

# Epidermal Growth Factor Receptor (EGFR) Ubiquitination as a Mechanism of Acquired Resistance Escaping Treatment by the Anti-EGFR Monoclonal Antibody Cetuximab

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## Abstract

Cetuximab is an epidermal growth factor receptor (EGFR)-blocking antibody that has been approved for treatment of patients with metastatic colorectal cancer. In this study, we investigated biochemical changes in signaling pathways of a cetuximab-resistant subline of DiFi colorectal cancer cells (DiFi5) that was developed by exposing the parental sensitive cells to subeffective doses of cetuximab over an extended period of time. Compared with parental DiFi cells that express high levels of EGFR and in which cetuximab induces apoptosis, the cetuximab-resistant DiFi5 cells showed markedly lower protein levels of EGFR, an increased association of EGFR with Cbl, and an increased ubiquitination of EGFR. DiFi5 cells also had a markedly higher level of Src-Y416 phosphorylation both at baseline and on EGF stimulation. Although EGFR levels were low, DiFi5 cells responded to EGF stimulation with robust phosphorylation of EGFR on Y845 and strong phosphorylation of Akt and extracellular signal-regulated kinase, comparable to those of parental cells. Most importantly, inhibition of Src kinase activity with PP2 reversed the resistance of DiFi5 cells to cetuximab-induced apoptosis without affecting the levels of EGFR in the cells. Our results indicate that colorectal cancer cells may develop acquired resistance to cetuximab via altering EGFR levels through promotion of EGFR ubiquitination and degradation and using Src kinase-mediated cell signaling to bypass their dependency on EGFR for cell growth and survival. [Cancer Res 2007;67(17):8240–7]

## Introduction

The complexity of signaling networks developed in cancer cells frequently results in redundancy and overlap of cell survival and proliferation pathways, potentially allowing cancer cells to circumvent the therapeutic effects of targeting one single growth or survival signaling pathway. This capability poses a great challenge to the effectiveness of novel therapeutic agents rationally designed to target specific cell-surface receptors known to play a role in cancer initiation and progression, such as the epidermal growth factor receptor (EGFR; refs. 1, 2). The interacting or complementing signaling networks may render cancer cells inherently resistant to EGFR-targeted therapy, despite the expres-

sion and functional role of EGFR in the growth and survival of the cancer cells. Only in a small percentage of cancer patients, their cancer cells rely on EGFR for growth and survival to a great extent or even exclusively and, as a result, pharmacologic inhibition of EGFR in these patients elicits positive therapeutic responses (1, 2). The recent demonstration of objective antitumor activity in 10% to 20% patients from several pivotal clinical studies involving patients with several different types of solid tumors, including colorectal cancer, non-small cell lung cancer, and head and neck cancer, has led to the regulatory approval of cetuximab and panitumumab (3, 4),<sup>3</sup> two monoclonal antibodies (mAb) that block ligand binding to EGFR, and gefitinib and erlotinib (5–7), two small-molecule inhibitors that are ATP analogues of the EGFR tyrosine kinase.

Many studies have explored the inherent resistance of tumors to EGFR-targeted therapy, but little effort has been applied to whether acquired resistance, presumably caused by target alteration and subsequent activation of substitutive or alternative signaling pathways, allows cancer cells to bypass the therapeutic effects of EGFR inhibition. From a clinical practice point of view, acquired resistance happens when a drug is initially effective in controlling disease, decreasing tumor burden, or inhibiting tumor growth, but the patient has a relapse and becomes insensitive to further therapy. An earlier study reported phenotypic changes in variants of A431 vulvar squamous carcinoma cells that were obtained from recurrent tumor xenografts after two consecutive cycles of therapy with one of three different anti-EGFR mAbs: mR3, hR3, or cetuximab (8). Following initial response after a 2-week period of treatment, which led to regression of the A431 xenografts, the tumors reappeared at the site of injection after a prolonged latency period, with most of the recurrent tumors being refractory to a second round of the antibody therapy. Interestingly, the sublines established from the relapsed tumors retained high levels of EGFR expression, normal sensitivity to cetuximab treatment in culture, and an unaltered growth rate, indicating that the direct drug target (EGFR) was not modified on the tumor cells. However, the A431 cell variants exhibited an accelerated growth rate *in vivo*; five of the six variant sublines contained higher levels of vascular endothelial growth factor (VEGF) than did the parental cells (8). Another study reported similar results obtained from GEO colorectal cancer cell xenografts that recurred after an initial response to cetuximab and gefitinib treatment (9). Resistant sublines established from the recurrent xenografts exhibited marked increases in mitogen-activated protein kinase phosphorylation and increased expression of cyclooxygenase-2 and VEGF. The cetuximab- or gefitinib-resistant GEO cells were sensitive to ZD6474, an inhibitor of VEGF

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doi:10.1158/0008-5472.CAN-07-0589

<sup>3</sup> <http://www.fda.gov/bbs/topics/NEWS/2006/NEW01468.html>

receptor (9). These previous *in vivo* studies provided experimental evidence for a possible secondary “driving force” in cancers that relapse after an initial response to EGFR-targeted therapy and are important in developing clinically relevant strategies of novel therapeutics aimed at overcoming or preventing acquired resistance to EGFR-targeted therapy.

It is not clear from these studies, however, whether acquired resistance is a *de novo* process or represents selection of preexisting subclones. Furthermore, it is not clear whether the mechanisms are primarily mediated by the tumor or by tumor-stromal interactions, particularly in the cases in which sublines derived from the relapsed tumor cells remain sensitive to the anti-EGFR agents in cell culture (8).

Previously, we reported that DiFi colorectal cancer cells rely exclusively on EGFR-mediated cell signaling for growth and survival; treatment of the cells with either EGFR-blocking antibodies or small-molecule receptor tyrosine kinase inhibitors leads to cell death by triggering programmed cell death via apoptosis (10–14). To investigate potential mechanisms of acquired cetuximab resistance, we exposed DiFi colorectal cancer cells to cetuximab at doses that killed 80% to 90% of the cells in the first round of treatment and then maintained the surviving 10% to 20% of the cells in long-term uninterrupted subculture with cetuximab, gradually increasing the dose of cetuximab in cell culture. In this study, we report the successful establishment of a subline of cetuximab-resistant DiFi cells, termed DiFi5. In contrast to the results from previous studies by other groups (8, 9), we found that the resistant DiFi5 cells show marked biochemical changes, including enhanced ubiquitination and degradation of EGFR and an increased activity of Src kinase compared with parental DiFi cells. We also explored approaches that may resensitize DiFi5 cells to cetuximab, showing that the increased Src activity present in

DiFi5 plays a causal role in resistance to cetuximab. As inhibitors to the EGFR are in clinical use and Src inhibitors are in clinical trials, our results suggest a novel combinatorial therapy targeting either concurrently or sequentially EGFR and Src for cancer treatment.

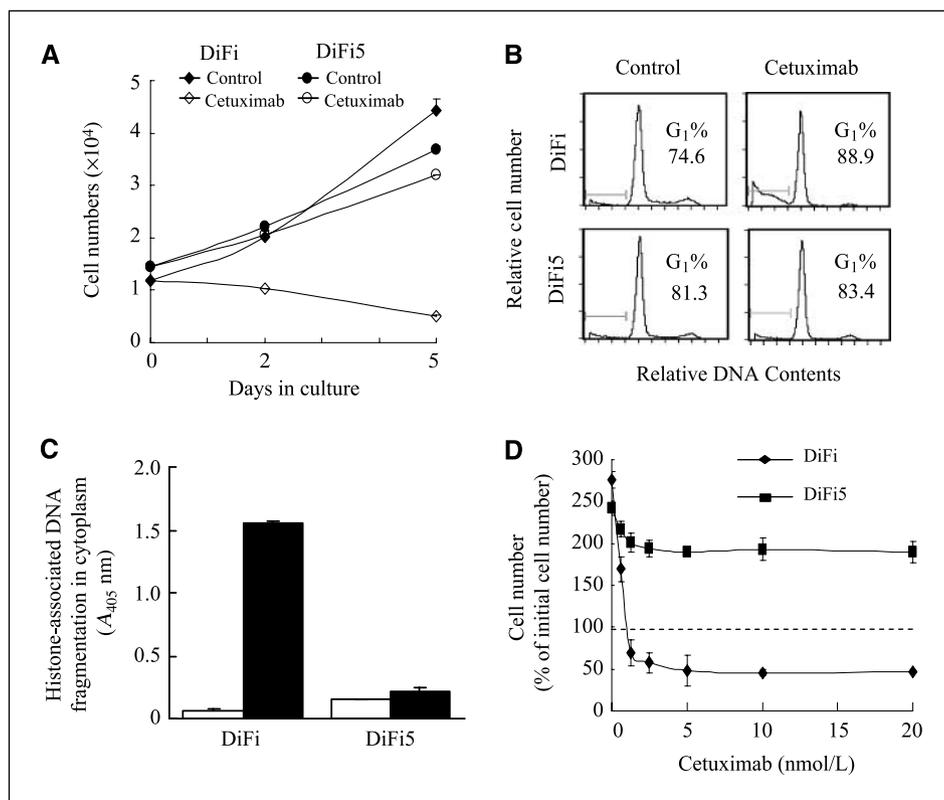
## Materials and Methods

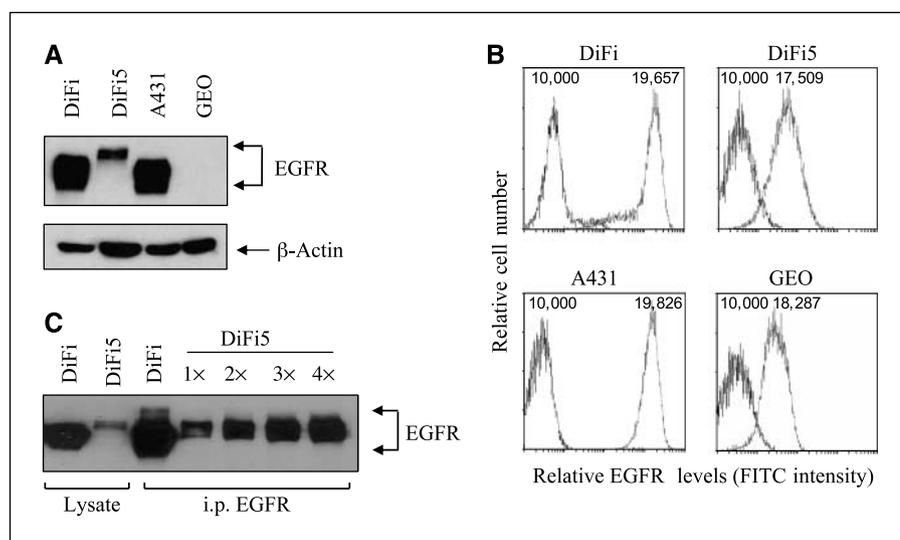
**Materials.** The human-mouse chimeric anti-EGFR antibody cetuximab was provided by ImClone Systems, Inc. Antibodies directed against total and phosphorylation-specific (S473) Akt and phosphorylation-specific (T202/Y204) extracellular signal-regulated kinase (ERK) were obtained from Cell Signaling Technology, Inc. Anti-ERK antibody was from Santa Cruz Biotechnology, Inc. The anti-EGFR antibody used for Western blot analysis was from Sigma-Aldrich Co. All other reagents were purchased from Sigma Chemical unless otherwise specified.

**Cells and cell culture.** The parental DiFi colorectal adenocarcinoma cell line has previously been described (10–14). The cetuximab-resistant DiFi cells (DiFi5) were created by exposing the parental DiFi cells to 10 nmol/L cetuximab, which killed >80% of the cells, followed by maintenance of the surviving cells for an extended period in gradually increased concentrations of cetuximab peaking at 100 nmol/L. The A431 vulvar squamous carcinoma cell line and GEO colorectal adenocarcinoma cell line have previously been described (15, 16). All cell lines were maintained in DMEM containing 10% fetal bovine serum (FBS), 2 mmol/L glutamine, 100 units/mL penicillin, and 100 µg/mL streptomycin and cultured in a humidified atmosphere of 95% air and 5% CO<sub>2</sub> at 37°C.

**Cell survival and proliferation.** Cell proliferation assays were done on 12- or 24-well culture plates. Absolute cell numbers were determined by harvesting the cells by trypsinization and counting the cells with a Coulter counter. Relative cell growth and survival were assessed with a colorimetric assay by adding 50 µL of 10 mg/mL 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) to 0.5 mL of culture medium and incubating the cells for 2 h at 37°C in a CO<sub>2</sub> incubator; this was followed by cell lysis overnight at 37°C with 500 µL of lysis buffer containing 20% SDS in dimethyl formamide/H<sub>2</sub>O (1:1, v/v), pH 4.7. The absorbance of cell lysates

**Figure 1.** Growth and survival of DiFi and DiFi5 cells in the presence and absence of cetuximab. **A**, DiFi cells ( $1.1 \times 10^4$  per well) and DiFi5 cells ( $1.4 \times 10^4$  per well) were seeded into 12-well culture plates in 0.5% FBS medium and cultured in the presence or absence of 10 nmol/L cetuximab for 5 d. Cells were counted on days 2 and 5 with a Coulter counter after being harvested by trypsinization. **B**, DiFi and DiFi5 cells were cultured overnight (16 h) in 0.5% FBS medium with or without 10 nmol/L cetuximab. The cells were then harvested and subjected to cell cycle distribution analysis by flow cytometry, as described in Materials and Methods. **C**, DiFi and DiFi5 cells were treated as in (B). After treatment, the cells were lysed and a 10-µg aliquot of cell lysate from each group was used to measure the levels of cytoplasmic histone-associated DNA fragments by using an ELISA kit. **D**, DiFi and DiFi5 cells seeded into 24-well culture plates were exposed to the indicated concentrations of cetuximab for 72 h in medium containing 0.5% FBS. After the treatment, cells were subjected to an MTT colorimetric assay (incubation with the MTT dye for 2 h at 37°C, followed by cell lysis with an MTT lysis buffer overnight in a 37°C oven). Relative cell growth and survival of the cells were determined by comparing the absorbance of the cell lysates in each group after normalization to the absorbance of the initial cells seeded. The data were plotted against the concentrations of cetuximab used.





**Figure 2.** Decreased levels of total and cell-surface EGFR in DiFi5 cells relative to parental DiFi cells. **A**, total levels of EGFRs in DiFi and DiFi5 cells were compared by subjecting the cell lysates to SDS-PAGE and Western blot analysis with anti-EGFR antibody. The level of  $\beta$ -actin was used as a reference for cell lysate proteins loaded in the Western blot analysis. **B**, cell-surface EGFRs were measured by FACS with flow cytometry. The cells were collected for FACS analysis of the distribution of FITC-positive cells. The data are plotted for cell numbers versus the intensity of fluorescence. Total numbers of cells counted in each cell line (stained respectively with a control IgG and the anti-EGFR mAb 528) are shown at the top. **C**, one aliquot (200  $\mu$ g) of DiFi cell lysate was compared with incrementally increased aliquots (up to four times) of DiFi5 cell lysate for levels of EGFRs immunoprecipitated by cetuximab. The immunoprecipitates were subjected to Western blot analysis with an anti-EGFR antibody as in (A).

was measured at a wavelength of 570 nm, and the absorbance values of treated cells are presented as a percentage of the absorbance of untreated cells.

**Immunoprecipitation and Western blot analysis.** Cells were lysed in a lysis buffer containing 50 mmol/L Tris (pH 7.4), 150 mmol/L NaCl, 0.5% NP40, 50 mmol/L NaF, 1 mmol/L  $\text{Na}_3\text{VO}_4$ , 1 mmol/L phenylmethylsulfonyl fluoride, 25  $\mu$ g/mL leupeptin, and 25  $\mu$ g/mL aprotinin and clarified by centrifugation (14,000  $\times g$  for 30 min at 4°C). The protein concentration of cell lysates was determined using the Bradford Coomassie blue method (Pierce Chemical). For immunoprecipitation studies, cell lysates were incubated with primary antibodies, and the resultant immune complexes were precipitated with protein A-Sepharose beads (Amersham Biosciences). Whole-cell lysates or immunoprecipitated proteins were then separated by SDS-PAGE, transferred onto nitrocellulose by Western blotting, and probed with various primary antibodies and horseradish peroxidase-labeled secondary antibodies. The signals were visualized with an enhanced chemiluminescence detection kit (Amersham Biosciences).

**Quantification of apoptosis by ELISA.** An apoptosis ELISA kit was used to quantitatively measure the levels of histone-associated DNA fragments (mononucleosomes and oligonucleosomes) in cytoplasm after induced cell death. This colorimetric enzyme immunoassay was done exactly according to the manufacturer's instructions (Roche Diagnostics).

**Flow cytometric analysis.** For cell cycle distribution analysis, cells grown in 100-mm culture dishes (50–70% confluence) were harvested by trypsinization, washed twice with cold PBS, and fixed with 70% ethanol. The cells were stained for DNA with a solution containing 50  $\mu$ g/mL propidium iodide and 100  $\mu$ g/mL RNase I in PBS for 30 min at 37°C. The cells were filtered through a nylon mesh (50- to 70- $\mu$ m pore size) to remove cell clumps and then analyzed for cell cycle distribution with a FACScan flow cytometer (Becton Dickinson). To measure levels of cell-surface EGFRs, cells were harvested by trypsinization, resuspended into serum-free medium, and then chilled on ice for 20 min before being incubated with a murine anti-EGFR antibody (mAb 528) for 1 h on ice. After incubation, the cells were washed gently with cold PBS thrice and then incubated with a FITC-labeled rabbit anti-mouse immunoglobulin G (IgG) antibody for an additional 1 h. The cells were then collected for fluorescence-activated cell sorting (FACS) analysis of the distribution of FITC-positive cells. The data were plotted as the number of cells versus the intensity of cell fluorescence.

## Results

**Characteristics of DiFi colorectal cancer cells with acquired resistance to cetuximab.** DiFi colorectal cancer cells behave as if they are "addicted" to the EGFR; exposure of the cells to receptor-

saturating concentrations of cetuximab was sufficient to induce cell death via apoptosis (10–14). Figure 1 compares the growth and survival of the parental DiFi cells and DiFi5 cells in cell culture in the presence and absence of cetuximab. When cultured in regular medium, DiFi5 cells proliferated slightly slower than the parental cells (Fig. 1A). Flow cytometric analysis of cell cycle phase distribution showed that a slightly higher percentage of DiFi5 cells than parental DiFi cells (81.3% compared with 74.6%) were in the  $G_1$  phase of the cell cycle, and exposure to cetuximab increased the cell cycle distribution of DiFi5 cells only modestly (from 81.3% to 83.4%), whereas the percentage of parental cells in the  $G_1$  phase increased from 74.6% to 88.9% after exposure to cetuximab. Furthermore, treatment of parental DiFi cells with cetuximab resulted in cell death via apoptosis, as evidenced by an increased percentage of pre- $G_1$  phase cells (Fig. 1B), a higher level of histone-associated DNA fragmentation in the cytoplasm (Fig. 1C), and a reduction in the number of cells after 72 h of continuous exposure to cetuximab starting at doses as low as 1.25 nmol/L (Fig. 1D). In contrast, DiFi5 cells exhibited considerable resistance to cetuximab; despite being slightly growth inhibited compared with untreated cells, they continued to proliferate in the presence of cetuximab (up to 20 nmol/L) without loss of viable cells.

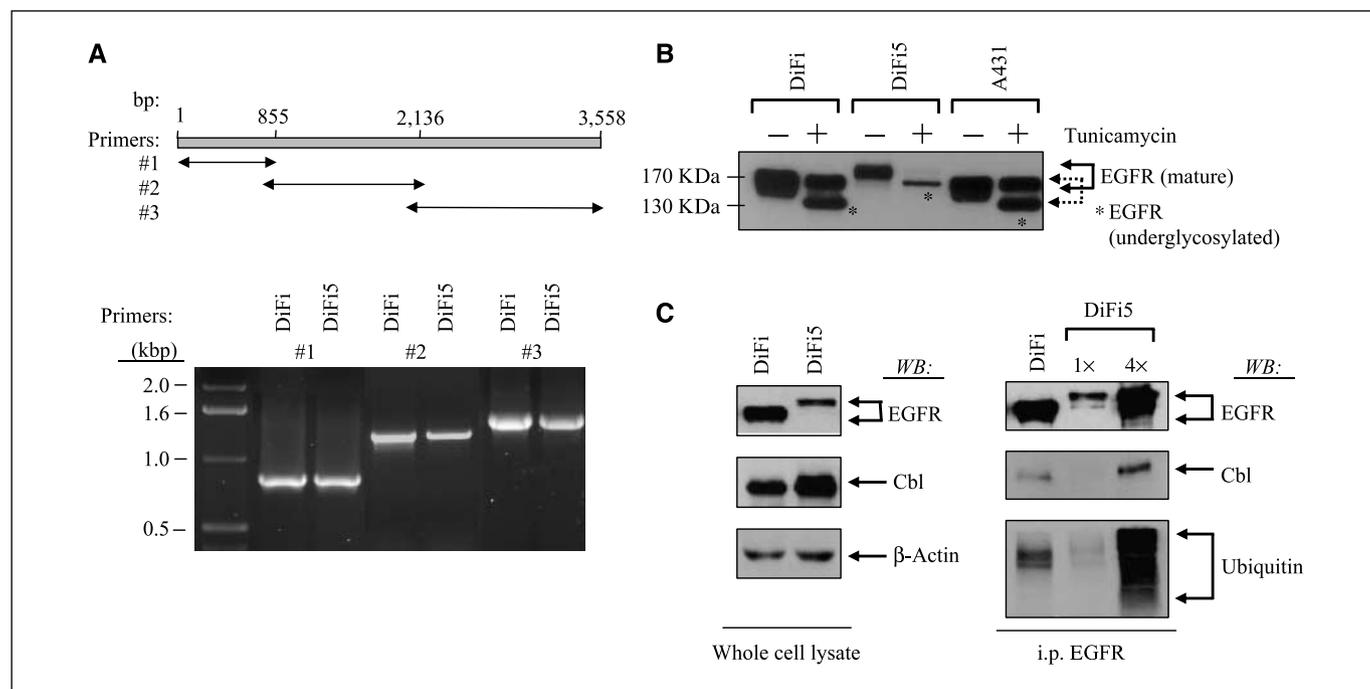
**Posttranslational modification of EGFR in cetuximab-resistant cells.** To determine whether the resistance of DiFi5 cells to cetuximab treatment was associated with alteration of EGFR cell-surface protein level or receptor binding by cetuximab, we first examined the total and cell-surface EGFR levels in DiFi5 cells relative to parental cells. Western blot analysis showed that the total level of EGFR in parental DiFi cells was comparable to that in A431 cells, a cell line well known for overexpression of EGFR (Fig. 2A). In contrast, the level of EGFR in DiFi5 cells, which was revealed as a "slow-migrating" band, was much lower than that in DiFi cells but still higher than that in GEO cells, another colon cancer cell line known to be sensitive to cetuximab treatment but with an EGFR level being barely detectable by conventional Western blot analysis (16, 17). Consistent with the results of Western blot analysis, FACS analysis with a different anti-EGFR antibody (mAb 528) showed that the level of cell-surface EGFR in DiFi5 cells was remarkably lower than that in parental DiFi cells or A431 cells but still higher than that in GEO cells (Fig. 2B). To

eliminate the possibility of potential structural alteration of EGFR in DiFi5 cells that would impair the recognition of the EGFR by cetuximab, we subjected one aliquot (200  $\mu$ g) of DiFi cell lysate and aliquots of DiFi5 cell lysates with incremental increases in protein up to four times (800  $\mu$ g) to EGFR immunoprecipitation by cetuximab and subsequent Western blot analysis with an EGFR antibody that recognizes the intracellular carboxyl domain of EGFR. We found that the total level of EGFR in DiFi5 cells was  $\sim$ 20% to 25% of that found in parental DiFi cells and the levels of EGFR immunoprecipitated by cetuximab were proportional to the amounts of cell lysates applied for the experiment, indicating that the EGFR in DiFi5 cells is recognizable by cetuximab. Information of the relative ratio (1:4 or 1:5) of EGFR contents in DiFi and DiFi5 cells was used in later studies when an adjustment of the EGFR content was needed to compare the levels of EGFR-associated proteins in DiFi and DiFi5 cells.

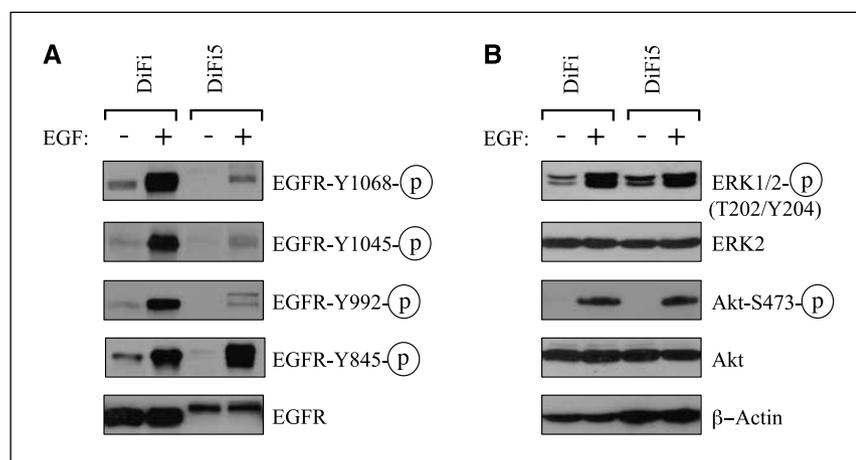
Slow-migrating forms of EGFR were previously reported in glioma cells as being caused by tandem duplication of exons in the extracellular or intracellular domains of EGFR (18–22). To determine whether the slow-migrating form of EGFR in DiFi5 cells may reflect EGFR mutation such as tandem duplication of exons, we used reverse transcription-PCR to amplify EGFR cDNA from DiFi and DiFi5 cells with three individual pairs of primers that collectively cover the whole range of EGFR-coding sequences. We found no difference between DiFi and DiFi5 cells in the size of the PCR products amplified with these primers (Fig. 3A). Sequence analysis of the PCR products did not reveal any mutation either (data not shown).

Because EGFRs are glycoproteins, we next examined whether the decreased electrophoretic mobility of EGFR in DiFi5 cells might be caused by altered glycosylation of the protein during its posttranslational maturation process. If this were indeed the case, the nascent (nonglycosylated) EGFRs would migrate during electrophoresis to the same position as that of EGFRs from parental DiFi or A431 cells. Tunicamycin, an inhibitor of *N*-acetylglucosamine transferases, inhibits the glycosylation of EGFR during its maturation process, leading to the appearance of two EGFR bands (nonglycosylated and glycosylated) on Western blot analysis (23). Figure 3B shows that in contrast to untreated DiFi, DiFi5, and A431 cells, the tunicamycin-treated cells contained a second, faster-migrating EGFR band, representing nascent EGFRs. However, the nascent EGFRs in DiFi5 cells still migrated slower than did the receptors in parental DiFi and A431 cells. These results suggest that the slow-migrating EGFRs in DiFi5 cells are unlikely to be a result of altered glycosylation of the receptor.

Another important posttranslational modification of proteins that may lead to a higher apparent molecule weight is protein ubiquitination due to the attachment of ubiquitin chains. Recent studies have shown that the Cbl protein selectively targets several protein tyrosine kinases, including EGFR, marking them for polyubiquitination and degradation via RING finger-mediated ubiquitin E2 and E3 activities (24). We then reasoned that the slow-migrating EGFRs may be caused by increased ubiquitination, particularly as the appearance of the slow-migrating EGFR was accompanied concomitantly by a reduced level of EGFR. Indeed, we found that the total level of Cbl protein was higher in DiFi5 cells



**Figure 3.** Analysis of EGFR cDNA transcript sizes and posttranslational modifications of the receptor in DiFi5 cells relative to parental DiFi cells. *A*, three pairs of primers selected to collectively span the whole range of the EGFR coding sequence were used for polychain reactions using the reverse transcription products from DiFi and DiFi5 cells as the respective templates. The reaction products were resolved on a 1% agarose gel. *B*, DiFi and DiFi5 cells were left untreated or treated with tunicamycin (2 mg/mL) overnight (16 h), along with A431 cells that served as a positive control. The cells were then harvested for Western blot analysis with an anti-EGFR antibody. \*, underglycosylated receptors in the cells. *C*, *left*, equal amounts of cell lysates from DiFi and DiFi5 cells were examined for the levels of total EGFR and Cbl by SDS-PAGE and Western blot analysis with respective antibodies. The level of  $\beta$ -actin served as a reference of protein loading. *Right*, one aliquot (200  $\mu$ g) of DiFi cell lysate was used along with equal (1 $\times$ ) and 4 $\times$  amounts of DiFi5 cell lysates for immunoprecipitation of EGFRs with cetuximab, followed by Western blot analysis with antibodies directed against EGFR, Cbl, or ubiquitin.



**Figure 4.** Comparison of site-specific phosphorylation of EGFR, Akt, and ERK in DiFi and DiFi5 cells following EGF stimulation. DiFi and DiFi5 cells were stimulated with 10 nmol/L EGF for 30 min in culture medium with 0.5% FBS. The cells were then lysed and equal amounts of cell lysates were subjected to SDS-PAGE and Western blot analysis with antibodies directed against total and several site-specific tyrosine-phosphorylated EGFRs, as indicated in (A), or with antibodies directed against total and site-specific phosphorylated Akt and ERK, as indicated in (B).

than in DiFi cells (Fig. 3C). Because the total EGFR content in DiFi5 cells was only 20% to 25% of that in DiFi cells, we used two sample sets of DiFi5 cell lysates, containing 1× and 4× the DiFi cell lysate protein, respectively, for immunoprecipitation of EGFRs to compare the levels of EGFR-associated Cbl protein in DiFi and DiFi5 cells. Subsequently, Western blot analysis of the immunoprecipitates was done with antibodies directed against Cbl, ubiquitin, and EGFR. Figure 3C shows that when the EGFR level of the DiFi5 cell lysate was adjusted to be comparable to that of the DiFi cell lysate, there was an increased amount of Cbl that was found in the EGFR content-adjusted immunoprecipitates of DiFi5 cells, indicating there is indeed an increased association between EGFR and Cbl protein in DiFi5 cells compared with DiFi. Furthermore, this was accompanied by increased ubiquitination of EGFR in DiFi5 cells. Taken together, our results indicate that the slow migration of EGFR as well as the low EGFR levels found in DiFi5 cells can be attributed, at least partially, to an increased level of EGFR ubiquitination in these cells.

**Altered responses to EGF-induced signal transduction in DiFi5 cells relative to parental DiFi cells.** Phosphorylation of EGFR on specific tyrosine residues on its intracellular domain by EGFR tyrosine kinase or other kinases such as Src is critical for transducing EGF-induced signals to downstream signal mediators and effector molecules as well as for functional regulation of the receptor itself (25, 26). To identify potential changes in signal transduction that may be associated with the acquired resistance of DiFi5 cells to treatment with cetuximab, we compared the levels of EGFR tyrosine phosphorylation in DiFi and DiFi5 cells at baseline and on stimulation with EGF by Western blot analysis with several antibodies that recognize EGFR tyrosine phosphorylation on specific residues, including Y992 (phospholipase C $\gamma$  binding site), Y1068 (growth factor receptor binding protein-2 binding site), Y845 (Src-dependent phosphorylation site), and Y1045 (Cbl binding site). Figure 4A shows that, compared with parental DiFi cells, DiFi5 cells had lower baseline levels of EGFR phosphorylation on all four tyrosine residues examined, which apparently can be attributed to the low total EGFR level in DiFi5 cells. However, despite low levels of EGFR present, DiFi5 cells showed robust phosphorylation on Y845 upon EGF stimulation compared with the other three tyrosine residues (Y992, Y1045, and Y1068). In contrast, all four EGFR tyrosine residues were highly phosphorylated on EGF stimulation in DiFi cells. Thus, EGFR tyrosine phosphorylation following EGF stimulation differs in DiFi and DiFi5 cells, with

efficient phosphorylation on Y845 (Src-dependent phosphorylation site) in DiFi5 cells.

We next examined the levels of basal and EGF-induced phosphorylation of downstream signal mediators that might be differentially activated by EGF stimulation in DiFi and DiFi5 cells. Figure 4B shows that despite the low levels of EGFR in DiFi5 cells, the basal and EGF-stimulated phosphorylation levels of ERK and Akt were similar in DiFi and DiFi5 cells, suggesting that the EGFR in DiFi5 cells was highly efficient in transducing signals to downstream signal mediators. This could be due to threshold effects, alterations in regulatory loops, and a balance of homeostatic effects that compensate for the reduced level of EGFR.

The *Src* nonreceptor kinase proto-oncogene is a well-known cooperating partner for transformation of EGFR (27, 28). Figure 5A shows the levels of phosphorylated Src and total Src kinase in unstimulated and EGF-stimulated DiFi and DiFi5 cells in the presence or absence of the Src kinase inhibitor PP2. We found that DiFi5 cells contained a higher basal level of phosphorylation of Src-Y416 (a positive regulatory site) and an enhanced response to EGF stimulation. No remarkable differences were found between DiFi5 and parental cells in phosphorylation of the Y527 auto-inhibitory site at basal level or on EGF stimulation (29). Figure 5B shows the effects of inhibiting Src kinase on the phosphorylation levels of EGFR in DiFi and DiFi5 cells. In both parental and DiFi5 cells, PP2 decreased both the basal and EGF-stimulated levels of EGFR phosphorylation on Y1068, Y1045, Y992, and Y845, although it was more effective in decreasing the basal level than the EGF-stimulated level. These results suggest that Src kinase activity is involved in the tyrosine phosphorylation of EGFR on cellular stimulation with EGF. Strikingly, whereas PP2 decreased phosphorylation of Y845, a Src phosphorylation site (30–32), in DiFi cells, PP2 induced twice as much inhibition in DiFi5 cells, suggesting a greater role for Src in phosphorylating the EGFR kinase in DiFi5 cells than in DiFi cells. PP2 did not alter total EGFR levels, suggesting that Src did not regulate ubiquitination and degradation of EGFR over the time course treated.

We next examined the effect of inhibiting Src with PP2 on basal and EGF-stimulated phosphorylation of Akt and ERK in DiFi and DiFi5 cells (Fig. 5C). Similar to the results shown in Figs. 4 and 5A and B, EGF induced comparable levels of activation-specific phosphorylation of Akt and ERK in DiFi and DiFi5 cells despite a lower level of EGFR in the latter. Interestingly, inhibition of Src with PP2 elicited a very different effect on EGF-induced phosphorylation

of Akt and ERK in DiFi and DiFi5 cells. Stimulation of the cells with EGF produced comparable levels of activation-specific phosphorylation of Akt, which was equally sensitive to PP2 treatment, suggesting that Src plays an important role in EGF-stimulated phosphorylation of Akt in both DiFi and DiFi5 cells. In contrast, PP2 had no inhibitory effect on EGF-stimulated phosphorylation of ERK in DiFi cells. The basal level of phosphorylated ERK was slightly higher in DiFi5 cells than in DiFi cells. Treatment of the DiFi5 cells with PP2 even led to increased ERK phosphorylation both at the basal level and on EGF stimulation. This finding suggests that in DiFi5 cells, Src is not only dispensable for EGFR-induced ERK phosphorylation but also exhibits a modest inhibitory effect on ERK phosphorylation; inhibition of Src released the inhibition and accordingly increased the level of ERK phosphorylation.

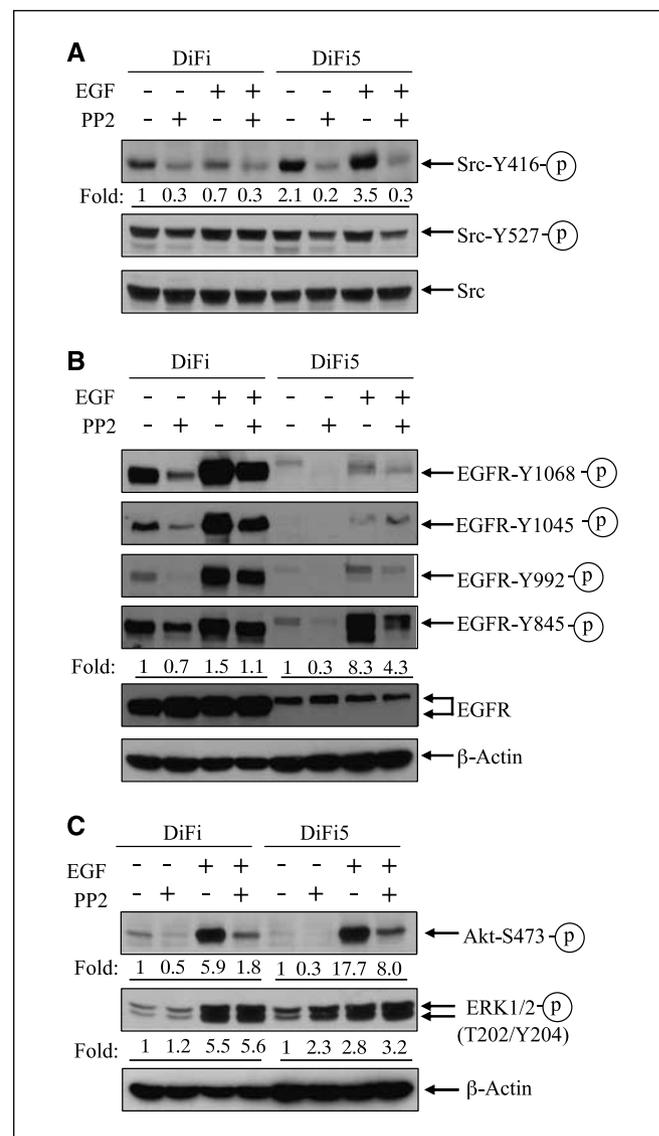
The higher level of Src activation-specific phosphorylation on Y416 in DiFi5 cells suggests that Src may perform an enhanced role in cell survival in collaboration with EGFR in DiFi5 cells. We therefore examined whether inhibition of Src with PP2 would reverse the resistance of DiFi5 cells to cetuximab treatment. We found that, compared with DiFi cells, DiFi5 cells were resistant to cetuximab-induced apoptosis but were equal or more sensitive to PP2-induced apoptosis, as assessed by two independent apoptosis assays, one measuring the levels of histone-associated DNA fragmentation in the cytoplasm by ELISA (Fig. 6A) and the other measuring poly(ADP-ribose) polymerase (PARP) proteolytic cleavage levels (Fig. 6B). Treatment of DiFi5 cells with a combination of cetuximab and PP2 substantially increased apoptosis. Compared with the levels of activation-specific phosphorylation of Akt at S473 in DiFi cells after cetuximab and PP2 treatment, cetuximab had no effect on the basal level of Akt phosphorylation in DiFi5 cells, but PP2 had a stronger effect in DiFi5 cells than in DiFi cells. Treatment of DiFi5 cells with a combination of cetuximab and PP2 inhibited Akt phosphorylation to a degree comparable to that in DiFi cells treated with cetuximab alone (which is sufficient to induce apoptosis). With regard to the activation-specific phosphorylation of ERK in DiFi cells, PP2 had no effect on or slightly increased ERK phosphorylation compared with cetuximab, which strongly inhibited ERK phosphorylation. Combination treatment with PP2 and cetuximab led to a lesser decrease in ERK phosphorylation than with cetuximab alone. The basal level of ERK phosphorylation was modestly higher, and cetuximab inhibited ERK phosphorylation to a lesser degree, if at all, in DiFi5 cells than in DiFi cells. Interestingly, PP2 treatment led to a higher level of ERK phosphorylation than that in untreated DiFi5 cells, but the combination of PP2 and cetuximab had an effect similar to that in DiFi cells, except that overall ERK phosphorylation was at a higher level in DiFi5 cells. Linking these results to the levels of apoptosis induced by treatment with cetuximab, PP2, or the combination, our findings suggest that the activity-specific inhibition of Akt phosphorylation is more important than that of ERK phosphorylation for the induction of apoptosis by cetuximab.

Because ubiquitination and degradation play a fundamental role in the phenotypic changes of DiFi5 cells by reducing the level of EGFR, we examined the effects of PP2 and cetuximab on cellular levels of Cbl in DiFi and DiFi5 cells. We found that treatment of DiFi cells with cetuximab led to a modest decrease in Cbl levels, whereas PP2 elevated the level of Cbl in DiFi cells. The exposure of DiFi cells to cetuximab and PP2 simultaneously offset their respective effects on Cbl. DiFi5 cells contained a higher basal level of Cbl, as shown in Fig. 2. Cetuximab had little or no effect on

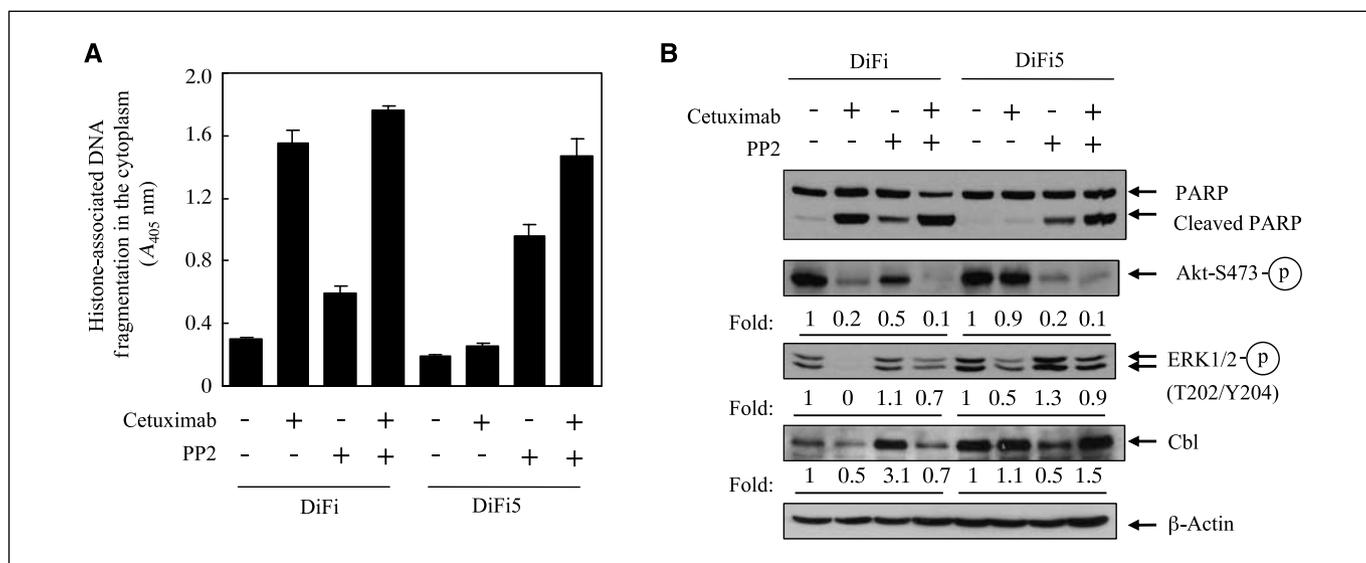
the level of Cbl protein, whereas PP2 decreased the level of Cbl compared with untreated cells; this effect was abolished and indeed modestly reversed when DiFi5 cells were simultaneously exposed to both cetuximab and PP2. This result suggests that the pathway(s) or mechanism(s) regulating the level of Cbl protein, an important ubiquitin ligase to both EGFR and Src, is altered in DiFi5 cells compared with the parental cells.

## Discussion

Acquired resistance commonly develops in cancer cells through modification of the structure or functionality of the drug target. The conventional approach to identifying structural or functional



**Figure 5.** Effects of the Src inhibitor PP2 on basal and EGF-stimulated phosphorylation of Src, EGFR, Akt, and ERK in DiFi and DiFi5 cells. DiFi and DiFi5 cells were left untreated or treated with 5  $\mu$ mol/L PP2 overnight (16 h) in culture medium with 0.5% FBS. Before harvesting, the PP2-treated and untreated cells were either stimulated with 10 nmol/L EGF for 15 min or not. The cells were then lysed and equal amounts of cell lysates were subjected to SDS-PAGE and Western blot analysis with various antibodies directed against total or site-specific phosphorylation of EGFR, Src, Akt, and ERK, as shown. Densitometric values of cell signaling are indicated with a group line underneath selected gel pictures and shown as relative folds to untreated DiFi or DiFi5 cells.



**Figure 6.** Resensitization of DiFi5 cells by PP2 to cetuximab-induced apoptosis. **A**, DiFi and DiFi5 cells were left untreated or treated with 20 nmol/L cetuximab or 5  $\mu$ mol/L PP2, alone or in combination, overnight (16 h) in culture medium with 0.5% FBS. After treatment, the cells were harvested and lysates were prepared for quantification of the levels of histone-associated DNA fragmentation in the cytoplasm by ELISA. **B**, DiFi and DiFi5 cells received the same treatment as in (A) and were examined by Western blot analysis for levels of PARP cleavage and changes in the phosphorylation levels of Akt and ERK and cellular level of Cbl protein after treatment. Densitometric values of cell signaling are indicated with a group line underneath selected gel pictures and shown as relative folds to untreated DiFi or DiFi5 cells.

changes associated with acquired resistance is to compare congenic sensitive cells with counterparts that are clinically or experimentally induced to become resistant to the treatment. In this study, we found increased ubiquitination and degradation of EGFR in a cetuximab-resistant subline (DiFi5) of EGFR-over-expressing DiFi cells. We found that Src plays a critical role in concert with EGFR to support the survival of DiFi5 cells on cetuximab treatment and that inhibition of Src kinase with PP2 resensitized the resistant DiFi5 cells to cetuximab treatment.

Both Src and EGFR play important roles in human malignancies. Oncogenic collaboration between Src and EGFR in cell transformation is well established (27, 28). Earlier studies have shown that a high level of Src potentiated the mitogenic and tumorigenic capacity of EGFR (25, 30). The kinase activity of Src is required for both biological synergy with EGFR and phosphorylation of EGFR, although kinase activity is not required for a physical association between Src and EGFR (25). Src-dependent phosphorylation of Y845 on EGFR is required for DNA synthesis induced not only by EGF but also by several transactivating agonists of EGFR, such as endothelin, lysophosphatidic acid, cytokines, and growth hormone, all of which act through G-protein-coupled receptors (33). Transient expression of the Y845F mutant of EGFR in murine fibroblasts resulted in the ablation of DNA synthesis induced by EGF or by transactivating agonists (25, 30, 33).

In the current study, we found that DiFi5 cells contain a higher basal level of activation-specific phosphorylation on Src-Y416 and an enhanced response to EGF-stimulated phosphorylation of Src-Y416. The high level of Src-Y416 phosphorylation was accompanied by a high level of Src-mediated Y845 phosphorylation of EGFR. Indeed, the levels of Y845 phosphorylation were quite remarkable when considering the low level of EGFR protein in DiFi5 cells. This observation suggests that Src plays a more important role in collaborating with EGFR in DiFi5 cells than in DiFi cells. We found that in both DiFi and DiFi5 cells, the EGF-stimulated phosphor-

ylation of Akt requires Src kinase activity because PP2 treatment inhibited Akt phosphorylation in both cells. The basal level of Akt phosphorylation was reduced by both cetuximab and PP2 in DiFi cells but only by PP2 (to a greater extent) in DiFi5 cells, suggesting a more important role for Src in Akt phosphorylation in DiFi5 cells. In contrast, neither basal nor EGF-stimulated ERK phosphorylation required Src kinase activity in the cells, and inhibition of Src kinase with PP2 even led to higher levels of both basal and EGF-stimulated ERK phosphorylation in DiFi5 cells. These results may be due to PP2-mediated inhibition of Src with subsequent inhibition of Akt activity, leading to an increase in ERK phosphorylation, owing to the known negative effect of Akt on ERK phosphorylation via inhibition of Raf-1/B-Raf signaling (34, 35).

A recent study proposed a model through which Src collaborates with EGFR in oncogenesis (36). Src may promote ubiquitination and proteasomal degradation of Cbl by phosphorylating Cbl proteins on tyrosine residues, through which Src reduces the negative role of Cbl in EGFR stability (i.e., ubiquitination of EGFR for sorting to the endosome; ref. 36). We found that whereas this may be the case in DiFi cells, in which inhibition of Src with PP2 led to a higher level of Cbl protein, it does not seem to occur in the cetuximab-resistant DiFi5 cells because inhibition of Src with PP2 down-regulated rather than up-regulated the Cbl protein level. In addition, in contrast to this model, the increased activity of Src in DiFi5 cells was associated with a higher basal level of Cbl, which decreased EGFR stability in DiFi5 cells. Furthermore, PP2 did not alter EGFR levels over the time course assessed. Thus, Src-induced ubiquitination of Cbl and the subsequent down-regulation of Cbl levels do not seem to account for altered levels of EGFR in DiFi5 cells. The increase of Cbl protein level in DiFi5 cells found in our study is likely due to *de novo* synthesis, which warrants further investigation.

In summary, cetuximab-resistant DiFi5 colorectal cancer cells showed a remarkable decrease in the level of EGFR and an

enhanced role of Src kinase in collaboration with EGFR for supporting cell growth and survival. Our results suggest that the implementation of combination treatments targeting both EGFR and Src kinases may result in better therapeutic outcomes for patients with colorectal cancer.

## Acknowledgments

Received 2/14/2007; revised 4/3/2007; accepted 5/2/2007.

**Grant support:** An unrestricted grant from Bristol-Myers Squibb.

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# Cancer Research

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## Epidermal Growth Factor Receptor (EGFR) Ubiquitination as a Mechanism of Acquired Resistance Escaping Treatment by the Anti-EGFR Monoclonal Antibody Cetuximab

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*Cancer Res* 2007;67:8240-8247.

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