MET Amplification Leads to Gefitinib Resistance in Lung Cancer by Activating ERBB3 Signaling

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20. In patenting, we may observe weaker trends because (i) citing earlier work can limit a patent's scope, so that applicants may avoid citations, and (ii) patent examiners typically add the majority of citations, which makes patent citations different from paper citations (*26*, *27*).
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Figs. S1 to S5
Tables S1 to S5
References

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MET Amplification Leads to Gefitinib Resistance in Lung Cancer by Activating ERBB3 Signaling

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The epidermal growth factor receptor (EGFR) kinase inhibitors gefitinib and erlotinib are effective treatments for lung cancers with *EGFR* activating mutations, but these tumors invariably develop drug resistance. Here, we describe a gefitinib-sensitive lung cancer cell line that developed resistance to gefitinib as a result of focal amplification of the *MET* proto-oncogene. inhibition of MET signaling in these cells restored their sensitivity to gefitinib. *MET* amplification was detected in 4 of 18 (22%) lung cancer specimens that had developed resistance to gefitinib or erlotinib. We find that amplification of *MET* causes gefitinib resistance by driving ERBB3 (HER3)–dependent activation of PI3K, a pathway thought to be specific to EGFR/ERBB family receptors. Thus, we propose that *MET* amplification may promote drug resistance in other ERBB-driven cancers as well.

Tyrosine kinase inhibitors (TKIs) are an emerging class of anticancer therapies that have shown promising clinical activity. Gefitinib (Iressa) and erlotinib (Tarceva) inhibit the epidermal growth factor receptor (EGFR) kinase and are used to treat non–small cell lung cancers (NSCLCs) that have activating mutations in the *EGFR* gene (*1*–*4*). Although most *EGFR* mutant NSCLCs initially respond to EGFR inhibitors, the vast majority of these tumors ultimately become resistant to the drug treatment. In about 50% of these cases, resistance is due to the occurrence of a secondary mutation in *EGFR* (T790M) (*5*, *6*). The mechanisms that contribute to resistance in the remaining tumors are unknown.

To explore additional mechanisms of gefitinib resistance, we generated resistant clones of the gefitinib hypersensitive *EGFR* exon 19 mutant NSCLC cell line, HCC827, by exposing these cells to increasing concentrations of gefitinib for 6 months. The resultant cell line, HCC827 GR (gefitinib resistant), and six clones isolated from single cells were resistant to gefitinib in vitro ($IC_{50} > 10$ μM) (Fig. 1A). Unlike in the parental HCC827 cells, phosphorylation of ERBB3 and Akt in the HCC827 GR cells was maintained in the presence of gefitinib (Fig. 1B).

We previously observed that *EGFR* mutant tumors activate phosphoinositide 3-kinase (PI3K)/Akt signaling through ERBB3 and that down-regulation of the ERBB3/PI3K/Akt signaling pathway is required for gefitinib to induce apoptosis in *EGFR* mutant cells (*7*, *8*). In addition, persistent ERBB3 phosphorylation has also been associated with gefitinib resistance in ERBB2-amplified breast cancer cells (*9*). We therefore hypothesized that gefitinib resistance in *EGFR* mutant NSCLCs might involve sustained signaling via ERBB3. After excluding the presence of a secondary resistance mutation in *EGFR* (*10*), we investigated whether aberrant activation of another receptor might be mediating the resistance. We used a phospho–receptor tyrosine kinase (phospho-RTK) array to compare the effects of gefitinib on 42 phosphorylated RTKs in HCC827 and HCC827 GR5 cells (Fig. 1C). In the parental cell line, EGFR, ERBB3, ERBB2, and MET were all phosphorylated, and this phosphorylation was either completely or markedly reduced upon gefitinib treatment. In contrast, in the resistant cells, phosphorylation of MET, ERBB3, and EGFR persisted at higher levels in the presence of gefitinib (Fig. 1C).

We next performed genome-wide copy number analyses and mRNA expression profiling of the HCC827 GR cell lines and compared them with the parental HCC827 cells (fig. S1 and table S1). The resistant but not parental cell lines showed a marked focal amplification within chromosome 7q31.1 to 7q33.3, which contains the *MET* proto-oncogene (Fig. 1D). *MET* encodes a transmembrane tyrosine kinase receptor for the hepatocyte growth factor (scatter factor), and *MET* amplification has been detected in gastric and esophageal cancers (*11*, *12*). Analysis by quantitative polymerase chain reaction (PCR) confirmed that *MET* was amplified by a factor of 5 to 10 in the resistant cells (fig. S2), and sequence analysis provided no evidence of mutations in *MET*.

To determine whether increased MET signaling underlies the acquired resistance to gefitinib, we examined whether MET inhibition suppressed growth of the resistant cells. HCC827 GR cells were exposed to PHA-665752, a MET tyrosine kinase inhibitor, alone or in combination with gefitinib (*13*). Although the HCC827 GR5 cells were resistant to both gefitinib alone and PHA-665752 alone, combined treatment resulted

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in substantial growth inhibition (Fig. 2A) and induced apoptosis (fig. S3). In the resistant cells, gefitinib alone substantially reduced phosphorylation of EGFR, and it had only minimal effects on ERBB3 and Akt phosphorylation (Fig. 2B). However, gefitinib in combination with PHA-665752 fully suppressed ERBB3 and Akt phosphorylation in the resistant cells. These findings suggest that the observed resistance in HCC827 GR cells is mediated by increased MET signaling.

To investigate the mechanism by which PI3K/Akt becomes activated in the resistant cells, we immunoprecipitated the p85 regulatory subunit of PI3K and examined coprecipitating proteins. In the parental HCC827 cell line, two major phosphotyrosine proteins, ERBB3 (~240 KD) and growth-factor-receptor–bound protein 2 (Grb2)–associated binder 1 (Gab1) (~120 KD), a known PI3K adaptor protein (*14*), coprecipitated with p85 (Fig. 2C), and both interactions were disrupted by gefitinib alone. In contrast, in the resistant cells, both ERBB3 and Gab1 remained associated with p85 in the presence of gefitinib alone. However, the combination of gefitinib and PHA-665752 completely disrupted these interactions in the resistant cell lines (Fig. 2C). As shown in Fig. 2B, ERBB3 tyrosine phosphorylation was suppressed in the resistant cells only when they were in the presence of both inhibitors, which suggests that MET can trigger the activation of ERBB3 independent of EGFR kinase activity. In the course of these studies, we noted that, although PHA-665752 alone blocked Gab-1 association with p85, it had minimal effect on Akt phosphorylation (Fig. 2, B and C). This observation suggests that the association of Gab-1 with PI3K is not necessary for Akt phosphorylation in the resistant cell lines.

To determine whether a MET/ERBB3/PI3K signaling axis was mediating resistance in these cells, we used RNA interference (RNAi) technology. Down-regulation of ERBB3 by an *ERBB3*-specific short hairpin RNA (shRNA) led to substantial inhibition of Akt phosphorylation and significantly inhibited cell growth in both resistant and parental cells (Fig. 2, D and E) In addition, two shRNAs directed against two different regions of *MET* restored gefitinib sensitivity in the resistant cells (fig. S4) (*15*). Moreover, both of the *MET*-specific shRNAs down-regulated MET to the level found in the parental HCC827 cell line (see Fig. 2B) and restored the ability of gefitinib to down-regulate both ERBB3 and Akt phosphorylation in these cells (Fig. 2F). Finally, overexpression of MET in HCC827 cells was sufficient to confer gefitinib resistance (fig. S5). Together, these findings suggest that *MET* amplification leads to persistent activation of PI3K/Akt signaling in the presence of gefitinib by maintaining ERBB3 phosphorylation.

Notably, gastric cancer cell lines with *MET* amplification exhibit an increased sensitivity to PHA-665752 (*11*). Therefore, we investigated whether other cell lines with *MET* amplification might also activate PI3K/Akt signaling through ERBB3. Interestingly, we readily detected ERBB3/p85 complexes in SNU638 and MKN45 gastric cancer cells, as well as H1993 NSCLC cells, which are known to harbor an amplified *MET* allele (Fig. 3A). In all cases, the ERBB3/p85 complexes could be disrupted by PHA-665752 but not by gefitinib, lapatinib (a dual EGFR/ERBB2 inhibitor), or CL-387,785 (an irreversible EGFR/ERBB2 inhibitor). Accordingly, phosphorylation of ERBB3 and Akt was inhibited only by PHA-665752 but not by the other compounds (Fig. 3A). Finally, *ERBB3*-specific shRNAs also resulted in a marked decrease in phosphorylation of Akt (Fig. 3B) and significantly inhibited cell growth of SNU-638 cells (Fig. 3C). Thus, we conclude that *MET* amplification leads to ERBB3 phosphorylation and PI3K activation in an EGFR- and ERBB2-independent manner. More generally, these studies suggest that ERBB3-mediated activation of PI3K/Akt might be a common feature of cancer cells that have *MET* amplification.

To investigate how MET activates ERBB3 tyrosine phosphorylation, we first expressed ERBB3 alone or in combination with MET in

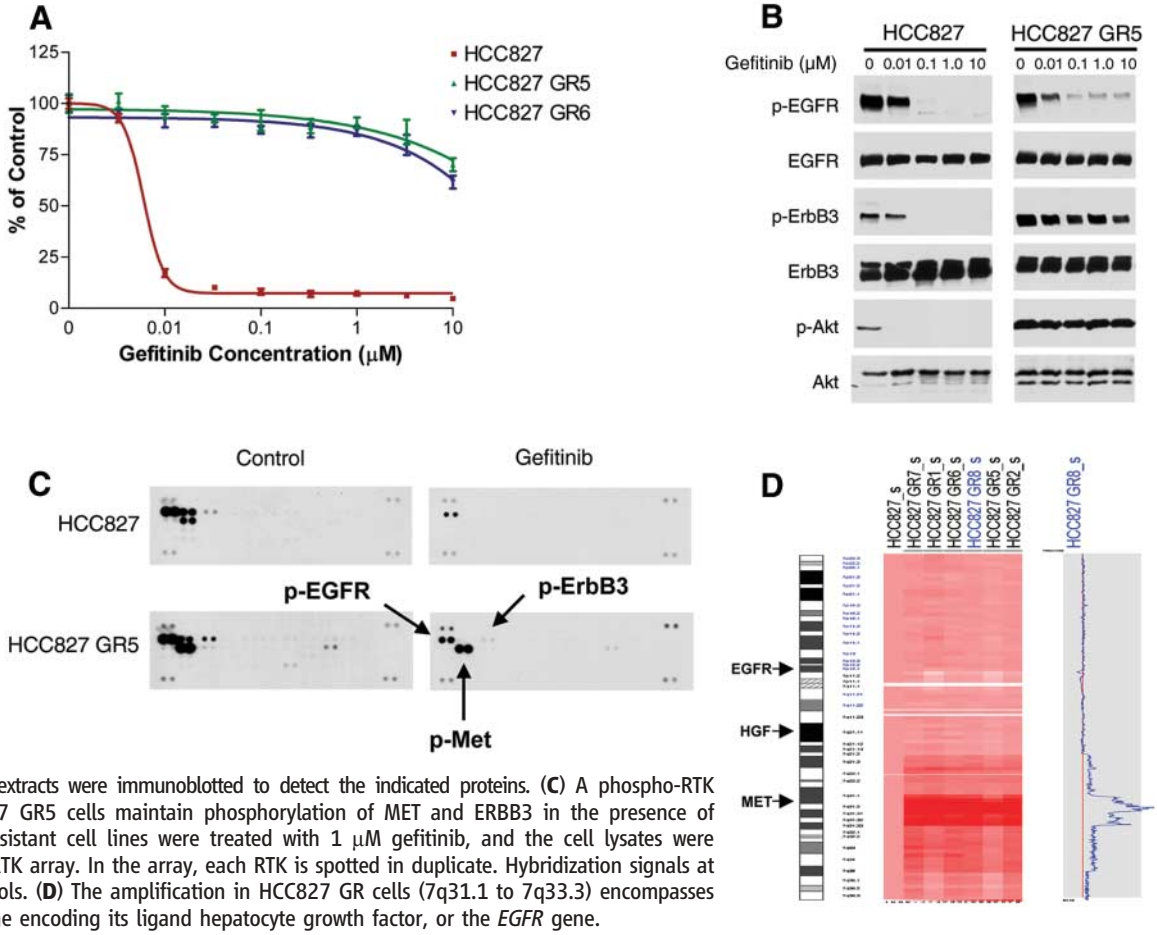

Fig. 1. HCC827 GR cells are resistant to gefitinib in vitro and show *MET* amplification. (**A**) The *EGFR* mutant HCC827 human lung cancer cell line was made resistant to gefitinib by growing it in increasing concentrations of gefitinib (*8*). Parental and resistant HCC827 GR5 and GR6 cells were treated with gefitinib at the indicated concentrations, and viable cells were measured after 72 hours of treatment. The percentage of viable cells is shown relative to untreated controls. (**B**) Gefitinib-resistant cells maintain ERBB3 and Akt phosphorylation in the presence of gefitinib. HCC827 and HCC827 GR5 cells were exposed to increasing concentrations of gefitinib for 6 hours. Cell extracts were immunoblotted to detect the indicated proteins. (**C**) A phospho-RTK array reveals that HCC827 GR5 cells maintain phosphorylation of MET and ERBB3 in the presence of gefitinib. Parental and resistant cell lines were treated with 1 μM gefitinib, and the cell lysates were hybridized to a phospho-RTK array. In the array, each RTK is spotted in duplicate. Hybridization signals at the corners serve as controls. (**D**) The amplification in HCC827 GR cells (7q31.1 to 7q33.3) encompasses *MET* but not *HGF*, the gene encoding its ligand hepatocyte growth factor, or the *EGFR* gene.

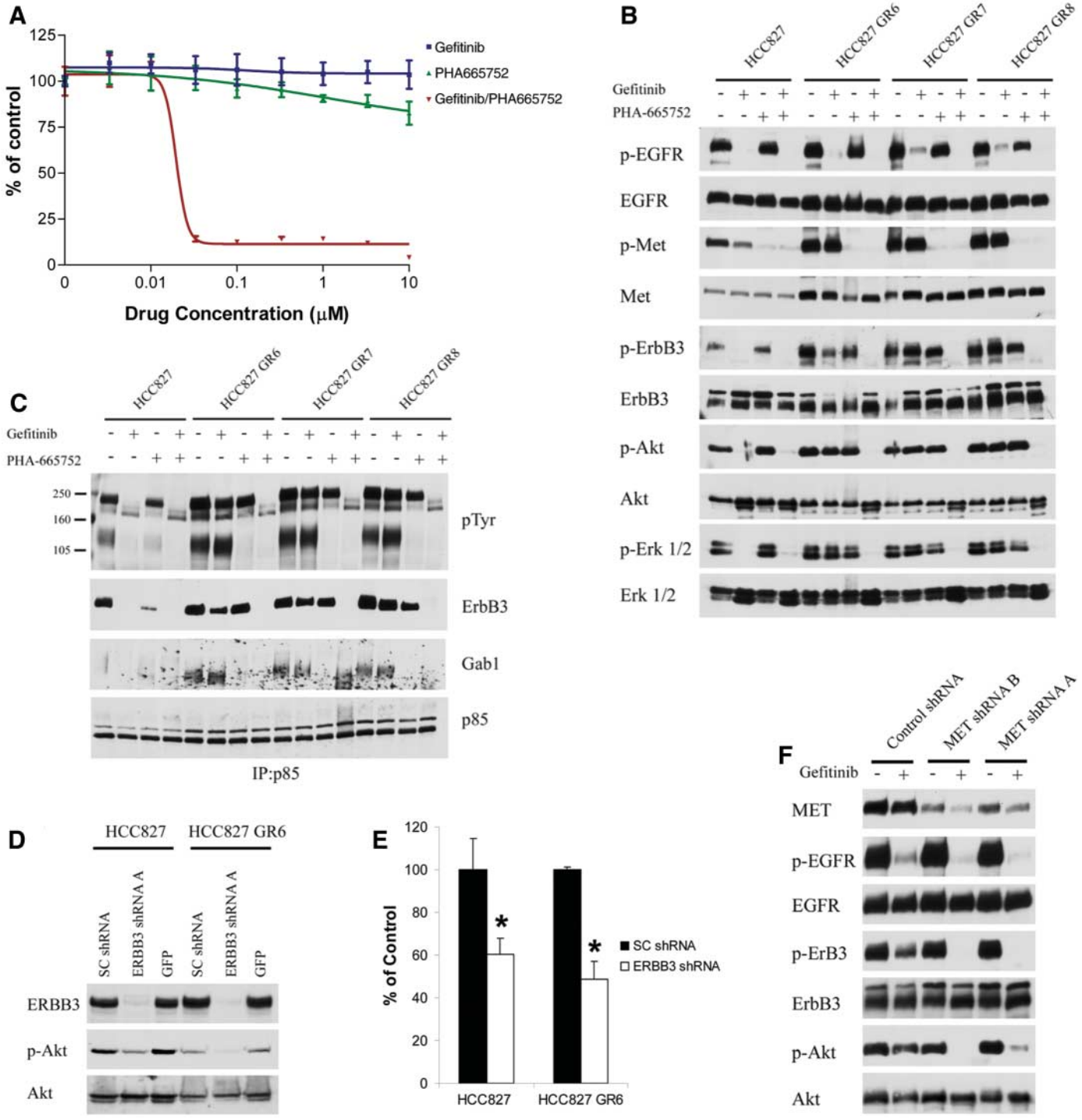

Fig. 2. Concurrent inhibition of MET and EGFR suppresses growth of HCC827 GR cells and leads to down-regulation of ERBB3/PI3K/AKT signaling. (**A**) The HCC827 GR5 cells were treated with increasing concentrations of gefitinib alone, PHA-665752 alone, or the two drugs in combination. Growth was assessed by the MTS survival assay. (**B**) The phosphorylation of ERBB3, Akt, and MET is substantially reduced only by the combination of gefitinib and PHA-665752 in the resistant cells. Parental and resistant cells were treated for 6 hours with gefitinib alone, the MET inhibitor PHA-665752 alone, or the two drugs in combination. Cells were lysed, and the indicated proteins were detected by immunoblotting. (**C**) The association of ERBB3 with p85 is blocked only by the combination of gefitinib and PHA-665752 in the resistant cells. Parental and resistant cells were treated as in (B). Cell extracts were immunoprecipitated with an antibody to p85. The precipitated proteins were determined by immunoblotting with the indicated antibodies. (**D**) Down-regulation of ERBB3 by an *ERBB3*-specific shRNA results in loss of Akt phosphorylation in both HCC827 and HCC827 GR6 cells. Control or *ERBB3*-specific shRNAs were introduced into parental or resistant cells. Cell extracts were prepared 96 hours later and immunoblotted with the indicated antibodies. SC, scrambled; GFP, green fluorescent protein. (**E**) The viability of cells from (D) was measured using an MTS assay. Viability of cells expressing the *ERBB3*-specific shRNA is shown relative to cells expressing control shRNA. Error bars indicate SD. *, $P < 0.05$ (paired *t* test). (**F**) Down-regulation of MET by *MET*-specific shRNAs restores gefitinib-induced down-regulation of ERBB3 and Akt phosphorylation. Control or *MET*-specific shRNAs were introduced into HCC827 GR6 cells. The cells were treated with 1 μM gefitinib, and cell extracts were immunoblotted with indicated antibodies.

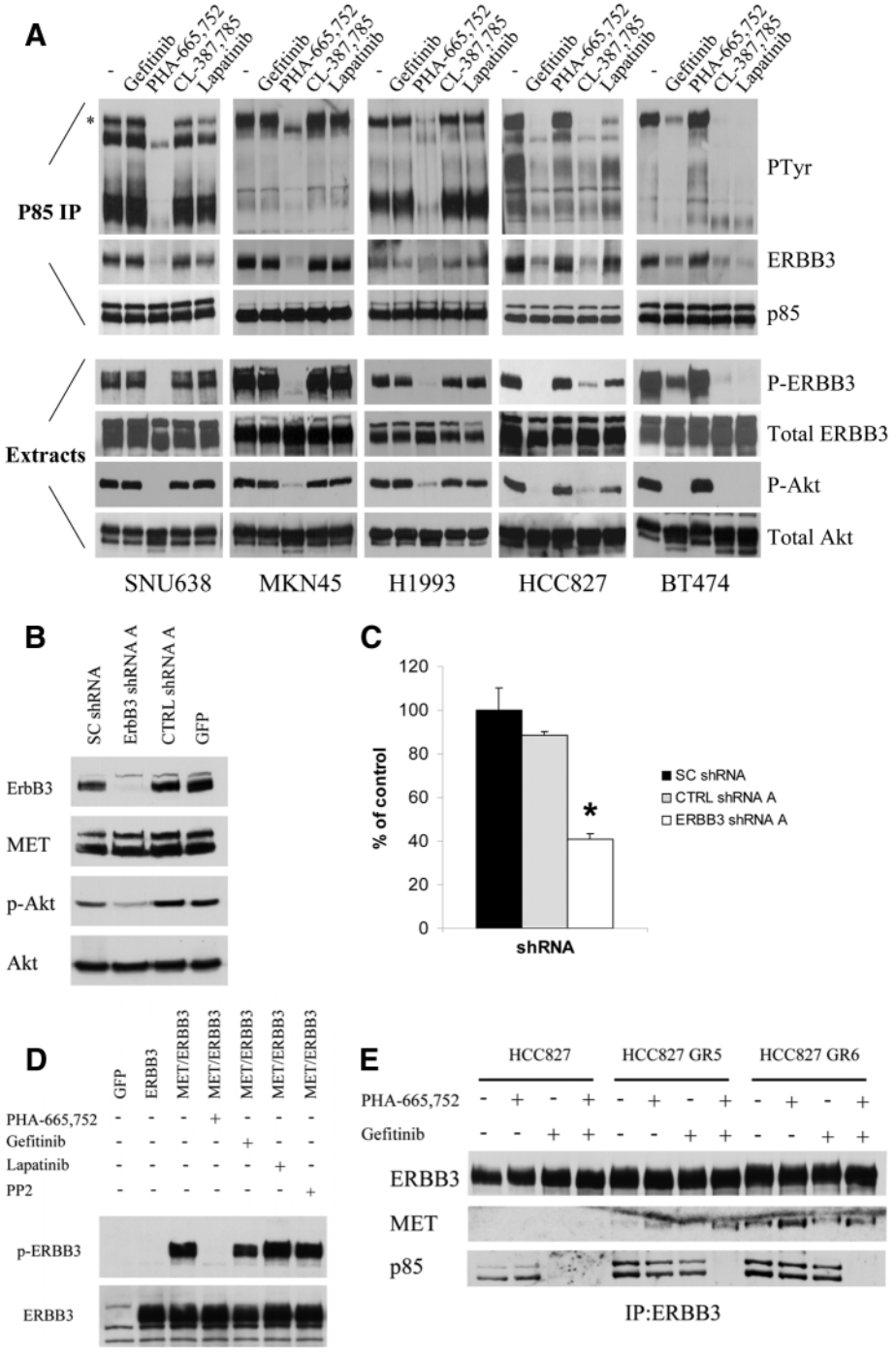

Fig. 3. MET activates ERBB3/PI3K signaling in tumor cell lines with *MET* amplification. (**A**) *MET*-amplified cell lines (with wild-type *EGFR*) also use ERBB3 to activate PI3K/Akt signaling. Cell lines with *MET* amplification (gastric cancer cell lines, SNU-638 and MKN-45, and the NSCLC cell line H1993), with an *EGFR* mutation (NSCLC cell line HCC827), or with *ERBB2* amplification (breast cancer cell line BT474) were treated with the indicated drugs for 6 hours. Cell extracts were immunoprecipitated with an antibody to p85. The precipitated proteins were determined by immunoblotting with the indicated antibodies. In parallel, whole-cell extracts were immunoblotted to detect the indicated proteins. *, ERBB3. (**B**) Down-regulation of ERBB3 by an *ERBB3*-specific shRNA results in loss of Akt phosphorylation in SNU-638 cells. SC, scrambled; GFP, green fluorescent protein; CTRL, control. (**C**) The viability of cells from (B) was measured using an MTS assay. *, $P < 0.05$ (paired t test). (**D**) MET induces ERBB3 phosphorylation. cDNAs encoding for *GFP*, *ERBB3*, or *MET* were introduced into CHO cells. The cells were treated with the indicated drugs for 6 hours, and cell extracts were immunoblotted to detect indicated proteins. (**E**) ERBB3 coprecipitates with MET and p85 from the resistant but not the parental HCC827 cells. HCC827 and HCC827 GR cells were treated with gefitinib alone, PHA-665752 alone, or both drugs in combination. Cell extracts were immunoprecipitated with an antibody to ERBB3. The precipitated proteins were identified by immunoblotting with the indicated antibodies.

Chinese hamster ovary (CHO) cells, which normally do not express detectable levels of EGFR, ERBB2, or ERBB3. Coexpression of MET and ERBB3 resulted in marked phosphorylation of ERBB3 (Fig. 3D). This phosphorylation could be blocked with PHA-665752 but not with high concentrations of gefitinib (3 μM), lapatinib (3 μM) or the SRC family kinase inhibitor PP2 (10 μM). In addition, phosphorylated ERBB3 coimmunoprecipitated with p85 in a MET kinase–dependent manner (fig. S6). We also found that endogenous ERBB3 coprecipitates with MET and p85 in the HCC827 GR cells (Fig. 3E). Similarly, the interaction between ERBB3 and p85 was blocked only with the combination of gefitinib and PHA-665752 in the resistant cells.

To assess the clinical relevance of this resistance mechanism, we examined whether *MET* amplification could be detected in *EGFR* mutant NSCLCs that had become resistant to gefitinib. We analyzed tumors from 18 patients (tables S2 and S3), all of whom had shown partial response to gefitinib or erlotinib during initial treatment but showed signs of tumor regrowth (i.e., resistance) while still receiving these drugs. *MET* copy status was assessed either by quantitative PCR when only tumor-derived DNA was available (n = 11) or by fluorescence in situ hybridization (FISH) when tumor sections were available (n = 7) (fig. S7). For eight patients, we were able to obtain paired tumor specimens from before treatment and after the development of resistance to gefitinib. For the other 10 patients, tumor specimens were available only after the development of resistance to gefitinib or erlotinib. Overall, *MET* amplification was detected in 4 out of 18 (22%) gefitinib/erlotinib–resistant tumor specimens. Of the eight paired tumor samples, two showed *MET* amplification in the resistant specimens but not in the before-treatment samples. In patient 1, the level of *MET* amplification in the post-treatment specimen was similar to that observed in the HCC827 GR cell lines (table S2 and fig. S2). *MET* amplification was also detected in two other patients for whom only post-treatment specimens were available (patients 12 and 13). Of the four resistant tumors with *MET* amplification, one had a concurrent *EGFR* T790M mutation; the other three did not. Interestingly, two independent resistant tumors from patient 12 were analyzed, and one had an *EGFR* T790M while the other had a *MET* amplification (table S2).

Mechanisms of acquired resistance to kinase inhibitors in NSCLC, chronic myelogenous leukemia (CML), and gastrointestinal stromal tumor include secondary mutations in the kinase itself (*EGFR*, *KIT*, or *BCR-ABL*), amplification of the target kinase (*KIT* or *BCR-ABL*), or overexpression of other kinases downstream of the target kinase (for example, *LYN* in CML) (*5*, *16*–*19*). However, *MET* amplification provides an example of a resistance mechanism characterized by

gene amplification of a kinase that is not a direct or downstream target of gefitinib or erlotinib. Moreover, MET has not previously been shown to signal through ERBB3. These findings may have important clinical implications for NSCLC patients who develop acquired resistance to gefitinib or erlotinib. Our findings also suggest that irreversible EGFR inhibitors, which are currently under clinical development as treatments for patients whose tumors have developed acquired resistance to gefitinib and erlotinib, may be ineffective in the subset of tumors with a *MET* amplification even if they contain an *EGFR* T790M mutation. Therefore, combination therapies with MET kinase inhibitors, which are in early-stage clinical trials, and irreversible EGFR inhibitors should be considered for patients whose tumors have become resistant to gefitinib or erlotinib. Notably, a small percentage of NSCLCs from EGFR TKI–naïve patients have been reported to contain both an *EGFR*-activating mutation and *MET* amplification (*20*, *21*). This situation is analogous to the observation that untreated NSCLCs occasionally have an *EGFR* T790M. These concurrent genetic alterations may help explain why some NSCLCs with *EGFR*-activating mutations fail to respond when initially treated with gefitinib (*22*).

It will continue to be important to study NSCLC primary tumors and cell lines with acquired resistance to EGFR inhibitors for insights into additional resistance mechanisms. Our findings illustrate the value of studying genetic alterations that produce persistent PI3K/Akt signaling in the presence of gefitinib rather than focusing solely on mutations in the *EGFR* gene itself. It will also be important to determine whether *MET* amplification contributes to resistance in other *EGFR*-dependent cancers such as glioblastoma multiforme, head and neck cancer, and colorectal cancer after treatment with EGFR-directed therapies. Finally, since ERBB2-amplified breast cancers also activate PI3K/Akt signaling through ERBB3, it will be interesting to explore whether *MET* amplification also occurs in breast cancers that develop resistance to drugs that target ERBB2, such as trastuzumab and lapatinib (*9*, *23*).

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Supporting Online Material

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Materials and Methods
Figs. S1 to S7
Tables S1 to S4
References

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Wilms Tumor Suppressor WTX Negatively Regulates WNT/β-Catenin Signaling

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Aberrant WNT signal transduction is involved in many diseases. In colorectal cancer and melanoma, mutational disruption of proteins involved in the degradation of β-catenin, the key effector of the WNT signaling pathway, results in stabilization of β-catenin and, in turn, activation of transcription. We have used tandem-affinity protein purification and mass spectrometry to define the protein interaction network of the β-catenin destruction complex. This assay revealed that WTX, a protein encoded by a gene mutated in Wilms tumors, forms a complex with β-catenin, AXIN1, β-TrCP2 (β-transducin repeat–containing protein 2), and APC (adenomatous polyposis coli). Functional analyses in cultured cells, *Xenopus*, and zebrafish demonstrate that WTX promotes β-catenin ubiquitination and degradation, which antagonize WNT/β-catenin signaling. These data provide a possible mechanistic explanation for the tumor suppressor activity of WTX.

In the absence of WNT ligands, cytosolic β-catenin is constitutively degraded through phosphorylation-dependent ubiquitination and subsequent proteosomal clearance. A complex of proteins including adenomatous polyposis coli (APC), AXIN, casein kinase 1α (CK1α), and glycogen synthase kinase 3 (GSK3) phosphorylates N-terminal serine residues in β-catenin, which creates a substrate efficiently ubiquitinated by the Skp1, Cullin1, F-box protein β-TrCP ($SCF^{\beta TrCP}$) ubiquitin ligase (*1*). The engagement of a Frizzled receptor with WNT ligand initiates a signaling cascade, culminating in the inactivation of the β-catenin destruction complex. Consequently, β-catenin levels increase in the nucleus, where it functions as a transcriptional coactivator for members of the TCF-LEF family of transcription factors (*2*, *3*). Although mutations in APC are common in colorectal cancer, many human malignancies harboring active WNT/β-catenin signaling have no identified causative mutation(s) (*4*, *5*).

To identify proteins associated with the β-catenin destruction complex, we performed a tandem-affinity purification (TAP) of $\beta\text{-catenin}^{(SA)}$, AXIN1, APC (amino acids 1 to 1060), β-TrCP1, and β-TrCP2 in mammalian cells (*6*). The $\beta\text{-catenin}^{(SA)}$ mutant has alanine substituted for serine at codon 37. Specifically, cDNA for each of these "bait" proteins was cloned into the pGlue vector encoding a dual-affinity tag containing streptavidin-binding protein (SBP), calmodulin-binding protein (CBP), and the hemagglutinin (HA) epitope (*7*). Lines of human embryonic kidney cells (HEK293T) expressing low levels of each of the tagged-bait fusion proteins were generated, then detergent-solubilized, subjected to two rounds of affinity purification, trypsinized,

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