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

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Running Title: Nuclear EGFR inhibits *Arf* transcription

Abstract

Epidermal growth factor receptor (EGFR) is a cell surface receptor that plays 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 siRNA 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.

Keywords: EGFR, nuclear trafficking, Vps34, ARF, lung tumor

Introduction

Epidermal growth factor receptor (EGFR) belongs to a large family of membrane-bound receptor tyrosine kinases that serve as mediators of cell signalling by extra-cellular growth factors, and play 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 (EGFR-TKI, Gefitinib)(3-5). Binding of growth factor such as EGF induces EGFR homo- or heterodimerization and autophosphorylation of the intracellular domain. This promotes the recruitment of adaptor molecules which trigger the activation of signal transduction pathways including the RAS/MAPK, the phosphoinositide-3-kinase (PI3K)/AKT and the signal transducer and activator of transcription (STAT) pathways (6-10). These downstream signaling activities regulate proliferation, survival, mobility and differentiation in many different cell types. Results of several recent studies show that receptor tyrosine kinase such as EGFR can also transmit signal from sub-cellular localisations 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) (15-17). The nuclear presence of EGFR is emerging as an important marker in many human cancer (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 EBV-encoded latent membrane protein 1 (LMP1) was found to enhance transactivation of *cyclin D1* and *cyclin E* by nuclear EGFR thereby fostering cell proliferation (19). In lung

cancer, the nuclear presence of EGFR was recently associated with poor clinical prognosis (18). However, the molecular mechanisms by which a nuclear signalisation 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. Due to splicing events, separate promoters, and unique first exons, the two proteins share no homology and have distinct functions (20, 21). P14ARF is 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* signaling 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 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 signaling in lung tumor cells expressing mutant EGFR. These results provide the first 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 WT EGFR) and H1975 (mutant EGFR-L858R) cells (25). Here, we investigated the molecular mechanisms involved. To answer, we first neutralized

EGFR expression using specific small interfering RNA (siRNA) and studied *Arf* expression by western blot and RT/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 the accumulation of the 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 hours and *Arf* expression was studied after EGF stimulation. In all cellular models, the downregulation of the p14ARF protein was associated with a significant diminution of its transcripts (Figure 1B and supplementary Fig1). Same results were obtained when H1719 cells were treated with Amphiregulin (Areg), 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 2). Taken together, these data demonstrated that wild type and mutant EGFRs specifically downregulate *Arf* transcript level. Of note we observed that EGF does not decrease the stability of *Arf* mRNAs (supplementary Figure 3), 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 4). To go further, we used siRNA against *importin β* or Brefeldin A (BFA) 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 5). As siRNA against *importin β* and BFA do not target specifically EGFR trafficking, we took advantage of CHO cells stably expressing wild type 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 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 to CHO-EGFR cells in which EGFR was present in the nucleus (Figure 2C and D). In agreement with our previous results, expression of *INK4a* did not vary significantly in CHO-EGFR-pNLS cells (Supplementary Figure 2E 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 ATRSS as putative EGFR-targeting sequences. Therefore, we used in vivo 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 3 most proximal putative ATRSs 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 chromatin immunoprecipitation was performed with control 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 6). 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 to 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 7). 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/MAPK or STATs pathways using U0126 and cucurbitacin respectively did not prevent the nuclear trafficking of EGFR in response to EGF nor the p14ARF downregulation (Supplementary Figure 8A and 8B). In contrast, the PI3K/AKT inhibitor wortmanin strongly inhibited EGFR nuclear transport in the same conditions whatever the EGFR status (WT or mutant) (Figure 3A and 3B 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 (Figure 3B and 3E), nor the downregulation of *Arf* transcript (Figure 3D). These results were confirmed with another specific AKT inhibitor, namely MK2206 (Supplementary Figure 8C). 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 Vps 34 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 PI 3-kinases although members of the class II show decreased sensitivity. The class I PI3K is activated downstream of receptor tyrosine kinases such as EGFR and stimulates the AKT signaling 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 9). Interestingly, previous work in yeast had implicated class III PI 3-kinase in diverse intracellular trafficking events including endosome-to-Golgi retrograde transport (29). Therefore, we speculated that class III PI 3-kinase may play 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 human. Because of 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 (Figure 4A and 4B). 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 knock-down 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 the 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 signaling pathway. To address this issue we transfected H1975 cells with siRNA against Vps34. In these cells, we confirmed that 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 the accumulation of pSTAT3-Y705, the downregulation of Bcl-2 expression and the induction of apoptosis (Figure 5A and C). Importantly, silencing *Arf* expression using siRNA rescued Vps34 knock-down cells from

all these modifications (Figure 5A) and diminished apoptosis occurrence (Figure 5C). We also noticed a slight increase of cell proliferation after 72h of siRNA transfection (data not shown). Of note, silencing of INK4a had no effect on apoptosis caused by Vps34 downregulation (Supplementary Figure 10). Together, these results indicated that a Vps34-dependent nuclear signalisation 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 signaling 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 signaling 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 signaling.

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 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 the class III PI3K Vps34 as an important mediator of the 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 plays 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 PKC 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 which 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, iNOS, COX-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 (PcG) proteins have been reported to inhibit *Arf* transcription (44). Most important and well-characterized *Arf* repressors include the BMI-1

and Pokemon proteins which 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 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 the p14ARF protein in NSCLC (51, 52). In agreement with an EGFR/*Arf* connection we recently demonstrated that EGFR inhibits the expression of the p14ARF protein to promote cell survival (25). In this study, we further deepened these results by providing the first evidence that a nuclear EGFR signaling pathway represses *Arf* transcription and pro-apoptotic function, and by identifying the Vps34 protein as an important mediator of this pathway. It was recently reported an inverse correlation between nuclear expression of EGFR and overall survival in NSCLC patients (18). Therefore, our data provide a mechanistic explanation to these in situ observations, and unravel the Vps34/nuclearEGFR/*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 wild type 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-TKI treatment.

Materials and Methods

Cell culture and treatments

HCC1719, HCC827 and H1975 cell lines (kindly provided by Pr A. Gazdar) were derived from human lung adenocarcinoma. HCC1719 cells carry a wild type 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% CO₂ at 37°C. CHO-EGFR and CHO-EGFR-pNLS were kindly provided by Dr Mien Chie Hung. They were cultured in DMEM-F12 medium (GIBCO, Cergy Pontoise, France) supplemented with 10% fetal calf serum. All cell lines were mycoplasma free. For growth factor stimulation, cells were incubated in serum-free medium for 24 hours and after medium change Rh-EGF (Eurobio, Courtaboeuf, France) (50ng/mL in HCC1719 cells and 100ng/mL in HCC827 and H1975 cells) or rh-Amphiregulin (R&D SYSTEMS EUROPE, Lille, France) (50ng/ml) was added for 15 min or 24 hours. 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 PE-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, Le Pont de Claix, France).

Transfection of siRNA oligonucleotides

The sequences designed to specifically target human *EGFR*, *Importin β 1*, *Vps34*, *INK4a* and *Arf* RNAs were as follows: *EGFR*, 5'-CUCUGGAGGAAAAGAAAGU-3'; *Importin β 1*, 5'-GGACUUAUGUACAGCAUUU-3'; *Vps34*, 5'CCCAUGAGAUGUACUUGAACGUAAU-3', *INK4a*, 5'-CGCACCGAAUAGUUACGGU-3' ; *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 72H post-transfection.

Cellular fractionation

Cells were pelleted and washed twice with PBS 1X. Cells were resuspended in hypotonic buffer (10mM Tris-HCl pH 7.5, 10mM KCl, 1.5mM MgCl₂, 0.5mM dithiothreitol, 0.02M NaF, 1mM Na₃VO₄, 1X EDTA free Protease Inhibitor Cocktail (Roche Diagnostics) for 20 minutes on ice. Then, cells were centrifugated at 2000g for 5 minutes and pellets were resuspended in hypotonic buffer + 2% NP-40. Homogenates were incubated for 15 minutes on ice and centrifugated at 3000g during 15 minutes. The resulting supernatant formed the non-nuclear fraction. The nuclear pellets were washed four times with PBS 1X, resuspended in RIPA buffer (50mM Tris-HCl pH 7.5, 150mM NaCl, 0.5% Na deoxycholate, 1% NP-40, 0.1% SDS, 0.02M NaF, 1mM Na₃VO₄, 1X EDTA free Protease Inhibitor Cocktail) and

sonicated. Homogenates were centrifugated at 13500g for 30 minutes, the resulting supernatant formed the nuclear fraction. Total proteins extracts was performed in RIPA buffer as described previously (25). Analysis of protein expression was performed by western blotting. EGFR, p14ARF and laminB intensities were quantified using the Image J software and the relative densitometric areas for EGFR or p14ARF were determined according to Lamin B signal in each condition.

Antibodies

The 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). The anti- α tubulin (B-5-1-2), anti-p16INK4a (sc-759) and the anti-importin β 1 antibodies were purchased from Santa-Cruz (Clinisciences, Montrouge, France). The anti-Vps34 antibody was from Novusbio (R et D system Europe-Bio-Techne, Lille, France).

RNA extraction, reverse transcription and real-time quantitative PCR analysis

RNA was extracted using High Pure RNA isolation kit (Roche Diagnostics) according to the manufacturer's protocol and subjected to Reverse Transcription using iScript RT supermix (Biorad, Marnes-la-Coquette, France). Quantitative PCR (qPCR) was performed with iTaq Universal SYBR Green Supermix (Biorad). The primers used for cDNA amplification were as follows: *Arf* S: 5'-GCGCAGGTTCTTGGTGAC-3'; *Arf* AS: 5'-GGCTCCTCAGTAGCATCAGC-3'; *INK4a* S: 5'-GAGCAGCATGGAGCCTCC-3'; *INK4a* AS: 5'-GGCCTCCGACCGTAACTATT-3'; *Vps34* S: 5'-AAGCAGTGCCTGTAGGAGGA-3'; *Vps34* AS: 5'-TGTCGATGAGCTTTGGTGAG-3';

GAPDH S: 5'-CGAGATCCCTCCAAAATCAA-3'; GAPDH AS: 5'-ATCCACAGTCTTCTGGGTGG-3'. Amplification of GAPDH was performed in all experiments and used as 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 +/- SD of at least three independent experiments.

Chromatin Immunoprecipitation assay

Cells were crosslinked with 1% formaldehyde for 10 minutes and subsequently processed using the ChIP-IT^R 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 Chromatin IP DNA Purification kit (Active Motif) and subjected to QPCR using primers specific to the ARF promoter which were sense 5'-GCGTGCAGCGGTTTAGTTTA-3' and anti-sense 5'-CTCTATCCGCCAAT-CAGGAG-3'. Human Negative Control Primer Set 1 (Active Motif) was used as negative control. Input DNA sample corresponding to 1% of immunoprecipitated chromatin was analysed in parallel in order to normalized 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 immunolocalisation studies, cells were fixed with 1% paraformaldehyde in PBS for 10 min at room temperature, washed once with PBS, and permeabilised with 0.1% Triton X-100 in PBS for 10 min. Blocking was performed for 45 min in 5% BSA. 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), visualised by fluorescent microscopy using Axioimager microscope (Carl Zeiss, Jena, Germany) with the AxioVision® software at a 60x magnification and processed with Image J software.

Statistical analysis

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

Conflict of interest

The authors declare no conflict of interest

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Figures legends

Figure 1 EGFR downregulates *Arf* mRNA level

A, Cells were transfected with control siRNA (Ctl) or siRNA against *EGFR* (siEGFR) for 72 hours. Expression of *Arf* transcript was studied by quantitative PCR. qPCR data are represented as fold increase relative to non treated cells which were arbitrarily assigned to 1. Protein expression was analysed by western blot using indicated antibodies. Tubulin was used as a loading control. B, Cells were incubated with EGF (50ng/ml) for 24 hours. Expression of *Arf* was studied by qPCR and western blotting as described above. C, H1719 cells were treated with Amphiregulin (Areg) (50ng/ml) for 24 hours. Expression of *Arf* was studied by qPCR and western blotting as described above. D, H1719 cells were stimulated with EGF or Areg (50ng/ml) for 24 hours. Expression of *INK4a* was assessed by qPCR and western blotting. All the data represent the mean \pm s.d. of three independent experiments. NS, not significant. *P \leq 0.05, **P \leq 0.01, ***P \leq 0.001. Quantification of p14ARF protein levels was performed as described in the methods section and is numerically indicated below each lane.

Figure 2 Nuclear EGFR inhibits *Arf* transcription

A, B, Cells were stimulated for 15 min with EGF (50ng/ml). Western blotting was performed using 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. Immunolocalisation of EGFR (D38B1, Red) was performed in H1719 cells using fluorescent pseudo-confocal (apoptome) microscopy. DAPI (Blue) was used to counterstained nuclei. C, D, Expression of EGFR and Arf was studied by western blotting and/or qPCR in CHO cells constitutively expressing wild type (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 3 most proximal putative ATRS sites within the *Arf* promoter are illustrated. Arrows represent primers used in the chromatin immunoprecipitation (ChIP)/qPCR study. HCC827 cells were stimulated for 30 min with EGF. The binding of EGFR to *Arf* promoter was analyzed by ChIP as described in the materials and methods section. IgG was used as a negative control for chromatin immunoprecipitation. qPCR with irrelevant primers (ChIP-IT^R Express kit, Active Motif) was performed to ensure the specificity of the results. Data represent the mean \pm s.d. of three independent experiments. * $P \leq 0.05$, ** $P \leq 0.01$, *** $P \leq 0.001$. NS, not significant.

Figure 3 PI3K but not AKT controls nuclear EGFR trafficking to inhibit *Arf* transcription

H1719 cells were pre-treated for 30 min with Wortmanin (0.1 μ M) or Triciribin (10 μ M) before EGF stimulation for 15 min (A, B, C, E) or 24 hours (D). A, E, Western blotting was performed after sub-cellular fractionation using 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 (apoptome) microscopy. DAPI (blue) was used to counterstain nuclei. C, Chromatin immunoprecipitation was performed as previously described. D, *Arf* expression was analysed by qPCR as described. Data represent the mean \pm s.d. of three independent experiments. * $P \leq 0.05$, ** $P \leq 0.01$, *** $P \leq 0.001$. NS, not significant.

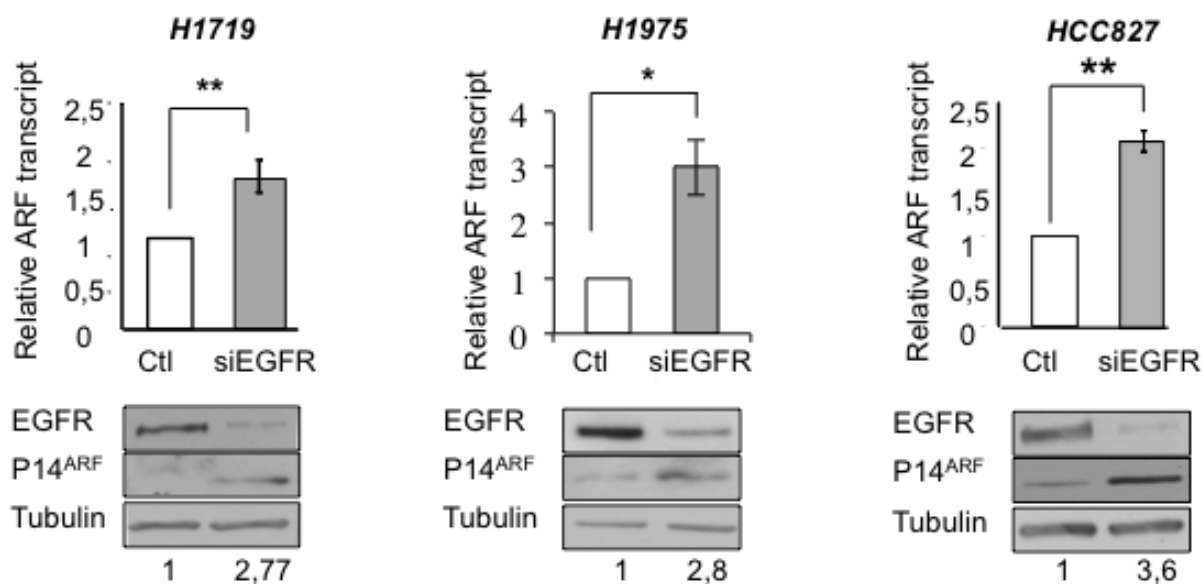
Figure 4 Class III PI3K Vps34 controls nuclear trafficking and accumulation of EGFR to *Arf* promoter

A,B,C, H1719 cells were transfected with control siRNA (Ctl) or siRNA against Vps34 (siVps34) for 72h prior to EGF stimulation (50ng/ml) for an additional 15 min (A upper pannel, B) or for 48h prior to EGF stimulation (50ng/ml) for an additional 24 hours (A, lower pannel, 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. Immunolocalisation of EGFR (Red) was performed using fluorescent pseudo-confocal (apoptome) microscopy. DAPI (Blue) was used to counterstained nuclei Expression of *Arf* transcript was studied by qPCR. D, HCC827 cells were transfected with control siRNA (Ctl) or siRNAs against Vps34 (siVps34) for 72h and stimulated with EGF (50ng/ml) for an additional 30 min. ChIP analysis was performed as previously described. All the data represent the mean \pm s.d. of three independent experiments. *P \leq 0.05, **P \leq 0.01, ***P \leq 0.001.

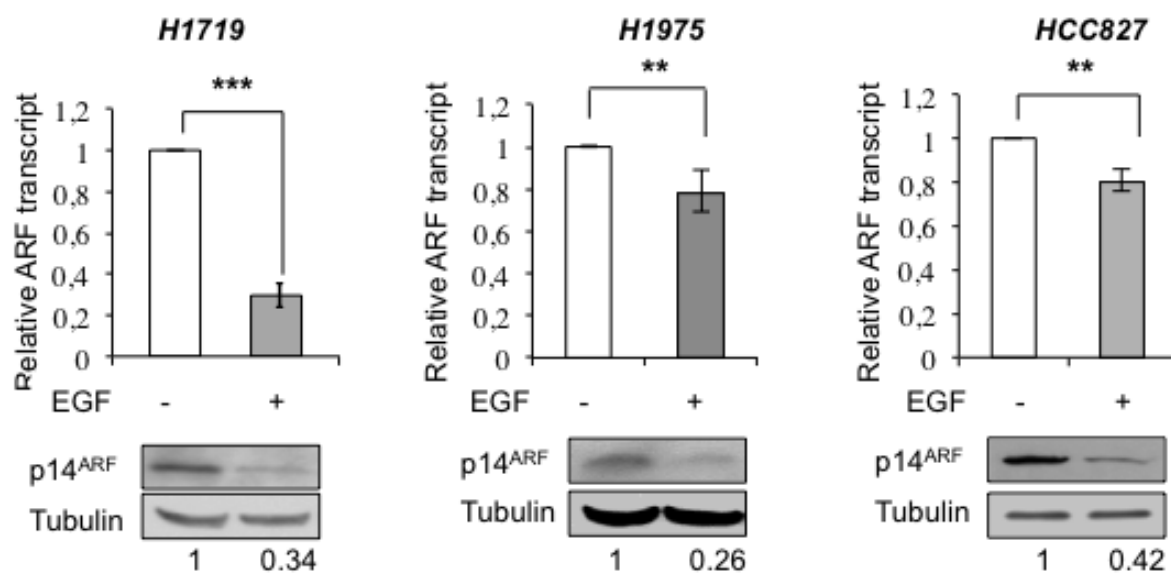
Figure 5 Nuclear trafficking of mutant EGFR by Vps34 inhibits p14ARF-dependent apoptosis in lung tumor cells

A, B, C, H1975 cells were transfected with control siRNA (Ctl) or siRNAs against Vps34 (siVps34) and/or *Arf* (siArf) for 72h. 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 FACS analysis. Data represent the mean \pm s.d. of three independent experiments. *P \leq 0.05, **P \leq 0.01, ***P \leq 0.001. D, A proposed model underlying the way by which the nuclear translocation of mutant EGFR through a Vps34-dependent mechanism negatively controls Arf expression to prevent cellular apoptosis.

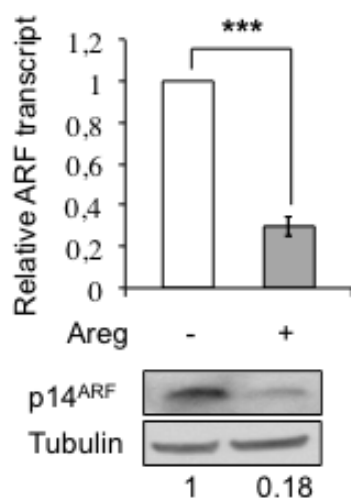
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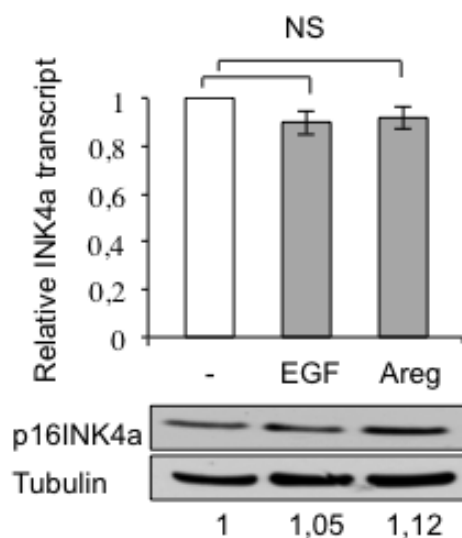
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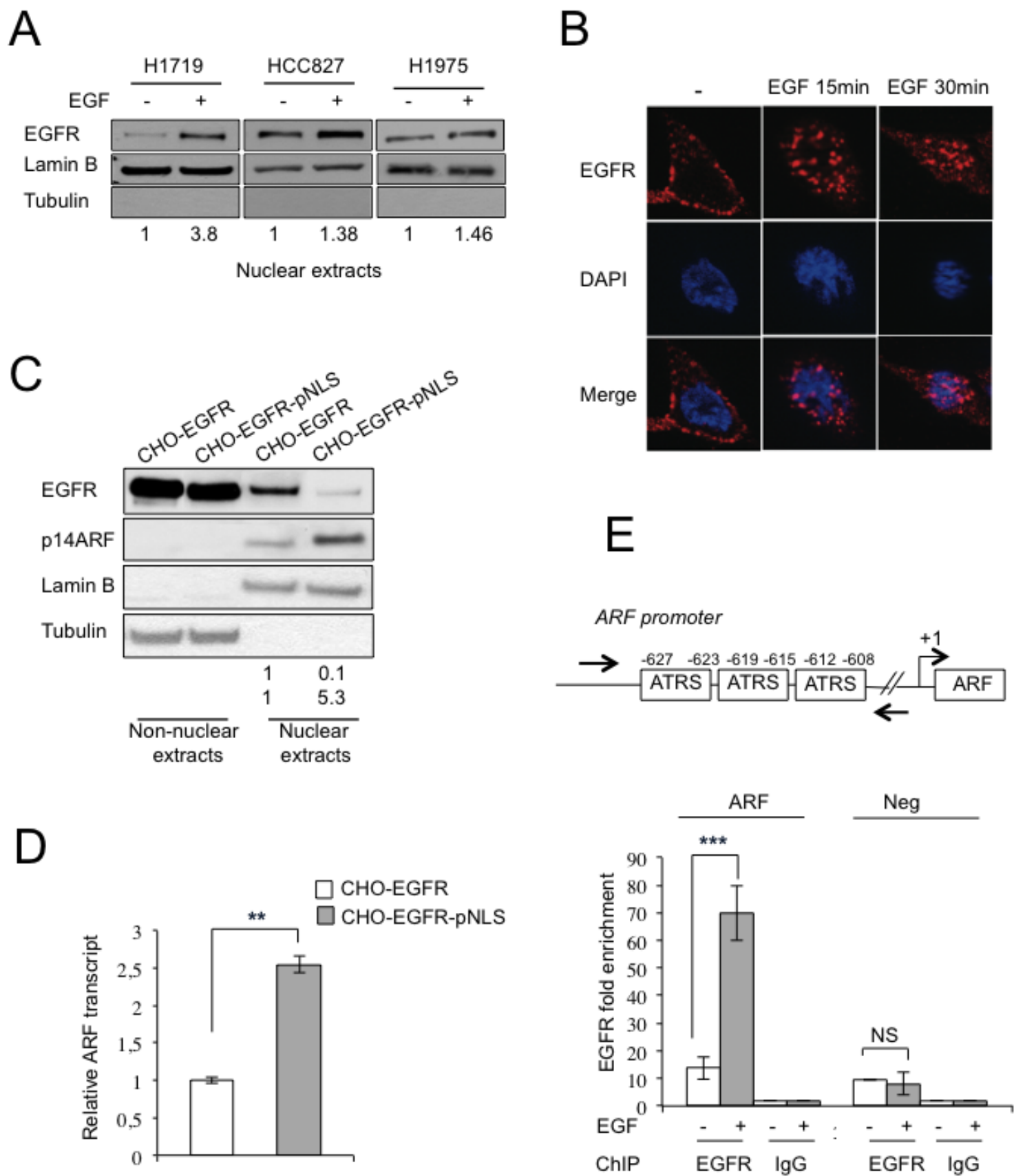


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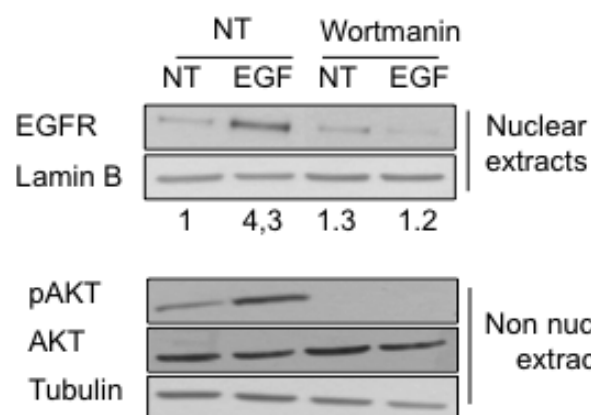


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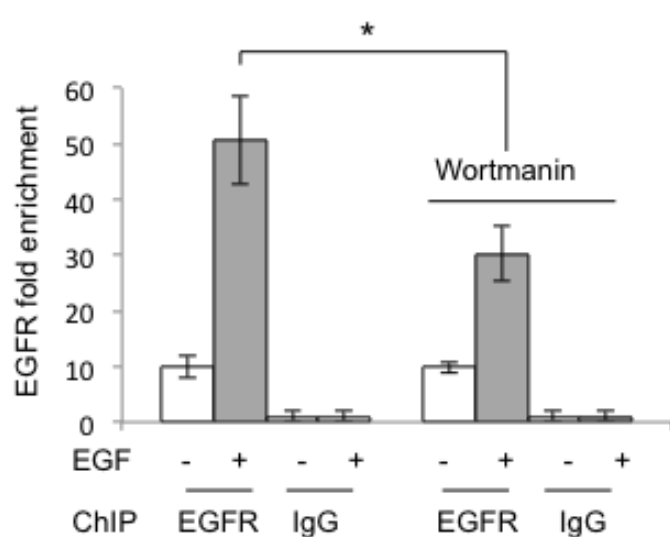




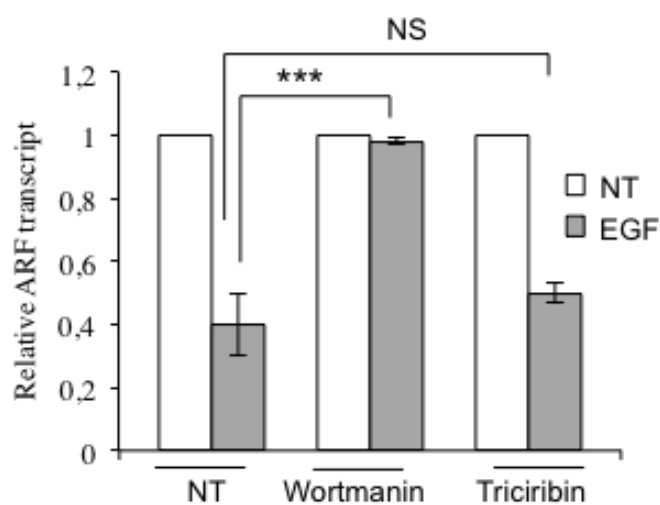
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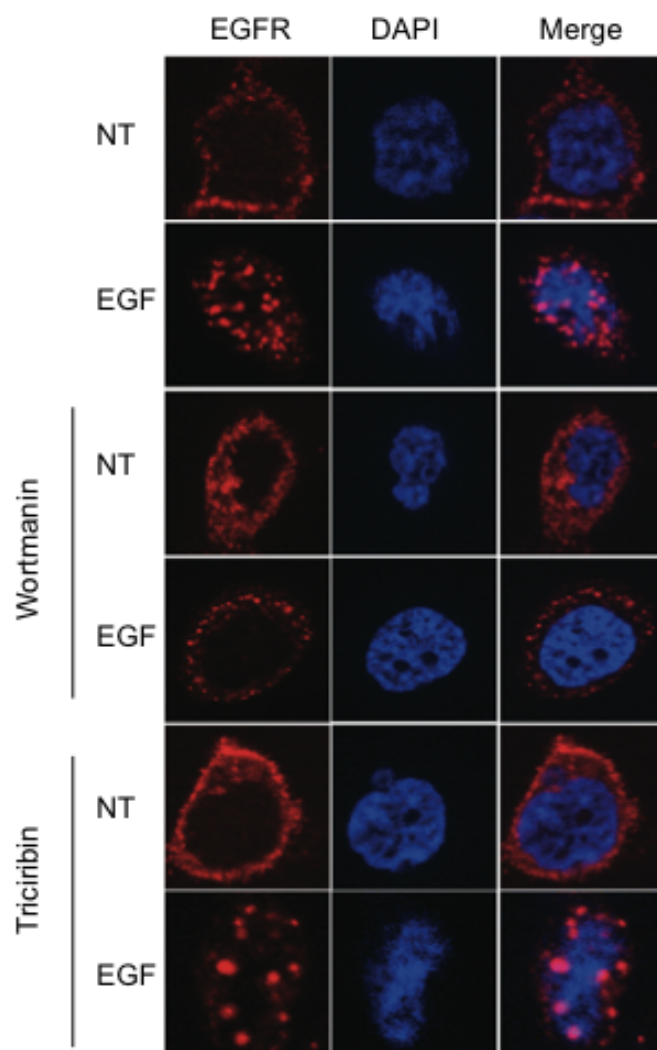
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