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# The PI3K/AKT pathway promotes gefitinib resistance in mutant *KRAS* lung adenocarcinoma by a deacetylase-dependent mechanism

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To select the appropriate patients for treatment with epidermal growth factor receptor tyrosine kinase inhibitors (EGFR-TKIs), it is important to gain a better understanding of the intracellular pathways leading to EGFR-TKI resistance, which is a common problem in patients with lung cancer. We recently reported that mutant *KRAS* adenocarcinoma is resistant to gefitinib as a result of amphiregulin and insulin-like growth factor-1 receptor overexpression. This resistance leads to inhibition of Ku70 acetylation, thus enhancing the BAX/Ku70 interaction and preventing apoptosis. Here, we determined the intracellular pathways involved in gefitinib resistance in lung cancers and explored the impact of their inhibition. We analyzed the activation of the phosphatidylinositol-3-kinase (PI3K)/AKT pathway and the mitogen-activated protein kinase/extracellular-signal regulated kinase (MAPK/ERK) pathway in lung tumors. The activation of AKT was associated with disease progression in tumors with wild-type *EGFR* from patients treated with gefitinib (phase II clinical trial IFCT0401). The administration of IGF1R-TKI or amphiregulin-directed shRNA decreased AKT signaling and restored gefitinib sensitivity in mutant *KRAS* cells. The combination of PI3K/AKT inhibition with gefitinib restored apoptosis via Ku70 downregulation and BAX release from Ku70. Deacetylase inhibitors, which decreased the BAX/Ku70 interaction, inhibited AKT signaling and induced gefitinib-dependent apoptosis. The PI3K/AKT pathway is thus a major pathway contributing to gefitinib resistance in lung tumors with *KRAS* mutation, through the regulation of the BAX/Ku70 interaction. This finding suggests that combined treatments could improve the outcomes for this subset of lung cancer patients, who have a poor prognosis.

**Key words:** EGFR-TKI resistance, lung cancer, PI3K-AKT, sirtuins, IGF1R

**Abbreviations:** EGFR: epidermal growth factor receptor; HDAC: histone deacetylases; IGF1R: insulin-like growth factor-type 1 receptor; IFCT: Intergroupe Francophone de Cancérologie Thoracique; *KRAS*: Kirsten rat sarcoma viral oncogene homolog; MAPK/ERK: mitogen-activated protein kinase/extracellular-signal regulated kinase; NSCLC: non-small cell lung cancers; PDK: phosphatidylinositol-3-kinase-dependent kinase; PI3K: phosphatidylinositol-3-kinase; shRNA: small hairpin ribonucleic acid; siRNA: small interfering ribonucleic acid; TKI: tyrosine kinase inhibitor. Additional Supporting Information may be found in the online version of this article.

<sup>†</sup>Jacques Cadranel is an IFCT0401 Principal Investigator.

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The epidermal growth factor receptor (EGFR) is frequently overexpressed in nonsmall-cell lung cancers (NSCLCs) and is associated with a poor prognosis.<sup>1</sup> As a consequence, therapies targeting the tyrosine kinase activity of EGFR (EGFR-TKIs, such as gefitinib and erlotinib) have been developed, and are highly effective for the treatment of *EGFR*-mutated NSCLC.<sup>2</sup> For patients with *EGFR* wild-type tumors, first-line chemotherapy is still the standard of care.<sup>2</sup> *EGFR*-TKIs are approved for the second- and third-line treatment of advanced NSCLC or as maintenance therapy.<sup>2</sup> However, the limited response rates to *EGFR*-TKIs observed in *EGFR* wild type led to the investigation of mechanisms governing resistance to *EGFR*-TKI treatments.<sup>3</sup> Tumors with constitutive activation of *KRAS* small GTPase (30%) are hypothesized to be intrinsically resistant to *EGFR*-TKI.<sup>2</sup> Elevated level of circulating amphiregulin, an *EGFR* ligand, is correlated with a poor prognosis in NSCLC patients.<sup>4</sup> Our group has demonstrated that amphiregulin cooperates with the insulin-like growth factor-type 1 receptor (IGF1R)<sup>5,6</sup> to promote resistance to gefitinib-induced apoptosis in *KRAS* mutant NSCLC cells.<sup>7,8</sup> The overexpression of amphiregulin or IGF1R has been observed in the mucinous invasive adenocarcinoma subtype of NSCLC tumors, which often harbors *KRAS* mutations

**What's new?**

EGFR is frequently overexpressed in non-small cell lungcancers (NSCLCs) and is associated with poor prognosis. While therapies targeting the tyrosine kinase activity of EGFR (EGFR-TKIs, such as gefitinib) are highly effective for the treatment of EGFR mutated NSCLC, limited response rates are observed in EGFR wild-type NSCLC. Here the authors found that the PI3K/AKT pathway contributes to gefitinib resistance in mutant *KRAS* adenocarcinoma by a deacetylase-dependent mechanism. They showed for the first time that the PI3K/AKT pathway induces survival of wild-type EGFR NSCLCs with *KRAS* mutations, suggesting a new therapeutic target for treating this subset of lung cancer patients.

and is resistant to EGFR-TKI treatments.<sup>8,9</sup> In addition, amphiregulin prevents gefitinib-induced cell death through the inhibition Ku70 protein acetylation, thus enhancing the interaction between the proapoptotic protein BAX and Ku70.<sup>7,10</sup> However, the survival-signaling pathway that is induced by amphiregulin and IGF1R in the presence of an EGFR-TKI, leading to the regulation of acetylation, is not fully understood. Receptor tyrosine kinases, such as EGFR or IGF1R, mainly activate the phosphatidyl inositol-3 kinase (PI3K)/AKT and mitogen-activated protein kinase (MAPK)/extracellular-signal regulated kinase (ERK1/2) pathway. PI3K/AKT signaling is frequently deregulated in NSCLC.<sup>11</sup> Recent studies indicate that the PI3K/AKT pathway plays a crucial role in the resistance to various types of TKI, including EGFR-TKIs.<sup>12–14</sup>

Acetylation is a reversible modification controlled by the antagonistic actions of two types of enzymes, histone acetyltransferases and histone deacetylases (HDACs). HDACs regulate the function of histones and many nonhistone proteins by modulating their acetylation status and have emerged as crucial transcriptional co-repressors in highly diverse physiological and pathological systems.<sup>15</sup> To date, 18 human HDACs have been identified and grouped into four classes.<sup>15</sup> Class I, class II (subdivided into classes IIa and IIb) and class IV HDACs are named “classical” HDACs. Class III HDACs are also called sirtuins. The expression of HDACs is deregulated in many cancer types. Interestingly, class I and II HDACs are involved in lung carcinogenesis.<sup>15,16</sup> The use of class I/II HDACs inhibitor (trichostatin A) or class III HDACs inhibitor (nicotinamide) results in the increased acetylation of Ku70 and the dissociation of BAX/Ku70.<sup>10,17</sup> Recently, the tubulin deacetylase HDAC6 has been shown to deacetylate Ku70 and to regulate BAX/Ku70 binding in neuroblastoma.<sup>18</sup>

In the present study, we analyzed the intracellular pathways associated with EGFR-TKI response in *EGFR* wild-type lung adenocarcinoma. In particular, we examined whether the activation of AKT or ERK1/2 can predict the responses of patients with adenocarcinoma to gefitinib as well as whether the inhibition of these signaling pathways can overcome EGFR-TKI resistance induced by amphiregulin and IGF1R in mutant *KRAS* adenocarcinoma cells. In this setting, we investigated the relationships between AKT or ERK1/2 activation and acetylation-dependent regulation of BAX/Ku70 interaction.

**Material and methods****Immunohistochemistry of NSCLC tumors**

The experiments were performed on 62 formalin-fixed paraffin-embedded human adenocarcinoma samples. Twenty-eight were taken from the site of the surgical resections of lung tumors, and 34 were collected from patients enrolled in the prospective multicenter phase II trial that was conducted to evaluate gefitinib as a first-line therapy for non-resectable adenocarcinoma (IFCT0401, NCT00198380).<sup>9</sup> All patients enrolled in this trial provided informed consent. Tissue banking and research conduct were approved by the ministry of research (approval AC-2010-1129) and by the regional IRB (CPP 5 Sud-Est). *EGFR* exons 18–21 and *KRAS* exon 2 were amplified and sequenced in both directions, as previously described.<sup>8</sup> Mucinous invasive adenocarcinoma were categorized as mucinous type ( $n = 15$ ), whereas others adenocarcinoma were categorized as nonmucinous type ( $n = 47$ ), as previously described.<sup>8</sup>

Immunostaining analysis was performed with 3- $\mu$ m-thick tissue sections on an automated instrument (BenchMark, Ventana Medical Systems). Sections were incubated with antibody against phosphorylated-AKT or -ERK1/2 (Cell Signaling Technology, 1/50 and 1/100, respectively). An indirect biotin avidin system and the Ventana Basic DAB detection kit (Ventana Medical Systems) were used, according to the manufacturer's instructions. The omission of the primary antibody and/or incubation with same species and isotype IgG at the same concentration of the primary antibody served as negative controls. Pathologists blinded to the clinicopathological variables, mutation status and treatment response independently evaluated the immunostaining. Differential scores (0–300) were ascribed by multiplying the percentage of stained cells (0–100%) by the staining intensity (1+, 2+, 3+). A score >0 was considered positive.

**Cell culture and drug treatments**

The human H358 and H322 NSCLC cell lines were obtained from the American Type Culture Collection (ATCC, Manassas, VA) and were maintained in RPMI 1640 medium (Gibco, Cergy Pontoise, France), supplemented with 10% heat-inactivated fetal bovine serum in a humidified atmosphere with 5% CO<sub>2</sub>. Amphiregulin-directed or control shRNA lentiviral particles (Santa Cruz Biotechnology) were used to infect H358 cells. Stable cell lines were selected by culturing cells in

233 2 µg/mL puromycin (Calbiochem, La Jolla, CA) for 2 weeks.  
 234 Western blotting or qPCR was used to determine the effects  
 235 of gene expression knockdown as described.<sup>7</sup>

236 Tubastatin A, LY294002, gefitinib, PD98059 and OSI-906  
 237 were from Selleckchem (Munich, Germany); trichostatin A,  
 238 nicotinamide, U0126 and sodium butyrate were from Sigma-  
 239 Aldrich (St Quentin-Fallavier, France).

### 242 Apoptosis assays

243 The morphological changes related to apoptosis were assessed  
 244 by fluorescence microscopy after Hoechst 33342 (5 µg/mL,  
 245 Sigma) staining of cells. The percentage of apoptotic cells was  
 246 scored after counting at least 500 cells. Active caspase-3 was  
 247 detected by immunoblotting or by flow cytometry using a  
 248 phycoerythrin-conjugated monoclonal active caspase-3 anti-  
 249 body kit (BD Pharmingen, Le Pont de Claix, France), follo-  
 250 wing the manufacturer's instructions. The analysis was  
 251 performed on a BD-Accuri C6 flow cytometer with CFlow-  
 252 Plus software (BD Biosciences).

### 253 Immunoprecipitation and immunoblotting

254 Endogenous BAX immunoprecipitation and immunoblotting  
 255 experiments were performed as previously described<sup>7,10</sup> using  
 256 antibodies against Ku70 (N3H1), IGF1Rβ (Santa Cruz Bio-  
 257 technology), BAX (BD Pharmingen), cleaved caspase-3  
 258 (Asp<sup>175</sup>), actin, phospho-EGFR-Y<sup>1068</sup>, EGFR, phospho-  
 259 IGF1R-Y<sup>1135/1136</sup>, phospho-AKT-S<sup>473</sup>, pan-AKT, phospho-  
 260 ERK1/2-T<sup>202</sup>/Y<sup>204</sup> and ERK1/2 (Cell Signaling Technology, St  
 261 Quentin en Yvelines, France). The relative intensity, meas-  
 262 ured using ImageJ (NIH software), of co-precipitated Ku70  
 263 was normalized to the respective immunoprecipitated Bax.

### 264 Statistical analyses

265 Difference in treatments and continuous variables were  
 266 compared using the Kruskal–Wallis test or Mann–Whitney  
 267 *U*-test. Two-sided *p* values <0.05 were considered statistically  
 268 significant. All analyses were performed using Statview 4.1  
 269 software (Abacus Concept, Berkeley, CA).

## 270 Results

### 271 AKT and ERK1/2 activation in patients

272 We previously showed that gefitinib resistance is associated  
 273 with the overexpression of IGF1R and amphiregulin in muc-  
 274 inous invasive adenocarcinoma.<sup>8</sup> We investigated the intracellu-  
 275 lar pathways involved in this resistance by analyzing the  
 276 phosphorylation of AKT (p-AKT) and ERK1/2 (p-ERK1/2) in  
 277 62 lung adenocarcinoma samples. p-ERK1/2 and p-AKT were  
 278 undetectable in normal tissues distant from cancer tissues and  
 279 moderately detectable in normal bronchial epithelia adjacent to  
 280 tumor cells. p-AKT and p-ERK1/2 displayed diffuse cytoplas-  
 281 mic staining and less frequent (~32% of positive tumors)  
 282 nuclear staining patterns (Fig. 1a). Twenty-five (49%) and 49  
 283 (82%) tumors had a staining score >0 for p-AKT and p-  
 284 ERK1/2, respectively (Fig. 1b). The medians of the staining  
 285 scores for p-AKT and p-ERK1/2 are shown in Table 1. High p-  
 286

292 ERK1/2 levels were strongly associated with the presence of  
 293 lymph-node metastasis (*p* = 0.0053); p-AKT levels were also  
 294 higher in tumors with lymph-node metastasis (not significant).  
 295

296 Five tumors (8%) had *EGFR* exon-19 or -21 mutations.  
 297 All were non-mucinous subtypes, as previously described,<sup>8</sup>  
 298 and had higher levels of p-AKT (*p* = 0.0732), highlighting  
 299 the continuous activation of the mutated *EGFR*.

300 Among *EGFR* wild-type tumors, 11 (19%) had a *KRAS*  
 301 exon 2 mutation and higher p-AKT and p-ERK1/2 levels (not  
 302 significant). No additional relationships were observed between  
 303 p-AKT and p-ERK1/2 (Table 1) or between *EGFR*, IGF1R,  
 304 amphiregulin expression (Supporting Information Table S1)  
 305 and other clinical parameters in *EGFR* wild-type tumors.

### 306 p-AKT, p-ERK1/2 and the gefitinib response

307 Among the adenocarcinoma samples, we analyzed the  
 308 responses to gefitinib of the 34 patients with surgical samples  
 309 who were enrolled in the IFCT0401 phase II clinical trial, ini-  
 310 tially conducted to evaluate gefitinib as a first-line therapy for  
 311 nonresectable adenocarcinoma.<sup>9</sup> Twelve patients achieved dis-  
 312 ease control at 3 months with gefitinib treatment.<sup>8</sup> Among  
 313 them, four patients with an *EGFR* mutation had a partial  
 314 response to treatment. Consistent with the enhanced p-AKT  
 315 levels in patients with mutated *EGFR* among the 62 tumors,  
 316 a high p-AKT was associated with a partial response to gefiti-  
 317 nib (*p* = 0.0378, Table 1). No significant relationship was  
 318 observed between p-ERK1/2 level and disease control with  
 319 gefitinib (*p* = 0.43, Table 1).

320 Interestingly, in patients with wild-type *EGFR*, a high  
 321 p-AKT level was associated with disease progression (*p* =  
 322 0.0475, Fig. 1c), whereas the eight patients with stable dis-  
 323 eases had wild-type *EGFR* and *KRAS* and did not express  
 324 p-AKT. No significant relationship was observed between  
 325 p-ERK1/2 level and disease progression in patients with wild-  
 326 type *EGFR* (*p* = 0.32, Fig. 1c). These results suggest that  
 327 high p-AKT levels are associated with gefitinib resistance in  
 328 lung adenocarcinoma with wild-type *EGFR*, independently of  
 329 *KRAS* mutational status.

### 330 Amphiregulin and IGF1R activate AKT and promote 331 gefitinib resistance

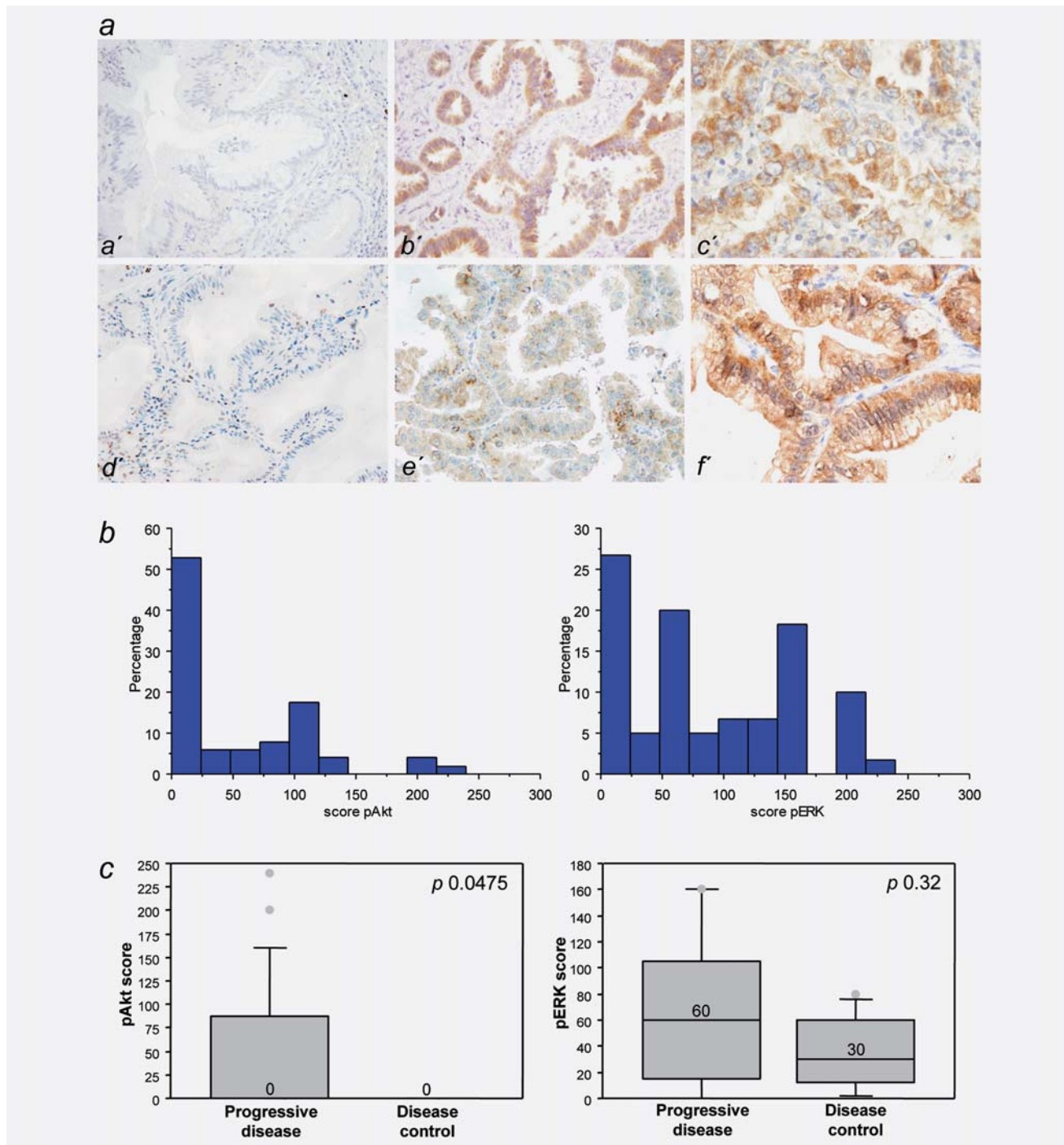
332 Amphiregulin and IGF1R control gefitinib resistance in adeno-  
 333 carcinoma cells with wild-type *EGFR*,<sup>7,8</sup> as seen using the mutant  
 334 *KRAS* adenocarcinoma cell line H358, which overexpresses  
 335 amphiregulin<sup>6</sup> and is resistant to gefitinib.<sup>7,8</sup> Small hairpin RNA  
 336 (shRNA) were used to stably silence amphiregulin expression  
 337 (Fig. 2a) and to restore the sensitivity to gefitinib at the same  
 338 level as that of the wild-type *EGFR* and *KRAS*, amphiregulin-  
 339 lacking NSCLC cell line H322 (Fig. 2b). We examined the phos-  
 340 phorylation of *EGFR*, IGF1R and their downstream pathways  
 341 (the PI3K/AKT and MAPK/ERK1/2 pathways) by western blot-  
 342 ting. As previously shown,<sup>8</sup> gefitinib decreased the level of p-  
 343 *EGFR* and increased the levels of p-IGF1R, p-AKT and p-ERK1/  
 344 2 after 96 hr of treatment (Figs. 2c and 2e, second column).  
 345 Amphiregulin-directed shRNA prevented gefitinib resistance by  
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**Figure 1.** p-AKT and p-ERK1/2 immunostaining in NSCLC tumors. (a) Immunohistochemistry showing p-AKT expression (a, negative score, b, score of 100, c, score of 240) and p-ERK1/2 expression (d, negative score, e, score of 60, f, score of 160). Original magnification: 200×. (b) Distribution of p-AKT (left) and p-ERK1/2 (right) staining across all tumor samples: percentage of samples (Y-axis) showing the indicated scores of stained cells (X-axis). (c) Distribution of p-AKT scores (left) and p-ERK1/2 (right) scores, according to disease control with gefitinib in patients with wild-type *EGFR*. Numbers: median score staining. Statistical analysis was carried out using the Mann-Whitney *U*-test.

inhibiting p-IGF1R and p-AKT, whereas ERK1/2 phosphorylation was not affected (Fig. 2c). OSI-906, an IGF1R-TKI, inhibited cell proliferation (Supporting Information Fig. S1) and restored gefitinib-induced apoptosis in H358 cells (Fig. 2d). OSI-906 also strongly blocked p-IGF1R and the gefitinib resistance by decreasing

ing p-AKT but not p-ERK1/2 (Fig. 2e). These results further suggest that amphiregulin and IGF1R control resistance to gefitinib through the activation of downstream PI3K/AKT.

To confirm the importance of the PI3K/AKT pathway in gefitinib resistance, we examined whether the inhibition of

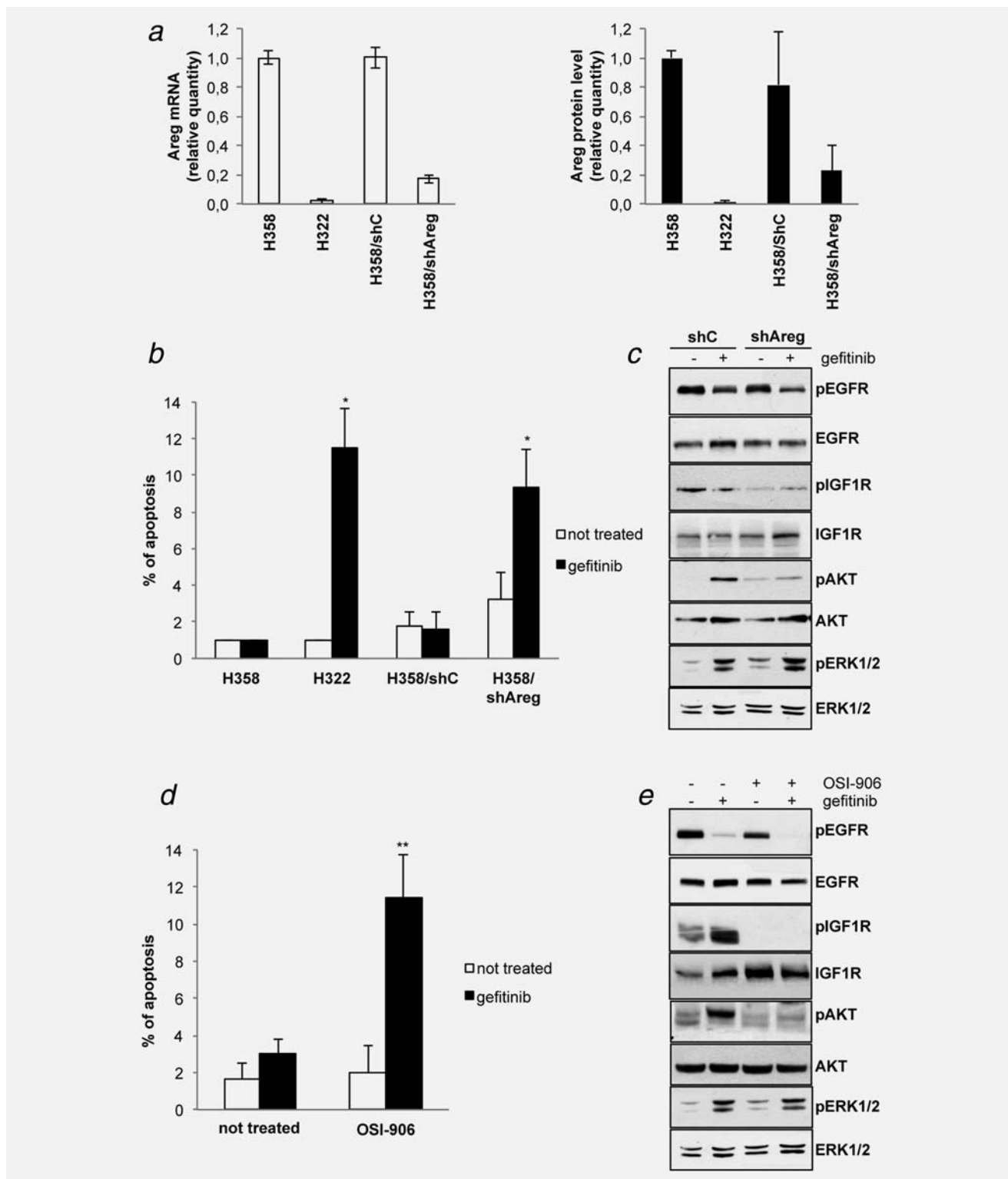
**Table 1.** Immunohistochemical analysis of p-AKT and p-ERK1/2 in lung adenocarcinoma

	n (%)	p-AKT		p-ERK1/2	
		Median (range)	P	Median (range)	p
<b>All patients</b>	62 (100%)	0 (0–240)		60 (0–240)	
<b>Gender</b>			0.26		0.63
Male	39 (63%)	30 (0–240)		100 (0–210)	
Female	23 (37%)	0 (0–200)		60 (0–240)	
<b>Age (years)</b>			0.80		0.25
Median (range)	65.7 (36–80.4)				
<70	44 (71%)	10 (0–240)		80 (0–240)	
≥70	18 (29%)	0 (0–200)		60 (0–160)	
<b>Smoking status</b>			0.57		0.18
Smokers	49 (79%)	25 (0–240)		80 (0–240)	
Never a smoker	13 (21%)	0 (0–200)		50 (0–160)	
<b>Stage</b>			0.84		0.57
I–II	23 (37%)	15 (0–240)		60 (0–210)	
III–IV	39 (63%)	0 (0–200)		90 (0–240)	
<b>Node metastasis</b>			0.19		0.0053
No	38 (61%)	0 (0–240)		50 (0–210)	
Yes	24 (39%)	45 (0–120)		120 (0–240)	
<b>Metastasis</b>			0.98		0.14
No	37 (60%)	20 (0–240)		80 (0–240)	
Yes	25 (40%)	0 (0–200)		60 (0–200)	
<b>Cytological subtype</b>			0.10		0.94
Mucinous	15 (24%)	0 (0–200)		100 (0–160)	
Nonmucinous	47 (76%)	30 (0–240)		60 (0–240)	
<b>EGFR mutation</b>			0.0732		0.33
Mutation	5 (8%)	100 (0–200)		40 (20–150)	
No mutation	57 (92%)	0 (0–240)		80 (0–240)	
<b>KRAS mutation</b>			0.60		0.78
Mutation	11 (19%)	40 (0–240)		110 (0–200)	
No mutation	47 (81%)	10 (0–200)		70 (0–240)	
Noninterpretable	4				
<b>IFCT0401 patients</b>	34 (100%)	0 (0–240)		30 (0–160)	
<b>Disease control with gefitinib</b>			0.0378*		0.43*
Partial response	4 (12%)	90 (0–200)		35 (20–50)	
Stable disease	8 (24%)	0 (0)		30 (0–80)	
Progressive disease	22 (64%)	0 (0–240)		60 (0–160)	

Statistical analysis was performed using Mann–Whitney *U*-test or \*Kruskall–Wallis test.

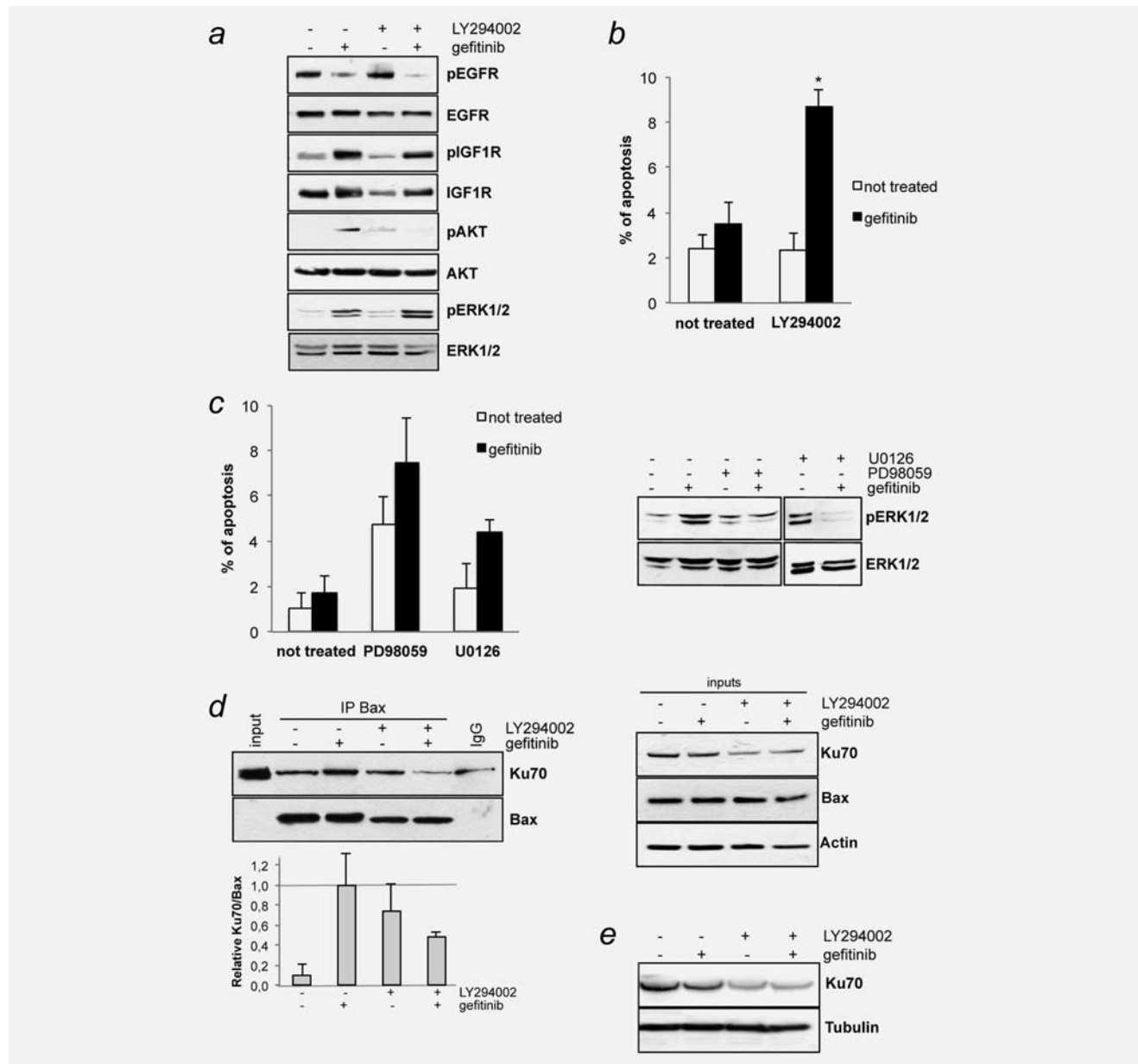
F3 this pathway could affect gefitinib-induced apoptosis *in vitro*. LY294002, a PI3K inhibitor, strongly inhibited the activation of AKT without affecting the levels of p-EGFR, p-IGF1R or p-ERK1/2 (Fig. 3a). The resistance of H358 cells to gefitinib was abolished when the cells were co-treated with LY294002, producing a significant induction of apoptosis (Fig. 3b) and inhibition of cell proliferation (Supporting Information Fig. S1). The same results were obtained using the PI3K inhibitor

wortmannin (Supporting Information Fig. S1). In contrast, MAPK/ERK1/2 pathway inhibition, using the specific inhibitors PD98059 or U0126, did not significantly enhance gefitinib-induced apoptosis (Fig. 3c, left panel). The effect of PD98059 and U0126 on gefitinib-induced ERK1/2 phosphorylation inhibition was confirmed by western-blot (Fig. 3c, right panel). These results confirm that PI3K/AKT activation is a major pathway contributing to gefitinib resistance



**Figure 2.** Amphyregulin or IGF1R inhibition restored gefitinib sensitivity and decreased p-AKT levels. (a) Amphyregulin (Areg) mRNA (left), assessed by quantitative real-time RT-PCR for amphyregulin mRNA on total RNA extracted from cells, and protein levels (right), detected by enzyme-linked immunosorbent assay, in H358, H322 and H358 stably expressing control shRNA (shC) or amphyregulin-directed shRNA (shAreg). Results are expressed as a rate of amphyregulin mRNA or amphyregulin released by H358 cells and as the mean  $\pm$  SD. (b and c) H358, H322 or H358/shC or H358/shAreg were treated with 0.5  $\mu$ mol/L gefitinib for 96 hr. (d-e) H358 cells were treated with 1  $\mu$ mol/L OSI-906 and/or 0.5  $\mu$ mol/L gefitinib for 96 hr. (b and d) Percentages of apoptosis were scored and expressed as the mean  $\pm$  SD ( $n \geq 3$ ). \* $p < 0.05$ , \*\* $p < 0.01$  for comparison between treated and control cells, and (c and e) representative immunoblots of EGFR, IGF1R, AKT and ERK1/2 and their respective phosphorylated forms are shown.





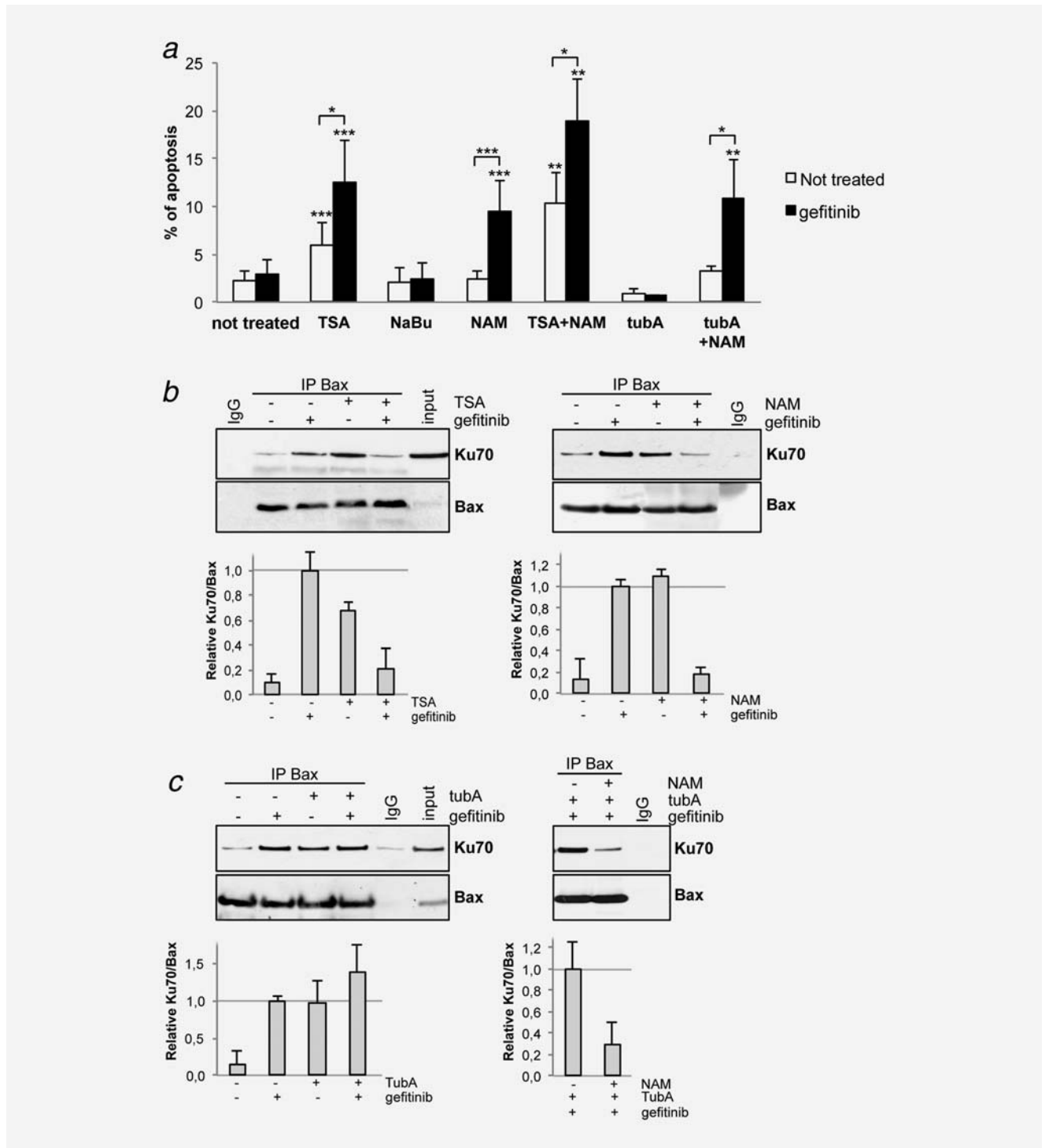
**Figure 3.** PI3K/AKT inhibition restored gefitinib sensitivity. H358 cells were treated with 25  $\mu\text{mol/L}$  LY294002, 20  $\mu\text{mol/L}$  PD98059 or U0126 and/or 0.5  $\mu\text{mol/L}$  gefitinib, as indicated. (a) Representative immunoblots of EGFR, IGF1R, AKT and ERK1/2 and their respective phosphorylated forms are shown. (b and c) Left panel: Percentages of apoptotic cells were scored and expressed as means  $\pm$  SD ( $n = 4$ ).  $*p < 0.05$ , for comparison between treated and control cells. (c) Right panel: Representative immunoblots of ERK1/2 and their respective phosphorylated forms. (d) Endogenous BAX immunoprecipitations (left panel) were performed and subjected to immunoblotting with Ku70-specific and BAX-specific antibodies. IgG: irrelevant immunoglobulin, used as negative control. Input: cell lysate not subjected to immunoprecipitation. Actin was used as a protein level control (right panel). The histogram shows the relative intensity of Ku70 protein bands of samples to that of gefitinib treated cells, after being normalized to the respective Bax and represents the means  $\pm$  SD of two independent experiments. (e) Representative immunoblot of Ku70. Tubulin was used as a protein level control.

and that PI3K/AKT inhibition can overcome gefitinib resistance.

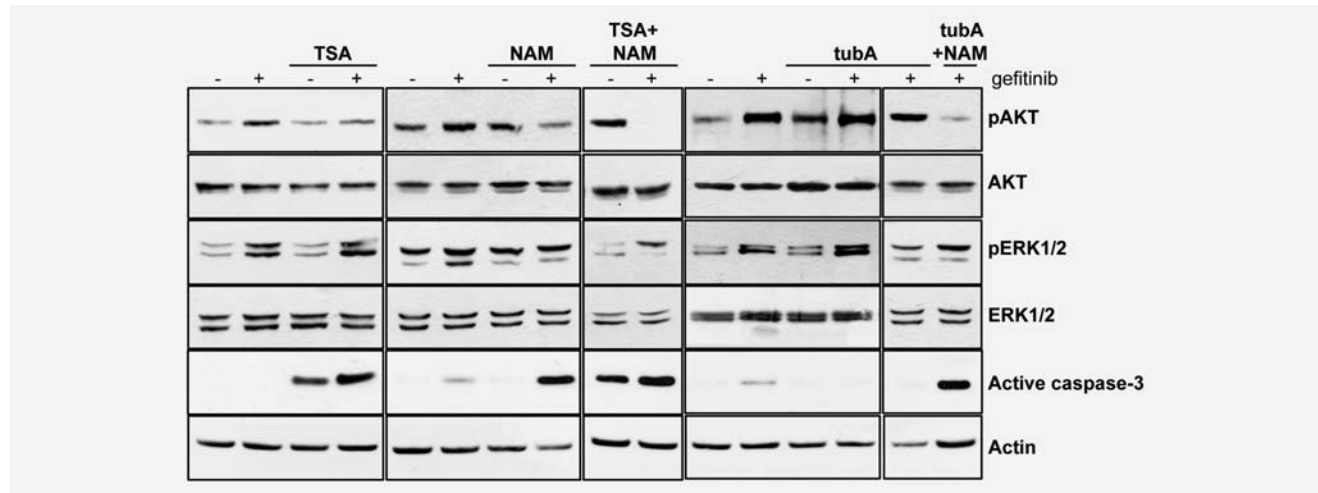
**AKT activation controls BAX/Ku70 interaction in the gefitinib resistance mechanism**

We have previously shown that amphiregulin and IGF1R mediate gefitinib resistance through increasing the interaction

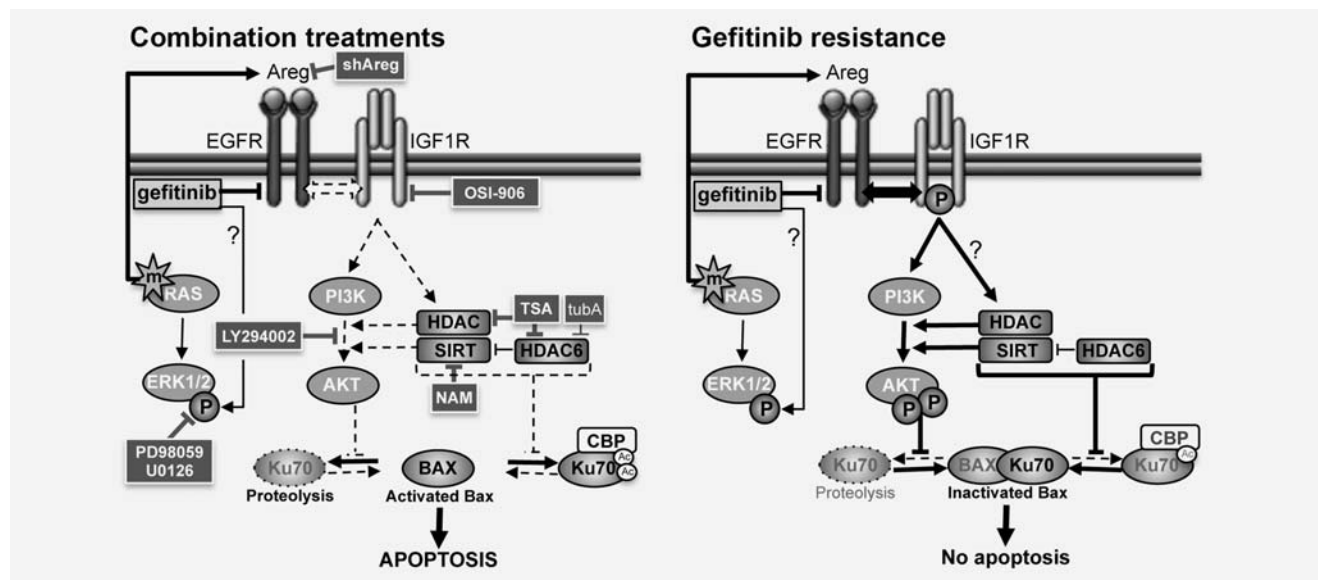
between the proapoptotic protein BAX and Ku70.<sup>7,10</sup> The inhibition of Ku70 acetylation enhances BAX/Ku70 binding and prevents gefitinib-induced apoptosis. In contrast, the acetylation of Ku70 releases BAX from Ku70 and restores the sensitivity to gefitinib.<sup>10</sup> We investigated the involvement of the PI3K/AKT pathway in the interaction between BAX and Ku70. We observed that PI3K/AKT inhibition by LY294002 decreased



**Figure 4.** HDACs inhibited gefitinib-induced apoptosis in NSCLC cells via a decrease in BAX/Ku70 interaction. H358 cells were treated with 2 mmol/L sodium butyrate (NaBu), 200 ng/mL trichostatin A (TSA), 5 mmol/L nicotinamide (NAM), 5  $\mu$ mol/L tubastatin A (tubA), 0.5  $\mu$ mol/L gefitinib or a combination of inhibitors for 96 hr as indicated. (a) Percentages of apoptotic cells were scored and expressed as means  $\pm$  SD ( $n \geq 3$ ). \* $p < 0.05$ , \*\* $p < 0.01$ , \*\*\* $p < 0.001$  for comparisons between treated and control cells or as indicated. (b and c) Endogenous BAX immunoprecipitations were performed and subjected to immunoblotting with Ku70-specific and BAX-specific antibodies. IgG: irrelevant immunoglobulin, used as negative controls. Input: cell lysate not subjected to immunoprecipitation. The histograms show the relative intensity of Ku70 protein bands of samples to that of gefitinib treated cells, after being normalized to the respective Bax and represent the means  $\pm$  SD of two or three independent experiments.



**Figure 5.** HDACs inhibited gefitinib-induced apoptosis in NSCLC cells *via* activation of AKT. H358 cells were treated as described in Figure 4. Representative immunoblots of cleaved caspase-3, AKT and ERK1/2 and of their respective phosphorylated forms are shown. Actin was used as a protein-level control.



**Figure 6.** Hypothesized model for regulation of gefitinib resistance by PI3K/AKT and HDAC in mutant KRAS lung adenocarcinoma cells. In the presence of gefitinib (left panel), the level of p-ERK1/2 is increased, probably through the Ras/Raf/MAPK pathway activation by mutant KRAS. Amphiregulin (Areg), whose level of expression and secretion is upregulated by mutant KRAS, and IGF1R cooperate to enhance the level of p-AKT, which inhibits the proteolysis of Ku70. Ku70 levels are thus high enough to suppress BAX activation and apoptosis. Classes I/II/III HDACs also increase the activation of p-AKT, and in addition, inhibit Ku70 acetylation, thus enhancing BAX/Ku70 binding and preventing gefitinib-induced apoptosis. HDAC6 may regulate sirtuins activation. In the presence of combination treatments with gefitinib (right panel), leading to inhibition of active AKT signals (shAreg, OSI-906, LY294002), Ku70 is targeted for degradation. In addition, HDACs inhibitors (trichostatin A (TSA) or nicotinamide (NAM)) inhibit the activation of AKT in an additive manner, and enhance Ku70 acetylation by histone acetyltransferase such as CBP. These conditions allow the release of BAX from inhibition, and BAX is activated. In contrast, tubastatin A (TubA) removes sirtuins inhibition, thus reinforcing p-AKT activation and Ku70 deacetylation. The level of p-ERK1/2 is still increased through Ras/Raf/MAPK pathway, except in presence of PD98059 or U0126.

gefitinib-induced BAX/Ku70 binding in the immunoprecipitation assay using a BAX-specific antibody (Fig. 3d, left panel). In addition, LY294002 downregulated the level of Ku70 (Figs. 3d, right panel, and 3e). These data suggest that the PI3K/AKT pathway controls gefitinib-induced apoptosis, at least in part through the regulation of Ku70 levels and interaction with BAX.

**HDACs control BAX/Ku70 interaction *via* AKT activation**

We next examined the relationship between acetylation, which controls the interaction between BAX and Ku70, gefitinib resistance and the PI3K/AKT pathway. Trichostatin A (classes I/II HDAC inhibitor) and nicotinamide (class III/sirtuin deacetylases inhibitor) strongly sensitized H358 cells to

gefitinib-induced apoptosis,<sup>10</sup> in contrast to sodium butyrate (classes I/IIa HDAC inhibitor) (Fig. 4a). In addition, the combination of trichostatin A and nicotinamide treatment induced apoptosis and significantly sensitized cells to gefitinib compared to gefitinib treatment with trichostatin A or nicotinamide alone (Fig. 4a). As a control of their activity, we verified that administration of trichostatin A or nicotinamide increased the acetylation of  $\alpha$ -tubulin,<sup>19,20</sup> whereas sodium butyrate promoted the accumulation of the cyclin-dependent kinase inhibitor p21<sup>WAF1</sup> protein<sup>21</sup> and trichostatin A or sodium butyrate strongly inhibited cell proliferation (Supporting Information Fig. S2).

When gefitinib, trichostatin A, or nicotinamide were administered alone, the interaction between BAX and Ku70 was strongly enhanced,<sup>10</sup> whereas combination treatments (gefitinib with either trichostatin A or nicotinamide) decreased the interaction between BAX and Ku70,<sup>10</sup> in agreement with the induction of apoptosis (Fig. 4b). Together, these data suggest that gefitinib in combination with trichostatin A or nicotinamide leads to BAX-dependent NSCLC cell death, whereas sodium butyrate induces cellular growth arrest.

The effects of trichostatin A and sodium butyrate suggested the involvement of a class IIb HDAC. As the class IIb HDAC HDAC6 has been shown to deacetylate Ku70 and to regulate BAX/Ku70 binding,<sup>18</sup> we analyzed its role in gefitinib resistance. Specific HDAC6 inhibition using tubastatin A enhanced BAX/Ku70 binding (Fig. 4c, left panel), without affecting the HDAC6 level, cell proliferation (Supporting Information Fig. S2) or apoptosis induction (Fig. 4a), in either the presence or absence of gefitinib. These results were confirmed using specific siRNA targeting either HDAC6 or the other class IIb HDAC HDAC10 (Supporting Information Fig. S3). Interestingly, nicotinamide sensitized the cells to gefitinib-induced apoptosis (Fig. 4a) and decreased the interaction between BAX and Ku70 (Fig. 4c, right panel), even in presence of tubastatin A. Overall, these results suggest that inhibition of class IIb HDACs is not sufficient to restore gefitinib-induced apoptosis. In contrast, HDACs and sirtuin deacetylases prevented gefitinib-dependent BAX-mediated cell death.

Finally, we assessed the effect of HDACs and sirtuin deacetylases on the PI3K/AKT pathway with respect to gefitinib resistance. The effects of gefitinib and/or HDAC inhibitors on apoptosis were controlled by cleaved caspase-3 detection by western-blot (Fig. 5). Trichostatin A or nicotinamide inhibited the gefitinib-induced activation of p-AKT through an additive effect (Fig. 5). In contrast, both tubastatin A and gefitinib increased the p-AKT level; this effect was blocked by NAM. HDAC6- or HDAC10-directed siRNA also enhanced the p-AKT level (Supporting Information Fig. S3). The level of activation of p-ERK1/2 was not affected by HDAC inhibitor treatment. These results suggest that trichostatin A or nicotinamide lead to BAX-dependent apoptosis through p-AKT inactivation.

## Discussion

The intracellular pathways leading to EGFR-TKI resistance in NSCLC patients with wild-type *EGFR* should be investigated in order to search for new combination therapies. We have previously shown that amphiregulin and IGF1R both induce gefitinib resistance in mucinous invasive adenocarcinoma<sup>8</sup> through the inhibition of Ku70 acetylation, which enhances the BAX/Ku70 interaction.<sup>10</sup> Here, we investigated which downstream pathways underlie this mechanism in 62 human lung tumors and showed that the activation of AKT is associated with progressive disease during gefitinib treatment in patients with wild-type *EGFR*. The inhibition of amphiregulin, IGF1R or deacetylase expression *in vitro* decreases PI3K/AKT signaling and releases BAX from Ku70, thus restoring apoptosis under gefitinib treatment (Fig. 6). PI3K/AKT inhibition strongly sensitizes wild-type *EGFR* and mutant *KRAS* mucinous adenocarcinoma cells to gefitinib, suggesting that this treatment could overcome the resistance of these tumors to EGFR-TKIs (Fig. 6).

AKT activation in tumors and its correlation with clinicopathological parameters have already been investigated.<sup>22,23</sup> Overall, these studies show that elevated AKT activity is prevalent in high-grade, advanced tumors and is associated with metastasis, radioresistance and reduced patient survival.<sup>22,24</sup> Most cases of NSCLC that harbor mutations in the *EGFR* show hyperphosphorylated AKT,<sup>11,24,25</sup> and their gefitinib responsiveness can be predicted by AKT activation.<sup>26</sup> The present study confirms that elevated AKT activity is associated with *EGFR* mutation and plays an essential role downstream of continuous activation of mutated *EGFR*. Interestingly, we observed that AKT activation is also associated with disease progression under gefitinib treatment in adenocarcinoma patients with wild-type *EGFR*. This underlines the importance of blocking the PI3K/AKT pathway while treating adenocarcinoma with EGFR-TKIs. Resistance to TKI has been associated with the presence of a *KRAS* mutation,<sup>27</sup> most likely through the direct activation of downstream Ras effector pathways MEK/ERK1/2 and PI3K/AKT.<sup>28</sup> The *EGFR*/Ras axis is often thought of a simple one-directional signaling cascade where Ras activation leads to changes in gene expression. However, several studies have suggested that tumors with aberrant Ras signaling may require an *EGFR* autocrine feedback loop to promote tumor growth and progression.<sup>29</sup> In agreement, the upregulated expression and secretion of *EGFR* ligands including amphiregulin have been observed in *KRAS* mutant cells.<sup>29</sup> Depending on mutant *KRAS* amino-acid substitutions, PI3K/AKT signaling is constitutively activated or is growth factor dependent.<sup>30</sup> To support these observations, we established here the involvement of PI3K/AKT signaling by amphiregulin and IGF1R in wild-type *EGFR* and mutant *KRAS* mucinous invasive adenocarcinoma cells (Fig. 6). This suggests that inhibiting *EGFR* may be more effective if combined with selective inhibition of the downstream pathways for treating wild-type *EGFR* and mutant *KRAS* tumor cells, in agreement with previous studies.<sup>14,29,30</sup>



AKT activation is dependent on the stimulation of growth factor receptors and is initiated through translocation to the plasma membrane and phosphorylation at Thr<sup>308</sup> by PI3K-dependent kinase (PDK1) and at Ser<sup>473</sup> by PDK2.<sup>22</sup> Subsequently, AKT translocates to distinct cellular compartments, phosphorylates its substrates and regulates diverse cellular functions, such as survival, cell-cycle progression and growth.<sup>22</sup> AKT has been shown to suppress BAX-mediated apoptosis through cytosolic Ku70 proteolysis inhibition.<sup>31</sup> In agreement, we observed a decrease in the Ku70 level after PI3K/AKT inhibition. The activation of the PI3K/AKT pathway under treatment with an EGFR-TKI inhibits Ku70 degradation, thus reinforcing the BAX/Ku70 interaction. This results in BAX inhibition, absence of apoptosis and thus to resistance to the treatment (Fig. 6).

The interaction between BAX and Ku70 is also regulated by an acetylation-dependent mechanism. Ku70 is targeted for deacetylation by classes I/II and III/sirtuin deacetylases.<sup>17,18</sup> Class I/II HDACs are involved in the development of lung cancer<sup>32,33</sup> and are associated with poor prognosis.<sup>16,34</sup> Class III/sirtuin deacetylases are implicated in important cellular processes, such as aging, metabolism, stress response and cancer.<sup>35,36</sup> Deacetylation of Ku70 by the sirtuin SIRT1 or SIRT3 promotes the interaction of Ku70 with BAX,<sup>37-39</sup> and high SIRT1 expression may be important in the pathogenesis of lung cancer.<sup>40</sup> In agreement with these studies, we showed that the class I/II HDAC inhibitor trichostatin A and the class III/sirtuin deacetylase inhibitor nicotinamide both sensitize NSCLC cells to gefitinib<sup>10</sup> through the inhibition of p-AKT and the release of BAX from Ku70. Surprisingly, the administration of trichostatin A or nicotinamide alone enhanced the interaction of BAX and Ku70, suggesting that the acetylation level of Ku70 is not strong enough to release BAX and requires the combination of both trichostatin A and nicotinamide, as previously demonstrated.<sup>17</sup> This could indicate that HDAC activity can compensate for sirtuins inhibition and vice versa and that both inhibitors are required concomitantly in order to induce cell death in NSCLC cells. In addition, HDAC inhibitors may allow these resistant cells to maintain a reservoir of active BAX, ready for apoptosis induction after additional exposure to gefitinib, as already proposed.<sup>41</sup>

In contrast to trichostatin A, the class I/IIa HDAC inhibitor sodium butyrate did not restore gefitinib sensitivity, suggesting the involvement of class IIB HDACs in resistance to EGFR-TKIs. HDAC6, a class IIB HDAC mainly localized in the cytoplasm, directly deacetylates tubulin, cortactin, Hsp90 and Ku70.<sup>18,42</sup> In particular, HDAC6 interacts with cytoplasmic Ku70 and regulates its acetylation and BAX-dependent cell death in neuroblastoma.<sup>18</sup> Little is known about the other class IIB HDAC HDAC10.<sup>15,16</sup> HDAC10 has been recently reported to be involved in autophagy-mediated cell survival.<sup>43</sup> In our hands, specific HDAC6 inhibition using the highly specific tubastatin A<sup>44</sup> or siRNA, or HDAC10 invalidation, induced

AKT phosphorylation, but maintained the BAX/Ku70 interaction, and thus failed to restore apoptosis in adenocarcinoma cells, in contrast to that observed in neuroblastoma cells.<sup>18</sup> One likely explanation is that other HDACs prevent Ku70 acetylation, thus compensating for the specific inhibition of class IIB HDAC. Consistent with this possibility, nicotinamide inhibited p-AKT, induced BAX/Ku70 dissociation and restored gefitinib-induced apoptosis, even in presence of tubastatin A. This finding suggests that the activation of PI3K/AKT following HDAC6 inhibition could be mediated by sirtuins (Fig. 6). Synergistic relationships between HDACs and sirtuins have been reported in several cancers.<sup>20,45,46</sup> Our results support an important role for sirtuins in EGFR-TKI resistance mediated through PI3K/AKT signaling. Consistent with this idea, direct binding of SIRT1 to AKT has been demonstrated in the presence of growth factor stimulation, mediating the activating deacetylation of AKT/PDK1.<sup>47</sup> The specific sirtuin isoform involved in the regulation of the BAX/Ku70 interaction in adenocarcinoma cells in response to gefitinib remains to be identified.

Our results support a potential therapeutic role of co-targeting EGFR and the PI3K pathway to counteract gefitinib resistance and suggest that this approach should be evaluated further for lung cancer patients with wild-type *EGFR* and mutant *KRAS*. A large number of PI3K inhibitors are currently being developed.<sup>23,48,49</sup> Perifosine, an allosteric inhibitor of AKT, reduces the levels of activated AKT in breast and ovarian cancer cells.<sup>50</sup> Other trials of triciribine phosphate, which inhibits AKT phosphorylation and its recruitment to the plasma membrane,<sup>51</sup> and of BKM120<sup>52</sup> have been initiated. However, the inhibition of AKT frequently induces the expression of upstream receptor tyrosine kinases and their activity by relieving feedback inhibition.<sup>53</sup> In addition, EGFR can function both upstream and downstream of Ras,<sup>29</sup> reinforcing the use of combinatorial therapy using EGFR-TKI and AKT signaling pathway inhibitors in mutant *KRAS* tumor cells.

In summary, we have shown that PI3K/AKT activation is a major pathway leading to gefitinib resistance and that it contributes to maintaining the BAX/Ku70 complex, at least in part by inhibiting the proteolysis of cytosolic Ku70. HDACs and sirtuin deacetylases participate in this resistance through the control of PI3K/AKT activation and the BAX/Ku70 interaction. These findings suggest new prospects for combining both PI3K/AKT and EGFR inhibitors for the treatment of resistant mutant *KRAS* adenocarcinoma; these possibilities should be evaluated in clinical trials for patients with lung adenocarcinoma.

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