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IMPAIRED SHP2-MEDIATED ERK ACTIVATION CONTRIBUTES TO GEFITINIB SENSITIVITY OF LUNG CANCER CELLS WITH EGFR-ACTIVATING MUTATIONS

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Abstract

Most non-small cell lung cancers (NSCLC) display elevated expression of epidermal growth factor receptor (EGFR), but response to EGFR kinase inhibitors is predominantly limited to NSCLC harboring EGFR-activating mutations. These mutations are associated with increased activity of survival pathways including PI3K/AKT and STAT3/5. We report that EGFR-activating mutations also surprisingly lead to decreased ability to activate ERK compared to wild-type EGFR. In NSCLC cells and mouse embryonic fibroblasts expressing mutant EGFR, this effect on ERK correlates with decreased EGFR internalization and reduced phosphorylation of SHP2, a tyrosine phosphatase required for the full activation of ERK. We further demonstrate that ERK activation levels impact cellular response to gefitinib. NSCLC cells with EGFR mutation display reduced gefitinib sensitivity when ERK activation is augmented by expression of constitutively active mutants of MEK. Conversely, in an NSCLC cell line expressing wild-type EGFR, gefitinib treatment along with or following MEK inhibition increases death response compared to treatment with gefitinib alone. Our results demonstrate that EGFR-activating mutations may promote some survival pathways but simultaneously impair others. This multivariate alteration of the network governing cellular response to gefitinib, which we term “oncogene imbalance”, portends a potentially broader ability to treat gefitinib-resistant NSCLC.

Keywords

endocytosis; oncogene addiction; MEK; non-small cell lung cancer

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DISCLOSURE OF POTENTIAL CONFLICTS OF INTEREST

None of the authors declares a conflict of interest.

INTRODUCTION

Up to 80% of non-small-cell lung cancers (NSCLC) display elevated expression of EGFR (1), but response to EGFR kinase inhibitors is predominantly limited to the 10–20% of tumors that harbor somatic EGFR-activating mutations (2–6). Consistent with patient response data, NSCLC cell lines bearing EGFR-activating mutations display enhanced sensitivity to gefitinib and erlotinib in culture (3,6–8). Studies of these cell lines demonstrate increased basal phosphorylation of EGFR and increased activity of survival-associated signaling pathways including PI3K/AKT and STAT3/5 (3,7,9). Exposure of mutant-bearing NSCLC cells to EGF results in more protracted increases in EGFR phosphorylation than for NSCLC cells expressing wild-type EGFR (3), consistent with the reduced endocytosis of EGFR mutants (10,11). Elevated expression of ERBB3 has been observed in some gefitinib-sensitive cells, including those lacking EGFR mutation (7,12). ERBB3 phosphorylation is apparently promoted by EGFR in these cells and is the primary mediator of PI3K/AKT activity (7).

EGFR-activating mutations also perturb gefitinib and ATP binding, and promote receptor inhibition at lower gefitinib concentrations (3,13,14). This effect propagates to multiple signaling pathways and in some cases may involve differential inhibition of ERBB3. For example, the phosphorylation of AKT and ERBB3 are equally or more responsive to gefitinib than EGFR phosphorylation in certain gefitinib-sensitive cell lines (7). Activating mutations may result in signaling after EGFR inhibition that favors apoptosis over survival (15). Each of these features may partially explain increased sensitivity to gefitinib of cells with EGFR-activating mutations.

In tumors that are initially responsive to gefitinib and erlotinib, resistance mechanisms may arise that abrogate inhibition of survival signaling. In approximately 50% of acquired resistance cases, a secondary T790M mutation disfavors the binding of gefitinib to EGFR (14,16,17). In other cases, *MET* amplification maintains ERBB3/PI3K/AKT activity after treatment with gefitinib (12). Mutations of *BRAF* and *KRAS* also correlate with primary resistance to gefitinib (18–21).

Because these mutations increase EGFR kinase activity (22), the finding that the activities of some downstream survival pathways are elevated is not surprising. These alterations apparently lead to cellular dependence on EGFR, or EGFR “oncogene addiction”, as demonstrated by the finding that EGFR inhibition or knockdown leads to apoptosis in NSCLC cells expressing mutant, but not wild-type, EGFR. How elevated survival signaling leads to EGFR dependence, however, remains poorly understood.

We report that activation of ERK is impaired by the expression of EGFR mutants compared to wild-type. Reduced EGF-elicited activation of ERK in mutant EGFR-bearing cells correlates with diminished EGFR internalization and reduced phosphorylation of the protein tyrosine phosphatase SHP2, a positive regulator of ERK activity (23). Moreover, the effect on SHP2 phosphorylation is linked to defective EGFR internalization. We further demonstrate that ERK activity impacts cellular sensitivity to gefitinib. NSCLC cells expressing an EGFR mutant exhibit reduced death response to gefitinib when ERK activation is augmented by constitutively active MEK. Conversely, NSCLC cells expressing wild-type EGFR are more sensitive to gefitinib when cotreated or pretreated with the MEK inhibitor U0126. Our results suggest that EGFR-activating mutations are associated with enhancement of some survival signals but impairment of others, with the integrated effects influencing cellular response to gefitinib.

MATERIALS AND METHODS

Cells

Wild-type *Egfr* homozygous (*Egfr*^{WT/WT}), L858R homozygous (*Egfr*^{L858R/L858R}), and heterozygous (*Egfr*^{WT/L858R}) primary mouse embryonic fibroblasts (MEFs) were created as described elsewhere (Lane and Jacks, in preparation). 3T3-immortalized MEFs expressing CreER and with one truncated (null) *Shp2* allele and one intact allele with a section of exon 11 flanked by LoxP sites (denoted *Shp2*^{fl/-}) were created as described elsewhere (Chan and Neel, in preparation). H1666 (obtained from American Type Culture Collection) and H3255 (Dr. Passi Janne, functionally characterized by gefitinib sensitivity, as described herein) cells were grown in ACL4. HeLa cells with conditional expression of wild-type or K44A dynamin (Dr. Sandra Schmid, functionally characterized as described herein) were cultured as described elsewhere (24,25). Primary MEFs and *Shp2*^{fl/-} MEFs were grown in DMEM. All media contained 100 units/mL penicillin, 100 µg/mL streptomycin, 1 mM L-glutamine, and 10% FBS. *Shp2*^{fl/-} MEFs were cultured in 1 µM 4-hydroxy-tamoxifen (4-OHT) for 24 hrs to induce *Shp2* deletion and returned to media without 4-OHT for 36 hrs prior to experiments. Otherwise, cells were plated in six-well dishes and grown for 24–48 hrs prior to serum starving (in media containing 0.1% FBS for 12–16 hrs) or treatment with inhibitors.

Egfr expression

Wild-type and L858R *Egfr* cDNA was generated from mRNA isolated from *Egfr*^{WT/WT} and *Egfr*^{L858R/L858R} mouse lines, respectively, and inserted into pMSCV expression vectors with puromycin or hygromycin resistance. Plasmids were transfected into ecotropic Phoenix cells (Dr. Gary Nolan, Stanford University), and viral supernatants were used to infect *Egfr*^{WT/WT} and *Shp2*^{fl/-} MEFs. Target cells were selected in 2 µg/mL puromycin or 200 µg/mL hygromycin.

MEKDD expression

VSV-G pseudotyped retrovirus was produced by transfecting HEK 293FT cells with pBABEpuro-*MEK1DD* (26) or pBABEpuro-*MEK2DD* (27) and the packaging plasmids pMD-G and pMD-g/p. Virus was harvested 48 and 72 hrs after transfection, concentrated by ultracentrifugation, and used to infect H3255 cells. Target cells were selected in 2 µg/mL puromycin.

Immunoblotting

Lysates were prepared in a standard buffer containing detergents, buffer salts, and protease and phosphatase inhibitors. Lysates were cleared by centrifugation at 4°C and 13,200 rpm for 10 min, and protein concentration was determined by micro-BCA assay. 20 µg of denatured and reduced protein was loaded per lane on 10% polyacrylamide gels and transferred to 0.2 µm nitrocellulose. Membranes were blocked in Odyssey blocking buffer (Licor), and all antibodies were used according to manufacturers' recommendations. Where needed, blots were stripped with 0.2 N NaOH.

Antibodies

Antibodies for EGFR, EGFR pY1068, ERK, ERK pT202/Y204, AKT pS473, SHP2 pY542, SHP2 pY580, MEK 1/2, and MEK 1/2 pS217/S221 were purchased from Cell Signaling Technologies. Antibodies for human and mouse *Shp2* were purchased from Epitomics and Santa Cruz Biotechnology, respectively. The GAPDH antibody was purchased from Calbiochem. Infrared-dye-conjugated secondary antibodies were purchased from Rockland Immunochemicals.

Other reagents

Gefitinib and U0126 were purchased from WuXi Pharmatech and Promega, respectively. Human epidermal growth factor (EGF) and platelet derived growth factor (PDGF) were purchased from Peprotech. Puromycin, propidium iodide, and 4-OHT were purchased from Sigma. Hygromycin B was purchased from Clontech. FBS, penicillin/streptomycin, L-glutamine, geneticin, and all media were purchased from Invitrogen.

EGFR internalization assay

Rate constants for the endocytosis of ^{125}I -EGF (k_e) were measured as described previously (28,29).

Propidium iodide permeability

Cells were plated in six-well dishes, grown for 24–72 hrs, and switched to complete media containing appropriate concentrations of gefitinib, U0126, and DMSO. After an additional 3–6 days, adherent and floating cells were pooled and resuspended in PBS-PI. Flow cytometry data was acquired on a Becton Dickinson FACSCalibur and analyzed using FlowJo.

RESULTS

Previous data demonstrate that ERK phosphorylation may be reduced in cells expressing EGFR mutants compared to counterparts expressing wild-type EGFR (EGFR^{WT}) (3,30), but subsequent studies have focused mainly on the enhancement of pathways including PI3K/AKT and STAT3/5 (3,7,9). To explore the possibility that ERK phosphorylation is reduced in the context of EGFR-activating mutations relative to EGFR^{WT}, we compared the phosphorylation of EGFR, ERK, and AKT in the human NSCLC cell lines H1666 (EGFR^{WT}) and H3255 (EGFR^{L858R}) stimulated with 10 ng/mL EGF for up to 1 hr. We chose H3255 for comparison because it is exquisitely sensitive to gefitinib (3,7) and because it carries EGFR^{L858R}, one of the most common NSCLC-associated EGFR mutations (6,31–33). Western blot analysis is shown in Fig. 1 and Supplementary Fig. S1. As expected, EGFR phosphorylation, monitored at Y1068, was basally elevated and prolonged in response to EGF in H3255 cells compared to H1666. Consistent with previous observations (3,7), the phosphorylation of AKT at S473 was basally elevated in H3255 cells, and AKT phosphorylation actually decreased in response to EGF. In H1666 cells, AKT phosphorylation increased in response to EGF and returned to baseline levels within 60 min. The phosphorylation of ERK at T202/Y204 was uniformly lower in H3255 cells than in H1666 cells, with lower basal levels and delayed increases in response to EGF.

H1666 and H3255 lysates were also probed for phosphorylation of the protein tyrosine phosphatase SHP2 at Y542 (Fig 1). SHP2 is required for complete ERK activation (23) and its phosphorylation at Y542 is required for its normal activity downstream of several growth factor receptors (34). SHP2 Y542 phosphorylation was induced in H1666 cells in response to EGF by 5 min, but its phosphorylation was not induced in H3255 cells at any time point. Samples were also probed for SHP2 phosphorylation at Y580, a site of secondary importance for SHP2 regulation (34). An EGF-inducible band at the appropriate molecular weight was observed for H1666 cells but not for H3255 (Supplementary Fig. S2A). EGF-induced SHP2 Y542 phosphorylation was modest in a number of other NSCLC cell lines with EGFR-activating mutations, including PC9, HCC827, and H1975 (Supplementary Fig. S2B). The mutant-bearing, *PTEN*-null cell line H1650 did exhibit inducible SHP2 Y542 phosphorylation. Experiments using MDA-468 cells, which are *PTEN*-null, with and without *PTEN* reconstitution, demonstrated no general effect of *PTEN* status on SHP2 Y542 phosphorylation (Supplementary Fig. S3).

To determine how ERK activity impacts H3255 sensitivity to gefitinib, we created H3255 cells expressing constitutively active mutants of MEK, MEK1DD (26) or MEK2DD (27). Interestingly, MEKDD expression slightly decreased basal ERK phosphorylation below that observed in controls (Supplementary Fig. S4A). Treatment with 1 and 10 μM gefitinib for 10 min, however, resulted in complete inhibition of ERK phosphorylation in control cells but only partial inhibition in MEKDD-transduced cells (Fig 2A), suggesting that both MEKDDs were active. To examine the effect of sustained ERK phosphorylation on H3255 sensitivity to gefitinib, we measured cell permeability to propidium iodide (PI) 72 hrs after treatment with gefitinib (Fig. 2B). At 1 μM gefitinib, the fraction of dead (Fig 2B) and floating (Supplementary Fig. S4B) cells increased significantly above baseline for control cells but not for either MEKDD-expressing line. At higher gefitinib concentrations, PI permeability of control cells increased monotonically but remained at approximately baseline levels for MEKDD-expressing cells. While proliferation was obviously impeded by gefitinib in MEKDD-expressing cells, significant numbers of those cells remained adherent even at 10 μM gefitinib.

To assess the potential for increasing cellular sensitivity of NSCLC cells expressing wild-type EGFR to gefitinib through the manipulation of ERK phosphorylation, we measured the response of H1666 cells to treatment with gefitinib alone or in combination with the MEK inhibitor U0126. 30 min exposure of H1666 cells to concentrations of U0126 ≥ 1 μM resulted in complete inhibition of ERK phosphorylation (Supplementary Fig. S5A). Treatment of H1666 cells with gefitinib alone (1 or 10 μM) for 5 and 6 days resulted in little to no increase in the fraction of PI positive cells versus treatment with DMSO. Response to 10 μM U0126 was similarly modest (Fig. 3A and 3B). When gefitinib was combined with U0126, PI permeability 5 and 6 days post-treatment increased beyond that observed for treatment with gefitinib or U0126 alone, especially for 10 μM gefitinib (Fig. 3A and 3B). Microscopy revealed a high percentage of floating cells for combination treatments (Supplementary Fig S5B). To attempt to mimic the condition of chronic low-level ERK phosphorylation encountered in H3255 cells, H1666 cells were cultured in the presence or absence of 10 μM U0126 for 3 weeks and subsequently treated for 72 hrs with DMSO, 1 μM gefitinib, 10 μM U0126, or 1 μM gefitinib and 10 μM U0126 in combination (Fig. 3C and Supplementary Fig. S5C). Control cells that had been maintained in DMSO showed no increase in PI permeability in response to 1 μM gefitinib alone and only a modest increase in response to 1 μM gefitinib in combination with 10 μM U0126. In contrast, for cells that had been cultured in 10 μM U0126 PI permeability increased more than two-fold above control for treatment with 1 μM gefitinib and more than five-fold above control for treatment with 1 μM gefitinib and 10 μM U0126.

We attempted to recapitulate the ERK and SHP2 phosphorylation trends observed in NSCLC lines in primary mouse embryonic fibroblasts (MEFs). *Egfr*^{WT/WT} MEFs expressing similar levels of murine wild-type and L858R *Egfr* were stimulated with 10 ng/mL EGF for up to 1 hr and analyzed by Western blot (Figs. 4A and 4B, and Supplementary Fig. S6A). Phosphorylation of Shp2 Y542 and Erk T202/Y204 was more modest in cells expressing *Egfr*^{L858R} than in cells expressing *Egfr*^{WT}, but the differences were not as pronounced as in H1666 and H3255 cells. In contrast to what has been observed in NSCLC cells, Akt phosphorylation was not enhanced in cells expressing *Egfr*^{L858R} and was actually decreased compared to cells expressing *Egfr*^{WT} at 60 min (Supplementary Fig. S6B).

The correlation between SHP2 and ERK phosphorylation levels in NSCLC cells and MEFs expressing EGFR^{L858R} suggests, but does not guarantee, that SHP2 may be less active in these cells (34). We investigated the functional role of Shp2 in promoting Erk phosphorylation by expressing *Egfr*^{WT} or *Egfr*^{L858R} in *Shp2*^{fl/-} 3T3-immortalized MEFs and probing for response to EGF for up to 1 hr (Figs. 4C and 4D, and Supplementary Fig. S7A). *Shp2*^{fl/-} MEFs constitutively express CreER and contain only one intact *Shp2* allele which has a portion of exon 11 flanked by LoxP recombination sites. As described in *Materials and Methods*,

treatment of these cells with 4-hydroxy-tamoxifen (4-OHT) results in loss of *Shp2* and its product. For *Shp2^{fl/-}* cells not treated with 4-OHT (i.e., cells retaining one functional *Shp2* allele), Erk phosphorylation was induced more gradually (similar to vector-transduced control cells) in cells expressing *Egfr^{L858R}* compared to *Egfr^{WT}*. When this panel of cells was treated with 4-OHT, induction of Erk phosphorylation was decreased for all three cell types, with the largest deviations from baseline (without 4-OHT treatment) observed for cells transduced with vector and *Egfr^{WT}*. These findings suggest that *Shp2* plays a more prominent role in promoting Erk phosphorylation downstream of *Egfr^{WT}* than *Egfr^{L858R}*. Interestingly, *Shp2^{fl/-}* cells became more responsive to gefitinib after treatment with 4-OHT (Supplementary Fig. S7B), suggesting that impairment of *Shp2* activity may promote cellular sensitivity to gefitinib.

Reduced phosphorylation of ERK downstream of *EGFR^{L858R}* may be linked to the previously established relationship between EGFR endocytosis and ERK activation (25) given that activating mutations of EGFR are endocytosis-impaired (10,11). Rate constants for the ¹²⁵I-EGF endocytosis rate constant (k_e) measured using a well-established technique (28,29) were previously reported for the NSCLC cell lines A549 (*EGFR^{WT}*, gefitinib-resistant) and PC9 (*EGFR^{del-E7460-A750}*, gefitinib-sensitive) as 0.14 and 0.04 min⁻¹, respectively (10). Using the same technique, we measured k_e values for H1666 and H3255 of 0.21 and 0.02, respectively (Fig. 5A). We found that this trend was reproduced in a panel of *Egfr^{WT/WT}*, *Egfr^{WT/L858R}*, and *Egfr^{L858R/L858R}* primary mouse embryonic fibroblasts, where k_e decreased monotonically with increasing relative abundance of the mutant allele (Fig. 5B). Reductions in k_e relative to control were also observed when *Egfr^{WT}* and *Egfr^{L858R}* were expressed in the *Egfr^{WT/WT}* background, with the greatest reduction observed for *Egfr^{L858R}* expression (Fig. 5C). These results indicate that both receptor expression level and mutational status play a role in determining k_e . For *Shp2^{fl/-}* cells (without 4-OHT treatment), k_e for vector control cells was significantly lower than in the *Egfr^{WT/WT}* background, but increased *Egfr* expression resulted in further reduced k_e values, with the lowest observed k_e for *Egfr^{L858R}* expression (Fig. 5D). No change was observed in the measured k_e for *Shp2^{fl/-}* cells treated with 4-OHT (Supplementary Fig. S7C).

To determine if SHP2 Y542 phosphorylation depends upon EGFR endocytosis, we investigated SHP2 phosphorylation in HeLa cells with inducible expression of wild-type and dominant negative K44A dynamin (*Dyn^{WT}* and *Dyn^{K44A}*, respectively) (25). Dynamin is a GTP-ase involved in clathrin-mediated endocytosis of receptor tyrosine kinases, and its ectopic expression is induced in the HeLa cells we used in the absence of tetracycline. *Dyn^{K44A}* expression has been previously shown to reduce rates of EGFR endocytosis compared to *Dyn^{WT}* (25), a phenotype we confirmed with the ¹²⁵I-EGF-based assay (Supplementary Fig. S8A). Western blot analysis of these cells treated with 10 ng/mL EGF for up to 1 hr is shown in Figs. 6A, 6B, and S8B. Phosphorylation of EGFR Y1068 was prolonged (Supplementary Fig. S8B) and that of ERK impaired (Fig. 6B) in cells expressing *Dyn^{K44A}* compared to *Dyn^{WT}*. Slight reduction in AKT phosphorylation at S473 was also observed with *Dyn^{K44A}* expression (Supplementary Fig. S8B). *Dyn^{K44A}* expression also resulted in reduced *Shp2* Y542 phosphorylation (Fig. 6A), but the effect was more modest than observed in NSCLC lines. Interestingly, a similar SHP2 Y542 phosphorylation trend was observed after stimulation with PDGF (Supplementary Fig. S8C). *Dyn^{K44A}* expression also increased cellular sensitivity to gefitinib compared to expression of *Dyn^{WT}* (Fig. 6C and 6D). *Dyn^{WT}* and *Dyn^{K44A}* HeLa cells maintained in tetracycline exhibited no increase in PI permeability when treated with up to 20 μ M gefitinib for 72 hrs. In the absence of tetracycline, however, cells expressing *Dyn^{K44A}* demonstrated increased death response to gefitinib concentrations \geq 15 μ M.

DISCUSSION

Our study demonstrates that the EGFR^{L858R} mutant promotes ERK activity less effectively than EGFR^{WT} in human NSCLC cells and MEFs, and that this effect associates with impaired endocytosis of EGFR mutants and concomitant reduction in SHP2 Y542 phosphorylation. Although previous data support the notion that NSCLC-associated EGFR mutants may not promote ERK activity as efficiently as wild-type EGFR (3,30), this effect had not been pursued. Moreover, while connection between EGFR internalization and ERK activation had been established (25), a link between this effect and the impairment of mutant EGFR internalization (10,11) had not been raised. To the best of our knowledge, our present study also represents a first report of differences in the role of SHP2 in promoting ERK activity downstream of wild-type and mutant EGFR.

SHP2 is required for complete activation of ERK (23), and its phosphorylation at Y542 is necessary for its full activity downstream of some, but not all, growth factors. For example, Shp2 Y542 phosphorylation has been found to be less important for mediating Erk activation downstream of Egfr than downstream of Fgfr or Pdgfr in mouse fibroblasts (34). Those studies did not explore the effects of receptor expression levels, however, and it has been argued that such effects may alter the occurrence and relevance of SHP2 Y542 phosphorylation in response to EGF (34). In addition to our finding of differential SHP2 phosphorylation, the finding that *Shp2* deletion in MEFs leads to less relative impairment of EGF-mediated Erk activity downstream of Egfr^{L858R} than Egfr^{WT} suggests that Shp2 activity may be less efficiently promoted by Egfr^{L858R}. Thus, ERK activity may be reduced downstream of mutant EGFR expression because of defects in receptor trafficking and the functional role (but possibly not phosphatase activity) of SHP2. Data from HeLa cells with inducible Dyn^{WT} or Dyn^{K44A} expression indicate that EGFR trafficking and SHP2 phosphorylation are linked.

Further studies indicated that, beyond the apparent link to receptor internalization, the mechanism underlying differential SHP2 involvement is beyond the scope of our investigation. Studies of GAB1 phosphorylation at Y627, a site involved in the binding and activation of SHP2, revealed constitutive phosphorylation in H3255 cells compared to ligand-induced phosphorylation in H1666 cells (Supplementary Fig. S9), suggesting that differential SHP2 involvement may not result from an inability to complex with GAB1. Still, another marker of defective SHP2 activity (i.e., impaired phosphorylation of SRC at Y418 (35)) was identified in H3255 cells (Supplementary Fig. S9).

We note that we were not able to fully recapitulate the differential phosphorylation of SHP2 and ERK observed in H1666 and H3255 cells in the model systems we explored, for a variety of reasons. Whereas EGF-induced SHP2 Y542 phosphorylation was not detectable in H3255 cells, Shp2 Y542 phosphorylation increased in response to EGF in *Egfr*^{WT/WT} cells infected with retroviruses encoding Egfr^{L858R} and HeLa cells expressing Dyn^{K44A}. Those increases were more modest, however, than those observed in *Egfr*^{WT/WT} cells infected with retroviruses encoding Egfr^{WT} and HeLa cells expressing Dyn^{WT}, respectively. Similarly, relative reductions or delays in ERK phosphorylation observed in these systems with the expression of Egfr^{L858R} or Dyn^{K44A} were less substantial than the difference observed between H1666 and H3255. However, H1666 and H3255 are not isogenic, so differences in the expression of proteins other than EGFR may affect SHP2 and ERK phosphorylation. Elevated ERBB3 expression in H3255 (and other gefitinib-sensitive NSCLC cell lines), for example, may play a role in modulating SHP2 and ERK phosphorylation dynamics. H1666 cells also carry an uncommon BRAF mutation (G465V), although implication for altered BRAF or ERK activity in H1666 cells is unclear (36). EGFR expression level can also influence receptor trafficking and signal persistence; we find that EGFR expression in H3255 exceeds that in H1666, in agreement with reports of relative *EGFR* copy number in these cell lines (30). Finally,

differences in cell background (lung epithelium versus other types) may also affect the extent to which EGFR mutants alter SHP2 and ERK phosphorylation.

The failure of MEKDD expression to increase ERK phosphorylation in H3255 cells raises the possibility that additional factors may suppress ERK activity. We explored the possibility that MEKDD expression might increase the activity of MEK phosphatases by probing for MEK phosphorylation at S217/S221. Since both MEKDD isoforms have been mutated at these sites, any reduction in total MEK activity should manifest within the pool of endogenous MEK. We observed no reduction in MEK phosphorylation with MEKDD expression (Supplementary Fig. S10). Interestingly, recent studies demonstrated that transcript levels of MKP3, considered a principal ERK phosphatase, increased more in human bronchial epithelial cells expressing the E746-A750 deletion and L858R EGFR mutants than in those expressing similar levels of wild-type EGFR (37). Thus, the role of MKP3 in determining ERK activity in NSCLC cells with or without EGFR mutation should be explored.

Our findings demonstrate an important role for ERK in determining cellular response to gefitinib. The observation that MEKDD expression reduced the death response of H3255 cells to gefitinib suggests that even low ERK activity may promote resistance to EGFR kinase inhibition. This is consistent with reports that primary mutations of *BRAF* and *KRAS* that increase ERK activity are associated with gefitinib resistance (18). That cotreatment of H1666 cells with gefitinib and U0126 promoted more cell death than treatment with gefitinib alone also suggests a role for ERK activity levels in determining cellular death response to gefitinib. Our finding that prolonged exposure to U0126 prior to gefitinib administration resulted in further enhanced cell death response may suggest scheduling strategies for the therapeutic combination of MEK and EGFR inhibitors.

Our results also raise the notion that the effects of EGFR-activating mutations might be most appropriately characterized as “oncogene imbalance”, since the ERK pathway is altered in converse direction to AKT and STAT. This idea resonates with a proposal holding that EGFR mutant expression results in transient pro-death imbalance of survival and apoptotic signaling in response to EGFR inhibition (15). Whereas NSCLC cells expressing EGFR mutants may have abundant PI3K/AKT and STAT activity, they may be put into especially precarious state by simultaneous impairment of ERK since this pathway can consequently be reduced below critical threshold more easily.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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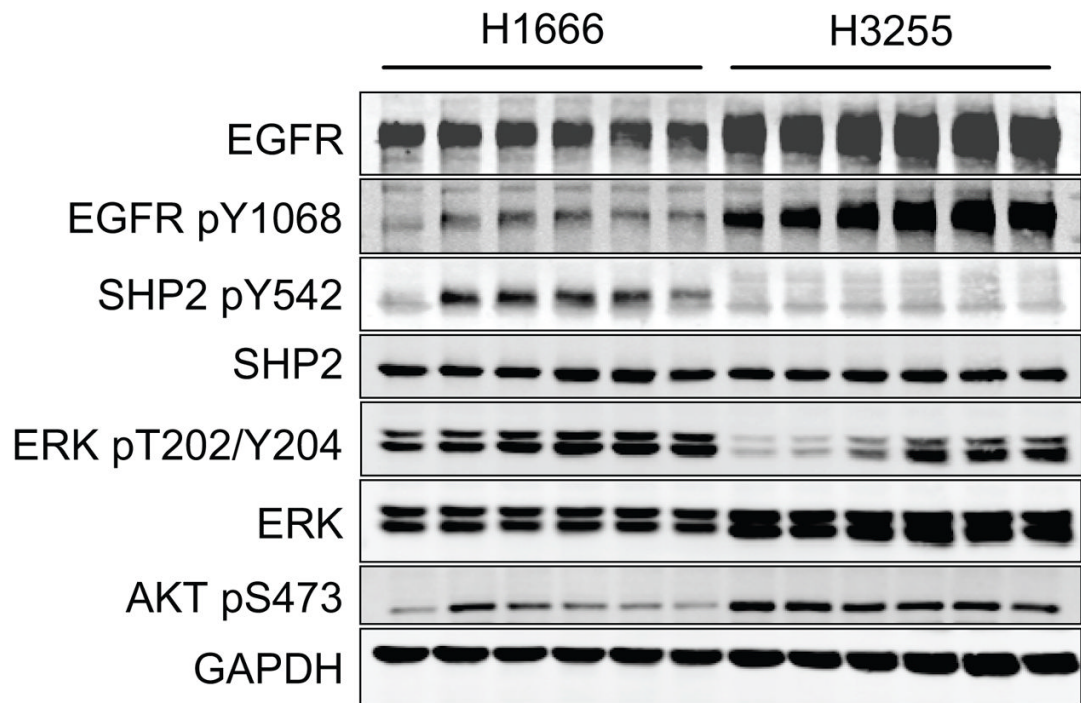


Figure 1. SHP2 and ERK phosphorylation are reduced, whereas EGFR and AKT phosphorylation are increased, in H3255 cells compared to H1666. Cells were treated with 10 ng/mL EGF for up to 1 hr, and lysates were analyzed by Western blotting with antibodies against indicated proteins. Images are representative of three sets of biological replicates.

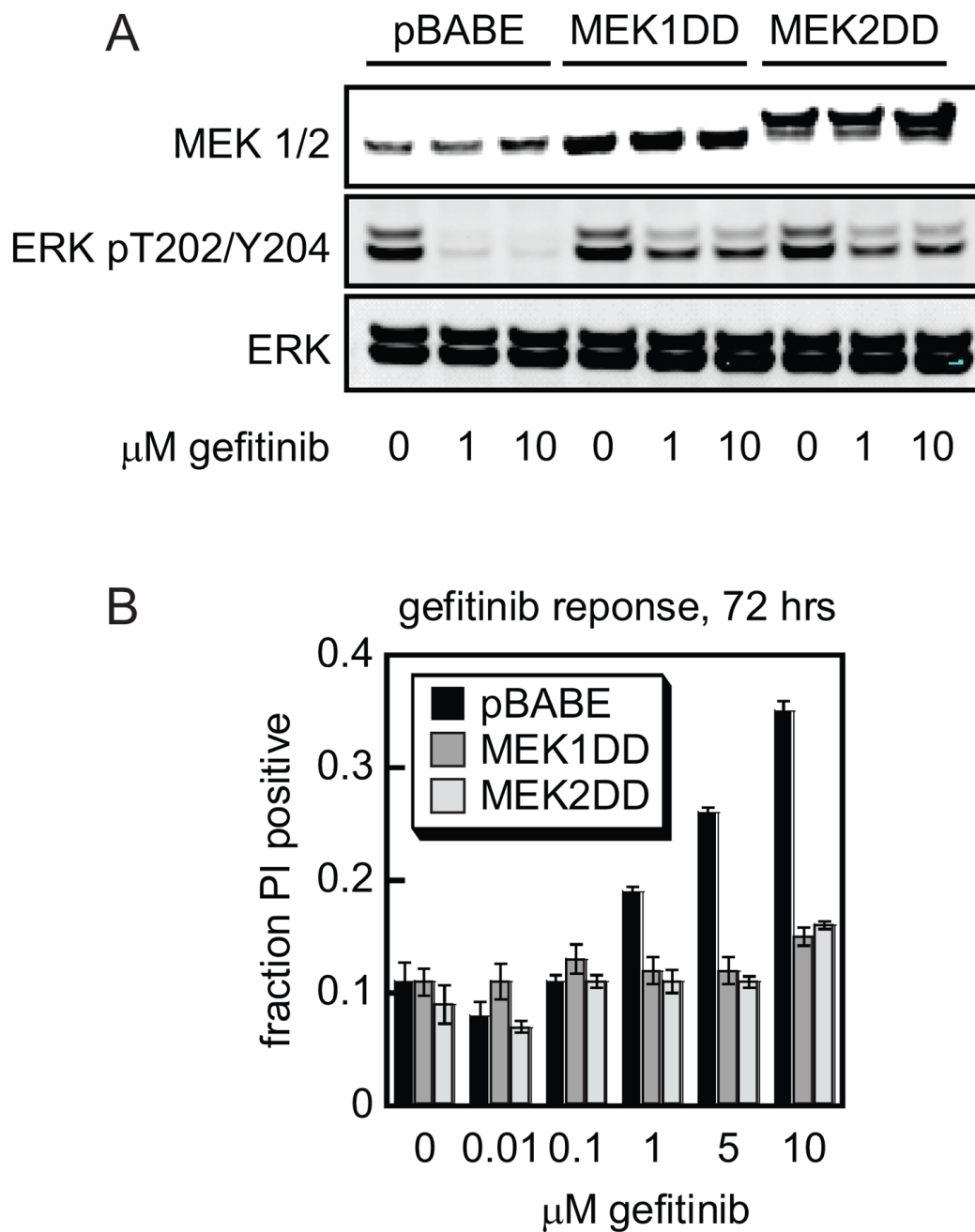
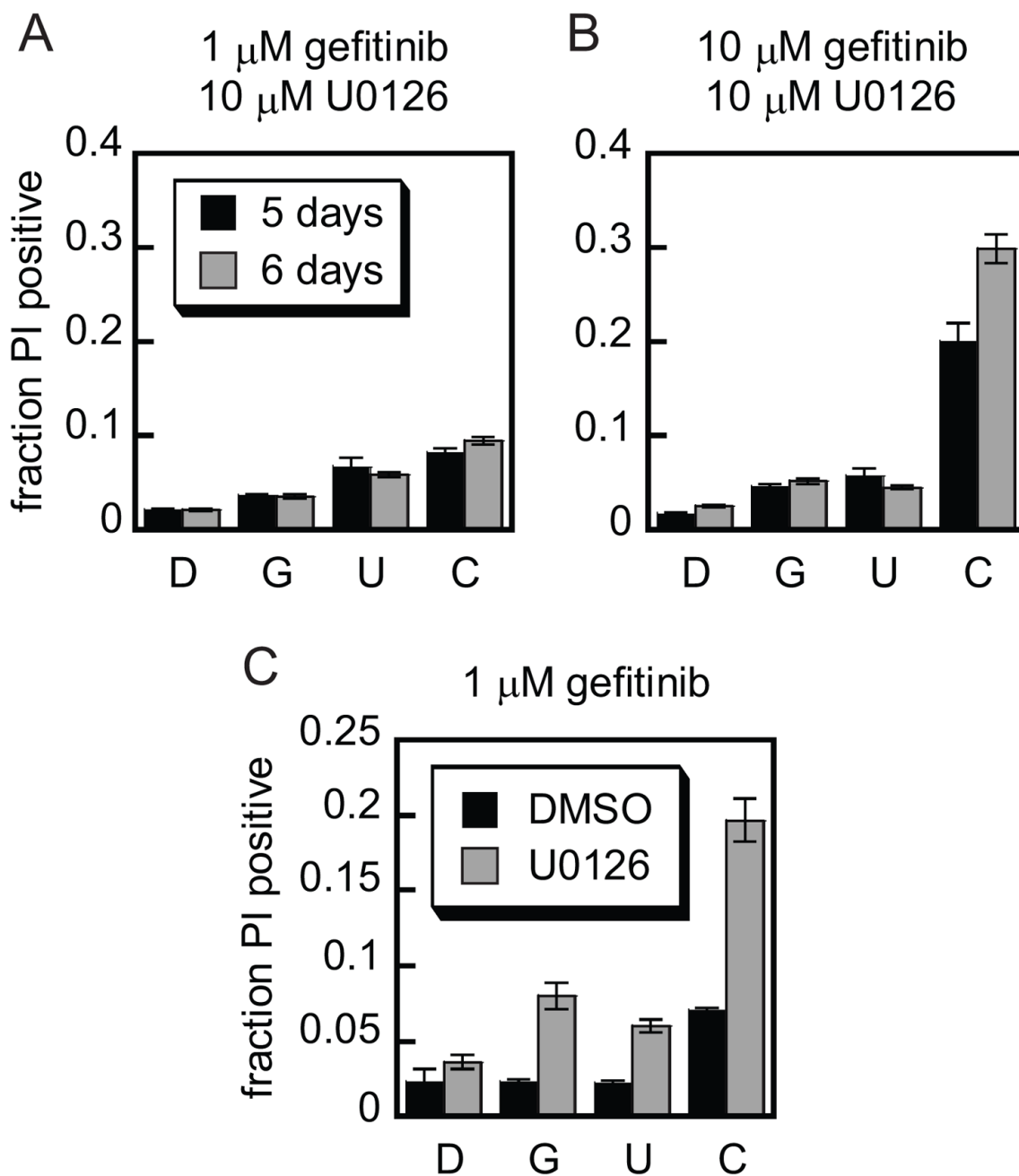


Figure 2. Constitutively active MEK mutants reduce H3255 response to gefitinib. *A*, pBABE-, *MEK1DD*-, and *MEK2DD*-transduced H3255 cells were treated with up to 10 μ M gefitinib for 10 min and lysates were analyzed by Western blotting with antibodies against indicated proteins. *B*, Retrovirally-transduced H3255 cells were treated with up to 10 μ M gefitinib for 72 hrs and analyzed for propidium iodide permeability. Data are presented as average \pm SEM for three sets of biological replicates.

**Figure 3.**

Cotreatment with and chronic exposure to U0126 enhances gefitinib-mediated cell death in H1666 cells. *A* and *B*, H1666 cells were treated for 5 and 6 days with DMSO (D), 1 or 10 μ M gefitinib (G) alone, 10 μ M U0126 (U) alone, or gefitinib and U0126 in combination (C) and subsequently analyzed for propidium iodide permeability. *C*, H1666 cells were cultured in DMSO or 10 μ M U0126 for three weeks and then treated for 72 hrs with DMSO, 1 μ M gefitinib, 10 μ M U0126, or 1 μ M gefitinib in combination with 10 μ M U0126. For all conditions, data are presented as average \pm SEM for three biological replicates.

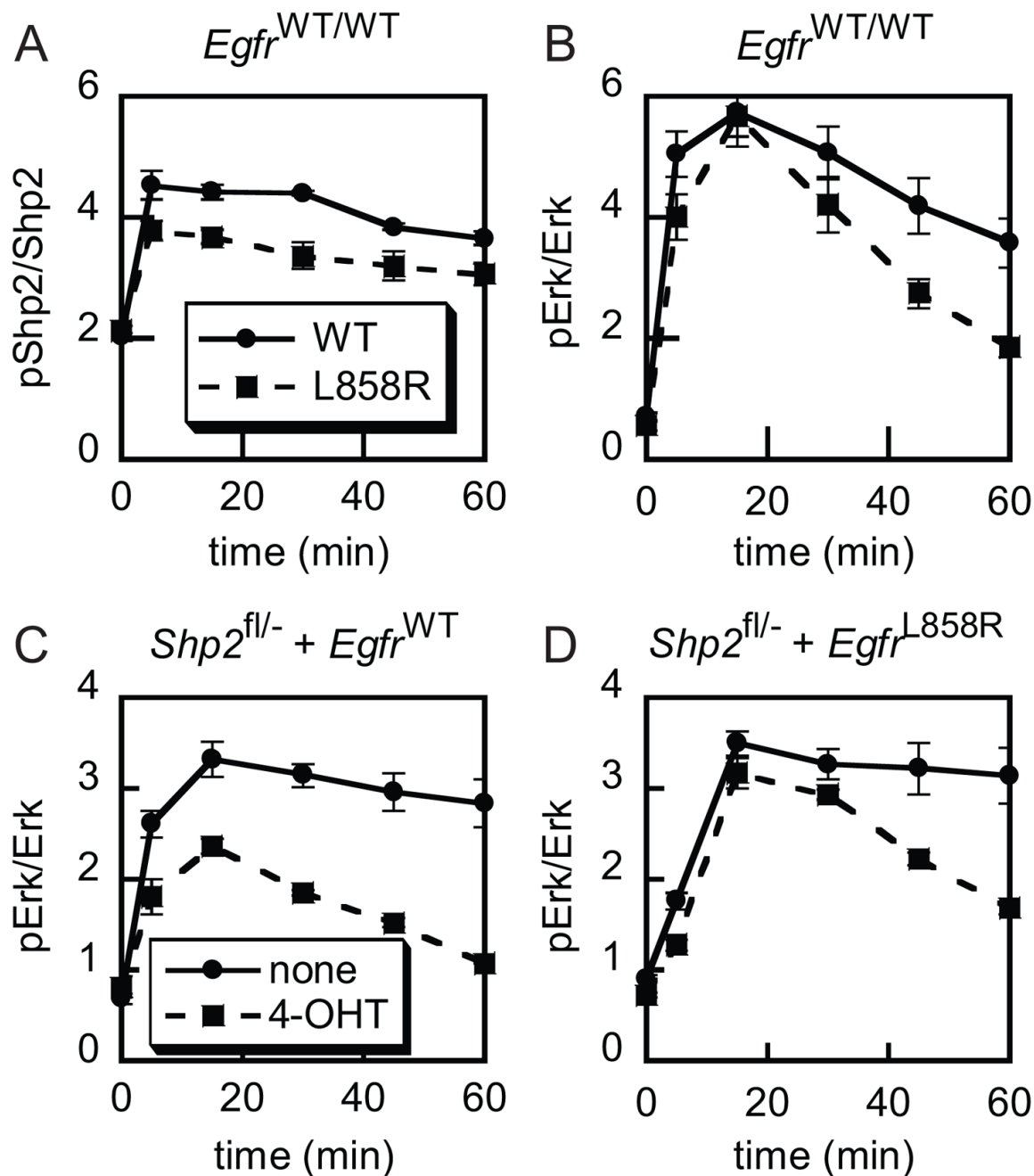


Figure 4. Compared to expression of *Egfr*^{WT}, expression of *Egfr*^{L858R} in MEFs results in decreased EGF-mediated Shp2 and Erk phosphorylation and diminished effect of Shp2 deletion on Erk phosphorylation. *A* and *B*, *Egfr*^{WT/WT} primary MEFs and, *C* and *D*, *Shp2*^{fl/-} MEFs transduced with vectors encoding for *Egfr*^{WT} (WT) or *Egfr*^{L858R} (L858R) were stimulated with 10 ng/mL EGF, and lysates were analyzed by Western blotting. Densitometry data are presented as average \pm SEM.

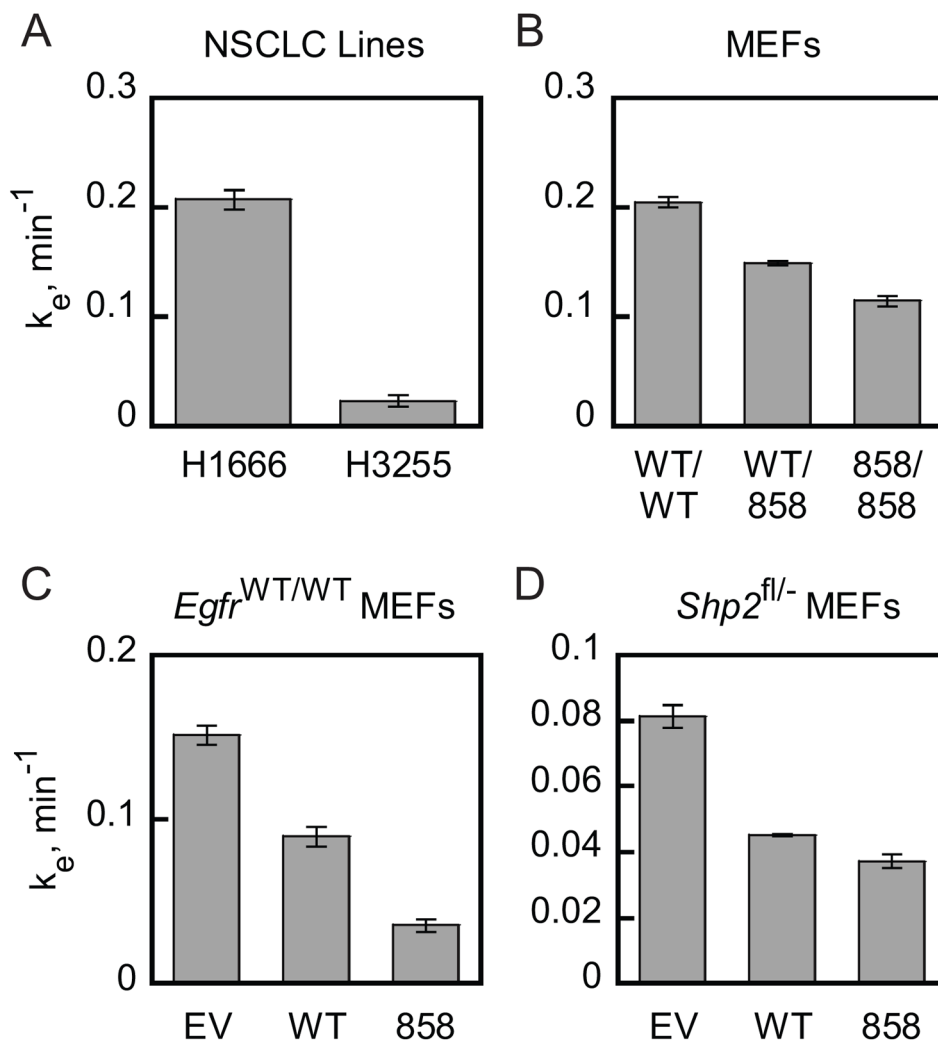
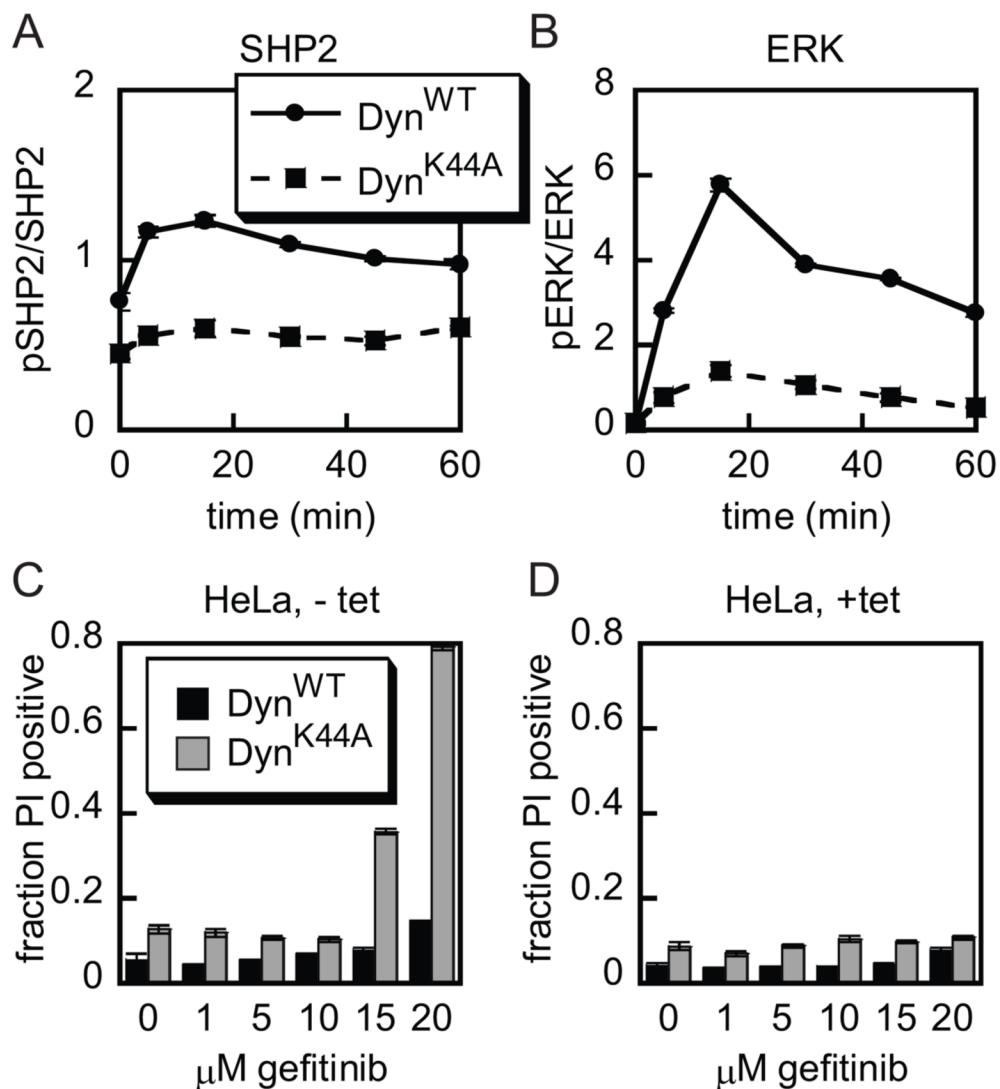


Figure 5. EGFR internalization is decreased by activating mutations in NSCLC cells and MEFs. Rate constants for EGF-mediated internalization of EGFR (k_e) were determined as described in *Materials and Methods* for: A, H1666 and H3255 NSCLC cells; B, $Egfr^{WT/WT}$ (WT/WT), $Egfr^{WT/L858R}$ (WT/858), and $Egfr^{L858R/L858R}$ (858/858) MEFs; and C, $Egfr^{WT/WT}$ MEFs and D, $Shp2^{fl/-}$ MEFs transduced with empty vector (EV), $Egfr^{WT}$ (WT) or $Egfr^{L858R}$ (858). Data are presented as average \pm SEM for the three sets of biological replicates.

**Figure 6.**

Impaired EGFR internalization in HeLa cells decreases SHP2 and ERK phosphorylation and increases gefitinib-mediated death. *A* and *B*, HeLa cells with tetracycline-regulated dynamin expression were cultured without tetracycline and stimulated with 10 ng/mL EGF for up to 1 hr, and lysates were analyzed by Western blotting. Densitometry data are presented as average \pm SEM for three sets of biological replicates. *C* and *D*, Dyn^{WT} and Dyn^{K44A} HeLa cells cultured with or without tetracycline were treated with up to 20 μ M gefitinib for 72 hrs and analyzed for propidium iodide permeability. Data are presented as average \pm SEM for the three sets of biological replicates.