciribin, a specific AKT inhibitor. Surprisingly, inhibition of AKT did not prevent the nuclear translocation of EGFR (Figures 3b and e) nor the downregulation of *Arf* transcript (Figure 3d). These results were confirmed with another specific AKT inhibitor, namely MK2206 (Supplementary Figure S8C). Altogether, these data indicated that activation of a PI3K-dependent AKT-independent pathway drives the nuclear translocation of EGFR and the inhibition of *Arf* transcription.

Class III PI3K Vps34 facilitates EGFR nuclear transport and binding to the *Arf* promoter

The family of PI3K enzymes comprises three different classes, I, II and III. Wortmamin and LY294002 are broad inhibitors against PI3Ks although members of the class II show decreased sensitivity. The class I PI3K is activated downstream of RTKs such as EGFR and

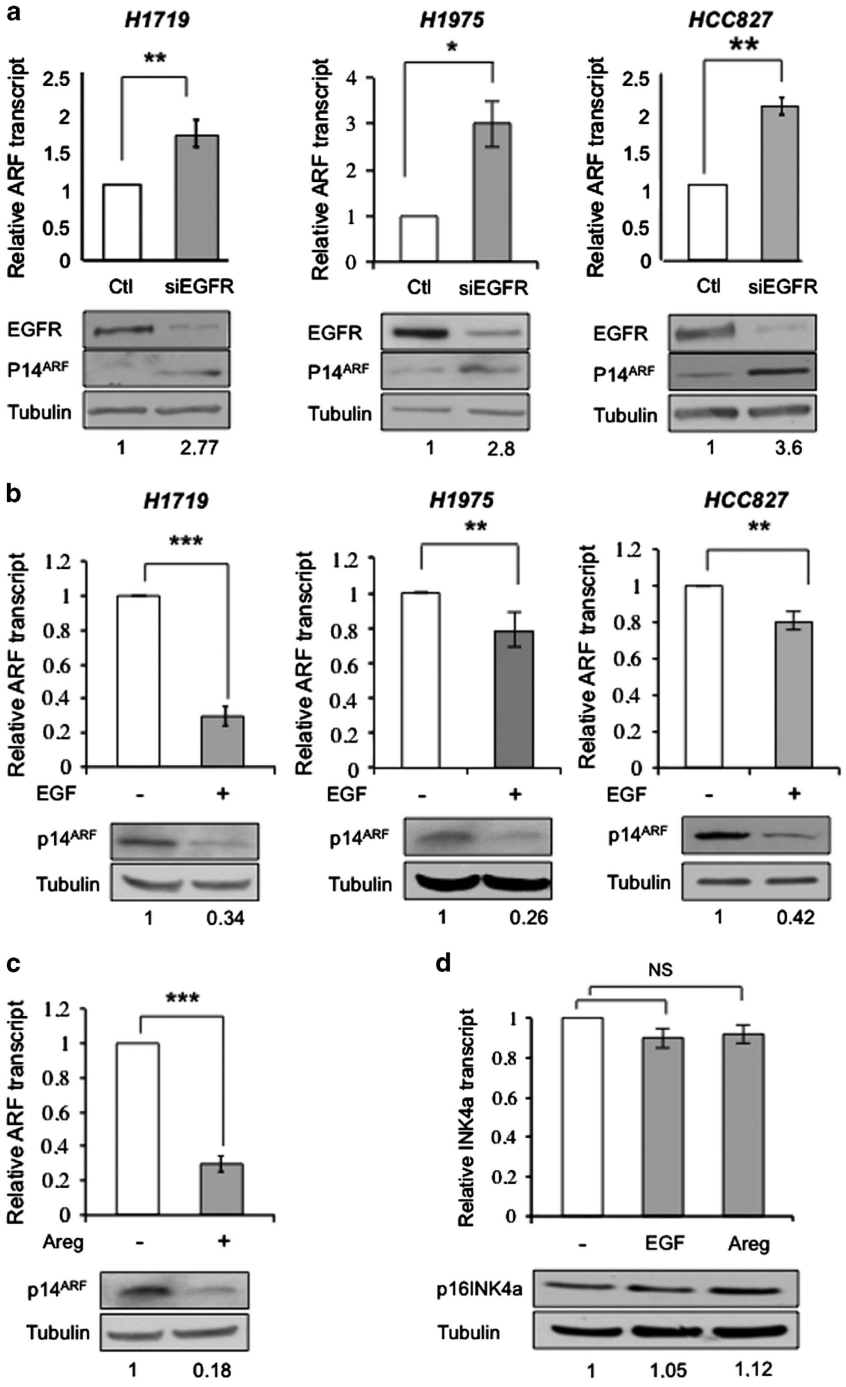

Figure 1. EGFR downregulates *Arf* mRNA level. (**a**) Cells were transfected with control siRNA (Ctl) or siRNA against *EGFR* (siEGFR) for 72 h. Expression of *Arf* transcript was studied by qPCR. qPCR data are represented as fold increase relative to non-treated cells, which were arbitrarily assigned to 1. Protein expression was analysed by western blotting using the indicated antibodies. Tubulin was used as a loading control. (**b**) Cells were incubated with EGF (50 ng/ml) for 24 h. Expression of *Arf* was studied by qPCR and western blotting as described above. (**c**) H1719 cells were treated with Amphiregulin (Areg) (50 ng/ml) for 24 h. Expression of *Arf* was studied by qPCR and western blotting as described above. (**d**) H1719 cells were stimulated with EGF or Areg (50 ng/ml) for 24 h. Expression of *INK4a* was assessed by qPCR and western blotting. All the data represent the mean ± s.d. of three independent experiments. NS, not significant. $^{*}P \leqslant 0.05$, $^{**}P \leqslant 0.01$, $^{***}P \leqslant 0.001$. Quantification of p14ARF protein levels was performed as described in the Materials and methods section and is numerically indicated below each lane.

stimulates the AKT signalling pathway. In agreement with our previous results (Figure 3), treating cells with the selective class I PI3K inhibitor GDC-0941 did not prevent EGFR nuclear transport in response to EGF nor p14ARF downregulation (Supplementary Figure S9). Interestingly, previous work in yeast had implicated class III PI3K in diverse intracellular trafficking events, including endosome-to-Golgi retrograde transport.[29] Therefore, we speculated that class III PI3K may have a role in the nuclear trafficking of EGFR in our cellular models. The Vacuolar sorting protein 34 (VPS34) is the single class III PI3K isoform in humans. Because of

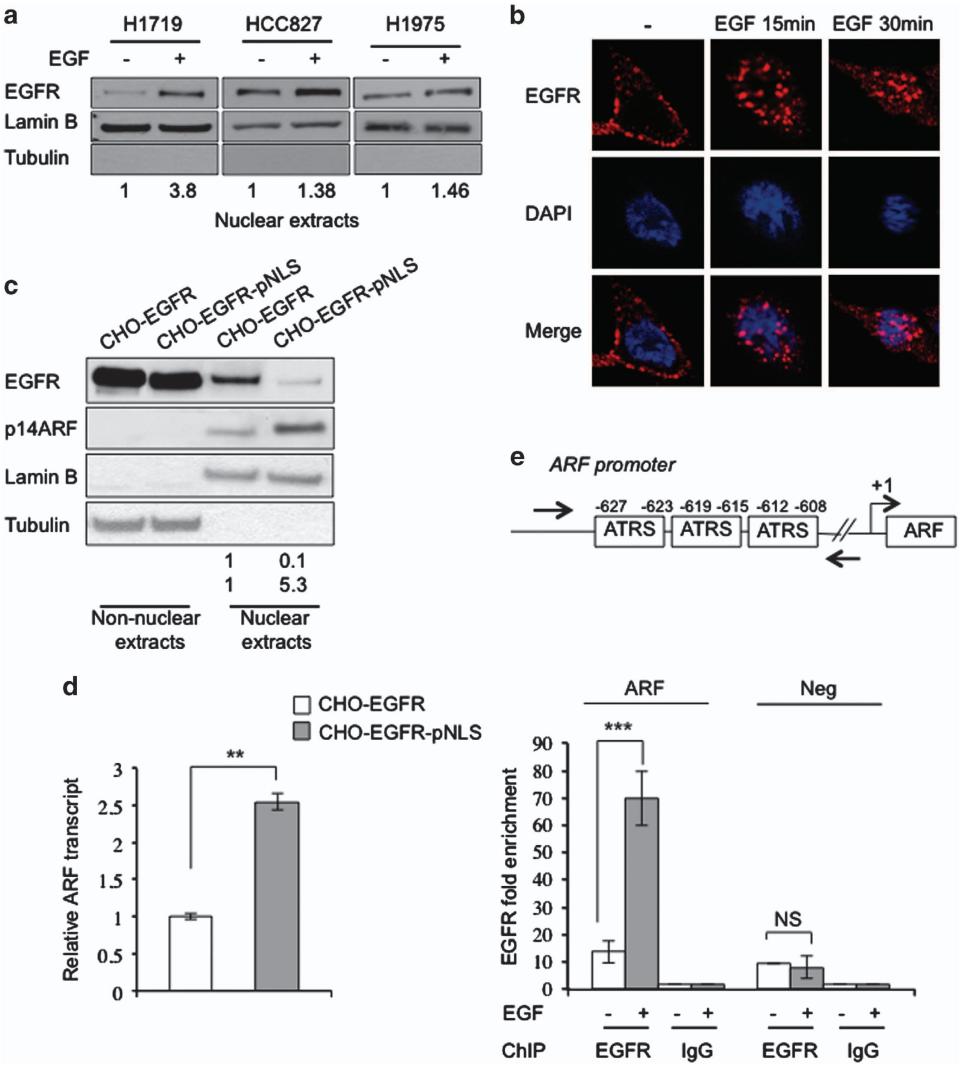

Figure 2. Nuclear EGFR inhibits *Arf* transcription. (**a**, **b**) Cells were stimulated for 15 min with EGF (50 ng/ml). Western blotting was performed using the indicated antibodies after subcellular fractionation. Levels of tubulin and lamin B were used as markers for cytosolic and nuclear fractions, respectively. Quantification of EGFR protein levels is numerically indicated below each lane. Immunolocalization of EGFR (D38B1, Red) was performed in H1719 cells using fluorescent pseudo-confocal (apotome) microscopy. DAPI (Blue) was used to counterstain nuclei. (**c**, **d**) Expression of EGFR and Arf was studied by western blotting and/or qPCR in CHO cells constitutively expressing WT (EGFR WT) or mutant EGFR (EGFRpNLS). Quantification of EGFR (top line) and Arf (bottom line) protein levels is numerically indicated below each lane. (**e**) The three most proximal putative AT-rich sequence sites within the *Arf* promoter are illustrated. Arrows represent primers used in the ChIP/qPCR study. HCC827 cells were stimulated for 30 min with EGF. The binding of EGFR to *Arf* promoter was analysed by ChIP as described in the Materials and methods section. IgG was used as a negative control for ChIP. qPCR with irrelevant primers (ChIP-IT Express Kit, Active Motif) was performed to ensure the specificity of the results. Data represent the mean ± s.d. of three independent experiments. $^{**}P \leqslant 0.01$, $^{***}P \leqslant 0.001$. NS, not significant.

the lack of specific pharmacological inhibitors of human Vps34, we used siRNA to disturb Vps34 expression in the presence or absence of EGF stimulation. The results showed that neutralization of Vps34 expression in H1719 cells prevents the nuclear transport of EGFR following EGF stimulation as visualized by western blotting following subcellular fractionation or by confocal microscopy (Figures 4a and b). Importantly, this was accompanied by a partial but significant and reproducible rescue of *Arf* transcript (Figure 4c) and the p14ARF protein was not decreased in Vps34 knockdown cells following EGF stimulation (Figure 4a). In addition, we observed that depriving cells from Vps34 using siRNA diminished the recruitment of EGFR to the *Arf* promoter following EGF treatment (Figure 4d), thereby indicating that Vps34 is important for the recruitment of nuclear EGFR to the *Arf* promoter. Altogether, these data strongly suggested that the Class III Vps34 contributes to the nuclear trafficking of EGFR in lung tumor cells.

Inhibition of Vps34 prevents nuclear trafficking of mutant EGFR and restores p14ARF-dependent apoptosis in lung adenocarcinoma cells

We previously reported that the EGFR-L858R mutant inhibits the expression of p14ARF protein to promote cell survival and showed that Arf knockdown reduces apoptosis caused by siEGFR.[25] As we demonstrated in this study that nuclear EGFR downregulates Arf transcript level, we speculated that nuclear translocation of the EGFR-L858R mutant might inhibit p14ARF-dependent apoptotic signalling pathway. To address this issue, we transfected H1975 cells with siRNA against Vps34. In these cells, we confirmed that

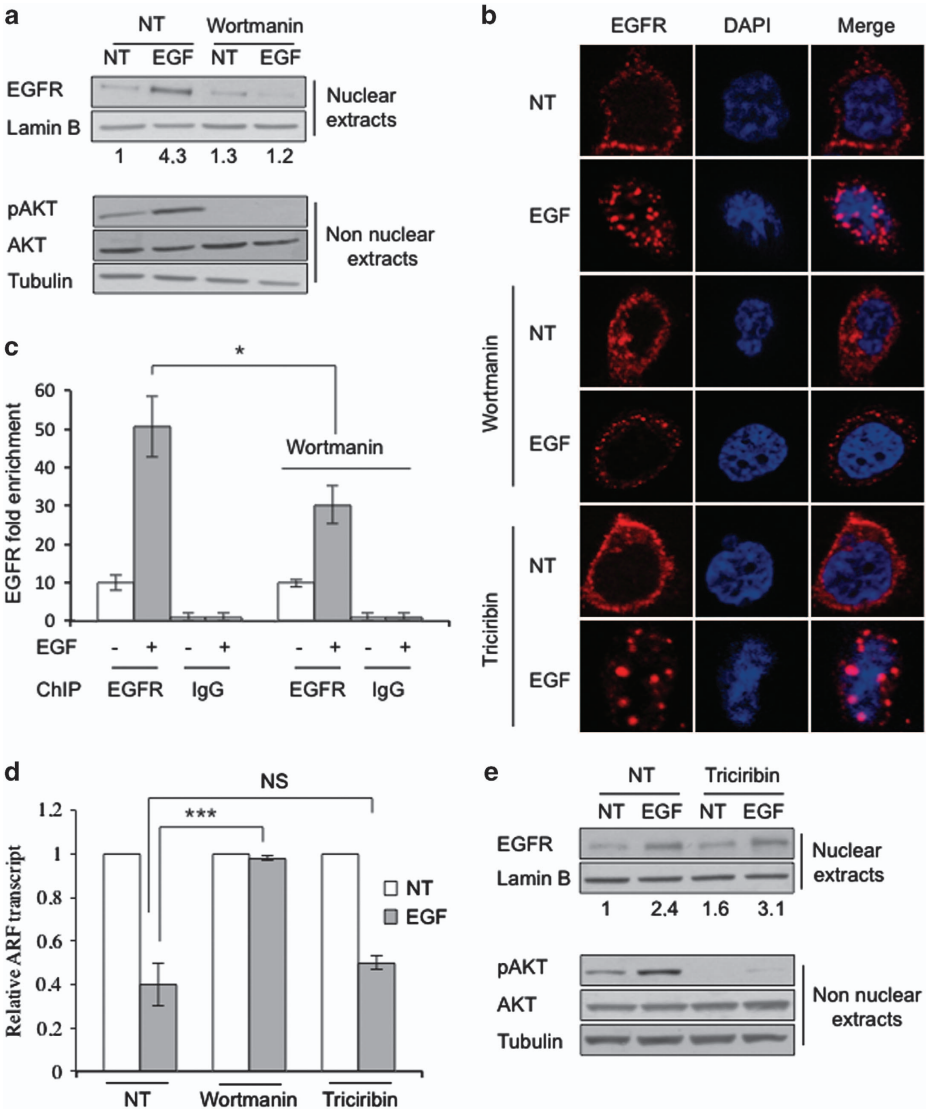

Figure 3. PI3K but not AKT controls nuclear EGFR trafficking to inhibit *Arf* transcription. H1719 cells were pretreated for 30 min with Wortmanin (0.1 μM) or Triciribin (10 μM) before EGF stimulation for 15 min (**a**, **b**, **c**, **e**) or 24 h (**d**). (**a**, **e**) Western blotting was performed after subcellular fractionation using the indicated antibodies. Quantification of EGFR protein levels is numerically indicated below each lane. (**b**) Expression of EGFR (D38B1, Red) was analysed by immunofluorescence and pseudo-confocal (apotome) microscopy. DAPI (blue) was used to counterstain nuclei. (**c**) ChIP was performed as previously described. (**d**) *Arf* expression was analysed by qPCR as described. Data represent the mean ± s.d. of three independent experiments. $*P \leqslant 0.05$, $***P \leqslant 0.001$. NS, not significant.

neutralization of Vps34 decreases the nuclear expression level of EGFR (Figure 5a) and upregulates those of *Arf* transcript (Figure 5b). Our previous results demonstrated that p14ARF promotes apoptosis of H1975 cells by activating an original pSTAT3(705)-dependent pro-apoptotic pathway that downregulates Bcl-2.[25] In Vps34 knocked down cells, we showed that *Arf* upregulation was accompanied by accumulation of pSTAT3-Y705, downregulation of Bcl-2 expression and induction of apoptosis (Figures 5a and c). Importantly, silencing *Arf* expression using siRNA rescued Vps34 knockdown cells from all these modifications (Figure 5a) and diminished apoptosis occurrence (Figure 5c). We also noticed a slight increase of cell proliferation after 72 h of siRNA transfection (data not shown). Of note, silencing of INK4a had no effect on apoptosis caused by Vps34 downregulation (Supplementary Figure S10). Together, these results indicated that a Vps34-dependent nuclear signalization of mutant EGFR promotes the survival of lung adenocarcinoma cells by counteracting the pro-apoptotic function of the *Arf* tumor suppressor (Figure 5d).

DISCUSSION

In recent years, a novel mode of EGFR signalling has emerged by which EGFR translocates to the nucleus after endocytosis where it displays unique functions. Nuclear accumulation of EGFR has been linked to various diseases, including cancer, and was associated with poor clinical outcome in that case.[18,30–32] However, a few studies have addressed the molecular determinants accounting for the nuclear trafficking and signalling pathways of EGFR in tumors. The present study identifies a mechanism by which nuclear EGFR might contribute to lung adenocarcinoma progression. We show that nuclear EGFR accumulates on the *Arf* promoter and inhibits *Arf* transcription. Moreover, we demonstrate that the vesicular trafficking protein Vps34 mediates the nuclear transport of EGFR to inhibit p14ARF-dependent apoptosis, thereby unravelling Vps34 as a new regulator of EGFR signalling.

The well-characterized trafficking of cell surface EGFR is routed, via endocytosis and endosomal sorting, to the lysosomes for degradation or to the plasma membrane for recycling. In addition

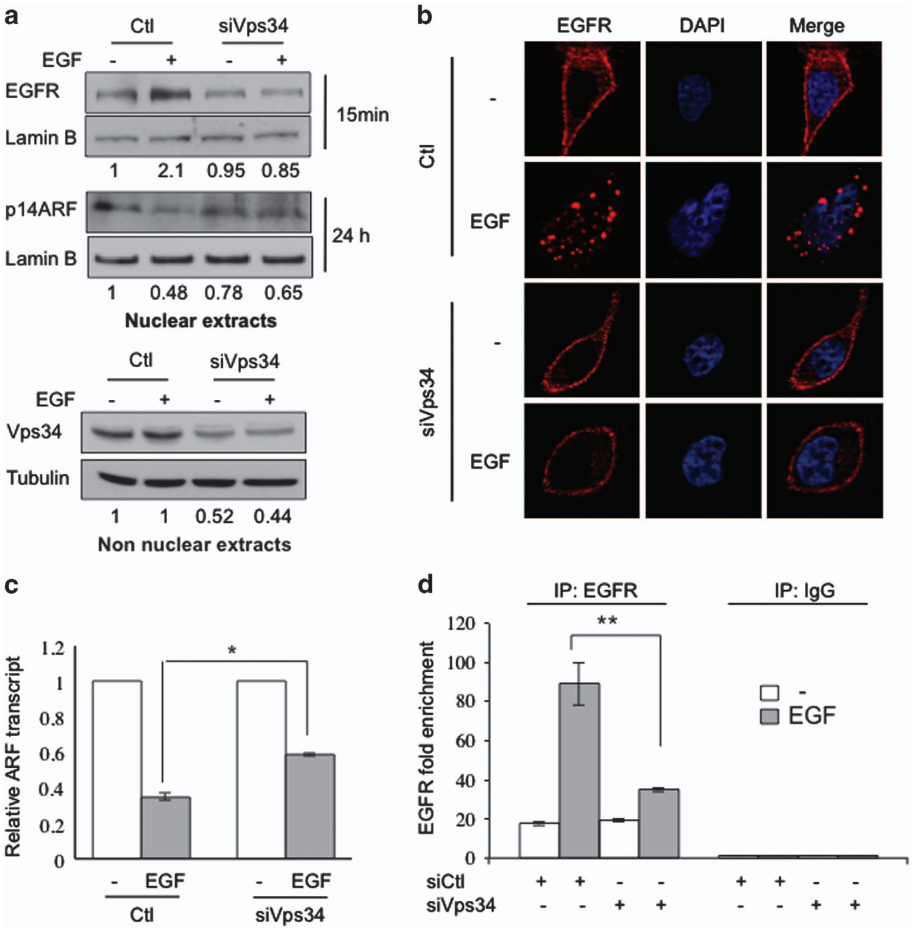

Figure 4. Class III PI3K Vps34 controls nuclear trafficking and accumulation of EGFR to *Arf* promoter. (**a**–**c**) H1719 cells were transfected with control siRNA (Ctl) or siRNA against Vps34 (siVps34) for 72 h prior to EGF stimulation (50 ng/ml) for an additional 15 min (**a**, upper panel, (**b**)) or for 48 h prior to EGF stimulation (50 ng/ml) for an additional 24 h (**a**, lower panel, (**c**)). EGFR and Vps34 expression was studied by western blotting after subcellular fractionation. Quantification of EGFR and Arf protein levels is numerically indicated below each lane. Immunolocalization of EGFR (Red) was performed using fluorescent pseudo-confocal (apotome) microscopy. DAPI (Blue) was used to counterstain nuclei. Expression of *Arf* transcript was studied by qPCR. (**d**) HCC827 cells were transfected with control siRNA (Ctl) or siRNAs against Vps34 (siVps34) for 72 h and stimulated with EGF (50 ng/ml) for an additional 30 min. ChIP analysis was performed as previously described. All the data represent the mean ± s.d. of three independent experiments. $*P \leqslant 0.05$, $**P \leqslant 0.01$.

to the above well-characterized trafficking routes, alternative fate for endocytosed activated EGFR involves transport to different compartments within the cells, including the nucleus.[11,33,34] Although nuclear detection of EGFR has been associated with tumor aggressiveness, the mechanisms underlying the nuclear accumulation of EGFR in cancer are not understood. In this study, we identify class III PI3K Vps34 as an important mediator of nuclear translocation of EGFR as we showed that its neutralization prevents the translocation of both ligand-stimulated and mutant EGFR in the nucleus of lung tumor cells. Vps34 has an important role in the control of vesicular protein sorting, a phenomenon that was first discovered in yeast.[35] In all eukaryotes, Vps34 has central functions in endosomal protein sorting, endosome–lysosome maturation, autophagy and phagocytosis.[36–38] Our results suggest that Vps34 might also facilitate the endosome-to-golgi sorting of RTK such as EGFR to allow nuclear transport. Previous studies have involved the AKT and protein kinase C kinases in the aberrant accumulation of EGFR in the nucleus in response to therapy in breast and lung tumor cell lines, respectively.[15,39] Our results show that these kinases do not control the nuclear translocation of EGFR in response to ligand stimulation (Figure 3 and data not shown). Collectively, these results support the fact that signalling pathways that mediate EGFR nuclear transport may vary according to the tumor type and/or the cellular stress.

We demonstrated that nuclear EGFR inhibits the expression of *Arf*. As other members of the HER family that are expressed in our cellular models such as HER2 are subjected to nuclear trafficking,[40] we cannot exclude the possibility that they could also negatively regulate Arf expression. We demonstrated that nuclear EGFR accumulates on the *Arf* promoter and inhibits the expression of *Arf* transcripts. It has been previously shown that, in the nucleus, EGFR functions as a co-transcription factor to enhance transcription of tumor-promoting genes, such as cyclin D1, inducible nitric oxide synthase, cyclooxygenase 2, Aurora A and c-Myc.[27,28,41–43] In contrast, our results suggested for the first time that nuclear EGFR may also repress transcription of tumor-suppressor genes to control tumor growth. Several transcription factors and complexes, including Polycomb group proteins, have been reported to inhibit *Arf* transcription.[44] Most important and well-characterized *Arf* repressors include the BMI-1 and Pokemon proteins whose aberrant overexpression have been reported to contribute to lung carcinogenesis.[45–48] Interestingly, previous studies reported that ligand-activated EGFR increases BMI-1 or Pokemon expression in head and neck and prostate cancer cells, respectively.[49,50] We investigated the role of these proteins in the transcriptional inhibition of *Arf* by EGFR in our lung tumor models. However, the results showed that downregulation of *Arf* mRNA following EGFR activation was maintained when expression of either BMI-1 or

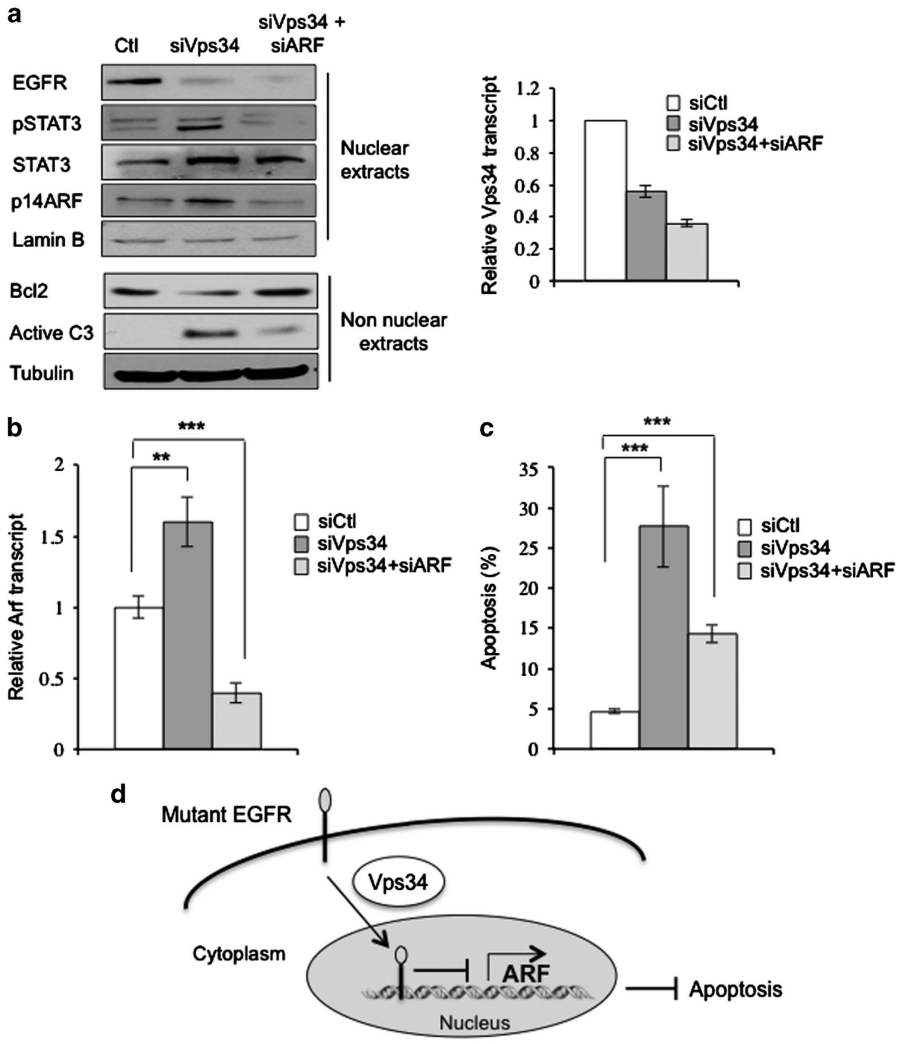

Figure 5. Nuclear trafficking of mutant EGFR by Vps34 inhibits p14ARF-dependent apoptosis in lung tumor cells. (**a**–**c**) H1975 cells were transfected with control siRNA (Ctl) or siRNAs against Vps34 (siVps34) and/or *Arf* (siArf) for 72 h. Western blotting was performed after subcellular fractionation. Neutralization efficiency of Vps34 expression was assessed by qPCR. Expression of *Arf* mRNAs was studied by qPCR as described above. Apoptosis was quantified by staining of active caspase3 followed by fluorescence-activated cell sorting analysis. Data represent the mean ± s.d. of three independent experiments. $^{**}P \leqslant 0.01$, $^{***}P \leqslant 0.001$. (**d**) A proposed model underlying the way by which the nuclear translocation of mutant EGFR through a Vps34-dependent mechanism negatively controls the *Arf* expression to prevent cellular apoptosis.

Pokemon genes was neutralized using specific siRNA, indicating that they are probably not involved in that case (data not shown). Similar results were obtained when expression of other well-identified *Arf* repressors such as p53, E2F3b, Tbx2 and Tbx3 was knocked down (data not shown). Therefore, molecular mechanisms underlying transcriptional inhibition of *Arf* gene by nuclear EGFR require further investigation.

Expression of p14ARF is decreased in many tumors, including lung cancer, and previous studies have described a coupling between mutations of EGFR and downregulation of p14ARF protein in non-small-cell lung cancer.[51,52] In agreement with an EGFR/Arf connection, we recently demonstrated that EGFR inhibits the expression of p14ARF protein to promote cell survival.[25] In this study, we further deepened these results by providing the first evidence that a nuclear EGFR signalling pathway represses Arf transcription and pro-apoptotic function and by identifying the Vps34 protein as an important mediator of this pathway. An inverse correlation between nuclear expression of EGFR and overall survival in non-small-cell lung cancer patients was recently reported.[18] Therefore, our data provide a mechanistic explanation to these *in situ* observations and unravel the Vps34/nuclear EGFR/Arf network as a potential mechanism of lung cancer progression. Future studies are now required to validate the Vps34/nuclear EGFR connection as an oncogenic pathway in human tumor samples, especially as Vps34 inhibitors have been recently developed.[53] Moreover, as nuclear accumulation of WT EGFR was also ascribed to resistance to anti-EGFR monoclonal antibody therapy (Cetuximab) in lung cancer models,[54] it is tempting to speculate that aberrant trafficking of mutant EGFR in the nucleus may also contribute to the resistance of lung adenocarcinoma patients to EGFR tyrosine kinase inhibitor treatment.

MATERIALS AND METHODS

Cell culture and treatments

HCC1719, HCC827 and H1975 cell lines (kindly provided by Professor A Gazdar) were derived from human lung adenocarcinoma. HCC1719 cells carry a WT EGFR, whereas HCC827 harbour a LREA deletion in the exon 19 of EGFR and H1975 a missense substitution L858R in exon 21 of EGFR. All cells were maintained in RPMI-1640+GlutaMAX medium (GIBCO, Cergy Pontoise, France) supplemented with 10% foetal calf serum in a humidified

incubator with 5% CO_2 at 37 °C. CHO-EGFR and CHO-EGFR-pNLS were kindly provided by Dr Mien Chie Hung. They were cultured in Dulbecco's modified Eagle's medium–F12 medium (GIBCO) supplemented with 10% foetal calf serum. All cell lines were mycoplasma free. For growth factor stimulation, cells were incubated in serum-free medium for 24 h and after medium change Rh-EGF (Eurobio, Courtaboeuf, France) (50 ng/ml in HCC1719 cells and 100 ng/ml in HCC827 and H1975 cells) or rh-Amphiregulin (R&D SYSTEMS EUROPE, Lille, France) (50 ng/ml) was added for 15 min or 24 h. Pharmacological inhibitors were added 30 min prior EGF stimulation. Actinomycin D and Brefeldin A were purchased from Sigma-Aldrich (Saint Quentin Fallavier, France), Triciribin from Calbiochem (VWR, Fontenay-sous-bois, France), Gefitinib from AstraZeneca (Rueil-Malmaison, France), U0126 and LY294002 from Selleckchem (Souffelweyersheim, France), Cucurbitacin I and Wortmaninn from Santa Cruz (TEBU, Le Perray en Yvelines, France) and GDC0941 and MK2206 from Roche Diagnostics (Meylan, France). Apoptosis was studied on a total cell population using the Phycoerythrin-conjugated Monoclonal Active Caspase 3 Antibody Apoptosis Kit (BD Biosciences, Pharmingen, Le Pont de Claix, France) according to the manufacturer's protocol. Analysis was performed using a FACScan flow cytometer (BD Biosciences).

Transfection of siRNA oligonucleotides

The sequences designed to specifically target human *EGFR*, *Importin β1*, *Vps34*, *INK4a* and *Arf* RNAs were as follows: *EGFR*, 5′-CUCUG GAGGAAAAGAAAGU-3′; *Importin β1*, 5′-GGACUUAUGUACAGCAUUU-3′; *Vps34*, 5′CCCAUGAGAUGUACUUGAACGUAAU-3′, *INK4a*, 5′-CGCACCGAAU AGUUACGGU-3′; and *Arf*, 5′-GAACAUGGUGCGCAGGUUC-3′. For all interference experiments, the mismatch siRNA oligonucleotide used as a control was 5′-UCGGCUCUUACGCAUUCAA-3′. Cells were transfected with siRNA oligonucleotide duplexes using jetPrime reagent (OZYME, Saint Quentin en Yvelines, France) according to the manufacturer's protocol. The cells were analysed 72 h posttransfection.

Cellular fractionation

Cells were pelleted and washed twice with phosphate-buffered saline (PBS) 1×. Cells were resuspended in hypotonic buffer (10 mM Tris-HCl pH 7.5, 10 mM KCl, 1.5 mM $MgCl_2$, 0.5 mM dithiothreitol, 0.02 M NaF, 1 mM Na_3VO_4, 1× EDTA-free Protease Inhibitor Cocktail (Roche Diagnostics) for 20 min on ice. Then cells were centrifuged at 2000 *g* for 5 min, and pellets were resuspended in hypotonic buffer+2% NP-40. Homogenates were incubated for 15 min on ice and centrifugated at 3000 *g* for 15 min. The resulting supernatant formed the non-nuclear fraction. The nuclear pellets were washed four times with PBS 1×, resuspended in RIPA buffer (50 mM Tris-HCl pH 7.5, 150 mM NaCl, 0.5% Na deoxycholate, 1% NP-40, 0.1% sodium dodecyl sulphate, 0.02 M NaF, 1 mM Na_3VO_4, 1× EDTA-free Protease Inhibitor Cocktail) and sonicated. Homogenates were centrifugated at 13 500 *g* for 30 min; the resulting supernatant formed the nuclear fraction. Total protein extracts was performed in RIPA buffer as described previously.[25] Analysis of protein expression was performed by western blotting. EGFR, p14ARF and lamin B intensities were quantified using the ImageJ software (ImageJ, U.S. National Institutes of Health, Bethesda, MD, USA), and the relative densitometric areas for EGFR or p14ARF were determined according to lamin B signal in each condition.

Antibodies

Anti-EGFR (D38B1), anti-$p14^{ARF}$ (4C6/4), anti-AKT, anti-pAKT (D9E), anti-ERK1/2 (137F5), anti-pERK1/2 (D13.14.4E), anti-STAT3 (79D7), anti-pSTAT3 (D3A7), anti-Bcl-2, anti-cleaved caspase3 (Asp175) and anti-lamin B1 (D4Q4Z) antibodies were purchased from Cell Signaling (OZYME, Saint Quentin Yvelines, France). Anti-α tubulin (B-5-1-2), anti-p16INK4a (sc-759) and anti-importin β1 antibodies were purchased from Santa-Cruz (Clinisciences, Montrouge, France). Anti-Vps34 antibody was from Novusbio (R&D System Europe–Bio-Techne, Lille, France).

RNA extraction, reverse transcription and real-time qPCR analysis

RNA was extracted using the High Pure RNA Isolation Kit (Roche Diagnostics) according to the manufacturer's protocol and subjected to Reverse Transcription using iScript RT supermix (Bio-Rad, Marnes-la-Coquette, France). qPCR was performed with iTaq Universal SYBR Green Supermix (Bio-Rad). The primers used for cDNA amplification were as follows: *Arf* S: 5′-GCGCAGGTTCTTGGTGAC-3′; *Arf* AS: 5′-GGCTCCTC AGTAGCATCAGC-3′; *INK4a* S: 5′-GAGCAGCATGGAGCCTCC-3′; *INK4a* AS: 5′-GGCCTCCGACCGTAACTATT-3′; Vps34 S: 5′-AAGCAGTGCCTGTAGGAGGA-3′; *Vps34* AS: 5′-TGTCGATGAGCTTTGGTGAG-3′; GAPDH (glyceraldehyde 3-phosphate dehydrogenase) S: 5′-CGAGATCCCTCCAAAATCAA-3′; and *GAPDH* AS: 5′-ATCCACAGTCTTCTGGGTGG-3′. Amplification of GAPDH was performed in all experiments and used as a reference gene. Relative gene expression was calculated for each sample as the ratio of target cDNA copy number to GAPDH cDNA copy number. The data represent the mean ± s.d. of at least three independent experiments.

ChIP assay

Cells were crosslinked with 1% formaldehyde for 10 min and subsequently processed using the ChIP-IT Express Kit (Active Motif, La Hulpe, Belgium) according to the manufacturer's protocol. Immunoprecipitation was performed with either anti-EGFR antibody (Santa-Cruz) or rabbit IgG as a control at 4 °C overnight. The immunoprecipitated complexes were purified using the Chromatin IP DNA Purification Kit (Active Motif) and subjected to qPCR using primers specific to the ARF promoters, which were sense 5′-GCGTGCAGCGGTTTAGTTTA-3′ and anti-sense 5′-CTCTATCCGC CAATCAGGAG-3′. Human Negative Control Primer Set 1 (Active Motif) was used as a negative control. Input DNA sample corresponding to 1% of immunoprecipitated chromatin was analysed in parallel in order to normalize the results of each ChIP DNA sample to the corresponding input DNA sample. EGFR enrichment on ARF promoter was calculated after adjustment of normalized ChIP fraction for the normalized background (IgG).

Indirect immunofluorescence

For immunolocalization studies, cells were fixed with 1% paraformaldehyde in PBS for 10 min at room temperature, washed once with PBS and permeabilized with 0.1% Triton X-100 in PBS for 10 min. Blocking was performed for 45 min in 5% bovine serum albumin. Incubation with anti-EGFR antibody (D38B1) from Cell Signaling was carried out overnight at 4 °C in a humid chamber. Cells were then incubated for 45 min with Alexa 568 (Life Technologies, Saint Aubin, France), mounted in a solution containing 4,6-diamino-2-phenylindole (DAPI) (Roti-Mount FluorCare DAPI, ROTH), vizualized by fluorescent microscopy using Axioimager microscope (Carl Zeiss, Jena, Germany) with the AxioVision software at a ×60 magnification and processed with the ImageJ software.

Statistical analysis

All the data represent the mean ± s.d. of three independent experiments. All statistical analyses were performed using an unpaired Student's *t*-test (*$P \leqslant 0.05$, **$P \leqslant 0.01$, ***$P \leqslant 0.001$). $P \leqslant 0.05$ was considered as statistically significant.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

ACKNOWLEDGEMENTS

We thank Professor A Gazdar for providing us with the H1719, HCC827 and H1975 cellular models and Dr MC Hung for the CHO-EGFR and CHO-EGFR-pNLS cells. This work was supported by Institut National de la Santé et de la Recherche Médicale U823, the Fondation ARC pour la recherche sur le cancer (grant no. 20131200109), the Fondation de France and the Ligue Nationale contre Le Cancer Comité de Savoie. DD was supported by the Fond de Dotation pour la Recherche en Santé Respiratoire 2010 and the Fondation ARC pour la recherche sur le cancer. MG was supported by le Fonds Agir pour les Maladies Chroniques. A-SH was supported by the French Research Ministry.

REFERENCES

1 Nicholson RI, Gee JM, Harper ME. EGFR and cancer prognosis. *Eur J Cancer* 2001; **37**: S9–S15.
2 Krause DS, Van Etten RA. Tyrosine kinases as targets for cancer therapy. *N Engl J Med* 2005; **353**: 172–187.
3 Pao W, Miller V, Zakowski M, Doherty J, Politi K, Sarkaria I *et al.* EGF receptor gene mutations are common in lung cancers from "never smokers" and are associated with sensitivity of tumors to gefitinib and erlotinib. *Proc Natl Acad Sci USA* 2004; **101**: 13306–13311.

4 Paez JG, Janne PA, Lee JC, Tracy S, Greulich H, Gabriel S *et al.* EGFR mutations in lung cancer: correlation with clinical response to gefitinib therapy. *Science* 2004; **304**: 1497–1500.
5 Lynch TJ, Bell DW, Sordella R, Gurubhagavatula S, Okimoto RA, Brannigan BW *et al.* Activating mutations in the epidermal growth factor receptor underlying responsiveness of non-small-cell lung cancer to gefitinib. *N Engl J Med* 2004; **350**: 2129–2139.
6 Yarden Y, Sliwkowski MX. Untangling the ErbB signalling network. *Nat Rev Mol Cell Biol* 2001; **2**: 127–137.
7 Jorissen RN, Walker F, Pouliot N, Garrett TP, Ward CW, Burgess AW. Epidermal growth factor receptor: mechanisms of activation and signalling. *Exp Cell Res* 2003; **284**: 31–53.
8 Olayioye MA, Neve RM, Lane HA, Hynes NE. The ErbB signaling network: receptor heterodimerization in development and cancer. *EMBO J* 2000; **19**: 3159–3167.
9 Schlessinger J. Ligand-induced, receptor-mediated dimerization and activation of EGF receptor. *Cell* 2002; **110**: 669–672.
10 Lill NL, Sever NI. Where EGF receptors transmit their signals. *Sci Signal* 2012; **5***pe41*.
11 Wang YN, Yamaguchi H, Hsu JM, Hung MC. Nuclear trafficking of the epidermal growth factor receptor family membrane proteins. *Oncogene* 2010; **29**: 3997–4006.
12 Lo HW. Nuclear mode of the EGFR signaling network: biology, prognostic value, and therapeutic implications. *Discov Med* 2010; **10**: 44–51.
13 Brand TM, Iida M, Li C, Wheeler DL. The nuclear epidermal growth factor receptor signaling network and its role in cancer. *Discov Med* 2011; **12**: 419–432.
14 Wang YN, Hung MC. Nuclear functions and subcellular trafficking mechanisms of the epidermal growth factor receptor family. *Cell Biosci* 2012; **2**: 13.
15 Huang WC, Chen YJ, Li LY, Wei YL, Hsu SC, Tsai SL *et al.* Nuclear translocation of epidermal growth factor receptor by Akt-dependent phosphorylation enhances breast cancer-resistant protein expression in gefitinib-resistant cells. *J Biol Chem* 2011; **286**: 20558–20568.
16 Liccardi G, Hartley JA, Hochhauser D. EGFR nuclear translocation modulates DNA repair following cisplatin and ionizing radiation treatment. *Cancer Res* 2011; **71**: 1103–1114.
17 Chen DJ, Nirodi CS. The epidermal growth factor receptor: a role in repair of radiation-induced DNA damage. *Clin Cancer Res* 2007; **13**: 6555–6560.
18 Traynor AM, Weigel TL, Oettel KR, Yang DT, Zhang C, Kim K *et al.* Nuclear EGFR protein expression predicts poor survival in early stage non-small cell lung cancer. *Lung Cancer* 2013; **81**: 138–141.
19 Tao Y, Song X, Deng X, Xie D, Lee LM, Liu Y *et al.* Nuclear accumulation of epidermal growth factor receptor and acceleration of G1/S stage by Epstein-Barr-encoded oncoprotein latent membrane protein 1. *Exp Cell Res* 2005; **303**: 240–251.
20 Mao L, Merlo A, Bedi G, Shapiro GI, Edwards CD, Rollins BJ *et al.* A novel p16INK4A transcript. *Cancer Res* 1995; **55**: 2995–2997.
21 Quelle DE, Zindy F, Ashmun RA, Sherr CJ. Alternative reading frames of the INK4a tumor suppressor gene encode two unrelated proteins capable of inducing cell cycle arrest. *Cell* 1995; **83**: 993–1000.
22 Ozenne P, Eymin B, Brambilla E, Gazzeri S. The ARF tumor suppressor: structure, functions and status in cancer. *Int J Cancer* 2010; **127**: 2239–2247.
23 Sherr CJ. Divorcing ARF and p53: an unsettled case. *Nat Rev Cancer* 2006; **6**: 663–673.
24 Eymin B, Leduc C, Coll JL, Brambilla E, Gazzeri S. p14ARF induces G2 arrest and apoptosis independently of p53 leading to regression of tumours established in nude mice. *Oncogene* 2003; **22**: 1822–1835.
25 Ozenne P, Dayde D, Brambilla E, Eymin B, Gazzeri S. p14(ARF) inhibits the growth of lung adenocarcinoma cells harbouring an EGFR L858R mutation by activating a STAT3-dependent pro-apoptotic signalling pathway. *Oncogene* 2013; **32**: 1050–1058.
26 Carpenter G, Liao HJ. Receptor tyrosine kinases in the nucleus. *Cold Spring Harb Perspect Biol* 2013; **5**: a008979.
27 Lo HW, Hsu SC, Ali-Seyed M, Gunduz M, Xia W, Wei Y *et al.* Nuclear interaction of EGFR and STAT3 in the activation of the iNOS/NO pathway. *Cancer Cell* 2005; **7**: 575–589.
28 Lin SY, Makino K, Xia W, Matin A, Wen Y, Kwong KY *et al.* Nuclear localization of EGF receptor and its potential new role as a transcription factor. *Nat Cell Biol* 2001; **3**: 802–808.
29 Burda P, Padilla SM, Sarkar S, Emr SD. Retromer function in endosome-to-Golgi retrograde transport is regulated by the yeast Vps34 PtdIns 3-kinase. *J Cell Sci* 2002; **115**: 3889–3900.
30 Xia W, Wei Y, Du Y, Liu J, Chang B, Yu YL *et al.* Nuclear expression of epidermal growth factor receptor is a novel prognostic value in patients with ovarian cancer. *Mol Carcinog* 2009; **48**: 610–617.
31 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**: 338–348.
32 Psyrri A, Yu Z, Weinberger PM, Sasaki C, Haffty B, Camp R *et al.* Quantitative determination of nuclear and cytoplasmic epidermal growth factor receptor expression in oropharyngeal squamous cell cancer by using automated quantitative analysis. *Clin Cancer Res* 2005; **11**: 5856–5862.
33 Mosesson Y, Mills GB, Yarden Y. Derailed endocytosis: an emerging feature of cancer. *Nat Rev Cancer* 2008; **8**: 835–850.
34 Massie C, Mills IG. The developing role of receptors and adaptors. *Nat Rev Cancer* 2006; **6**: 403–409.
35 Schu PV, Takegawa K, Fry MJ, Stack JH, Waterfield MD, Emr SD. Phosphatidylinositol 3-kinase encoded by yeast VPS34 gene essential for protein sorting. *Science* 1993; **260**: 88–91.
36 Vanhaesebroeck B, Guillermet-Guibert J, Graupera M, Bilanges B. The emerging mechanisms of isoform-specific PI3K signalling. *Nat Rev Mol Cell Biol* 2010; **11**: 329–341.
37 Simonsen A, Tooze SA. Coordination of membrane events during autophagy by multiple class III PI3-kinase complexes. *J Cell Biol* 2009; **186**: 773–782.
38 Backer JM. The regulation and function of Class III PI3Ks: novel roles for Vps34. *Biochem J* 2008; **410**: 1–17.
39 Dittmann K, Mayer C, Fehrenbacher B, Schaller M, Kehlbach R, Rodemann HP. Nuclear EGFR shuttling induced by ionizing radiation is regulated by phosphorylation at residue Thr654. *FEBS Lett* 2010; **584**: 3878–3884.
40 Giri DK, Ali-Seyed M, Li LY, Lee DF, Ling P, Bartholomeusz G *et al.* Endosomal transport of ErbB-2: mechanism for nuclear entry of the cell surface receptor. *Mol Cell Biol* 2005; **25**: 11005–11018.
41 Hung LY, Tseng JT, Lee YC, Xia W, Wang YN, Wu ML *et al.* Nuclear epidermal growth factor receptor (EGFR) interacts with signal transducer and activator of transcription 5 (STAT5) in activating Aurora-A gene expression. *Nucleic Acids Res* 2008; **36**: 4337–4351.
42 Jaganathan S, Yue P, Paladino DC, Bogdanovic J, Huo Q, Turkson J. A functional nuclear epidermal growth factor receptor, SRC and Stat3 heteromeric complex in pancreatic cancer cells. *PLoS One* 2011; **6**: e19605.
43 Lo HW, Cao X, Zhu H, Ali-Osman F. Cyclooxygenase-2 is a novel transcriptional target of the nuclear EGFR-STAT3 and EGFRvIII-STAT3 signaling axes. *Mol Cancer Res* 2010; **8**: 232–245.
44 Maggi Jr LB, Winkeler CL, Miceli AP, Apicelli AJ, Brady SN, Kuchenreuther MJ *et al.* ARF tumor suppression in the nucleolus. *Biochim Biophys Acta* 2014; **1842**: 831–839.
45 Zhao ZH, Wang SF, Yu L, Wang J, Chang H, Yan WL *et al.* Overexpression of Pokemon in non-small cell lung cancer and foreshowing tumor biological behavior as well as clinical results. *Lung Cancer* 2008; **62**: 113–119.
46 Meng X, Wang Y, Zheng X, Liu C, Su B, Nie H *et al.* shRNA-mediated knockdown of Bmi-1 inhibit lung adenocarcinoma cell migration and metastasis. *Lung Cancer* 2012; **77**: 24–30.
47 Dovey JS, Zacharek SJ, Kim CF, Lees JA. Bmi1 is critical for lung tumorigenesis and bronchioalveolar stem cell expansion. *Proc Natl Acad Sci USA* 2008; **105**: 11857–11862.
48 Vonlanthen S, Heighway J, Altermatt HJ, Gugger M, Kappeler A, Borner MM *et al.* The bmi-1 oncoprotein is differentially expressed in non-small cell lung cancer and correlates with INK4A-ARF locus expression. *Br J Cancer* 2001; **84**: 1372–1376.
49 Abhold EL, Kiang A, Rahimy E, Kuo SZ, Wang-Rodriguez J, Lopez JP *et al.* EGFR kinase promotes acquisition of stem cell-like properties: a potential therapeutic target in head and neck squamous cell carcinoma stem cells. *PLoS One* 2012; **7**: e32459.
50 Aggarwal H, Aggarwal A, Agrawal DK. Epidermal growth factor increases LRF/Pokemon expression in human prostate cancer cells. *Exp Mol Pathol* 2011; **91**: 496–501.
51 Mounawar M, Mukeria A, Le Calvez F, Hung RJ, Renard H, Cortot A *et al.* Patterns of EGFR, HER2, TP53, and KRAS mutations of p14arf expression in non-small cell lung cancers in relation to smoking history. *Cancer Res* 2007; **67**: 5667–5672.
52 Cortot AB, Younes M, Martel-Planche G, Guibert B, Isaac S, Souquet PJ *et al.* Mutation of TP53 and alteration of p14(arf) expression in EGFR- and KRAS-mutated lung adenocarcinomas. *Clin Lung Cancer* 2014; **15**: 124–130.
53 Marsh T, Debnath J. Ironing out VPS34 inhibition. *Nat Cell Biol* 2015; **17**: 1–3.
54 Li C, Iida M, Dunn EF, Ghia AJ, Wheeler DL. Nuclear EGFR contributes to acquired resistance to cetuximab. *Oncogene* 2009; **28**: 3801–3813.

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