CANCER

Combining three antibodies nullifies feedback-mediated resistance to erlotinib in lung cancer

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Despite initial responses to targeted kinase inhibitors, lung cancer patients presenting with primary epidermal growth factor receptor (EGFR) mutations acquire resistance, often due to a second-site mutation (T790M). However, clinical trials found no survival benefits in patients treated with a monoclonal antibody (mAb) to EGFR that should block activation of the mutated receptor and thus bypass resistance to molecules that target the catalytic or ATP-binding site. Using cell lines with the T790M mutation, we discovered that prolonged exposure to mAbs against only the EGFR triggered network rewiring by (i) stimulating the extracellular signal–regulated kinase (ERK) pathway; (ii) inducing the transcription of *HER2* (human epidermal growth factor receptor 2) and *HER3*, which encode other members of the EGFR family, and the gene encoding HGF, which is the ligand for the receptor tyrosine kinase MET; and (iii) stimulating the interaction between MET and HER3, which promoted MET activity. Supplementing the EGFR-specific mAb with those targeting HER2 and HER3 suppressed these compensatory feedback loops in cultured lung cancer cells. The triple mAb combination targeting all three receptors prevented the activation of ERK, accelerated the degradation of the receptors, inhibited the proliferation of tumor cells but not of normal cells, and markedly reduced the growth of tumors in mice xenografted with cells that were resistant to combined treatment with erlotinib and the single function-blocking EGFR mAb. These findings uncovered feedback loops that enable resistance to treatment paradigms that use a single antibody and indicate a new strategy for the treatment of lung cancer patients.

INTRODUCTION

Lung cancer is the worldwide leading cause of cancer-related death (*1*). Patients often present with advanced-stage disease, and the prognosis is generally poor. The discovery in 2004 of mutant forms of the epidermal growth factor receptor (EGFR) in non–small cell lung cancer (NSCLC) identified groups of patients who are sensitive to tyrosine kinase inhibitors (TKIs), including gefitinib and erlotinib (*2*–*4*). EGFR (also called ERBB1) forms active heterodimers with other drug targets, namely, HER2 (human epidermal growth factor receptor 2; also called ERBB2), HER3 (ERBB3), and HER4 (ERBB4) (*5*–*7*). Despite the initial efficacy of EGFR-specific TKIs, all patients acquire resistance within about 1 year (*8*, *9*). The most common (>50%) mechanism of acquired (secondary) resistance involves a specific second-site mutation in EGFR, denoted T790M, which places a bulky amino acid, methionine, instead of a threonine in position 790 of EGFR (*10*–*12*). Amplification of the gene encoding another receptor tyrosine kinase, MET, occurs in 5 to 10% of cases of acquired resistance (*13*, *14*). It appears that *MET* amplification causes resistance because it stimulates HER3-dependent activation of the phosphatidylinositol 3-kinase (PI3K) pathway. Consistent with this model, activation of PI3K and a downstream kinase AKT by an ectopically expressed active mutant of PI3K conferred TKI resistance in a cellular model, and down-regulation of PTEN (phosphatase and tensin homolog), which inhibits PI3K signals, was found to be sufficient for emergence of TKI resistance in another model (*15*, *16*).

To overcome EGFR TKI resistance, several second-generation TKIs have been developed [reviewed in (*17*, *18*)]. For example, afatinib (Gilotrif), an orally administered drug, covalently binds with the kinase domains of EGFR and HER2, resulting in irreversible inhibition of autophosphorylation as well as transphosphorylation of HER3 (*19*, *20*). Another approach, which combines afatinib and an antibody to EGFR, has recently been reported (*21*, *22*). Although afatinib is approved as a first-line treatment of advanced NSCLC harboring activating EGFR mutations, third-generation inhibitors, such as AZD-9291, CO-1686, and HM-61713, which inhibit both EGFR-activating and resistance mutations while sparing wild-type EGFR, are in early-phase studies (*23*). Nevertheless, the therapeutic potential of EGFR-specific antibodies, such as cetuximab, remains unclear. A clinical trial that combined cetuximab and chemotherapy (cisplatin and vinorelbine) demonstrated relatively small but significant prolongations of patient survival (*24*). However, another study in unselected NSCLC patients failed to demonstrate a survival benefit of the combination of cetuximab and chemotherapy (carboplatin and taxane) (*25*). Specific antibodies can reduce the surface abundance of EGFR, and EGFR abundance is considered a predictor of patient survival (*26*–*28*). For example, analysis of EGFR abundance using immunohistochemistry (*29*) or fluorescence in situ hybridization (*30*) associated high EGFR abundance with patient response to cetuximab.

Because mutants of EGFR are associated with a small increase in *EGFR* gene copy number (*31*), these observations hint that monoclonal antibodies (mAbs) against EGFR might be effective on a subset of NSCLC patients. We addressed this possibility by using two models of TKI-resistant NSCLC. We found feedback regulatory loops that increase the abundance of HER2 and HER3 and activate MET in response to antibody-induced blockade of EGFR. Preventing this complex compensatory response effectively inhibited

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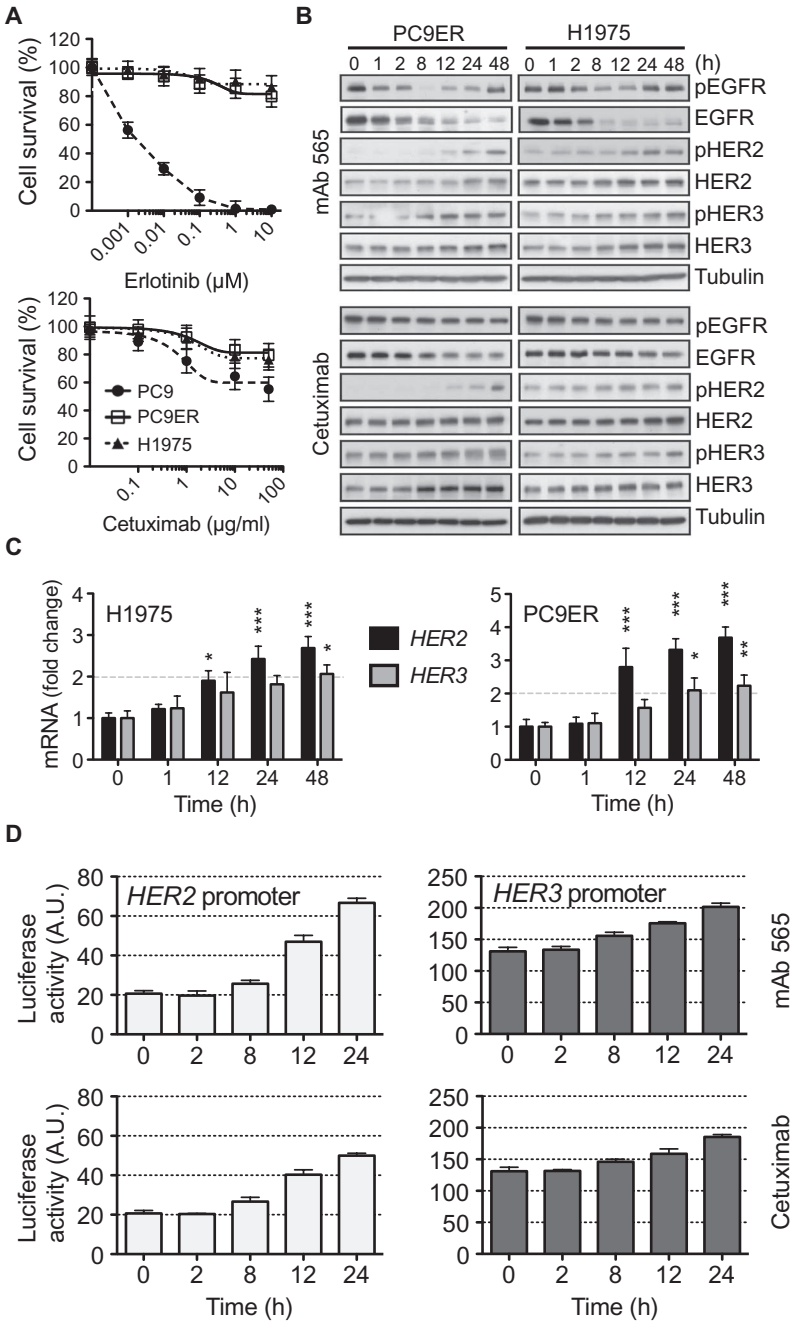

Fig. 1. Antibodies specific to EGFR increase the abundance of HER2 and HER3 in TKI-resistant NSCLC cells. (**A**) Survival assay of PC9, PC9ER, and H1975 human NSCLC cells treated for 72 hours with increasing concentration of erlotinib (top) or cetuximab (bottom). Data are means ± SD of three independent experiments. (**B**) Immunoblotting of erlotinib-resistant PC9ER and H1975 cells exposed to mAbs specific to EGFR (mAb 565 or cetuximab; 10 μg/ml) for the indicated times. Tubulin served as a loading control. Blots are representative of three experiments. (**C**) *HER2* and *HER3* mRNA levels of H1975 and PC9ER cells treated for the indicated time intervals with antibodies specific to EGFR (10 μg/ml). Data are means ± SD from three experiments. $*P \le 0.05$, $**P \le 0.01$, $***P \le 0.001$, two-way analysis of variance (ANOVA) with Tukey's comparison. (**D**) Relative luciferase activity in PC9ER cells stably cotransfected for 48 hours with luciferase reporter plasmids for the *HER2* (left) or *HER3* (right) promoter and treated with mAb 565 (top) or cetuximab (bottom). Data are means ± SEM from two experiments, each performed in technical triplicate. A.U., arbitrary unit.

NSCLC tumor growth in an animal model, thus offering a novel strategy to treat relatively aggressive lung tumors.

RESULTS

EGFR-specific antibodies increase the abundance of HER2 and HER3 in TKI-resistant NSCLC cells

EGFR mAbs avidly bind both the wild-type and mutant forms of EGFR; hence, antibody treatment is expected to overcome resistance to TKIs. To experimentally examine this possibility, we selected two NSCLC cell lines: patient-derived H1975 cells that express a double-mutant EGFR (L858R and T790M) and the PC9ER cell line, which is a derivative of PC9 cells that have a deletion in EGFR (del746–750 EGFR, also called LREA deletion) and have acquired in vitro the T790M secondary mutation (*32*). To evaluate the effect of EGFR-specific treatments, we monitored the survival of cells treated for 4 days with increasing doses of either erlotinib or cetuximab (Fig. 1A). As expected, the survival of parental PC9 cells was strongly inhibited by erlotinib and weakly inhibited by cetuximab. However, in line with a previous study (*33*), PC9ER and H1975 cells expressing EGFR T790M showed resistance to both treatments. Concentrating on the resistant cell lines, we noted that long-term exposure to cetuximab or to another EGFR-specific antibody, mAb 565, which was generated in our laboratory (*34*, *35*), caused a gradual disappearance in the abundance of EGFR (Fig. 1B). This was accompanied by increased abundance of HER2 and HER3, as well as increased tyrosine phosphorylation of EGFR, HER2, and HER3 (Fig. 1B), suggesting the emergence of positive feedback that compensates for EGFR down-regulation. Similar to mAb treatment, depletion of EGFR abundance with small interfering RNA (siRNA) oligonucleotides was followed by increased abundance of HER2 and HER3 proteins (fig. S1A).

Because of their delayed responses, we assumed that the regulation of HER2 and HER3 was controlled at the transcriptional level. Two lines of evidence supported this scenario: First, quantitative polymerase chain reaction (PCR) analyses performed on H1975 and PC9ER cell extracts displayed moderate, time-dependent increases in the abundance of the respective transcripts (Fig. 1C). Second, promoter-reporter plasmids verified that cetuximab treatment strongly or weakly increased promoter activity of *HER2* or *HER3*, respectively (Fig. 1D and fig. S1B).

A combination of three antibodies against EGFR, HER2, and HER3 targets all three receptors for degradation and prevents increased activation of ERK

The evidence so far implied that a compensatory feedback loop enhances HER2 and HER3 at the transcriptional level when mutant EGFR is depleted. Hence, preventing the increase of HER2 and HER3 might augment

the effects of EGFR-specific mAbs. As a first step toward examining this prediction, we used siRNA oligonucleotides specific to HER2 and HER3. Although each siRNA effectively inhibited its own target, neither oligonucleotide prevented the increase of the other receptor when cells were treated for 12 hours with an EGFR-specific antibody (mAb 565; Fig. 2A). In addition, knockdown of either HER2 or HER3 weakly inhibited the stimulatory effect of mAb 565 on extracellular signal–regulated kinase (ERK) activation, but knocking down both HER2 and HER3 suppressed the phosphorylation of ERK below detection while exerting only a weak effect, if any, on AKT phosphorylation on either of the two residues we tested (Fig. 2A). In line with these results, PC9ER cells treated for 3 days with mAb 565 and the combination of siRNAs exhibited significantly lower survival than cells treated with either of the single siRNAs (Fig. 2B). Blocking all three receptors, using siRNAs to HER2 and HER3 and a mAb to EGFR, was more effective than the combination of the two siRNAs with erlotinib (Fig. 2B), suggesting that eliminating EGFR with a mAb is more effective than blocking the receptor's enzymatic activity with a TKI.

Unlike siRNA oligonucleotides, which inhibit protein expression, mAbs reduce the abundance of their target ERBB family members by sorting them to degradation in the lysosomes (*34*, *36*–*38*). To examine the prediction that mAb combinations might inhibit NSCLC cells, we selected three mAbs, collectively targeting EGFR, HER2, and HER3 (mAb 565, mAb 12, and mAb 33, respectively), on the basis of their ability to inhibit the survival of cultured NSCLC cells (fig. S2). Fluorescence microscopy revealed that none of the three mAbs or their paired combinations was able to deplete the detection of all three receptors, but the triple combination substantially reduced the detection of EGFR, HER2, and HER3 on the surface of H1975 cells (Fig. 3A). Similarly, additional quantitative analysis using fluorescence-activated cell sorting indicated that mAbs against EGFR increased the abundance of HER2 and HER3 in PC9ER cells, and that the triple combination—unlike the antibody pairs—reduced the surface abundance of all three receptors (Fig. 3B). The triple combination retained the ability to enhance the transcription of *HER2* and *HER3* (fig. S3, A and B), an effect we attribute to the EGFR-specific antibody in the mixture. This effect is then counteracted at the (surface) protein level by the HER2 and HER3 mAbs.

On the basis of previous observations (*39*), a delayed positive feedback loop that engages HER2 and HER3 is expected to hyperphosphorylate EGFR and activate downstream pathways, such as those involving ERK or AKT. Indeed, treatment of PC9ER cells with an EGFR-specific mAb initially reduced the phosphorylation of the constitutively active mutant receptor; however, although EGFR underwent gradual degradation and its abundance was particularly low after 12 hours of treatment, we observed strikingly strong rephosphorylation of the residual EGFR (Figs. 1B and 3C). This enhanced activation of EGFR might be related to the concomitant increase of HER2 and HER3, as well as to enhancement of their phosphorylated (active) forms (*39*). As expected, the time course of EGFR phosphorylation was mirrored by the kinetics of ERK activation, including a late peak (~24 hours) of markedly increased phosphorylation (Fig. 3C). However, AKT did not exhibit similar effects (Fig. 3C and fig. S3C), suggesting that AKT is not involved in the feedback loop. The reactivation of ERK was not prevented by any pair of mAbs (Fig. 3C and fig. S3C), but it was durably prevented by the triple mAb combination (Fig. 3C), in line with the decreased surface abundance of EGFR in our analysis (Fig. 3, A and B).

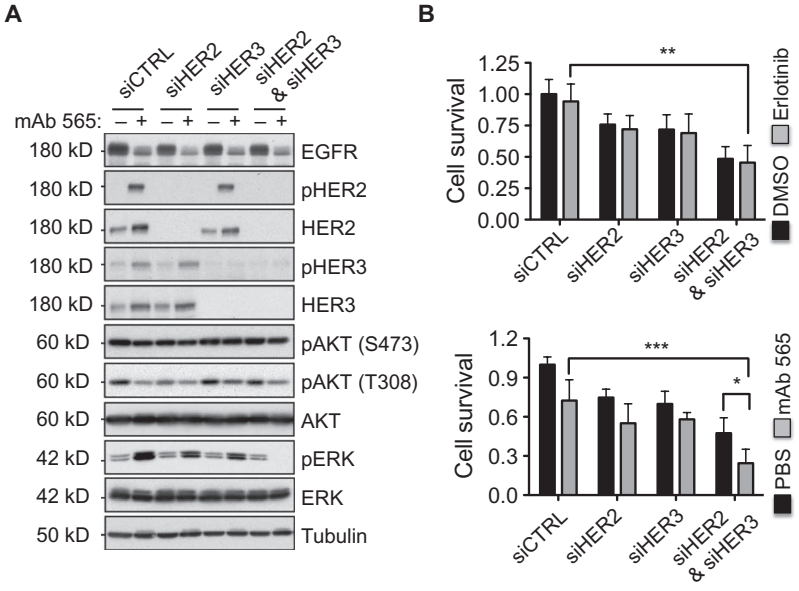

Fig. 2. Combining *HER2*- and *HER3*-specific siRNAs potentiates inhibition of NSCLC cell survival by an antibody against EGFR. (**A**) Immunoblotting of PC9ER cells transfected for 48 hours with *HER2*- or *HER3*-specific siRNAs (50 pM), their combination, or a control oligonucleotide and then treated for 12 hours with an antibody against EGFR (mAb 565; 10 μg/ml). Blots are representative of three experiments. (**B**) Cell survival assessed using a colorimetric assay in PC9ER cells transfected for 24 hours with siRNAs (80 pM) essentially as in (A) and then cultured for 3 days in RPMI 1640 (2% serum) containing erlotinib (1 μM) or its vehicle [dimethyl sulfoxide (DMSO)], or EGFR mAb 565 (10 μg/ml) or its vehicle [phosphate-buffered saline (PBS)]. Data are means ± SD from three experiments. $^{*}P \leq 0.05$, $^{**}P \leq 0.01$, $^{***}P \leq 0.001$, two-way ANOVA with Tukey's comparison.

A triple mAb combination inhibits compensatory MET activation and disrupts physical interactions with HER3

The relatively kinase-impaired member of the HER/ERBB family, HER3, has been implicated in TKI resistance (*40*). In addition, it has previously been reported that amplification of the *MET* gene leads to TKI resistance in lung cancer by activating HER3 signaling (*13*). To explore MET and HER3 involvement in resistance to EGFR-specific antibodies, we first depleted EGFR using siRNAs in PC9ER cells. When depleted of EGFR, PC9ER cells displayed increased phosphorylation of MET within the activation loop of the kinase domain (Fig. 4A). Similar to the effect of siRNA-mediated depletion of EGFR, treatment of PC9ER cells with an EGFR mAb activated MET, but this was abolished when antibodies to HER2 and HER3 were added (Fig. 4B). Increased MET phosphorylation subsequent to EGFR inhibition might be attributed to enhanced production of the corresponding ligand, the hepatocyte growth factor (HGF), as demonstrated by PCR analysis (fig. S4A), because MET displayed no changes at the transcript or protein level (fig. S4A and Fig. 3B, respectively).

An additional mechanism underlying MET activation likely involves dynamic crosstalk and physical interactions with members of the EGFR family. Coimmunoprecipitation experiments detected EGFR-MET complexes in unperturbed PC9ER cells, but either antibody-mediated depletion or siRNA-mediated reduction of EGFR induced the emergence of alternative

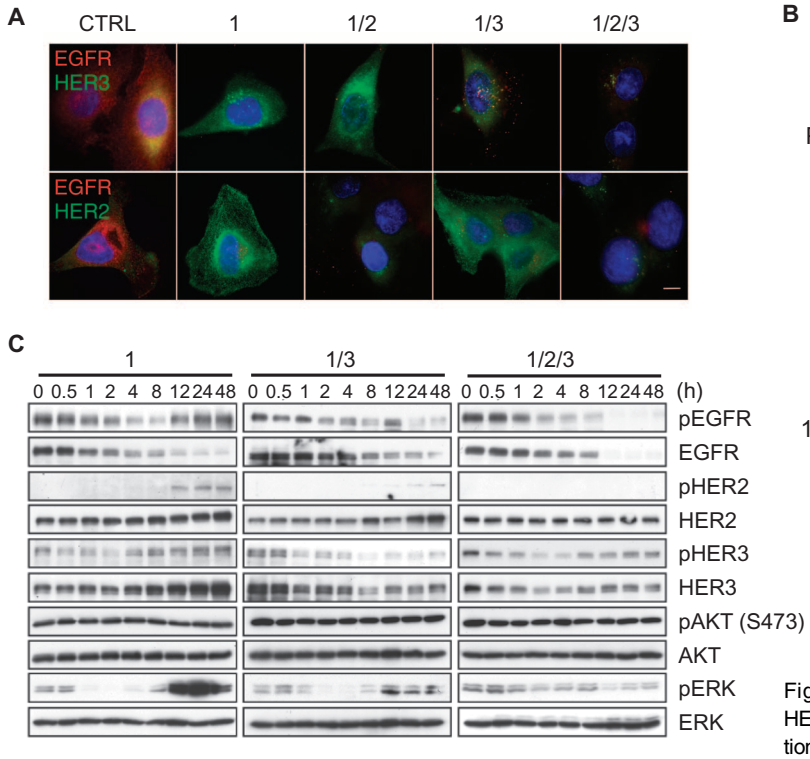

Fig. 3. A combination of antibodies against EGFR, HER2, and HER3 targets all three receptors for degradation and prevents delayed hyperactivation of ERK. (A) Immunostaining for EGFR, HER2, and HER3 in acid-washed, fixed H1975 cells. Cells were plated on coverslips and treated for 24 hours with either single, double, or triple combinations of antibodies against EGFR [mAb 565 (1)], HER2 [mAb 12 (2)], or HER3 [mAb 33 (3)], singly or in combination as indicated (total concentration, 10 μg/ml). Scale bar, 4 μm. (B) PC9ER cells were treated for 48 hours as described in (A) and then analyzed using flow cytometry for surface EGFR, HER2, HER3, and MET. Normalized data are means ± SEM of three independent experiments. (C) Immunoblotting in PC9ER cells treated with single, double, or triple combinations of the indicated antibodies as in (A) (total concentration, 10 μg/ml) for the indicated times. Blots and images are representative of two experiments.

HER3-MET complexes (Fig. 4C and fig. S4B). In line with these observations, immunofluorescence analyses detected colocalization of HER3 and MET, especially in EGFR-depleted cells (Fig. 4D). The mixture of three mAbs or three siRNAs (simultaneously depleting EGFR, HER2, and HER3) inhibited the formation of HER3-MET complexes (Fig. 4C and fig. S4B), as well as the colocalization of these two receptors (Fig. 4D), in line with the ability of the triple mAbs to suppress MET activity with no apparent effect on its stability (Fig. 4B). We hypothesize that EGFR normally forms inhibitory complexes with MET; when EGFR is down-regulated by a single antibody, MET engages HER3. The active HER3-MET complexes, however, undergo complete inactivation by the triple mAb mixture, indicating that this treatment can overcome resistance because of the MET-to-HER3 escape route.

A triple antibody mixture reduces the ability of stromal cells to support NSCLC cells and better inhibits tumor cells than normal cells

The ability of the triple mAb combination to inhibit positive feedback involving HER2, HER3, MET, and ERK might translate to retardation of tumor cell proliferation. A colorimetric assay showed that the EGFR-specific mAb 565 inhibited the proliferation of H1975 and PC9ER cells by about 40% after 4 days of incubation, and its combination with HER2 and HER3 antibodies was markedly more effective (exhibiting about 80% inhibition) compared to any paired combination or a triple combination of erlotinib with the HER2 and HER3 antibodies (Fig. 5, A and B). We corroborated these observations using a marker of proliferating cells (Ki67). Whereas most untreated or single mAb–treated H1975 cells stained positively for Ki67, only ~20% were positive after treatment with the triple antibody combination (Fig. 5C and fig. S5A). Despite strong inhibition, the triple antibody combination did not induce any signs of apoptosis, as determined with either a dye exclusion assay (fig. S5, B and C) or a caspase-3 cleavage blot (fig. S5, D and E).

One concern associated with combining drugs is additive adverse effects, which are primarily due to toxicity toward normal cells. To explore this concern and the mAb effects on tumor-stroma interactions, we selected two normal human cell lines of lung origin (NL20 epithelial cells and WI38 fibroblasts) and ascertained the endogenous abundance of EGFR, HER2, and HER3 using flow cytometry (fig. S6A). Then, we established fluorescent derivatives of the normal cells by stably expressing mCherry-*Renilla* (denoted -CherryRNL) and of the cancer cells (H1975 and PC9ER) by expressing green fluorescent protein (GFP)–firefly (-GFPFF). Whereas treatment of the labeled cancer cell lines with the triple mAb mixture confirmed dose-dependent inhibition of proliferation, the treatment had no or minimal effects on either of the normal cells (Fig. 5D), suggesting that the mixture might spare normal tissues. This is contrasted by the inhibitory effects of erlotinib toward both normal and TKI-sensitive cancer cells (fig. S6B).

This selective attribute of the triple combination therapy was further examined using tissue-like cocultures of tumor and normal cells. First, we cocultured labeled tumor cells with either unlabeled tumor cells (control) or unlabeled normal fibroblasts (fig. S6, C and D). The results showed that the normal fibroblasts enhanced the proliferation of the two

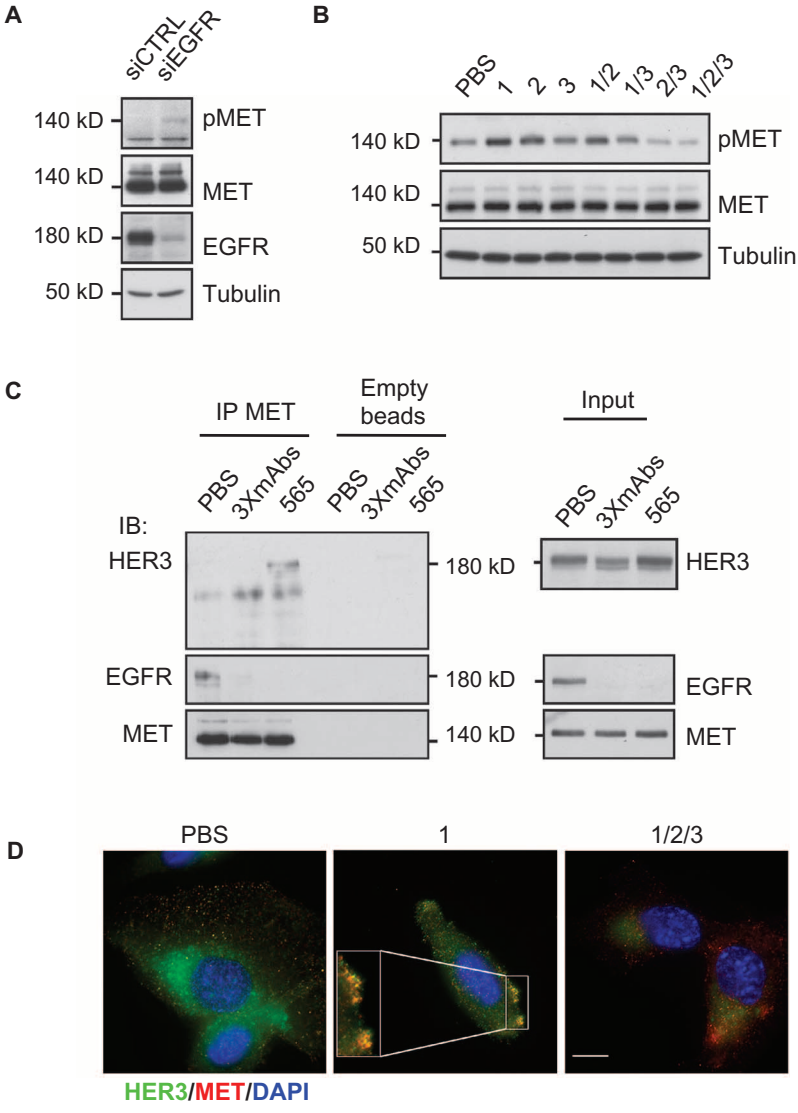

Fig. 4. A triple mAb combination inhibits MET activation and disrupts physical interactions of MET with HER3. (**A**) Immunoblotting in PC9ER cells transfected for 48 hours with *EGFR*-specific or control siRNA (50 pM). (**B**) Immunoblots of PC9ER whole-cell lysates treated for 24 hours with antibodies against EGFR (1), HER2 (2), or HER3 (3), singly or in combination (total concentration, 10 μg/ml). (**C**) Pull-down for MET (IP) and then immunoblotting (IB) in lysates from PC9ER cells treated for 24 hours with saline, an antibody against EGFR (mAb 565), or a triple combination of antibodies against EGFR, HER2, and HER3 (3XmAbs). Empty beads served as a control. Right: input. (**D**) Immunostaining for HER3 (green) or MET (red) in acid-washed, fixed H1975 cells plated on coverslips and treated for 24 hours with saline, an EGFR antibody, or a triple combination of antibodies as described in (B). Cells were counterstained with 4′,6-diamidino-2-phenylindole (DAPI). Inset of the middle panel is magnified twofold. Scale bar, 8 μm. Blots and images are representative of three experiments.

tumor cells. In contrast, reciprocal experiments indicated that the tumor cells might inhibit the proliferation of normal fibroblasts (fig. S6E). Assuming that these effects are mediated, on one hand, by growth factors secreted by stromal cells and, on the other hand, by growth inhibitory cytokines secreted by the tumor cells, we tested the impact of mAbs on this bidirectional crosstalk. Treating the cocultures with receptor-specific antibodies, especially the triple combination, reduced the ability of fibroblasts to enhance tumor cell proliferation and, at the same time, reduced the suppressive effects that the tumor cells imposed on stromal cells (Fig. 5, E and F, and fig. S6F). Together, our observations predict selective inhibition of NSCLC by the triple mAb combination, as well as low toxicity toward normal lung cells.

A combination of three mAbs enhances the inhibitory effects of new-generation TKIs, specifically at a low concentration range of the kinase inhibitors

A common way to increase tumor cell death is to combine a molecule-targeted therapy (like mAbs) with chemotherapeutic agents. Along this line, the triple mixture of EGFR/HER2/HER3 mAbs in PC9ER cells enhanced the cytotoxicity of cisplatin, a chemotherapeutic drug used to treat NSCLC patients (Fig. 6A). Because the second-generation EGFR TKIs, like the combination of three mAbs, are designed to inhibit HER2 and in general show promising effects in animal models and in patients (*21*, *23*, *41*, *42*), we compared them to the triple mAb combination. Compared to cetuximab alone, the triple mAb combination more effectively increased the inhibitory activity of afatinib, a second-generation TKI, in PC9ER cells. This effect was shared in combination with two third-generation TKIs, AZD-9291 and CO-1686. However, the additive effect of the triple mAb combination gradually diminished with increasing TKI concentrations. Presumably, at a lower range of concentrations, the TKIs have an EGFR-specific inhibitory effect, but at higher concentrations, the new TKIs nonspecifically engage molecular targets other than HER family members. An additional survival assay confirmed the additive effects of the TKIs AZD-9291 and CO-1686 when combined with mAbs and also indicated that the added benefit requires a triple, rather than double, combination of mAbs (Fig. 6B). Thus, combination treatment of novel TKIs and a triple mAb mixture holds promise to have additive or synergistic efficacy in drug-resistant NSCLC.

Simultaneous interception of EGFR, HER2, and HER3 is essential for inhibition of drug-resistant NSCLC cells both in vitro and in an animal model

The ability of the triple mAb combination to spare normal cells while strongly inhibiting tumor cells and possibly depriving them of stromal support prompted us to examine the triple combination both in vitro and in tumor-bearing animals. To address the parameters of antibody delivery, we treated H1975 cells for 3 days with an EGFR-specific antibody (either cetuximab or mAb 565) and later removed the antibody or fortified it by adding mAbs against HER2 (either trastuzumab or mAb 12) and/or HER3. The results indicated that simultaneous treatment with the three antibodies is essential for maximal inhibitory effects; no alternative combination or scheduling achieved superiority in terms of inhibition of cell proliferation (Fig. 7, A and B). Surprisingly, replacing the EGFR-specific antibody with the TKI erlotinib abolished the benefit gained by adding HER2- and HER3-specific mAbs, indicating potential advantage in

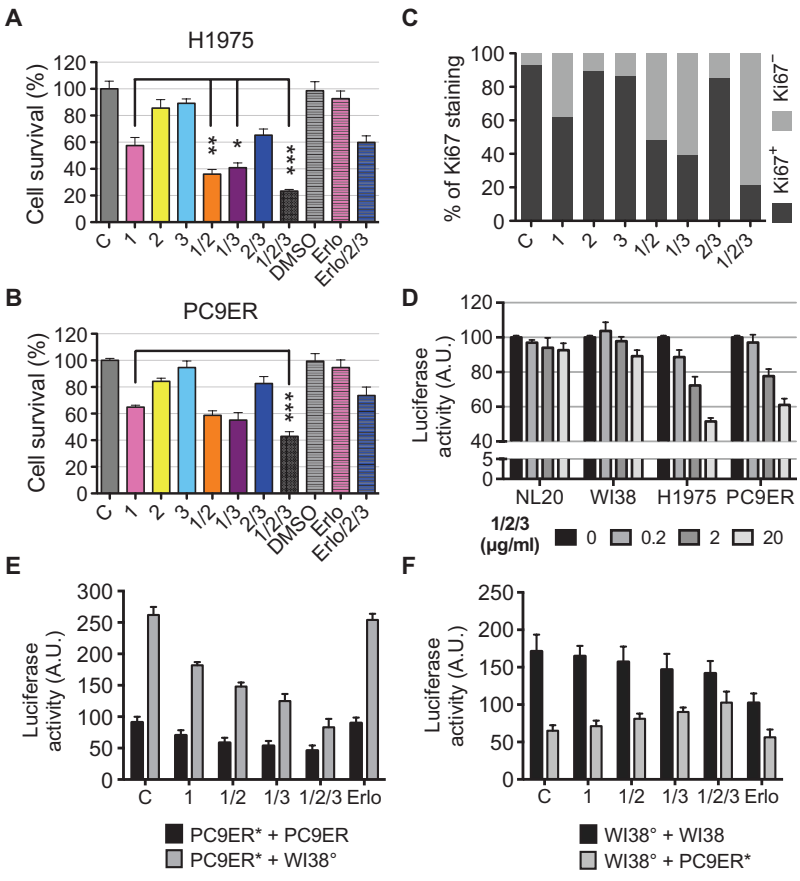

Fig. 5. A triple mAb combination reduces the ability of stromal cells to support tumor cells and more strongly inhibits NSCLC than normal cells. (**A** and **B**) Survival of cells cultured for 4 days with erlotinib (Erlo) (1 μM) or antibodies to EGFR (1), HER2 (2), or HER3 (3), singly or in combination. Data are means ± SD from three experiments. *$P \leq 0.05$, **$P \leq 0.01$, ***$P \leq 0.001$,one-way ANOVA with Bonferroni's comparison. (**C**) Quantification of Ki67-stained H1975 cells treated for 2 days with the indicated mAb combinations (total concentration, 10 μg/ml). Data are representative of two experiments. (**D**) Luciferase signals in NL20 and WI38 cells transfected with a Cherry-*Renilla* construct and in PC9ER and H1975 cells transfected with a GFP-firefly construct, each treated for 3 days with the indicated concentrations of the triple mAb combination (1/2/3). Data are means ± SD from three experiments. (**E** and **F**) Proliferation of PC9ER (left) and WI38 cells (right) assessed by luciferase signal in 1:1 cocultures with GFP-firefly–labeled PC9ER* cells and Cherry-*Renilla*–labeled WI38° cells treated for 5 days with the indicated antibody combinations (total concentration, 10 μg/ml) or erlotinib (1 μM). Data are means ± SD from three experiments.

antibody-only mixtures (fig. S7A) over those containing a mAb and a TKI (*22*).

For in vivo tests, we subcutaneously inoculated CD1-nu/nu mice with NSCLC cells (H1975). Eleven days later, tumor-bearing animals were randomized into groups of 10 mice, which were treated once every 3 days with antibodies to EGFR, HER2, and HER3, as well as with all possible combinations (Fig. 7C). In line with our cell culture results (Figs. 1A and 5A), the EGFR-specific mAb partly inhibited tumor growth when singly applied, but neither HER2-specific nor HER3-specific mAb, nor their combination, showed inhibitory activity in mice. Although the paired combinations were partially inhibitory, only the triple mAb combination exerted strong and lasting inhibitory effects on tumor growth as well as on animal survival (Fig. 7, C and D, and fig. S7B). Conceivably, the nearly complete tumor growth suppression we observed was due to the ability of the mAb mixture to induce the degradation of TKI-resistant EGFR.

DISCUSSION

Layered signaling networks, such as the HER/ERBB web, often respond to pharmacological and other perturbations by activating dormant feedback regulatory loops (*43*–*45*). Here, we report a previously uncharacterized example that involves an increase of HER2 and HER3 abundance, along with MET and ERK activation. A drug-induced compensatory increase in HER3 abundance was previously reported (*40*, *46*), and HER2 is implicated in the function of mutant EGFRs (*47*, *48*). We attribute the relatively weak response of lung cancer patients to cetuximab in several clinical trials [reviewed in (*49*)] to the compensatory increase of HER2 and HER3, MET activation, and consequent rebounds of active EGFR and ERK. As in other cases, overcoming compensatory feedback regulation requires either combining different drugs or applying broader-specificity drugs. Indeed, the second generation of EGFR inhibitors, such as the clinically approved afatinib, are designed to act as broader-specificity TKIs (*50*), and they show promising effects in the context of NSCLC (*21*, *22*).

Wider specificity might be achieved also by combining antibodies against different antigens. To nullify feedback activation of EGFR, we combined three antibodies, each targeting a distinct receptor. Similarly, combining antibodies to EGFR and HER2 inhibited different tumor models better than did the respective single antibodies (*34*, *51*). In analogy to heterocombinations (antibodies targeting different antigens), homocombinations—namely, mixtures of antibodies targeting different epitopes of the same antigen—are reported to act better than individual components of the mixture, as long as steric hindrance is avoided (*35*–*38*). Antibody mixtures bear clinical relevance: following experiments that showed strong tumor inhibition in animal models (*52*), relatively large breast cancer clinical trials successfully combined with chemotherapy two humanized mAbs against HER2, trastuzumab and pertuzumab (*53*). Similarly, a mixture of two EGFR-specific antibodies has been selected for ongoing clinical trials on the basis of both synergistic antitumor effects in animal models (*54*) and an ability to overcome resistance to cetuximab (*55*). In similarity to the triple mAb combination we tested herein, both homo- and heterocombinations of HER/ERBB mAbs resulted in enhanced receptor degradation (*35*–*38*, *54*), implying that target destruction underlies tumor retardation. Nevertheless, enhanced recruitment of immune effector cells (*56*) or inhibition of receptor interactions might also be involved in the improved antitumor effects of selected antibody mixtures.

It is worthwhile comparing mechanisms that confer resistance to TKIs and processes that putatively underlie resistance to EGFR-specific antibodies. One fundamental difference entails the fate of EGFR: whereas antibodies commonly target EGFR to a dynamin-dependent endocytic pathway and to degradation in lysosomes (*35*) [with the exception of irreversible TKIs, which target HER2 and EGFR to proteasomal degradation

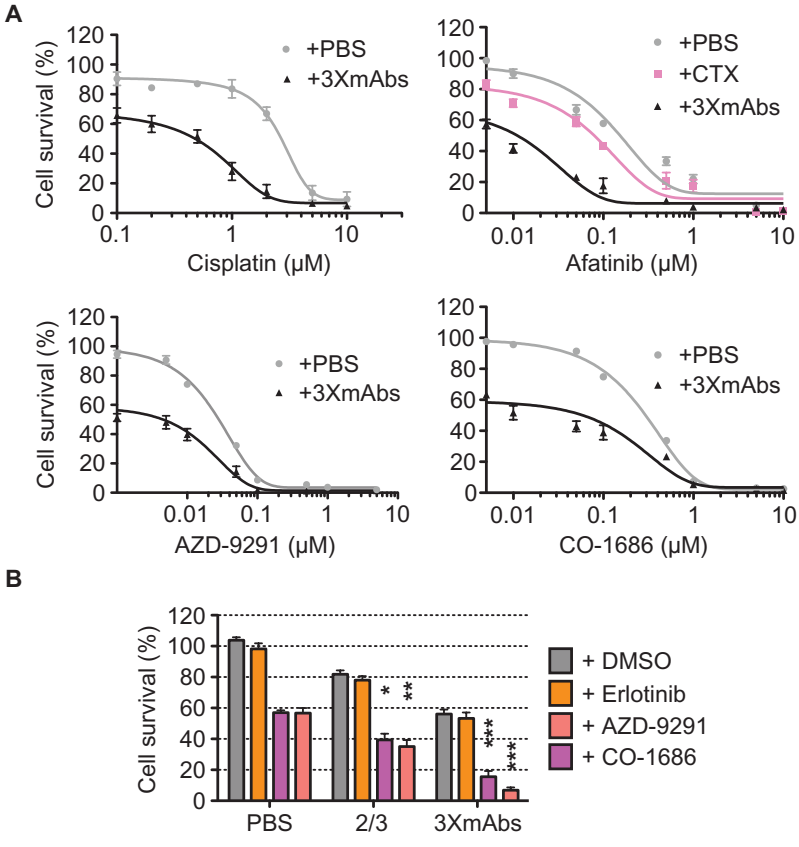

Fig. 6. A triple mAb combination sensitizes NSCLC cells expressing EGFR T790M to both a chemotherapeutic agent and novel TKIs. (A) Survival of PC9ER cells treated for 48 hours with increasing concentration of either cisplatin (upper left) or the indicated novel EGFR-specific TKIs. PBS was used as a control. In addition, cells were cotreated with cetuximab (CTX; 10 μg/ml) or with a triple combination of antibodies to EGFR, HER2, and HER3 (3XmAbs; 10 μg/ml). Data are means ± SD of three independent experiments. (B) Survival of PC9ER human NSCLC cells treated for 48 hours with the indicated TKIs [used at their specific half maximal inhibitory concentrations (IC_{50}), either alone or with the double combination of mAbs 12 and 33 (total concentration,10 μg/ml) against HER2 and HER3, respectively] or with the triple mAb combination [mAbs 565, 12, and 33 (total concentration, 10 μg/ml) against EGFR, HER2, and HER3]. Data are means ± SD of three independent experiments. *$P \leq 0.05$, **$P \leq 0.01$, ***$P \leq 0.001$, two-way ANOVA with Tukey's comparison.

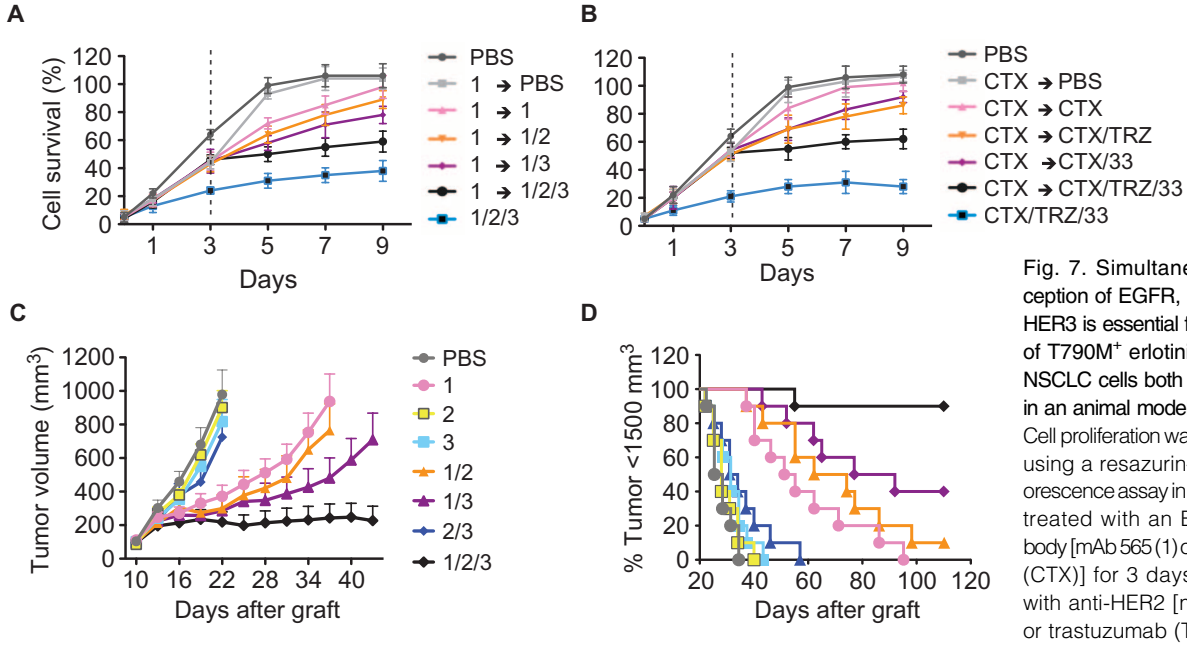

Fig. 7. Simultaneous interception of EGFR, HER2, and HER3 is essential for inhibition of T790M$^+$ erlotinib-resistant NSCLC cells both in vitro and in an animal model. (A and B) Cell proliferation was assessed using a resazurin-based fluorescence assay in H1975 cells treated with an EGFR antibody [mAb 565 (1) or cetuximab (CTX)] for 3 days and then with anti-HER2 [mAb 12 (2) or trastuzumab (TRZ)], anti-HER3 [mAb 33 (3)], or their combination. PBS and the triple mAb treatments (day 0) served as controls. Data are means ± SD from three experiments. (C) Tumor growth of subcutaneous H1975 NSCLC cell (3×10^6) xenografts in CD1-nu/nu mice treated once every 3 days with the indicated antibodies (0.2 mg per mouse per injection). Data are means ± SEM from 10 mice in each group. (D) Kaplan-Meier survival analysis of tumor-bearing mice in (C). Mice were euthanized when tumor volumes reached 1500 mm^3.

(*57*)], TKIs prevent rather than accelerate EGFR degradation, a process instigated by receptor autophosphorylation. Nonetheless, compensatory pathways stimulated by both TKIs and mAbs engage MET and HER3. For example, Engelman and colleagues (*13*) reported that the oncogenic receptor tyrosine kinase MET can phosphorylate HER3, leading to activation of the PI3K-AKT pathway. Our results raise the interesting possibility that mutation-activated EGFRs actually inhibit MET, but this inhibition was removed when EGFR is sorted by mAbs for degradation in lysosomes. Under these conditions, MET formed complexes with HER3 and undergoes activation, similar to the mechanism instigated by TKIs. However, unlike TKIs, we found that EGFR-specific antibodies activated ERK rather than the AKT pathway. ERK activation likely requires transcriptional increase of HER2 because this receptor, unlike HER3, strongly couples with SHC and the RAS-ERK pathway.

Future studies will likely identify sensors that propel compensatory responses to TKI and mAbs and also compare broader-specificity TKIs, like afatinib, and mAb mixtures in animals and in clinical settings. Notably, in contrast to TKIs, antibodies offer target destruction, absolute specificity, and the ability to cooperate with chemotherapy. Regardless of the exact mechanism of their action, the triple combination of mAbs we characterized in vitro and in animals offers a feasible pharmacological option for treating numerous lung cancer patients who inevitably develop resistance to the currently applied inhibitors of EGFR. Because mAbs targeting EGFR (like cetuximab) or HER2 (like trastuzumab) are already approved for clinical use and HER3-specific mAbs similar to NG33 (such as MM-121) are currently in clinical trials, our approach presents a realistic strategy for overcoming drug resistance.

MATERIALS AND METHODS

Antibodies and reagents

EGFR-specific mAbs 565 and 111, HER2-specific mAbs 12 (N12) and 26 (L26), and HER3-specific mAbs 33 (NG33) (*58*) and 252 (XC252) were generated in our laboratory. The total amount of antibodies used in all in vitro treatments was 10 μg/ml. For Western blotting, we used ERK2- and HER3-specific antibodies from Santa Cruz Biotechnology, antitubulin and phosphorylated ERK–specific antibodies from Sigma-Aldrich, and an EGFR-specific antibody from Alexis. Antibodies to phoshorylated EGFR (Tyr^{1068}), HER2, phoshorylated HER2 ($Tyr^{1221–1222}$), phosphorylated HER3 (Tyr^{1289}), phosphorylated MET ($Tyr^{1234–1235}$), MET, phosphorylated AKT (Thr^{3083} or Ser^{473}), and AKT were obtained from Cell Signaling Technology. The Ki67-specific antibody was from Cell Marque. Antibodies to EGFR, HER2, HER3, and MET were respectively conjugated with the following fluorophores: Alexa Fluor 488, allophycocyanin, and two different phycoerythrin dyes (BioLegend Inc.), and used for flow cytometric analysis. Erlotinib and afatinib were obtained from LC Laboratories, and AZD-9291 and CO-1686 were obtained from Selleckchem. 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) and resazurin were obtain from Sigma-Aldrich.

Cell cultures

The H1975 lung cancer cell line (with EGFR mutations L858R and T790M) was obtained from the American Type Tissue Culture Collection (ATCC). Erlotinib-resistant PC9ER cells (del746–750 + T790M) were derived in vitro from the PC9 (del746–750) cell line. NL20, an immortalized human lung epithelial cell line, was obtained from ATCC. WI38 lung fibroblasts were a gift from M. Oren. NSCLC cell lines were maintained in RPMI 1640 supplemented with 10% fetal calf serum (FCS; Life Technologies) and antibiotics. NL20 cells were maintained in F12K medium. Cocultures of WI38 and PC9ER or H1975 cells were maintained in Dulbecco's modified Eagle's medium supplemented with sodium pyruvate and 5% FCS (Life Technologies).

Western blotting

Cells were grown under specified conditions or treated as indicated, washed twice with cold PBS, and scraped into lysis buffer [50 mM Hepes (pH 7.5), 10% glycerol, 150 mM NaCl, 1% Triton X-100, 1 mM EDTA, 1 mM EGTA, 10 mM NaF, 0.1 mM Na_3VO_4, and a complete protease inhibitor cocktail]. Thereafter, lysates were centrifuged at 14,000*g* for 15 min at 4°C. The supernatant was used for subsequent procedures. Western blot analyses were conducted after protein separation using gel electrophoresis and transfer to nitrocellulose membranes. Immunoblotting was performed according to the antibody manufacturers' recommendation. Antibody binding to membrane blots was detected using horseradish peroxidase–secondary antibodies (Jackson ImmunoResearch Laboratories), followed by treatment with ECL Western blotting detection reagents (GE Healthcare).

Cell proliferation and survival assays

Cell proliferation was assessed using the MTT assay. Cells were plated in 96-well plates (2000 cells per well) in triplicate. Twenty-four hours later, the medium was changed, and cells were treated as indicated. After 2, 3, or 4 days, the MTT reagent was added to the cells, and 1 to 2 hours later, the formazan crystals that formed were dissolved in SDS-DMF (*N*,*N*′-dimethylformamide) solution. Absorbance was measured at 570 nm. All experiments were performed in medium containing 2% FCS unless otherwise indicated.

Real-time PCR

For mRNA expression, total RNA was isolated using the PerfectPure RNA Cultured Cell Kit (5 Prime). Complementary DNA was synthesized using the miScript kit (Qiagen). Specific *HER2*, *HER3*, and *GAPDH* (glyceraldehyde phosphate dehydrogenase) primers were designed using the Primer3 software as follows: HER2 forward, 5′-gaggtggtacttcaattgcgactca-3′; HER2 reverse, 5′-agggaaggcggacgcctgat-3′; HER3; forward, 5′-tgcagtggattcgagaagc-3′; HER3 reverse, 5′-agttcaacatgacgaagatg-3′; GAPDH forward, 5′-acagttgccatgtagacc-3′; GAPDH reverse, 5′-tttttggttgagcacagg-3′. Real-time quantitative PCR analyses were performed using SYBR Green (Qiagen or Applied Biosystems) and specific primers. Quantitative PCR signals (cT) were normalized to *GAPDH*.

Immunofluorescence analyses

Cells were grown on glass coverslips in 24-well plates. For Ki67 staining, H1975 cells were fixed for 20 min at room temperature with 3.7% paraformaldehyde (PFA), followed by permeabilization in 0.3% Triton X-100 for 5 min, and then rinsed three times in saline containing 0.1% Tween 20 (PBS-T). Cells were then incubated for 1 hour with 3% albumin in PBS-T, followed by incubation with a primary antibody in PBS-T containing 1% albumin (overnight at 4°C), washed in PBS-T, and incubated with a fluorescently labeled secondary antibody (Alexa Fluor 488) and DAPI for 1 hour at room temperature. After three additional washes, the coverslips were placed, cell face down, onto 10-μl drops of the ProLong Gold antifade reagent (Invitrogen) and left overnight protected from light. Samples were examined using a DeltaVision (Applied Precision) microscope. To analyze the increase in HER2 and HER3 abundance and to analyze MET and HER3 colocalization, cells were treated as indicated, acid-washed at 4°C for 30 min, rinsed in saline, and fixed for 20 min in 3.7% PFA. The acid wash step was performed using an acidic solution [150 mM NaCl, 0.1 mM glycine (pH 2.1)]. Samples were processed as

aforementioned, with the exception that Triton X-100 or Tween 20 was used only after incubation with the primary antibody.

siRNA transfection experiments

Specific On-Target EGFR-specific siRNA oligonucleotides and scrambled siRNAs (siCTRL), as well as On-Target HER2 and HER3 siRNAs, were purchased from Dharmacon (GE Healthcare). siRNA oligonucleotide transfection was performed using Oligofectamine (Invitrogen) according to the manufacturer's instruction.

Coimmunoprecipitation assays

For determination of complex formation, cells were treated as indicated and directly extracted (fig. S4B) or treated with an acidic solution for 30 min on ice before lysis in Triton X-100–containing buffer (Fig. 4C). Thereafter, complexes were immunoprecipitated from 500 µl of lysate with the indicated antibody and agarose beads conjugated to anti-rabbit immunoglobulin G $F(ab')_2$ fragments (Cell Signaling Technology) for 2 hours at 4°C. Complexes were washed three times in HNTG buffer [20 mM Hepes (pH 7.5), 0.15 M NaCl, 0.1% Triton X-100, and 10% glycerol], resuspended in gel sample buffer, and boiled for 10 min. Samples were subjected to electrophoresis in 8% polyacrylamide gels and electroblotted onto nitrocellulose membranes. Immunoblotting was then performed with the indicated antibodies.

Luciferase promoter reporter assays

Plasmids containing the promoters of HER2 (pLightSwitch_HER2prom) or HER3 (HPRM23883) were from Active Motif and GeneCopoeia, respectively. Cells were transfected with the pLightSwith_HER2prom or the *HER3* promoter reporter plasmid. In the case of the *HER2* promoter, cells were transfected also with pGL3-Control-Vector (from Promega), which contained a firefly luciferase under a constitutive SV40 promoter. Luciferase activity was assayed using the LightSwitch Luciferase Assay Kit (Active Motif) and Steady-Glo (Promega) or Secrete-Pair Dual Luminescence Assay Kit (GeneCopoeia). For the experiments presented in figs. S1B and S3, plasmids containing the HER2 (pNeuLite) or HER3 (HPRM23883) promoter regions were purchased from Addgene and GeneCopoeia, respectively. The *HER2* promoter region was amplified using *PfuTurbo* DNA polymerase (Stratagene) with the primers 5′-ggcgtcccggcgctaggaaggcctgcgcagagag-3 (forward) and 5-ctctcttcgcgcaggccttcctagcgccgggacgcc-3 (reverse) and cloned into pGM3-Basic-Vector (Promega; pHER2-prom-LucFF). The *HER3* promoter region was cloned into the pGM3-Basic-Vector (pHER3-prom-LucFF). *Renilla* luciferase from the pRL plasmid (Promega) cloned into pmCherry-C1 (pmCheRNL) was a gift from A. Levi. H1975 cells were cotransfected using Lipofectamine 2000 (Invitrogen) with pHER2-prom-LucFF and pmCheRNL or pHER3-prom-LucFF and pmCheRNL. Cells were cotransfected with pmCheRNL and pGL3-Control-Vector (positive control) or pGL3-Basic Vector (negative control). Cells were then selected for 3 weeks under puromycin (1 µg/ml). Stable derivative lines were used, and luciferase activity was measured using the Dual-Luciferase Reporter Assay Kit (Promega) and VictorX Luminometer (Promega).

Flow cytometric analyses

To determine surface receptor abundance, cells were treated with trypsin and washed twice in saline containing 1% (w/v) albumin. The cells were then incubated for 1 hour at 4°C with mAbs against EGFR, HER2, and HER3 (10 µg/ml). After two washes, the cells were incubated for 1 hour at 4°C with an anti-mouse antibody coupled to Alexa Fluor 488. Cells that were pretreated for 48 hours with antibodies were acid-washed (pH 2.2) for 5 min before trypsinization. The capacity of the HER mAbs to bind the corresponding receptor at the cell surface was correlated to the fluorescence intensity measured using the LSR II flow cytometer.

Cell survival analysis using luciferase assays

WI38 and NL20 cells were stably transfected with a pmCheRNL construct. Similarly, PC9ER and H1975 cells were infected with GFP–firefly luciferase lentiviral particles (GeneCopoeia). After 3 weeks of drug selection, labeled cells were treated with mAbs, and relative Firefly or *Renilla* luciferase signals were determined using *Renilla*-Glo or Steady-Glo Luciferase Assay kits (Promega).

Cell survival analysis using resazurin

Cells were plated in 96-well plates (400 cells per well) in triplicate. Twenty-four hours later, the medium was changed, and cells were treated as indicated. At the indicated time points, resazurin was added (10% of a well's volume), and cells were incubated for 2 to 3 hours at 37°C. Relative fluorescence intensity was determined using the VictorX microplate reader (Promega). Thereafter, the medium was replaced according to the indicated treatment, and the procedure was repeated every other day.

Tumorigenic cell growth in mice

All animal studies were approved by the Weizmann Institute's Review Board. Tumor-bearing CD1-nu/nu mice were randomized into groups of 10 and injected subcutaneously in the right flank with cancer cells (3×10^6 per mouse). mAbs were injected intraperitoneally at 200 µg per mouse per injection, once every 3 days. Tumor volume and body weight were evaluated twice and once per week, respectively. Mice were euthanized when tumor size reached 1500 mm^3.

Statistical and data analyses

Cell sorting data were analyzed using the FlowJo version 10.0.7 software (Tree Star Inc.). The other data were analyzed using the GraphPad Prism software. Other statistical analyses were performed using one-way ANOVA with Bonferroni's comparison test or two-way ANOVA with Tukey's test ($*P \leq 0.05$; $**P \leq 0.01$; $***P \leq 0.001$).

SUPPLEMENTARY MATERIALS

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Fig. S1. mAbs and siRNAs against *EGFR* up-regulate *HER2* and *HER3* in NSCLC cells.
Fig. S2. Selection of mAbs against HER family members on the basis of their ability to decrease survival of lung cancer cells.
Fig. S3. An antibody against EGFR activates the promoters of *HER2* and *HER3* and induces positive feedback regulation of ERK.
Fig. S4. Simultaneous inhibition of EGFR, HER2, and HER3 by using specific siRNAs disrupts HER-to-MET crosstalk.
Fig. S5. Combinations of mAbs against HER family members induce no apoptosis of lung cancer cells.
Fig. S6. Normal fibroblasts increase the proliferation of lung cancer cells, and the triple mAb mixture better inhibits the proliferation of cancer cells than that of normal cells.
Fig. S7. A triple mAb combination inhibits in vivo growth of NSCLC cells expressing a double-mutant form of EGFR.

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Acknowledgments: We thank M. Sela for continuous help, R. Rotkopf for statistical analyses, and A. Levi for advice and plasmids. **Funding:** Our laboratory is supported by the European Research Council, the Seventh Framework Programme of the European Commission (LUNGTARGET consortium), the German-Israeli Project Cooperation (DIP), the Israel Cancer Research Fund, and the Dr. Miriam and Sheldon G. Adelson Medical Research Foundation. Y.Y. is the incumbent of the Harold and Zelda Goldenberg Professorial Chair. Our studies were performed in the Marvin Tanner Laboratory for Research on Cancer. **Author contributions:** M.M. and Y.Y. designed the study; M.M., N.G., M.L., T.M.S., M.S.-S., M.D.'O., and A.A.-H. performed the experiments; M.M. and Y.Y. analyzed the data; J.D. provided lung cancer cell lines; and M.M., N.G., and Y.Y. wrote the manuscript. **Competing interests:** The authors declare that they have no competing interests. **Data and materials availability:** Requests for reagents may be addressed to Y.Y.

Submitted 13 October 2014
Accepted 12 May 2015
Final Publication 2 June 2015
10.1126/scisignal.aaa0725
Citation: M. Mancini, N. Gaborit, M. Lindzen, T. Meir Salame, M. Dall'Ora, M. Sevilla-Sharon, A. Abdul-Hai, J. Downward, Y. Yarden, Combining three antibodies nullifies feedback-mediated resistance to erlotinib in lung cancer. *Sci. Signal.* **8**, ra53 (2015).

Science Signaling

Combining three antibodies nullifies feedback-mediated resistance to erlotinib in lung cancer

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Sci. Signal. **8** (379), ra53.
DOI: 10.1126/scisignal.aaa0725

Triple teaming cancer

Patients with lung cancer that is driven by mutant epidermal growth factor receptor (EGFR) are resistant to EGFR-targeted kinase inhibitors, such as erlotinib. Mancini *et al.* found that resistance may be overcome by inhibiting erlotinib-induced feedback activation of other EGFR family members and the receptor tyrosine kinase MET. In culture and in mice, combining the three antibodies targeting three different EGFR family members inhibited the growth of tumor cells that survived treatment with erlotinib or a single antibody targeting only EGFR. Thus, attacking triple targets subverts the resistance-mediating network rewiring induced by single-agent therapies.