

ORIGINAL ARTICLE

The EGFR family members sustain the neoplastic phenotype of ALK + lung adenocarcinoma via EGR1

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In non-small cell lung cancer (NSCLC), receptor tyrosine kinases (RTKs) stand out among causal dominant oncogenes, and the ablation of RTK signaling has emerged as a novel tailored therapeutic strategy. Nonetheless, long-term RTK inhibition leads invariably to acquired resistance, tumor recurrence and metastatic dissemination. In ALK + cell lines, inhibition of ALK signaling was associated with coactivation of several RTKs, whose pharmacological suppression reverted the partial resistance to ALK blockade. Remarkably, ERBB2 signaling synergized with ALK and contributed to the neoplastic phenotype. Moreover, the engagement of wild-type epidermal growth factor receptor or MET receptors could sustain cell viability through early growth response 1 (EGR1) and/or Erk1/2; Akt activation and EGR1 overexpression prevented cell death induced by combined ALK/RTK inhibition. Membrane expression of ERBB2 in a subset of primary naive ALK + NSCLC could be relevant in the clinical arena. Our data demonstrate that the neoplastic phenotype of ALK-driven NSCLC relays 'ab initio' on the concomitant activation of multiple RTK signals via autocrine/paracrine regulatory loops. These findings suggest that molecular and functional signatures are required in *de novo* lung cancer patients for the design of efficacious and multi-targeted 'patient-specific' therapies.

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INTRODUCTION

Lung tumors are the most common form of cancer (12.3%), with 1.1 million deaths worldwide, accounting for 17.8% of all cancer fatalities. Despite major diagnostic improvements and novel tailored compounds, 90% of lung cancer patients die within 1–2 years from diagnosis.¹

Molecular studies have showed that nearly all lung cancers display multiple genetic defects^{2–4} and the abnormal activation of kinases is widely recognized as a frequent tumorigenic mechanism in non-small cell lung cancer (NSCLC).^{3,5–7} Epidermal growth factor receptor (EGFR) and its ligands are commonly deregulated, leading to receptor tyrosine kinase (RTK) phosphorylation fostering cell proliferation, inhibition of apoptosis and enhancing pro-angiogenesis and invasion signals.⁸ Tumor-acquired EGFR mutations have been documented in 5–10% of Caucasian NSCLC, often associated to unique clinico-pathologic features.⁹ Specific genetic alterations of KRAS, BRAF, PIK3CA, ALK and, more recently, ROS and RET have been described in well-defined subsets of NSCLC.^{6,7,10,11} These lesions represent 'driver mutations', as lung cancers depend on their constitutive activation.^{12,13} Thus, molecular screening of NSCLC is mandatory to guide the most appropriate therapies (that is, small molecule kinase inhibitors).^{14,15} Cancers harboring the same genetic alterations, however, respond differently to molecular targeted therapies, suggesting coexisting mutations or coactivation of other TKs.¹⁶

Finally, although molecularly targeted therapies (that is, gefitinib or erlotinib for EGFR and crizotinib for ALK fusions) are effective in NSCLC, intrinsic or acquired resistances inevitably lead to recurrence and/or metastatic dissemination. This is the case of MET activation/amplification,^{17,18} or acquired somatic mutations of EGFR (T790M)¹⁹ or ALK fusions (G1269A, L1196M and ALK amplification).^{20–22}

Chromosomal translocations involving the ALK gene have been described in 6% of NSCLC, where ALK is most frequently fused to the echinoderm microtubule-associated protein-like 4 (*EML4*).²³ As described for other ALK chimera, the *EML4*–ALK fusions contain a dimerization domain, which forces the ligand-independent oligomerization of the catalytic domain of ALK, resulting in its constitutive kinase activation. ALK fusions are oncogenic 'drivers' imposing a full cellular transformation, and are required for the maintenance of the neoplastic phenotype.^{24,25} Nevertheless, data from phase I clinical trials of ALK-rearranged NSCLC treated with the ALK inhibitor (ALKi) crizotinib showed an overall response rate of ~60%,^{26,27} suggesting that synergizing or overcoming mechanisms may occur.

Here we provide novel insights into ALK-driven transformation in NSCLC, demonstrating that tumor maintenance and survival of ALK + NSCLC cells, before any therapeutic selection, relay on the concomitant activation of multiple EGFR family RTKs. These data support the hypothesis that the degree of ALK addiction might

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differ *'ab initio'* among different ALK+ NSCLC patients and its identification should provide a novel rationale for the design of efficacious 'patient-specific' treatments.

RESULTS

ALK fusion proteins transform human immortalized lung epithelial cells and sustain the tumor phenotype of ALK+ NSCLC cell *in vitro* and *in vivo*

As it has been described that transformation of lung epithelial cells requires many hits, and that several oncogenes are unable to fully transform these elements, even in the presence of deregulated p53 and Rb-1 pathways, we assessed the transforming potential of ALK chimera in immortalized cell line (NuLi-1) derived from human normal airway epithelium. NuLi-1 cells were transduced with EML4-ALK variant 1 (EML4/exon13-ALK/exon20) or TFG-ALK constructs. Forced expression of EML4-ALK and TFG-ALK fusions led to constitutive phosphorylation of STAT3, SHC and Shp2 (Figure 1a), and imposed a transformed phenotype (Figure 1b).

To evaluate the biological effects of EML4-ALK, we transduced ALK-positive cell lines (H3122 and H2228) with ALK-specific inducible short hairpin RNA (shA5) or control short hairpin

RNA (shA5M) lentivirus cassettes. Alternatively, cells (ALK+ and ALK- (H1395)) were treated with ALK-selective inhibitors (CEP-14083 and CEP-26939, from Cephalon/Teva, West Chester, PA, USA).^{28,29} ALK inhibition led to a significant decrease in the phosphorylation of known ALK downstream targets (STAT3, Erk1/2, Shp2 and Akt; Supplementary Figure 1A).²⁴ As expected, ablation of ALK signaling was associated with growth impairment (Figure 1c and Supplementary Figure 1B) and a very high rate of apoptosis for the H3122 cells (Figure 1d and Supplementary Figure 1C). Consistently, *in vivo* treatment with CEP-26939 led to a decreased number of proliferating cells, without any significant loss of ALK protein expression (Supplementary Figures 1D and E), and to tumor mass regression. This treatment, however, did not result in tumor eradication (Supplementary Figure 1F).

Signaling through the EGFR-TK proteins contributes to the survival and growth of ALK+ NSCLC cells

On the basis of our previous phosphoproteomics findings³⁰ and on the different degree of ALK oncogenic addiction of ALK+ NSCLC lines and primary tumors,³¹ we investigated the association of anti-ALKi²⁹ with small anti-TK drugs currently into the clinics. Combined treatment of H2228 cells with ALKi and with the EGFR inhibitor gefitinib, or with an anti-EGFR monoclonal

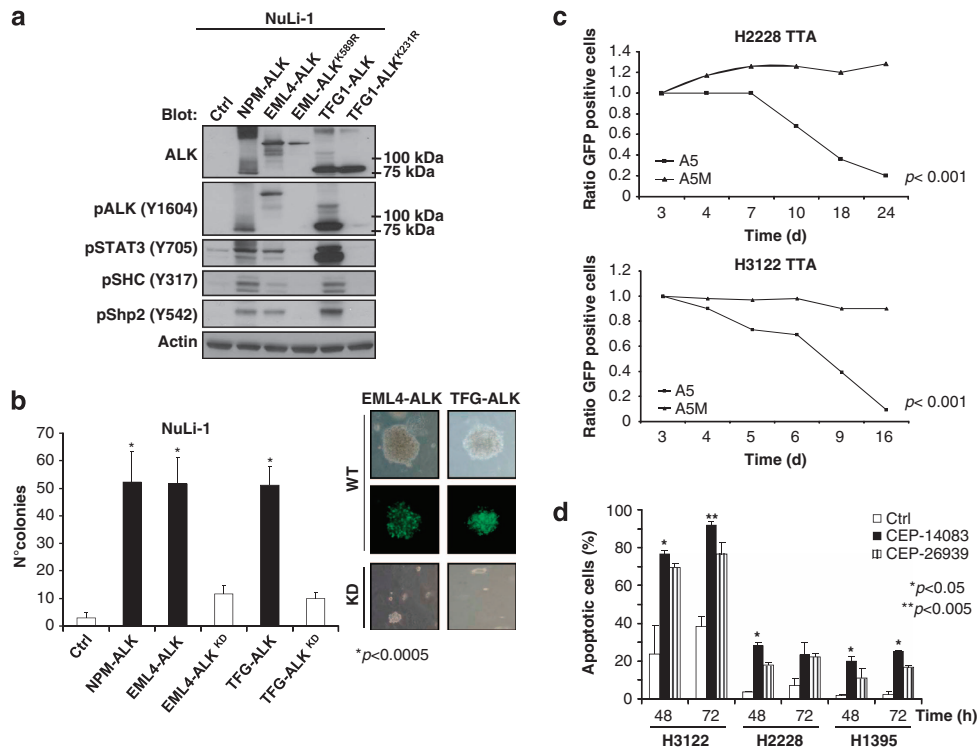


Figure 1. Lung-associated ALK fusion proteins transform human immortalized lung epithelial cells and are fundamental for the maintenance of the tumor phenotype. (a) NuLi-1 cells were infected with retrovirus expressing different ALK fusion proteins and were collected 96 h after transduction. NPM-ALK and the kinase-dead (EML4-ALK^{K589R} or TFG-ALK^{K231R}) fusion proteins were used as controls. Total cell lysates were immunoblotted with the indicated antibodies. (b) NuLi-1 cells transduced with the indicated constructs were plated in soft agar and cultured for 3 weeks. The histograms represent the average numbers of colonies grown in soft agar from the indicated cells and constructs. The number of colonies obtained in TFG-ALK, EML4-ALK or NPM-ALK transduced cells is significantly higher as compared with untransduced cells or to cells transduced with the kinase-dead constructs (* $P < 0.0005$; left panel). Colonies show green fluorescent protein (GFP) positivity (right panel). The number of colonies is normalized to the percentage of infection for every single construct. Cells were plated in triplicate. Results are representative of two different experiments. (c) H2228 TTA A5 and H2228 TTA A5M were cocultured with the untransduced counterpart, respectively, and grown in the presence of 1 $\mu\text{g}/\text{ml}$ doxycycline. GFP expression was quantified at indicated time points by fluorescence-activated cell sorting (FACS) analysis and normalized against the initial time point (day 0). Results are representative of two different experiments. (d) H3122, H2228 and H1395 cell lines were treated every 24 h with 300 nM CEP-14083 or CEP-26939. Tetramethylrodamine methyl ester staining followed by FACS analysis was performed after 48 and 72 h of treatment to check the percentage of apoptotic cells. Significance according to the analysis of variance test corresponded to * $P < 0.05$ and ** $P < 0.005$. Results are means of duplicates (s.e.m.), and graphics show one representative experiment from two independent assays.

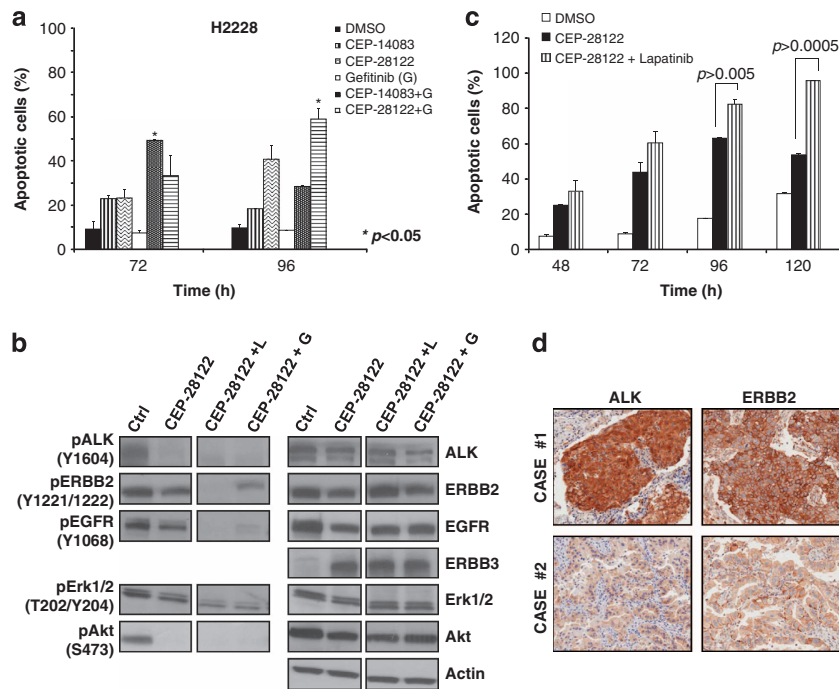


Figure 2. In H2228, apoptosis is increased by the combined treatment with ALK inhibitors and gefitinib or lapatinib. **(a)** H2228 cells were treated in 0.05% FCS with CEP-14083 or CEP-28122 (300 nM), and gefitinib (G; 1 μ M) as single agent or in combination. Apoptosis was measured by tetramethylrodamine methyl ester (TMRM) staining and fluorescence-activated cell sorting (FACS) analysis at the indicated time points. *Significance is referred to the corresponding single-agent treatment. **(b)** H2228 cells were treated with CEP-28122 alone or in combination with lapatinib (L) or gefitinib (G) in 0.05% FCS for 24 h. Total cell lysates were blotted with the indicated antibodies. **(c)** H2228 cells were treated with CEP-28122 alone or in combination with lapatinib in 0.05% FCS. TMRM staining followed by FACS analysis was performed at indicated times of treatment to check the percentage of apoptotic cells. **(d)** Primary ALK + NSCLC were stained with anti-ERBB2 or anti-ALK antibodies. Shown are two representative cases.

antibody (cetuximab), resulted in increased cell death. Similar findings were seen in H3122 cells after single anti-ALK treatment (Figure 2a and Supplementary Figures 2A and B), possibly related to higher protein expression and phosphorylation status of EGFR in only H2228 cells (Supplementary Figures 2C and D). Importantly, H2228 and H3122 cells were both insensitive to single treatment with gefitinib. As other members of the EGFR family are associated to RTK inhibitor resistance, we then investigated the functional role of ERBB2 and ERBB3 in the less-responsive H2228 cells.^{18,20} We observed a marked presence of ERBB2/ERBB3 and EGFR/ERBB3 heterodimers in H2228 in the absence of exogenous stimuli (Supplementary Figure 3A). Moreover, ERBB3 protein/mRNA levels were upregulated in H2228 when treated with anti-ALK small molecules (Figure 2b). Interestingly, we detected a constitutive phosphorylation of ERBB2 that was completely abrogated upon treatment with lapatinib (Figure 2b and Supplementary Figure 3B), and the combination of lapatinib with CEP-28122 suppressed cell growth and significantly reduced the number of viable cells as compared with CEP-28122 alone (Figure 2c). In contrast, the combined treatment had no further effect in H3122 cells (Supplementary Figure 3C). Remarkably, a significant subset of primary ALK + NSCLC (12/21, 57%; but ALK – NSCLC 38/180, 21%) shows a strong immunohistochemical membrane staining for ERBB2 (Figure 2d).

Overall, these data suggest that ALK inhibition might be overcome by alternative RTK pathways in ALK + NSCLC cells.

Ligand-mediated activation of tyrosine receptor kinases rescues ALK kinase inhibition through AKT and Erk1/2 pathways

As EGF can be secreted by lung epithelial cancer and/or stromal cells, we investigated whether an autocrine–paracrine

EGF-mediated activation of the EGFR signaling in H2228 cells could explain their partial resistance to ALK inhibition and overcome ALK inhibition of ALK-sensitive cells. Consistent with recently published data, upon stimulation with exogenous EGF the effects associated with the abrogation of the ALK signaling (CEP-28122) were overcome in both cell lines as documented by an increased cell viability and growth, which were restored to baseline levels (Figure 3a and Supplementary Figures 4A and B). Cells cotreated with gefitinib and/or expressing a mutant form of the EGFR (T790, data not show) could not be rescued by the exogenous EGF (Supplementary Figure 4A).^{20,32}

The molecular mechanisms operating in the EGF/EGFR-mediated rescue were first examined by the phosphorylation status of ALK and known ALK effectors. These were not affected. On the contrary, EGF stimulation could sustain the phosphorylated status of EGFR, Akt and Erk1/2, despite an ALK inhibition (Figure 3b). Finally, an increased expression of the proapoptotic protein BIM was seen only with the dual inhibitor cotreatment in H2228, whereas ALK inhibition alone was sufficient to induce a significant upregulation of BIM in H3122 (Figure 3b).³³ Similar results were obtained by heregulin stimulation. Upon heregulin stimulation the phosphorylation of ERBB3, Erk1/2 and Akt significantly increased (Figure 3c and Supplementary Figure 4B) and cell viability was rescued despite ALK inhibition (Figure 3d).

As hepatocyte growth factor (HGF)/Met signaling or Met overamplification can be involved in gefitinib resistance in lung cancer,^{18,34} we then explored the effects of HGF. HGF promoted a moderate proliferation and sustained cell viability upon ALK inhibition through Erk1/2 and Akt pathways (Supplementary Figures 5A and B). As expected, crizotinib, a dual ALK–Met inhibitor, abolished the prosurvival signals triggered by HGF (Supplementary Figure 5C).

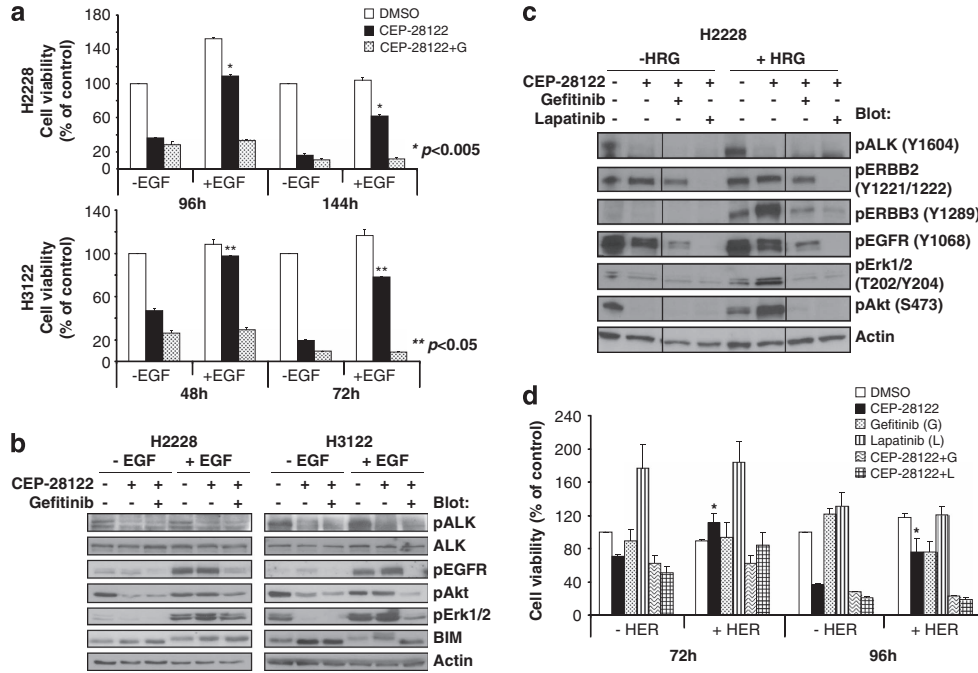


Figure 3. EGF or Heregulin stimulation rescues ALK-treated NSCLC cells through Erk1/2 and AKT. **(a)** Cell viability of treated H2228 and H3122 cells with or without EGF stimulation (10 ng/ml) was evaluated with Cell Titer-Glo Luminescent cell viability assay at the indicated time points. Significances * and ** is referred to the corresponding single-agent treatment. **(b)** H2228 and H3122 cells were treated for 6 h with 300 nM CEP-28122, alone or in combination with 1 μ M gefitinib (G) in 0.05% FCS. Cells were stimulated with EGF (10 ng/ml) for 15 min and then collected. Total cell lysates were blotted with the indicated antibodies. **(c,d)** H2228 cells were treated with CEP-28122 alone or combined with gefitinib or lapatinib for 24 h, and with or without Heregulin (HRG) stimulation (50 ng/ μ l) for 30 min and then collected. Total cell lysates were blotted with the indicated antibodies **(c)**. Cell viability was evaluated as previously described at the indicated time points. Significance according to Student's *t*-test corresponded to * $P < 0.05$ **(d)**. Significances * and ** are referred to the corresponding single treatment without EGF or Heregulin stimulation. Results are representative of three different experiments.

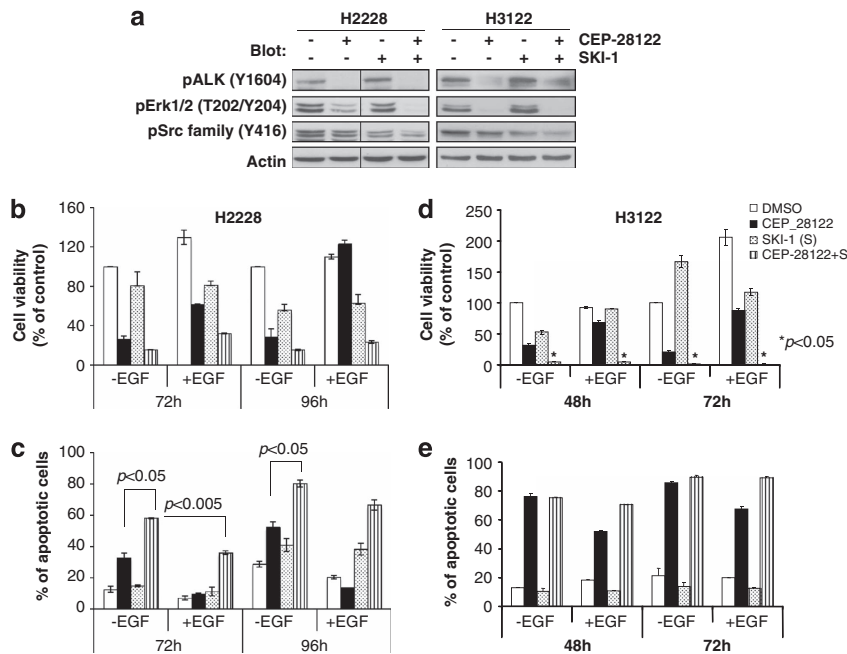


Figure 4. Src is a common node downstream of ALK and EGFR signaling pathways in NSCLC ALK-positive cells. **(a)** H2228 and H3122 cells were treated with CEP-28122 and with 5 μ M SKI-1 (S) in 0.05% FCS for 24 h and for 12 h, respectively. Total cell lysates were blotted with the indicated antibodies. **(b–e)** H2228 and H3122 cells were treated with CEP-28122 alone or in combination with 5 μ M Src kinase inhibitor-1 (SKI-1), and with or without EGF stimulation (10 ng/ml) every 24 h. The cell proliferation rate was evaluated with the Cell Titer-Glo Luminescent cell viability assay **(b, d)** and the percentage of apoptotic cells was quantified by tetramethylrodamine methyl ester staining followed by fluorescence-activated cell sorting analysis **(c, e)**.

As Src has a pathogenetic role in EGFR-mutated NSCLC³⁵ and is also a critical interactor in ALK+ anaplastic large cell lymphoma,²⁴ we applied a selective Src kinase inhibitor-1 as a single agent (Figure 4a) or in combination with CEP-28122. The combined treatment increased cell death only in H2228 that was partially rescued by EGF administration (Figures 4b–e). These data suggest that Src-independent pathways might be active in H2228 cells. On the contrary, EGF did not restore cell viability of H3122 cells treated with ALK and Src-TK inhibitor. Similar findings were observed using dasatinib, a pan Src inhibitor (data not shown).

Early growth response 1 (*EGR1*) gene is upregulated by active ALK and EGFR, and promotes survival

To identify ALK cooperating oncogene(s) in H2228 NSCLC cells, we interrogated their transcriptome before and after TK inhibitor treatment. Gene expression analysis showed that a single gene, early growth response 1 (*EGR1*), was significantly suppressed by the combined ALK/EGFR treatment (fold-change >2, $P < 0.001$; Supplementary Figure 6). Quantitative reverse transcription-PCR confirmed that the loss of ALK signaling or the dual treatment led to a substantial inhibition of the *EGR1* expression (Figure 5a), whereas gefitinib had no effect. Downregulation of *EGR1* could be inhibited by exogenous EGF that overcame cell death as well (Supplementary Figure 7A). Moreover, Src inhibition in association with CEP-28122 strongly reduced the level of expression of *EGR1* in both cell lines (Supplementary Figure 7B). Reversely, ectopic expression of *EGR1* in H2228 cells (Supplementary Figure 7C) prevented cell growth inhibition and cell death via CEP-28122 and/or gefitinib combination (Figures 5b and c). Finally, forced expression of *EGR1* could revert BIM levels to baseline (Figure 5d and Supplementary Figure 7D), meanwhile knockdown of *EGR1* in H2228 through short hairpin RNA impaired cell growth (data not shown). Overall, these findings indicate that both ALK and EGFR signaling are required for *EGR1* expression and demonstrated a central role of this transcription factor in ALK+ NSCLC cells.

To identify *EGR1*-regulated genes, we investigated the transcriptional profile of H2228 cells stably transduced with *EGR1* (*EGR1*+). This revealed a set of 780 genes linked to *EGR1* (fold-change >2, $P < 0.001$). Notably, *EGR1* overexpression resulted in *ERBB3* mRNA upregulation that was further increased by ALK inhibition (Supplementary Figure 8). Expression profiling of *EGR1*+ cells treated with ALKi and EGFR-TK inhibitor reported two clusters of genes behaving independently of ALK inhibition, as these genes showed a marked up- or downregulation in wild-type treated cells, and no change or even an opposite behavior in *EGR1*+ cells (Figure 6). In particular, seven genes were upregulated by *EGR1* transduction and remained significantly upregulated despite the treatment with TK inhibitor, suggesting direct and robust regulation by this transcription factor (that is, *TFF2*, *PPDPF*, *AGR2* and *CCL5*; Supplementary Figure 8).

DISCUSSION

The discovery of specific genetic lesions in lung cancer patients has profoundly changed their clinical management. Kinases are now recognized as critical tumorigenic events in human cancer. Constitutively active receptor and non-RTKs are excellent examples for the development of modern 'patient-specific' and/or 'tumor-restricted' therapies.³⁶ However, clinical responses are heterogeneous as a result of multiple mechanisms of resistance. It is believed that multi-targeted drugs may be more appropriate than highly restricted inhibitors. In NSCLC patients, ALK is a powerful driver/oncogene;³⁷ nevertheless, crizotinib, a dual ALK/Met inhibitor, is successful only in a subset of ALK+ patients (~60%). Moreover, responder patients inevitably become refractory.³⁸ Thus, the different responses to ALKi may underlie intrinsic mechanisms that undermine 'ab initio' their efficacy and/or favor the occurrence of resistance. Their identification provide the basis for a molecular stratification of ALK+ NSCLC patients and drive the selection of more compliant treatments.^{21,39}

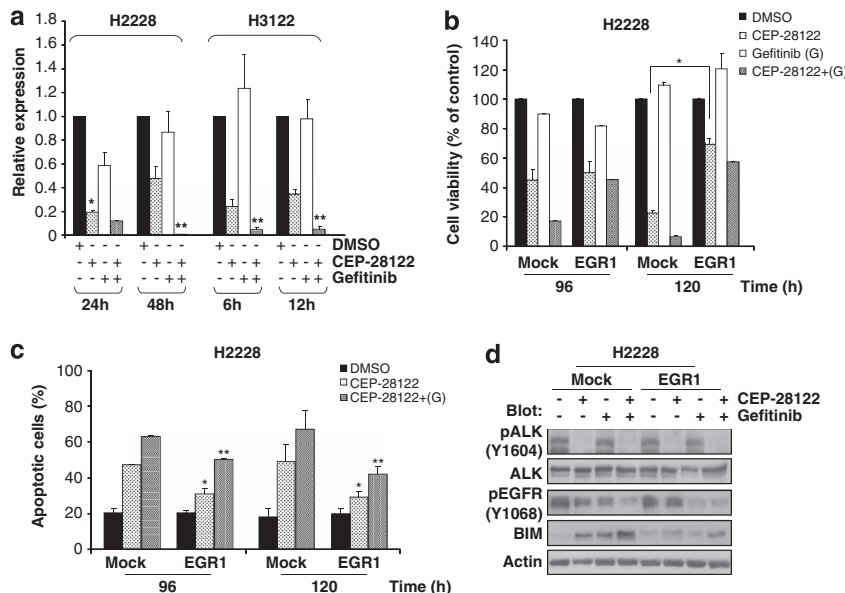


Figure 5. *EGR1* is regulated by ALK and EGFR, and promotes survival of H2228 cells. (a) H2228 and H3122 cells were treated with CEP-28122 and/or gefitinib (G) in 0.05% FCS for 24–48 h and 6–12 h, respectively. Total RNA was extracted and analyzed for *EGR1* expression by real-time PCR. Significance according to the *t*-test corresponded to * $P < 0.05$ and ** $P < 0.005$. (b, c) H2228 (mock) and H2228 transduced with *EGR1* cDNA (pWPI-*EGR1*) were treated with CEP-28122 and G, in single or in combination in 0.05% FCS for 96 h and 120 h, respectively. Cell viability was evaluated by Cell Titer-Glo Luminescent kit (b) and the percentage of apoptotic cells was measured by tetramethylrodamine methyl ester staining followed by fluorescence-activated cell sorting analysis (c). Significances * and ** are referred to the corresponding untransduced cells (mock). Results are representative of three different experiments. (d) Cells treated with CEP-28122 and/or G in 0.05% FCS for 24 h were collected for western blot analysis with the indicated antibodies.

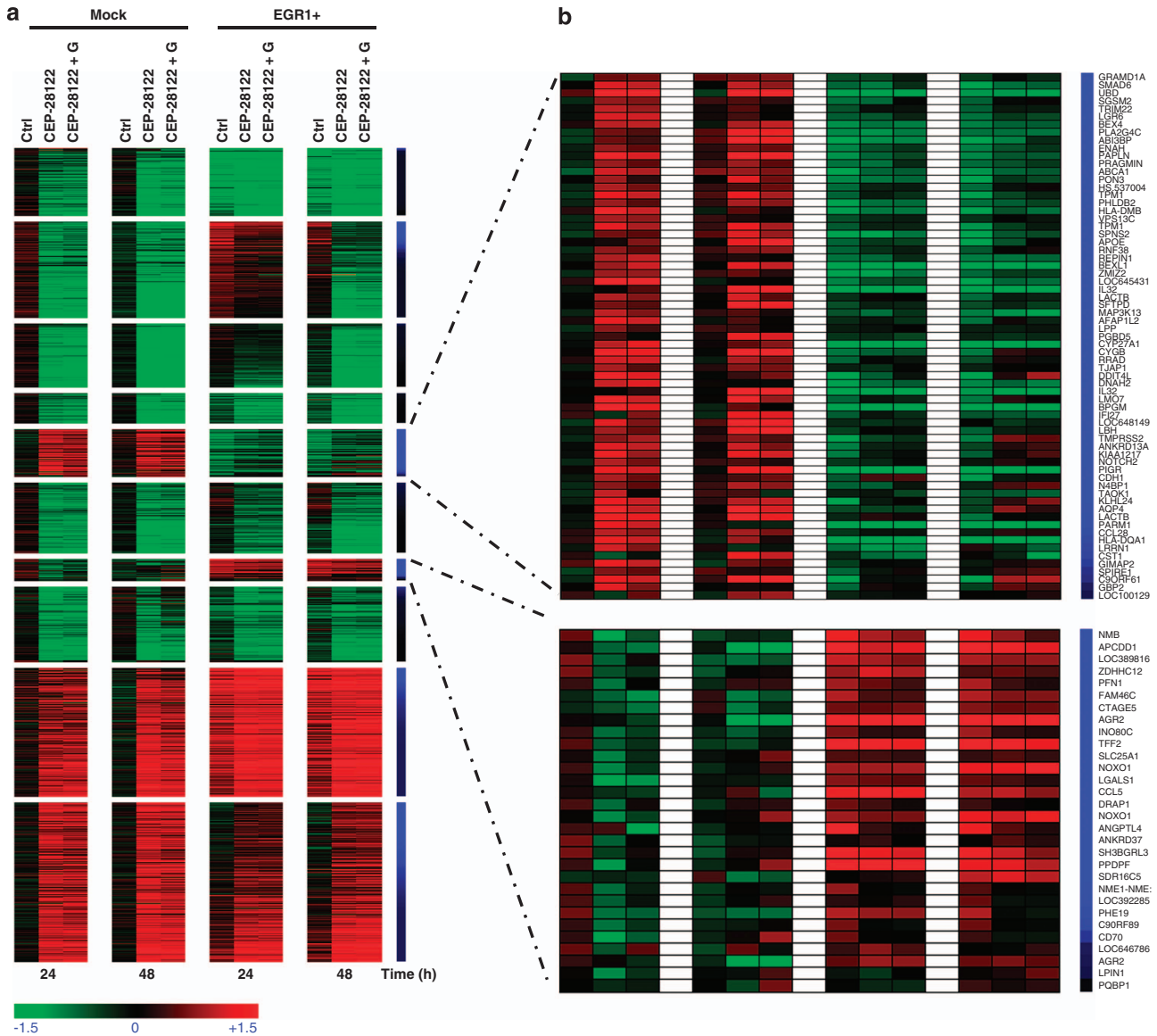


Figure 6. EGR1 overexpression regulates the transcription of genes independently of ALK inhibition. **(a)** Heat map of gene expression signature in response to ALK and/or EGFR inhibition in H2228 cells and in H2228 cells overexpressing EGR1. The color scale bar represents relative gene expression changes calculated as \log_2 (inhibited/non-inhibited). **(b)** List and expression changes of genes up- or downregulated by the overexpression of EGR1 independently of ALK or EGFR inhibition.

Here we studied the level of ALK oncogenic addiction of human ALK+ NSCLC and the cooperative role of several kinases. We show that the different responses of NSCLC cell lines to ALK are dependent on the activation of alternative pathways in which wild-type RTK can overcome ALK signaling blockade.

Using phosphoproteomics, different studies have demonstrated that lung cancer cells and primary tumors express kinases able to jointly participate to hierarchical networks or independently signal through separate parallel pathways.^{5,40} Our previous phosphomapping findings showed that ALK inhibition is rapidly followed by the parallel activation/phosphorylation of several other TKs (EGFR, Met, fibroblast growth factor receptor, Jak1 or insulin-like growth factor receptor) in H2228 cells, but not in H3122 cells, which are markedly ALK-addicted.³⁰ Interestingly, the dual inhibition with gefitinib or cetuximab overcomes this resistance feedback loop and increases cell death. The elevated levels of EGFR protein in H2228 compared with H3122, without

displaying any amplification or known mutation, might explain their partial addiction to ALK and the better responsiveness to the cotreatment. Notably, other members of the EGFR family, ERBB2 and ERBB3, can also overcome the partial resistance to ALKi, as the combination with lapatinib and an ALKi markedly reduced ERBB2 phosphorylation and cell viability. This suggests that forced EGFR family signaling (EGFR and ERBB2), even in naive/untreated NSCLC cells, may be critical, similar to what has been recently described for the acquired resistance to ALKi.^{20,41} Nevertheless, we demonstrated that this occurs in a context of wild-type EGFR and non-mutated ALK and, more importantly, in absence of drug selection.^{17,42} Our studies are in line with those described by Prahallad *et al.*⁴³, which have documented an intrinsic resistance to vemurafenib caused by a feedback activation of EGFR signaling in BRAF (V600E) mutant colon cancer cells. Thus, EGFR members can elicit an undermining mechanism that sustains the partial resistance seen in some ALK+ NSCLC patients, or

ultimately post-therapy resistance to ALKi.⁴¹ A greater complexity is provided by the observation that the engagement of wild-type EGFR or wild-type MET receptors could also restore cell viability of H2228 and H3122 cells via EGR1 and/or Erk1/2 and Akt activation.³² Remarkably, in H2228 the effects of HGF on cell survival were less significant, more likely for the partial dependence of these cells on EGFR signaling. We believe that EGFR member family expression levels or phosphorylation should be determined at diagnosis in order to benefit the most on combined therapies.

As cancer cells often trigger alternative pathways to bypass specific RTK inhibition, we further dissected other signaling pathways to find a common node of ALK and other RTKs. In ALK+ NSCLC cells, Src kinase represents a suitable target as in ALK+ anaplastic lymphoma its ablation has relevant effects in analogy to many human cancers.³⁵ In H2228 cells, Src inhibitors alone or in combination with ALKi increased the percentage of apoptotic cells similarly to ALK and gefitinib, suggesting that Src mediates ALK and EGFR signaling in NSCLC. These data are consistent with the notion that ALK and EGFR pathways functionally compensate each other. Nevertheless, other alternative signaling pathways might be operational in H2228, as EGF partially recovered cells from death. Indeed, rescue by EGF in H3122 was completely abrogated upon ALK and Src inhibition, suggesting that EGF/EGFR signaling triggers prosurvival responses through Src.

Next, to define additional putative downstream 'culprits', we used gene expression profiling analyses, which showed the *EGR1* gene as a key regulator. The cotreatment with ALKi and gefitinib downregulated the expression of a single transcript, *EGR1*. *EGR1* is a transcription factor, known to have a polyhedric/versatile role in human cancers,⁴⁴ engaged in proapoptotic or prosurvival signals, depending on the cellular context.⁴⁵ In solid tumors, including lung cancers, *EGR1* is a downstream target of EGFR through the mitogen-activated protein kinase signaling pathways.⁴⁶ Indeed, its overexpression, able to sustain cell viability in presence of ALK and EGFR inhibitors, was characterized by well-defined transcriptional signature. Among the upregulated genes, *TFF2* (trefoil factor 2) and *AGR2* (anterior gradient 2) seem to be relevant, as in human cancers they contribute to tumor growth, resistance to apoptosis and cell migration.^{47,48} Thus, *EGR1* could represent an ideal target for the design and implementation of new therapeutic approaches in ALK-positive NSCLC.

Our data indicate that the molecular characterization of primary lung cancers is required to foresee whether any given therapeutic protocol may be successful. The genetic heterogeneity of lung tumors implies that parallel pathways or different mechanisms of resistance may coexist 'ab initio' and that targeting a single molecule might be ineffective. This stratification is necessary to develop new combined treatment for NSCLC patients. Targeting common molecules downstream of RTKs, such as *EGR1* or Src, could bypass intrinsic or acquired drug resistance derived from the treatment with a single RTK inhibitor. Our data support the idea that the development of less-restricted drugs or the design of new therapeutic protocols making use of multiple TK inhibition might be more efficacious, even for the first-line treatment of NSCLC.

MATERIALS AND METHODS

Cell cultures, inhibitors and growth factors

Human lung cancer EML4-ALK-positive cell lines, H2228 (var 2: EML4/es6-ALK/es20) and H3122 (var 1: EML4/es13-ALK/es20), were grown in RPMI medium (Lonza, Basel, Switzerland) with 10% fetal calf serum (FCS). Human HEK-293T cells were cultured in Dulbecco's modified Eagle's medium with 10% FCS. Human immortalized airway epithelial cells (NuLi-1) were grown in bronchial epithelial cell basal medium (Lonza) with 10% FCS on human collagen type-VI-coated plates (Sigma, St Louis, MO, USA).

ALK inhibitors, CEP-14083, CEP-26939 and CEP-28122 (Cephalon), were used at 300 nM/l; gefitinib (ZD1839, Iressa) at 1 μ M/l; lapatinib at 2 μ M/l; Src kinase inhibitor-1 (Calbiochem, Millipore Corporation, Billerica, MA, USA) at 5 μ M/l; and PHA-665752 (Met inhibitor) at 250 nM. EGF was used at 10 ng/ml, Heregulin was used at 20 ng/ml, and HGF (Sigma) was used at 50 ng/ml.

Cell proliferation, cell cycle analysis, cell apoptosis and clonogenic assays

In 24-well plates, 8 \times 10⁴ cells/ml were grown in duplicates. The treatment with kinase inhibitors or with growth factors was done every 24 h in 0.05% FCS. Cell growth was analyzed using Cell Titer-GloMax assay (Promega, Fitchburg, WI, USA). For cell cycle analysis, cells were fixed for 1 h in 70% ethanol at 4 °C. After washing, cells were treated with RNase (0.25 mg/ml) and stained with propidium iodide (50 μ g/ml). The sub-G₀-phase fraction was calculated using the Modfit program from Becton-Dickinson (San Jose, CA, USA). Apoptosis was measured by flow cytometry after staining with the mitochondrion-permeable voltage sensitive dye tetramethylrhodamine methyl ester and fluorescence-activated cell sorting analysis. Clonogenic assay, were performed as described.²⁸

DNA constructs

EGR1 cDNA in pCMV-Sport6 was purchased from ImaGenes GmbH (Berlin, Germany). *EGR1* cDNA was then subcloned into the lentiviral vector pWPI for lentivirus production. pCMV-Sport6 containing *EGR1* construct was digested to obtain a *EcoRV/NotI* fragment, blunted with Klenow enzyme (Roche, Basilea, Switzerland) and cloned into the *PmeI* site of the lentiviral vector pWPI.

Lentivirus and short hairpin RNA production, cell infection and cell sorting

Lentivirus particles were obtained as described.²⁷ For cell sorting enrichments, EGFP-positive cells were sorted using a MoFlo High-Performance instrument (DAKO Cytomation, Glostrup, Denmark) and EGFP expression was checked by flow cytometry over time.

Cell lysis and western blot analysis

H3122 and H2228 were plated on six-well plates and treated with specific kinase inhibitors in Dulbecco's modified Eagle's medium with 0.05% FCS. Cells were lysed with GST-FISH (10 mM MgCl₂, 150 mM NaCl, 1% NP40, 2% Glycerol, 1 mM EDTA, 25 mM HEPES (pH 7.5)) buffer supplemented with protease inhibitors (NaF, phenylmethanesulfonylfluoride, PIC and Na₃VO₄) by scraping. The list of the antibodies is provided in Supplementary Information.

RNA extraction, cDNA synthesis and reverse transcription-PCR

Total RNA was isolated from cells with TRIZOL lysis solution (Invitrogen, Life Technologies Ltd, Paisley, UK). One microgram of total RNA was treated with 5 U/ μ l of DNase I recombinant RNase free (Roche) and it was subjected to reverse transcription with SuperScript III reverse transcriptase (Invitrogen). The cDNA obtained was subjected to PCR using specific primers for PBGD, B2M and ABL as control of cDNA quality.

cDNA microarray and analysis

Total RNA was extracted using the TRIZOL reagent (Invitrogen) and purified using the RNeasy total RNA Isolation Kit (Qiagen, Hilden, Germany). RNA integrity was verified by means of a Bioanalyzer 2100 (Agilent Technologies, Santa Clara, CA, USA). Hybridization was performed on HumanWG-12v4 BeadChips (Illumina, San Diego, CA, USA), using biological triplicates for each condition. cDNA and biotinylated cRNA were generated by Illumina TotalPrep RNA Amplification Kit (Ambion, Life Technologies Ltd, Paisley, UK). Data were processed with the Illumina GenomeStudio software using the following thresholds for significant differential expression: 'Illumina Custom' test *P*-value < 0.001, fold-change > 2. Gene expression data were clustered and visualized with the GEDAS software.²⁹

Real-time quantitative PCR

cDNA was transcribed using SuperSCRIPT III following the manufacturer's instructions (Invitrogen). Quantitative reverse transcription-PCR was

performed in triplicate on ICycler iQ Real-Time PCR Detection System (Bio-Rad Laboratories, Hercules, CA, USA) with SYBR green dye.

Immunofluorescence and immunohistochemistry

Cells were grown for 12 h on glass coverlips, fixed in phosphate-buffered saline containing 4% paraformaldehyde at room temperature (10 min), and then permeabilized with phosphate-buffered saline containing 0.3% Triton X-100 for 5 min. Anti-EGFR (1:100, Cell Signaling Technology, Danvers, MA, USA), ERBB2 (1:300, Dako) and ERBB3 (1:100, LabVision, ThermoScientific, Kalamazoo, MI, USA) were incubated O/N, and heterodimer formation was detected using DUOLINK II assay (Olink Bioscience, Uppsala, Sweden). Images were acquired by Imager.Z1 fluorescent microscope (Zeiss) and analysed with Isis and Metaphor software. Immunohistochemistry was performed on a semi-automated immunostainer as described previously.³⁰ The following antibodies were used: anti-ALK (clone D5F3; Cell Signaling Technology) and anti-ERBB2 (clone CB11; Novocastra, Leica, Milano, Italy).

Mice and *in vivo* bioluminescence experiments

CB17 scid and NOD-SCID mice (Charles River Laboratories Italia S.p.A., Wilmington, DE, USA) were inoculated subcutaneously in both flanks (single-cell suspension 1×10^7 in 0.2 ml phosphate-buffered saline). Mice were treated twice a day with 100 mg/kg of Comp-2. Tumor growth was measured over time. For imaging studies, luciferase-positive cell xenografts were analyzed *in vivo* by IVIS100 (Xenogen, Grantham, Lincolnshire, UK) instrument after intraperitoneal injection with D-luciferin (10 μ l/g body weight, 15 mg/ml). Mice were handled and treated in accordance with the European Community guidelines.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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