

Celecoxib Promotes c-FLIP Degradation through Akt-Independent Inhibition of GSK3

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Abstract

Celecoxib is a COX-2 inhibitor that reduces the risk of colon cancer. However, the basis for its cancer chemopreventive activity is not fully understood. In this study, we defined a mechanism of celecoxib action based on degradation of cellular FLICE-inhibitory protein (c-FLIP), a major regulator of the death receptor pathway of apoptosis. c-FLIP protein levels are regulated by ubiquitination and proteasome-mediated degradation. We found that celecoxib controlled c-FLIP ubiquitination through Akt-independent inhibition of glycogen synthase kinase-3 (GSK3), itself a candidate therapeutic target of interest in colon cancer. Celecoxib increased the levels of phosphorylated GSK3, including the α and β forms, even in cell lines, where phosphorylated Akt levels were not increased. Phosphoinositide 3-kinase inhibitors abrogated Akt phosphorylation as expected but had no effect on celecoxib-induced GSK3 phosphorylation. In contrast, protein kinase C (PKC) inhibitors abolished celecoxib-induced GSK3 phosphorylation, implying that celecoxib influenced GSK3 phosphorylation through a mechanism that relied upon PKC and not Akt. GSK3 blockade either by siRNA or kinase inhibitors was sufficient to attenuate c-FLIP levels. Combining celecoxib with GSK3 inhibition enhanced attenuation of c-FLIP and increased apoptosis. Proteasome inhibitor MG132 reversed the effects of GSK3 inhibition and increased c-FLIP ubiquitination, confirming that c-FLIP attenuation was mediated by proteasomal turnover as expected. Our findings reveal a novel mechanism through which the regulatory effects of c-FLIP on death receptor signaling are controlled by GSK3, which celecoxib acts at an upstream level to control independently of Akt. *Cancer Res*; 71(19); 6270–81. ©2011 AACR.

Introduction

The cellular FLICE-inhibitory protein (c-FLIP) is the major inhibitor of the extrinsic apoptotic pathway through inhibition of caspase-8 activation (1). c-FLIP has multiple splice variants, and 2 main forms have been well characterized: short form (c-FLIP_S) and long form (c-FLIP_L) c-FLIP (1). Generally speaking, elevated c-FLIP expression protects cells from death receptor-mediated apoptosis, whereas downregulation of c-FLIP by chemicals or siRNA augments death receptor-mediated apoptosis (1). Moreover, overexpression of c-FLIP

protects cells from apoptosis induced by certain cancer therapeutic agents such as etoposide and cisplatin (2–4).

c-FLIP is known to be subjected to rapid turnover, regulated by an ubiquitin–proteasome mechanism (5, 6). Certain cancer therapeutic agents stimulate downregulation of c-FLIP expression through this mechanism (5). However, the mechanism underlying drug-induced c-FLIP degradation is unclear. A recent study has shown that *c-jun*-NH₂-kinase-mediated activation of the E3 ubiquitin ligase Itch specifically ubiquitinates c-FLIP_L and induces its proteasomal degradation (7).

Glycogen synthase kinase-3 (GSK3) is a ubiquitous serine/threonine kinase that is present in mammals in 2 isoforms: α and β (8, 9). GSK3 was initially identified as an enzyme involved in the regulation of glycogen metabolism. Increasing evidence during the past decades indicates that GSK3 has a key role in regulating a diverse range of cellular functions including cell survival and death (8, 9). Thus, GSK3 inhibition has been considered an attractive therapeutic strategy for certain diseases such as diabetes, neurodegenerative diseases, and mental disorders (10, 11). It has been documented that GSK3 exerts opposing apoptosis-regulating effects: It inhibits the death receptor-mediated extrinsic apoptotic pathway while promoting cell death caused by the mitochondrial intrinsic apoptotic pathway (8). Inhibition of GSK3 with either small-molecule inhibitors or siRNA sensitizes cancer cells to TNF-related apoptosis-inducing ligand (TRAIL)-induced or agonistic death receptor 5 (DR5)

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antibody-induced apoptosis (12–14). However, it is largely unclear how GSK3 inhibition enhances death receptor-induced apoptosis (8). Recently, a study showed that GSK3 is involved in forming an antiapoptotic protein complex with DDX3 and cIAP-1, leading to inhibition of apoptotic signaling by preventing formation of the death-inducing signaling complex and caspase-8 activation (14). However, linkage between GSK3 and c-FLIP regulation has not been suggested.

Celecoxib, a marketed anti-inflammatory and anti-pain drug, is being tested in clinical trials for its chemopreventive and therapeutic effects against a broad spectrum of epithelial malignancies either as a single agent or in combination with other agents. The antitumor activity of celecoxib is thought to be associated with its ability to induce apoptosis in a variety of cancer cells (15). The molecular mechanisms underlying celecoxib-mediated apoptosis have not been fully elucidated, although it seems to be associated with the inactivation of PDK1/Akt, induction of endoplasmic reticulum stress involving upregulation of CHOP/GADD153, and increase in Ca^{2+} levels, or downregulation of the antiapoptotic protein survivin (16). Our previous results have shown that celecoxib induces apoptosis in non-small-cell lung cancer (NSCLC) cell lines involving the activation of the extrinsic death receptor pathway through both DR5 induction and c-FLIP downregulation (17, 18).

We have shown that celecoxib downregulates c-FLIP through facilitating ubiquitin/proteasome-dependent protein degradation (18). However, the signaling process leading to celecoxib-induced c-FLIP degradation is unknown. In an effort to show the mechanism underlying celecoxib-induced c-FLIP degradation, we have revealed a novel mechanism of c-FLIP degradation through GSK3 inhibition. To the best of our knowledge, this is the first study showing a linkage between GSK3 inhibition and c-FLIP downregulation, thus highlighting a new mechanism by which GSK3 modulates the extrinsic apoptotic pathway.

Materials and Methods

Reagents

Celecoxib, dimethyl-celecoxib (DMC), and antibodies against caspases and DR5 were the same as described previously (17, 19). Human recombinant TRAIL was purchased from PeproTech, Inc. Rapamycin and LY294002 were purchased from LKT Laboratories, Inc. Wortmannin and R831-8220 were purchased from Biomol. MG132, LiCl, SB216763, and SB415286 were purchased from Sigma Chemicals. Gö6983, Gö6979, GF109203X, and rottlerin were purchased from EMD Calbiochem. Rabbit polyclonal antibodies against phosphorylated (p)-Akt (S473), p-GSK3 β (S9), p-GSK3 α/β (S21/S9), and p-S6 (S235/S236) were purchased from Cell Signaling Technology, Inc. Rabbit polyclonal antibodies against GSK3 α/β and p-FOXO3 α (T32) were purchased from Upstate. Mouse monoclonal anti-FLIP antibody (NF6) was purchased from Alexis Biochemicals. Rabbit polyclonal anti-actin antibody was purchased from Sigma Chemicals. Wild-type (WT), constitutively active (CA; R85A), and kinase dead (KD; B9A) human GSK3 β in pCMV-Tag 5A expression vectors were

generously provided by Dr. B. P. Zhou (University of Kentucky School of Medicine, Lexington, KY).

Cell lines and cell culture

The human NSCLC cell lines used in this study were provided by Dr. R. Lotan (MD Anderson Cancer Center, Houston, TX) in 2003 and cultured as previously described (17). H157 and A549 cell lines were recently authenticated by Genetica DNA Laboratories, Inc. by analysis of the short tandem repeat DNA profile. The other cell lines used have not been authenticated. The stable H157-Lac Z-5, H157-FLIP_L-21, and H157-FLIP_S-1 transfectants were described previously (20). Through the entire study, the concentrations of dimethyl sulfoxide (DMSO; as a solvent control) did not exceed 0.05%.

Western blot analysis

Whole-cell protein lysates were prepared and analyzed by Western blotting as described previously (17, 21).

Cell survival assay

Cells were seeded in 96-well cell culture plates and treated the next day with the agents indicated. The viable cell number was determined by the sulforhodamine B (SRB) assay, as previously described (22).

Detection of apoptosis

Apoptosis was evaluated by Annexin V staining using Annexin V-phycoerythrin apoptosis detection kit purchased from BD Biosciences or by measuring cytoplasmic histone-associated DNA fragments with a Cell Death Detection ELISA^{Plus} kit following the manufacturer's instructions. We also detected caspase activation by Western blotting as an additional indicator of apoptosis.

Immunoprecipitation for detection of ubiquitinated c-FLIP

H157-FLIP_L-21 cells, which stably express FLIP_L, were transfected with HA-ubiquitin plasmid, using the FuGENE 6 transfection reagent (Roche Molecular Biochemicals) following the manufacturer's instructions. After 24 hours, the cells were treated with tested agent or MG132 plus the tested agent for 4 hours and then were lysed for immunoprecipitation of Flag-FLIP_L using Flag M2 monoclonal antibody (Sigma Chemicals) as previously described (23), followed by the detection of ubiquitinated FLIP_L with Western blotting using anti-HA antibody from Abgent.

siRNA-mediated gene silencing

GSK-3 α , GSK-3 β #1, and GSK-3 β #2 siRNAs, which target the sequences 5'-AAGTGATTGGCAATGGCTCAT-3', 5'-AAGAATCGAGAGCTCCAGATC-3', and 5'-AAGTAATC-CACCTCTGGCTAC-3' (12), respectively, were purchased from Qiagen. Itch #1 and Itch #2 siRNAs, which target the sequences 5'-AAGTGCTTCTCAGAATGATGA-3' and 5'-AACCACAACA-CACGAATTACA-3', respectively, were also purchased from Qiagen. The nonsilencing control siRNA duplexes were described previously (17). Transfection of these siRNA duplexes was conducted in 6-well plates using the HiPerfect

Transfection Reagent (Qiagen) following the manufacturer's manual. Gene-silencing effects were evaluated by Western blot analysis.

Reverse transcription PCR for detection of c-FLIP mRNA

Total cellular RNA was extracted from a given cell line with TRI-Reagent (Sigma Chemicals) and reverse transcribed into cDNA using iScript cDNA Synthesis Kit (Bio-Rad Laboratories) following the manufacturer's instructions. The cDNA was then amplified by PCR using the following primers: FLIP sense 5'-ACAGAGTGAGGCGATTTGAC-3'; FLIP antisense 5'-GAACAGACTGCTTGTACTTCT-3'; actin sense 5'-GGCACCCAGCACAATGAAGATCAA-3' and actin antisense 5'-ACTCGTCATACTCTGCTTGCTGA-3'. The 25- μ L amplification mixture contained 1 μ L of the cDNA, 0.5 μ L of deoxynucleotide triphosphate (25 mmol/L each), 1 μ L each of the sense and antisense primers (20 μ mol/L each), 5 μ L of TaqMaster PCR enhancer, 1 μ L of Taq DNA polymerase (5 units/ μ L; Eppendorf), 2.5 μ L \times 10 reaction buffer, and sterile H₂O. PCR was carried out for 35 cycles. After an initial step at 95°C for 3 minutes, each cycle consisted of 1 minute of denaturation at 94°C, 1 minute of annealing at 55°C, and 1 minute of extension at 72°C. This was followed by an additional extension step at 72°C for 7 minutes. The housekeeping gene *actin* was also amplified as an internal reference. PCR products were resolved by electrophoresis on 1.5% agarose gel, stained, and directly visualized under UV illumination.

Statistical analysis

The statistical significances among treatment groups were analyzed with 1-way ANOVA with GraphPad InStat 3 software (GraphPad Software). Results were considered to be statistically significant at $P < 0.05$.

Results

Celecoxib increases Akt and GSK3 phosphorylation in human NSCLC cells

It has been suggested that phosphoinositide 3-kinase (PI3K)/Akt signaling positively regulates c-FLIP expression in tumor cells (24, 25). Given that celecoxib was shown in some studies to inhibit PDK1/Akt signaling in certain types of cancer cells such as prostate cancer cells (26–29), we questioned whether there is a link between celecoxib-induced c-FLIP downregulation and Akt inhibition. To this end, we first determined whether celecoxib affects Akt phosphorylation in a panel of human NSCLC cell lines. In our cell systems, we did not find that celecoxib inhibited Akt phosphorylation in any tested NSCLC cell lines. Instead, we detected increased levels of p-Akt in some cell lines exposed to celecoxib (e.g., H157, Calu-1, and H358; Fig. 1A). In some cell lines such as H1792, we did not detect either basal levels or increased levels of p-Akt upon treatment with celecoxib (Fig. 1A). Furthermore, we examined the effects of celecoxib on the phosphorylation of 2 well-known Akt substrates, GSK3 β and FOXO3 α . As presented in Fig. 1A, celecoxib

weakly increased p-FOXO1 α levels in only 1 of 5 cell lines (i.e., Calu-1), whereas it increased p-GSK3 β levels in all the tested cell lines. Through detailed time course analysis, we found that the observed increase in p-Akt levels occurred 3 hours after celecoxib treatment and was sustained to 16 hours in both Calu-1 and H358 cell lines. Accordingly, p-FOXO1 α levels were weakly increased after 3 hours in Calu-1 cells and after 10 hours in H358 cells after exposure to celecoxib. In Calu-1 cells, celecoxib increased the levels of p-GSK3 β or α/β in a fashion similar to the p-Akt increase; however, in H358 cells, celecoxib increased p-GSK3 levels even at 1 hour posttreatment (Fig. 1B). Thus, these data clearly indicate that celecoxib exerts more pronounced effects on increasing the phosphorylation of GSK3 (both α and β) than on Akt in human NSCLC cells.

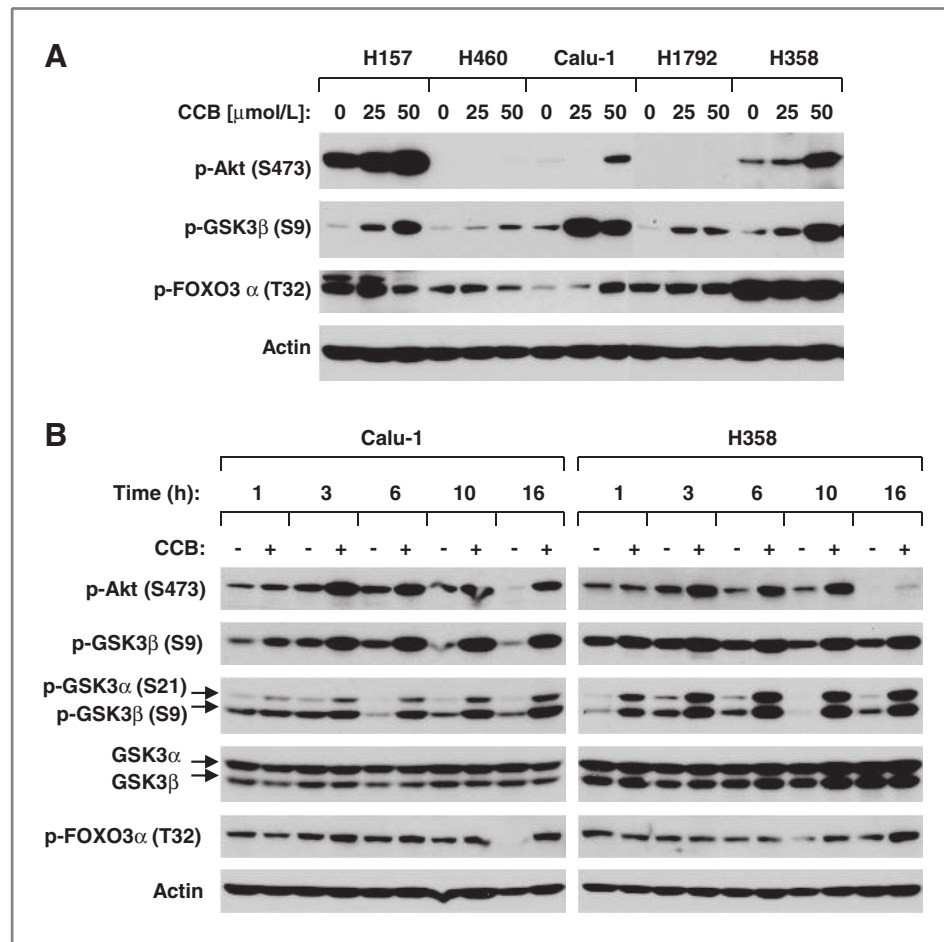
DMC is a celecoxib derivative lacking COX-2-inhibitory activity (30). It possesses more potent effects than celecoxib on induction of apoptosis, downregulation of c-FLIP, and enhancement of TRAIL-induced apoptosis (19). DMC even at 15 μ mol/L increased the levels of p-GSK3 β in H157, H460, and Calu-1 cells, whereas it increased p-Akt levels only at 30 μ mol/L in these cell lines (Supplementary Fig. S1A).

Celecoxib increases GSK3 phosphorylation independent of Akt and mTOR/p70S6K signaling

It is well known that Akt phosphorylates GSK3 resulting in its inactivation (31). To show whether the celecoxib-induced increase in GSK3 phosphorylation is due to an increase in Akt phosphorylation, we compared the effects of celecoxib on GSK3 phosphorylation in the absence and presence of the PI3K inhibitor LY294002 or wortmannin. Both LY294002 and wortmannin abrogated celecoxib-induced Akt phosphorylation but failed to prevent the increase in GSK3 phosphorylation (Fig. 2A). Similarly, LY294002 blocked DMC-induced Akt phosphorylation but failed to affect DMC-induced increase in p-GSK3 β (Supplementary Fig. S1B). These results indicate that celecoxib and DMC increase GSK3 phosphorylation independent of Akt.

It has been suggested that p70S6K also regulates or phosphorylates GSK3 under certain conditions (32, 33). Thus, we next asked whether this mechanism is involved in mediating celecoxib-induced GSK3 phosphorylation. To this end, we treated 2 NSCLC cell lines with celecoxib in the absence and presence of the mTOR inhibitor rapamycin, which is known to shut down mTOR/p70S6K signaling (34), and detected p-GSK3 and p-S6 (a readout of p70S6K activity) levels. As shown in Supplementary Fig. S2, rapamycin abolished basal levels of p-S6 despite the lack of increase in p-S6 levels by celecoxib, indicating the successful inhibition of p70S6K activity. However, rapamycin did not affect celecoxib-induced GSK3 phosphorylation at all. These results suggest that celecoxib also induces GSK3 phosphorylation independent of mTOR/p70S6K. We noted that rapamycin alone strongly increased p-Akt levels in both cell lines, as we previously reported (35); however, it either did not increase p-GSK3 β levels (i.e., Calu-1) or induced a weaker p-GSK3 β elevation than celecoxib (i.e., H358; Supplementary Fig. S2).

Figure 1. Celecoxib (CCB) increases Akt and GSK3 phosphorylation in human NSCLC cells. The indicated cell lines were treated with the given concentrations of celecoxib for 6 hours (A) or with 50 $\mu\text{mol/L}$ celecoxib for the given times (B). The cells were then subjected to preparation of whole-cell protein lysates and subsequent Western blot analysis for the indicated proteins.



Celecoxib induces protein kinase C-dependent GSK3 phosphorylation

Protein kinase C (PKC) has been documented to phosphorylate GSK3 (36–41). Thus, we next determined whether PKC is involved in mediating GSK3 phosphorylation by celecoxib. As presented in Fig. 2B, the presence of the pan-PKC inhibitor R δ 31-8220 abolished the ability of celecoxib to increase GSK3 phosphorylation in both Calu-1 and H358 cells. Moreover, we examined the effects of other PKC inhibitors on celecoxib-induced GSK3 phosphorylation and found that another pan-PKC inhibitor GF1092303X, the PKC α and β inhibitor G δ 9679, and the PKC α - ξ inhibitor G δ 6983 could abolish celecoxib-induced GSK3 phosphorylation. In contrast, the PKC δ inhibitor rottlerin did not inhibit celecoxib-induced GSK3 phosphorylation (Fig. 2C). Collectively, these results clearly suggest that celecoxib induces GSK3 phosphorylation through a PKC-mediated mechanism, likely involving PKC α and β . We also examined p-Akt levels in cells exposed to these treatments and found that the presence of these PKC inhibitors except for G δ 6976 actually exerted enhanced effects on Akt phosphorylation (Fig. 2B and C). This result further supports that celecoxib-induced GSK3 phosphorylation is separated from the increase in Akt phosphorylation.

Inhibition of GSK3 enhances the ability of celecoxib to downregulate c-FLIP

To determine the impact of GSK3 phosphorylation on celecoxib-induced c-FLIP downregulation, we used GSK3 siRNAs to knock down GSK3 α and GSK3 β , respectively, and then examined their effects on celecoxib-induced c-FLIP reduction. In H358 cells, GSK3 α siRNA reduced the levels of GSK3 α only whereas GSK3 β siRNA (#1) reduced the levels of not only GSK3 β but also GSK3 α (Fig. 3A). Silencing of GSK3 with both GSK3 α and GSK3 β siRNAs reduced basal levels of FLIP_L, suggesting that GSK3 regulates c-FLIP. Treatment of these cells, particularly GSK3 α siRNA- or GSK3 β #1 siRNA-transfected cells, with celecoxib resulted in further reduction of FLIP_L levels, which were lower than in cells treated with celecoxib alone or GSK3 siRNA transfection alone (Fig. 3A). These results indicate that silencing of GSK3 enhances the effect of celecoxib on downregulation of c-FLIP (i.e., FLIP_L).

We further examined the effects of celecoxib combined with a GSK3 inhibitor on c-FLIP downregulation. Both celecoxib and SB216763 alone decreased the levels of c-FLIP; however, the combination of celecoxib and SB216763 was even more potent than either agent alone in decreasing c-FLIP levels (Fig. 3B). Moreover, the combination of celecoxib with SB216763 was also much more effective than either celecoxib

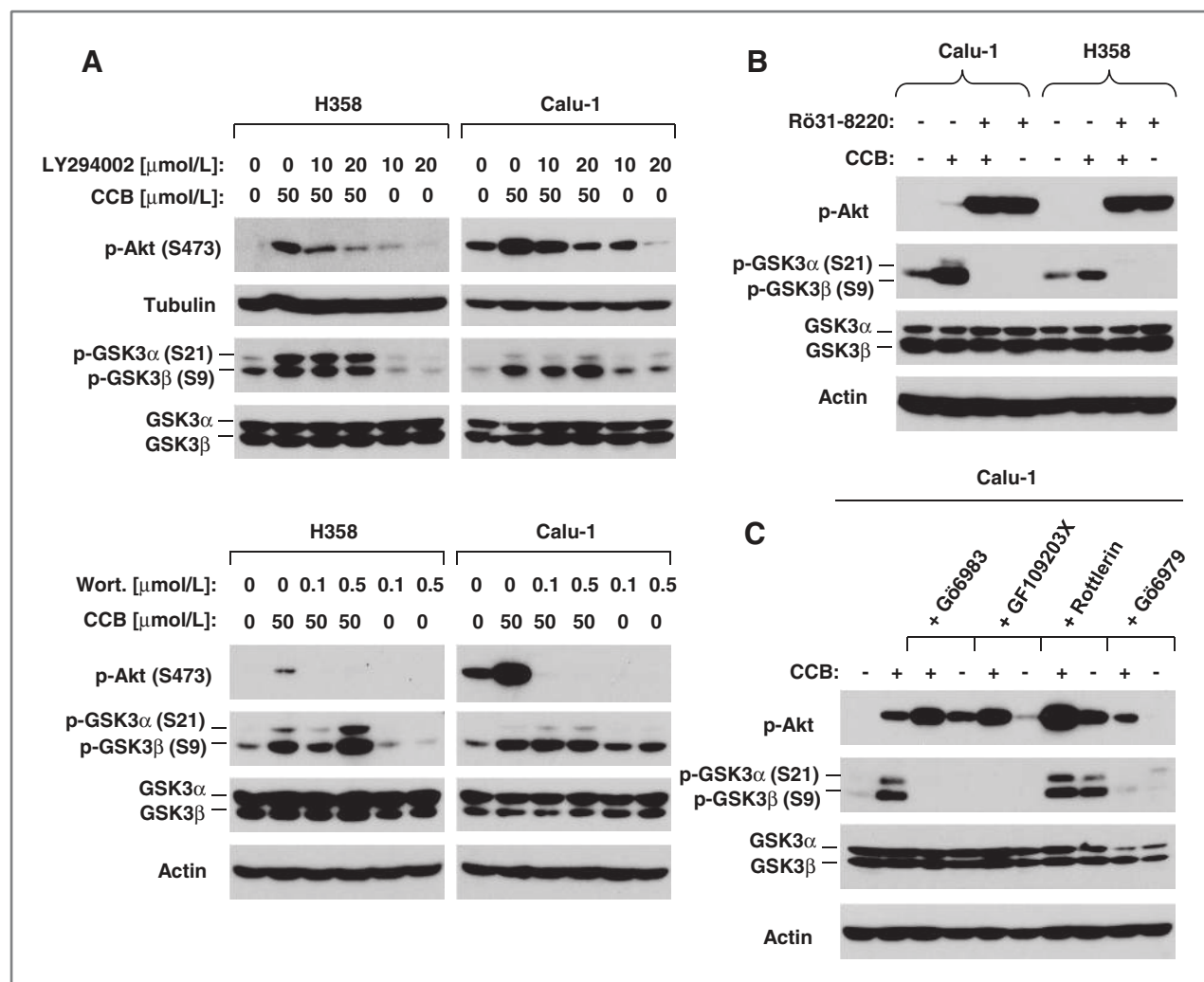


Figure 2. Celecoxib (CCB) induces PKC-mediated GSK3 phosphorylation (B and C) independent of Akt (A). A, the indicated cell lines were exposed to the given treatments as indicated for 6 hours. LY294002 or wortmannin (Wort.) was preincubated with the cells for 30 minutes before the addition of celecoxib. B, the indicated cell lines were pretreated with 2 $\mu\text{mol/L}$ R631-8220 for 30 minutes and then cotreated with 50 $\mu\text{mol/L}$ celecoxib for 6 hours. C, Calu-1 cells were pretreated with 20 $\mu\text{mol/L}$ Gö6983, 2 $\mu\text{mol/L}$ GF109203X, 20 $\mu\text{mol/L}$ rottlerin, or 0.5 $\mu\text{mol/L}$ Gö6979 for 30 minutes and then cotreated with 50 $\mu\text{mol/L}$ celecoxib for 6 hours. After the aforementioned treatments, the cells were harvested for preparation of whole-cell protein lysates and subsequent Western blot analysis for the indicated proteins.

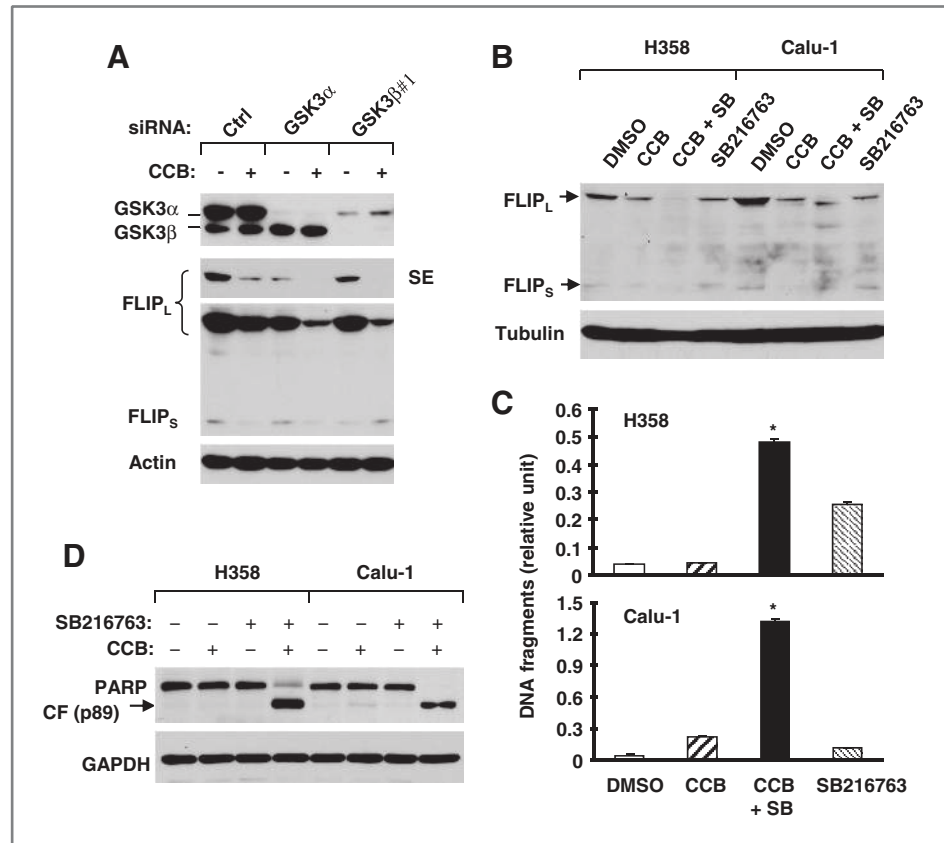
or SB216763 alone in increasing DNA fragmentation (Fig. 3C) and in inducing PARP cleavage (Fig. 3D). For example, the mean arbitrary units for DNA fragments induced by celecoxib, SB216763, and their combination were 0.224, 0.115, and 1.320, respectively, in comparison with 0.045 in control cells treated with DMSO. Thus, it is clear that the combination of celecoxib and SB216763 increases DNA fragmentation to a greater level than the sum of that caused by celecoxib or SB216763 alone, suggesting that celecoxib combined with a GSK3 inhibitor results in more than additive (i.e., synergistic) apoptosis-inducing effects in human NSCLC cells.

Modulation of GSK3 activity alters c-FLIP levels

The above data on reduction of c-FLIP by GSK3 inhibition (Fig. 3) suggest that GSK3 positively regulates c-FLIP levels.

Thus, we carried out more detailed experiments to validate this finding. To this end, we first treated 4 human NSCLC cell lines with different pharmacologic GSK3 inhibitors including LiCl, SB216763, and SB415286 and then detected c-FLIP levels in cells exposed to these treatments. As shown in Fig. 4A, all 3 GSK3 inhibitors exerted dose-dependent effects on reducing the levels of c-FLIP including FLIP_L and FLIP_S. Reduction of c-FLIP by GSK3 inhibition with a GSK3 inhibitor such as SB216763 occurred early, 3 hours after exposure to SB216763 in both Calu-1 and H358 cells (Fig. 4B), indicating that c-FLIP downregulation is an early event following GSK3 inhibition. Moreover, we further inhibited GSK3 by knocking down its expression using GSK3 siRNAs against the α and β forms, respectively, in both NSCLC cell lines. As presented in Fig. 4C, silencing of GSK3 α minimally decreased the levels of

Figure 3. The combination of celecoxib (CCB) with a GSK3 siRNA (A) or a small-molecule GSK3 inhibitor (B–D) exerts enhanced effects on downregulation of c-FLIP (A and B) and apoptosis (C and D). A, H358 cells were transfected twice with the given siRNAs in a 48-hour interval. Twenty-four hours after the second transfection, the cells were treated with 50 $\mu\text{mol/L}$ celecoxib for 8 hours (A) and then harvested for preparation of whole-cell protein lysates and subsequent Western blot analysis. Ctrl, control. B–D, the indicated cell lines were treated with 50 $\mu\text{mol/L}$ celecoxib alone, 20 $\mu\text{mol/L}$ SB216763 (SB) alone, or their combination. After 8 hours (B) or 24 hours (C and D), the cells were harvested for preparation of whole-cell protein lysates and subsequent Western blot analysis (B and D) or for analysis of apoptosis using the Cell Death Detection ELISA^{PLUS} kit (C). Columns, means of triplicate determinations; bars, \pm SDs. *, $P < 0.001$ compared with DMSO, celecoxib, or SB216763 with ANOVA.



FLIP_L, but not FLIP_S, in H157 cells; however, it reduced the levels of both FLIP_L and FLIP_S in A549 cells. Silencing of GSK3 β with 2 different siRNAs minimally decreased the levels of FLIP_L in A549 cells but reduced the levels of FLIP_S to a greater extent in both H157 and A549 cells. Alternatively, we enforced expression of WT, KD, and CA GSK3 β in H1299 cells and then examined their impact on c-FLIP levels. As presented in Fig. 4D, expression of WT, particularly CA GSK3 β , but not KD GSK3 β , increased the levels of c-FLIP. Thus, it seems that activation of GSK3 β elevates c-FLIP levels. Collectively, these results clearly indicate that GSK3 positively regulates c-FLIP.

Inhibition of GSK3 reduces c-FLIP levels by facilitating its ubiquitination and proteasome-mediated degradation

Given that c-FLIP protein is subjected to rapid turnover through ubiquitin/proteasome-dependent degradation (5, 6) and that celecoxib downregulates c-FLIP levels through this mechanism (18), we examined whether inhibition of GSK3 results in ubiquitin/proteasome-mediated c-FLIP degradation. Before these experiments, we determined whether inhibition of GSK3 affects c-FLIP at the mRNA level. Using RT-PCR, we did not detect any changes in c-FLIP mRNA levels in cells exposed to SB216763 (Fig. 5A), indicating that GSK3 inhibition-induced c-FLIP reduction does not occur at the transcriptional level. In the absence of the proteasome inhib-

itor MG132, SB216763 reduced c-FLIP levels; however, this effect was abolished by the presence of MG132 in both H157 and H358 cells (Fig. 5B). By immunoprecipitation/Western blotting, we detected the highest levels of ubiquitinated FLIP_L in cells treated with SB216763 plus MG132 compared with cells exposed to SB216763 alone or MG132 alone (Fig. 5C), indicating that SB216763 increases c-FLIP ubiquitination. Collectively, we conclude that inhibition of GSK3 facilitates ubiquitin/proteasome-mediated c-FLIP degradation, leading to c-FLIP downregulation.

Inhibition of GSK3 induces c-FLIP degradation independent of the E3 ligase Itch

The E3 ligase Itch has been suggested to be involved in TNF α -induced FLIP_L degradation (7). We then asked whether Itch is involved in mediating ubiquitin/proteasome-dependent degradation of c-FLIP induced by GSK3 inhibition. Transfection of 2 different Itch siRNAs into H157 cells substantially reduced the levels of Itch, indicating successful knockdown of Itch (Fig. 5D). However, knockdown of Itch neither increased basal levels of c-FLIP nor prevented c-FLIP reduction induced by SB216763 (Fig. 5D). Similar results were also generated in cells exposed to celecoxib (Supplementary Fig. S3). These results clearly indicate that Itch is unlikely to be the E3 ligase that mediates GSK3 inhibition-induced ubiquitin/proteasome-dependent c-FLIP degradation.

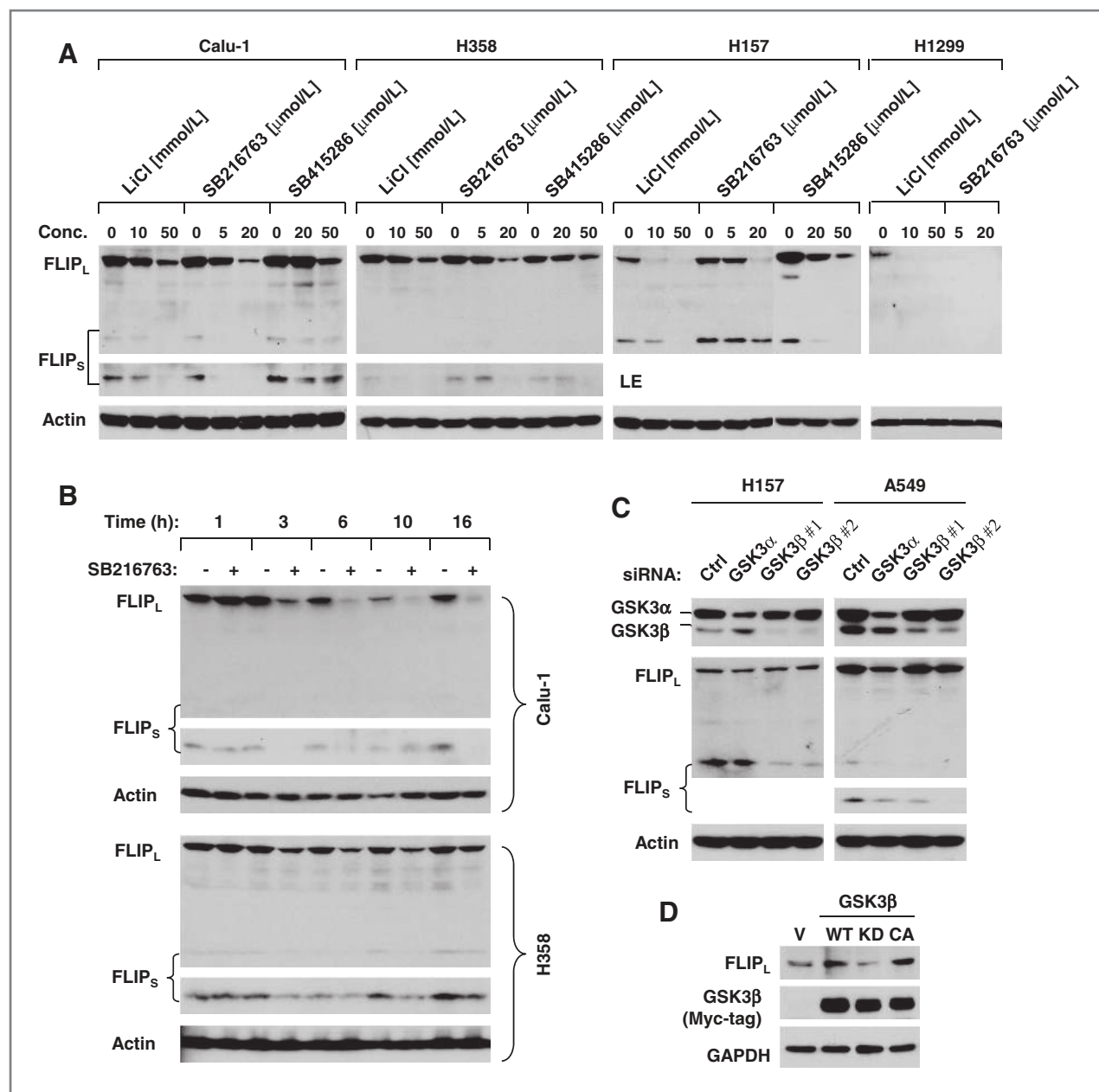


Figure 4. Inhibition of GSK3 with either small-molecule inhibitors (A and B) or GSK3 siRNAs (C) or activation of GSK3 (D) modulates c-FLIP levels. A and B, the given cell lines were treated with the given concentration of the respective GSK3 inhibitors for 8 hours (A) or 20 $\mu\text{mol/L}$ SB216763 for the indicated times (B). C, the given cell lines were transfected with the indicated siRNAs for 48 hours; D, H1299 cells were transfected with empty vector (V) or expression plasmids carrying WT, KD, or CA GSK3 β , using Lipofectamine 2000 for 48 hours. After the aforementioned treatment or transfection, the cells were harvested for preparation of whole-cell protein lysates and subsequent Western blot analysis for the indicated proteins. LE, longer exposure.

Inhibition of GSK3 enhances TRAIL-induced apoptosis

Given that c-FLIP is the major inhibitor of the extrinsic apoptotic pathway, it is plausible to speculate that downregulation of c-FLIP by inhibition of GSK3 will sensitize cancer cells to TRAIL-induced apoptosis as celecoxib does (17). Indeed, the combination of TRAIL with a GSK3 inhibitor such as SB415286 or SB216763 exerted much more potent effects than TRAIL or the inhibitors alone in decreasing the survival of human NSCLC cells (Fig. 6A and B). In agreement, the

combinations were also more potent than each single agent alone in inducing cleavage of caspase-8, caspase-9, caspase-3, and PARP (Fig. 6C), that is, activation of caspase cascades. Collectively, these results indicate that inhibition of GSK3 (e.g., with a small-molecule inhibitor) augments TRAIL-induced apoptosis.

Moreover, we tested whether downregulation of c-FLIP by GSK3 inhibition indeed contributes to TRAIL-induced apoptosis. We further compared the effects of TRAIL combined

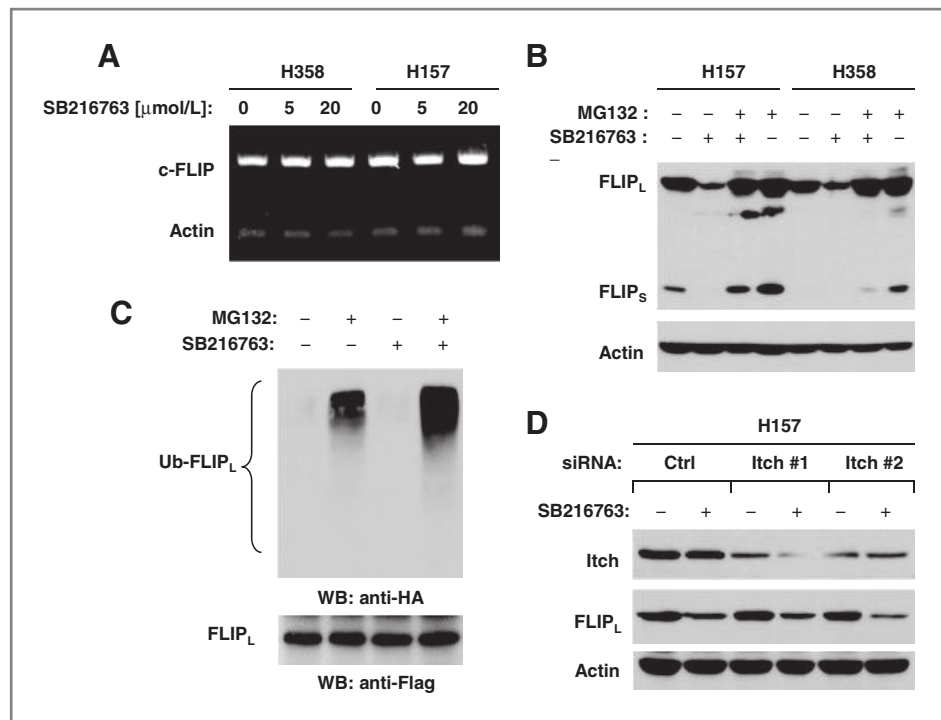


Figure 5. Inhibition of GSK3 does not alter c-FLIP mRNA levels (A), rather facilitates ubiquitin/proteasome-mediated c-FLIP degradation (B and C) independent of Itch (D). A, the indicated cell lines were treated with the given concentrations of SB216763 for 6 hours and then subjected to preparation of total cellular RNA and subsequent detection of c-FLIP using RT-PCR. B, the given cell lines were pretreated with 20 $\mu\text{mol/L}$ MG132 for 30 minutes prior to the addition of 20 $\mu\text{mol/L}$ SB216763. After cotreatment for 4 hours, the cells were harvested for preparation of whole-cell protein lysates and subsequent Western blot (WB) analysis. C, H157-FLIP_L-21 cells, which stably express ectopic flag-FLIP_L, were transfected with HA-ubiquitin plasmid using FuGENE 6 transfection reagent for 24 hours. The cells were then pretreated with 20 $\mu\text{mol/L}$ MG132 for 30 minutes and then cotreated with 20 $\mu\text{mol/L}$ SB216763 for 4 hours. Whole-cell protein lysates were then prepared for immunoprecipitation using anti-Flag antibody followed by Western blotting using anti-HA antibody for detection of ubiquitinated FLIP_L (Ub-FLIP_L) and anti-Flag antibody for detection of ectopic FLIP_L. D, H157 cells were transfected with the indicated siRNAs for 48 hours and then treated with 20 $\mu\text{mol/L}$ SB216763 for additional 8 hours. The cells were harvested for preparation of whole-cell protein lysates and subsequent Western blot analysis for detection of the indicated proteins.

with a GSK3 inhibitor, SB216763, on cell survival and caspase activation in H157 cell lines, which express Lac Z (as a control), FLIP_S, and FLIP_L. As presented in Fig. 7A, the combination effectively decreased the survival of H157-Lac Z-5 cells (e.g., by >50% compared with SB216763 or TRAIL alone) but not that of H157-FLIP_S-1 cells. The combination reduced the survival of H157-FLIP_L-21 cells only by less than 10% compared with SB216763 or TRAIL alone, although the reduction was statistically significant. Consistently, the SB216763 and TRAIL combination was more effective than either agent alone in inducing cleavage of caspase-8, caspase-9, caspase-3, and PARP in H157-Lac Z-5 cells, but this effect was substantially attenuated in both H157-FLIP_L-21 and H157-FLIP_S-1 cells (Fig. 7B). Thus, enforced expression of ectopic FLIP_S or FLIP_L abolished or attenuated the ability of GSK3 inhibition to sensitize cancer cells to TRAIL-induced apoptosis.

Discussion

The mechanisms by which celecoxib and its analogues induce apoptosis have long been a subject of intensive research. One such mechanism seems to be the inhibition

of PDK1/Akt signaling as documented in some studies (26–29). However, other studies have failed to show such a mechanism (30, 42, 43), thus leaving this as a controversial issue (16). In our studies primarily involving human NSCLC cell lines, we have never observed inhibition of p-Akt levels by celecoxib or its analogues such as DMC when they are used at growth arrest and apoptosis-inducing concentration ranges (up to 50 $\mu\text{mol/L}$). Instead, we detect increased p-Akt levels in some cell lines when they were exposed to celecoxib, as presented in Fig. 1. Thus, our data do not support a role for Akt inhibition in mediating celecoxib-induced growth arrest and apoptosis, at least in NSCLC cells.

Interestingly, the phosphorylation of GSK3 including both α (at Ser 21) and β (at Ser 9) isoforms, which are well known to be phosphorylated and inhibited by Akt (31, 44), was increased by celecoxib in dose- and time-dependent manners in the tested NSCLC cells, even in those without an increase in Akt phosphorylation (e.g., H1792; Fig. 1). Given that phosphorylation of GSK3 at Ser 21/Ser 9 results in inactivation of GSK3 (9, 11), our findings thus imply that celecoxib actually inhibits GSK3 function. Although celecoxib increases the phosphorylation of both Akt and GSK3 in some of our tested cell lines (e.g., H358 and Calu-1), inhibition of celecoxib-induced Akt

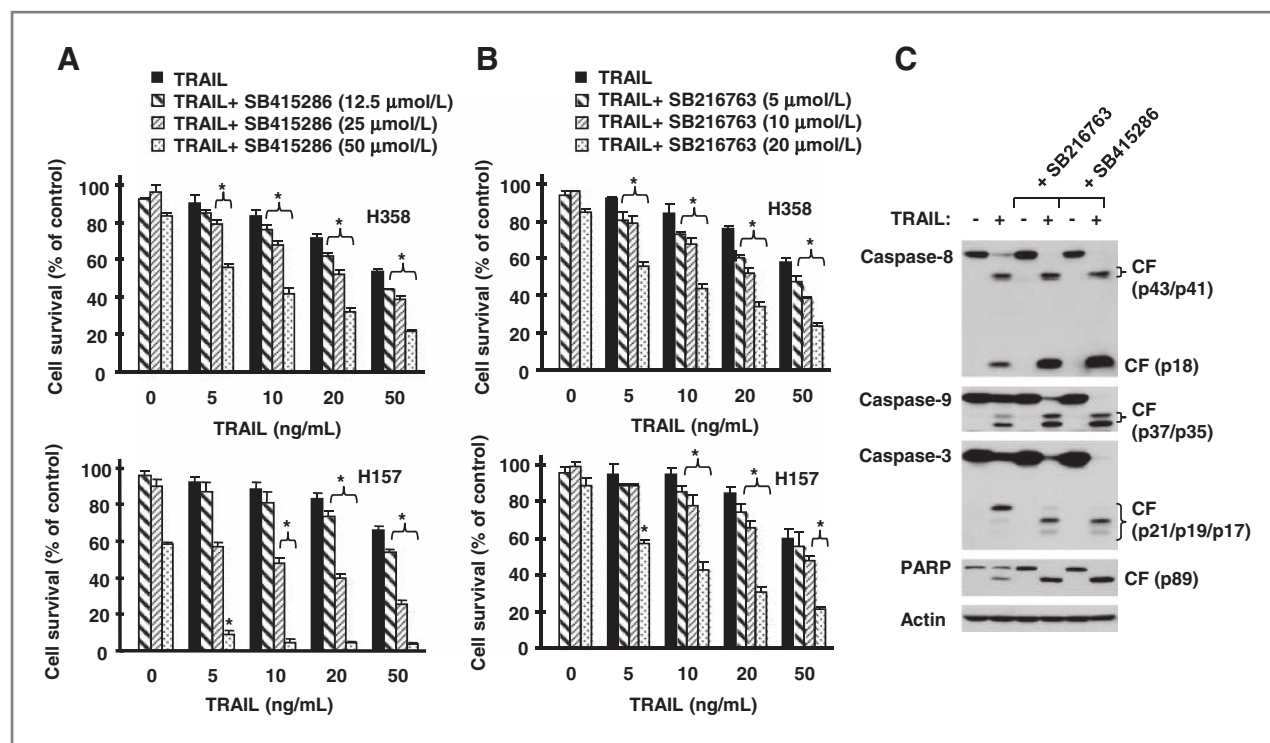


Figure 6. Inhibition of GSK3 with a GSK3 inhibitor augments TRAIL-induced apoptosis. A and B, the given cell lines were plated on 96-well plates and treated next day with the given doses of TRAIL alone, different concentrations of SB415286 (A) or SB216763 (B) alone, or their respective combinations. After 24 hours, the cells were subjected to estimation of cell number using the SRB assay. Columns, means of triplicate determinations; bars, \pm SDs. *, $P < 0.01$ compared with both TRAIL alone and a GSK3 inhibitor alone with ANOVA. C, H157 cells were treated with 50 ng/mL TRAIL alone, 20 μ mol/L SB216763, or 50 μ mol/L SB415286 alone, or their respective combinations. After 8 hours, the cells were harvested for preparation of whole-cell protein lysates and subsequent Western blot analysis. CF, cleaved form.

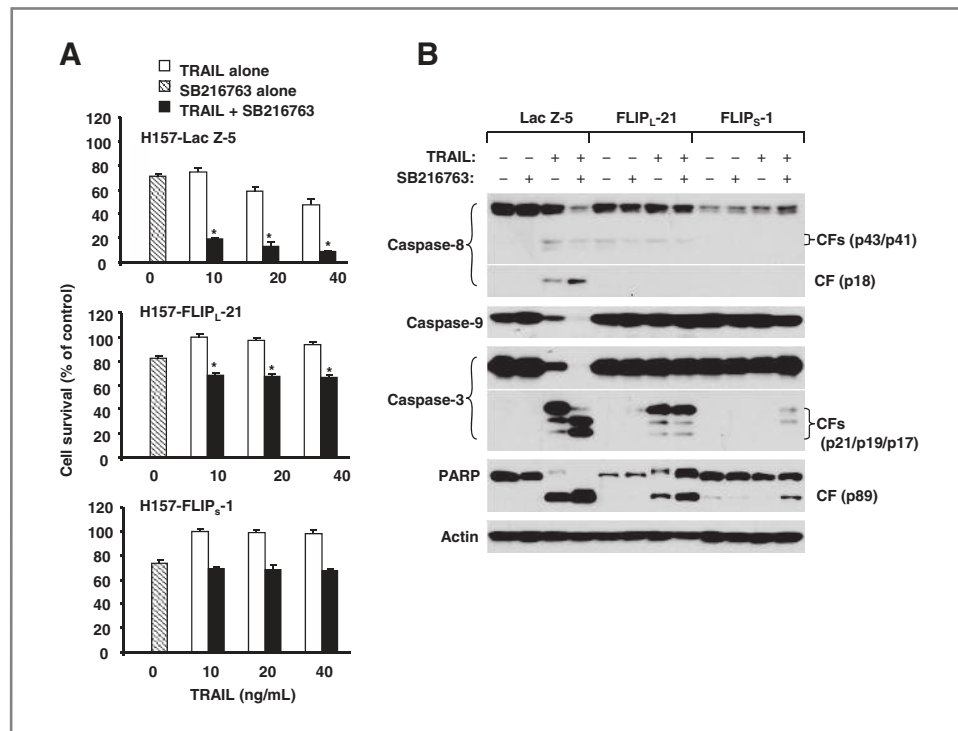
phosphorylation with the PI3K inhibitor LY294002 or wortmannin did not accordingly abrogate celecoxib-induced GSK3 phosphorylation (Fig. 2), suggesting that celecoxib induces Akt-independent GSK3 phosphorylation or inhibition. To the best of our knowledge, this is the first report of celecoxib inhibition of GSK3.

In addition to Akt, other kinases, such as p70S6K and PKC, can also phosphorylate GSK3 (9, 32, 33, 36–41). In our study, we did not show a role for mTOR/p70S6K in celecoxib-induced GSK phosphorylation because rapamycin effectively inhibited the basal levels of p-S6 but did not prevent the increase in Akt phosphorylation by celecoxib (Fig. 2). However, both R δ 31-8220 and GF109203X, which are pan-PKC inhibitors, abolished celecoxib-induced GSK3 phosphorylation, suggesting that celecoxib induces PKC-dependent GSK3 phosphorylation or inhibition. It is well known that PKC comprises multiple isoforms (45). Among these isoforms, PKC α , β , and δ isoforms have been suggested to regulate GSK3 phosphorylation (36, 41). In our study, we found that both G δ 6983, a specific PKC lacking activity against the μ isoform, and G δ 6979, a specific PKC α/β inhibitor, but not rottlerin, a specific PKC δ inhibitor, were as effective as the pan-PKC inhibitors in abolishing celecoxib-induced GSK3 phosphorylation (Fig. 2). Thus, we suggest that the PKC α/β isoforms may be important for mediating celecoxib-induced GSK3 phosphor-

ylation. These findings warrant further study in this direction. Our finding on celecoxib activation of PKC is novel, although we have yet to define the mechanism by which celecoxib activates PKC, warranting further investigation of this subject.

It has been shown that GSK3 β inhibition with either small-molecule inhibitors or siRNAs potentiates TRAIL-induced apoptosis in human prostate cancer cells (12). However, the underlying mechanisms are unknown. In our study, we could reproduce this biologic phenomenon in human NSCLC cells (Fig. 6). Very importantly, we found that inhibition of GSK3 with either siRNAs or small-molecule inhibitors downregulated c-FLIP levels, clearly indicating that GSK3 inhibition results in downregulation of c-FLIP levels. Complementarily, enforced expression of CA GSK3 increased c-FLIP levels (Fig. 4D). Thus, our findings clearly show that GSK3 regulates c-FLIP levels. To the best of our knowledge, this is the first study showing GSK3-dependent regulation of c-FLIP. Given that enforced ectopic c-FLIP expression protects cells from induction of apoptosis induced by GSK inhibition plus TRAIL (Fig. 7), it is plausible to conclude that c-FLIP downregulation should be a major event accounting for GSK3 inhibition-mediated enhancement of TRAIL-induced apoptosis. Thus, our findings on GSK3 regulation of c-FLIP provide a reasonable mechanism by which GSK inhibition potentiates TRAIL-induced apoptosis.

Figure 7. GSK3 inhibition-induced augmentation of TRAIL-induced apoptosis can be abrogated by enforced expression of ectopic c-FLIP. **A**, the indicated transfectants were seeded in 96-well plates and treated next day with 20 $\mu\text{mol/L}$ SB216763 alone, the given concentrations of TRAIL alone, and the respective combinations of SB216763 and TRAIL. After 24 hours, the cells were subjected to estimation of cell number using the SRB assay. Columns, means of triplicate determinations; bars, \pm SDs. *, $P < 0.001$ compared with both TRAIL alone and SB216763 alone with ANOVA. **B**, the indicated transfectants were treated with 20 $\mu\text{mol/L}$ SB216763 alone, 20 ng/mL TRAIL alone, and their combination. After 18 hours, the cells were harvested for preparation of whole-cell protein lysates and subsequent Western blot analysis. CF, cleaved form.



It is known that c-FLIP proteins, including FLIP_L and FLIP_S, are subjected to rapid turnover regulated through ubiquitin/proteasome-mediated protein degradation (5–7). However, the signaling event that triggers c-FLIP degradation has not been characterized. Our previous studies have shown that celecoxib and its analogue DMC downregulate c-FLIP levels through facilitating ubiquitination and proteasome-mediated degradation of c-FLIP (18, 19). In the current study, we found that the inhibition of GSK3 with SB216763 did not increase c-FLIP mRNA levels and that the presence of the proteasome inhibitor MG132 prevented SB216763-induced c-FLIP downregulation. Moreover, SB216763 substantially increased c-FLIP ubiquitination (Fig. 5). Collectively, these results indicate that GSK3 inhibition-induced c-FLIP downregulation occurs at a posttranslational level via promoting ubiquitin/proteasome-mediated protein degradation. Given that celecoxib inhibits GSK3, as discussed earlier, and reduces c-FLIP levels through the same mechanism as we have previously shown (18), we suggest that celecoxib inhibits GSK3, leading to facilitation of c-FLIP degradation. The E3 ligase Itch has been suggested to be involved in TNF α -induced c-FLIP (i.e., FLIP_L) degradation (7). In our study, we found that silencing of Itch expression with Itch siRNAs neither increased basal levels of c-FLIP nor blocked c-FLIP downregulation induced by either SB216763 or celecoxib (Fig. 4 and Supplementary Fig. S2), suggesting that Itch is unlikely to be involved in GSK3 inhibition-induced c-FLIP degradation.

Previous work has shown that c-FLIP downregulation contributes to celecoxib-induced apoptosis and enhancement of TRAIL-induced apoptosis (18). In agreement, we found in this study that siRNA-mediated silencing of GSK3 β enhanced

the ability of celecoxib to downregulate c-FLIP (i.e., FLIP_L). Similar results were generated when cells were cotreated with celecoxib and a GSK3 inhibitor (e.g., SB216763; Fig. 3). Thus, our results further support an important role of c-FLIP downregulation, which is mediated by GSK3 inhibition, in celecoxib-induced apoptosis.

We have previously shown that celecoxib downregulates c-FLIP independent of its COX-2 inhibitory activity by using COX-2 siRNA and DMC, which lacks COX-2 inhibitory activity (18, 19). In this study, we further showed that DMC also increased p-GSK3 levels; this effect could not be abrogated by LY294002 (Supplementary Fig. S1). Thus, celecoxib-induced GSK3 phosphorylation, and subsequent downregulation of c-FLIP, is unlikely to be secondary to COX-2 inhibition.

In summary, this study shows a novel mechanism by which celecoxib induces c-FLIP degradation through Akt-independent phosphorylation or inhibition of GSK3. Through this study, we are able to show, for the first time, that inhibition of GSK3 is associated with the induction of c-FLIP degradation, thus providing a reasonable explanation for how GSK3 inhibits the extrinsic death receptor-mediated apoptotic pathway.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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References

1. Wajant H. Targeting the FLICE inhibitory protein (FLIP) in cancer therapy. *Mol Interv* 2003;3:124-7.
2. Kamarajan P, Sun NK, Chao CC. Up-regulation of FLIP in cisplatin-selected HeLa cells causes cross-resistance to CD95/Fas death signalling. *Biochem J* 2003;376:253-60.
3. Longley DB, Wilson TR, McEwan M, Allen WL, McDermott U, Galligan L, et al. c-FLIP inhibits chemotherapy-induced colorectal cancer cell death. *Oncogene* 2006;25:838-48.
4. Abedini MR, Qiu Q, Yan X, Tsang BK. Possible role of FLICE-like inhibitory protein (FLIP) in chemoresistant ovarian cancer cells *in vitro*. *Oncogene* 2004;23:6997-7004.
5. Kim Y, Suh N, Sporn M, Reed JC. An inducible pathway for degradation of FLIP protein sensitizes tumor cells to TRAIL-induced apoptosis. *J Biol Chem* 2002;277:22320-9.
6. Poukkula M, Kaunisto A, Hietakangas V, Denessiouk K, Katajamaki T, Johnson MS, et al. Rapid turnover of c-FLIPshort is determined by its unique C-terminal tail. *J Biol Chem* 2005;280:27345-55.
7. Chang L, Kamata H, Solinas G, Luo JL, Maeda S, Venuprasad K, et al. The E3 ubiquitin ligase itch couples JNK activation to TNF α -induced cell death by inducing c-FLIP(L) turnover. *Cell* 2006;124:601-13.
8. Beurel E, Jope RS. The paradoxical pro- and anti-apoptotic actions of GSK3 in the intrinsic and extrinsic apoptosis signaling pathways. *Prog Neurobiol* 2006;79:173-89.
9. Frame S, Cohen P. GSK3 takes centre stage more than 20 years after its discovery. *Biochem J* 2001;359:1-16.
10. Jope RS, Yuskaitis CJ, Beurel E. Glycogen synthase kinase-3 (GSK3): inflammation, diseases, and therapeutics. *Neurochem Res* 2007;32:577-95.
11. Cohen P, Goedert M. GSK3 inhibitors: development and therapeutic potential. *Nat Rev Drug Discov* 2004;3:479-87.
12. Liao X, Zhang L, Thrasher JB, Du J, Li B. Glycogen synthase kinase-3 β suppression eliminates tumor necrosis factor-related apoptosis-inducing ligand resistance in prostate cancer. *Mol Cancer Ther* 2003;2:1215-22.
13. Rottmann S, Wang Y, Nasoff M, Deveraux QL, Quon KC. A TRAIL receptor-dependent synthetic lethal relationship between MYC activation and GSK3 β /FBW7 loss of function. *Proc Natl Acad Sci U S A* 2005;102:15195-200.
14. Sun M, Song L, Li Y, Zhou T, Jope RS. Identification of an antiapoptotic protein complex at death receptors. *Cell Death Differ* 2008;15:1887-900.
15. Thun MJ, Henley SJ, Patrono C. Nonsteroidal anti-inflammatory drugs as anticancer agents: mechanistic, pharmacologic, and clinical issues. *J Natl Cancer Inst* 2002;94:252-66.
16. Schonthal AH. Antitumor properties of dimethyl-celecoxib, a derivative of celecoxib that does not inhibit cyclooxygenase-2: implications for glioma therapy. *Neurosurg Focus* 2006;20:E21.
17. Liu X, Yue P, Zhou Z, Khuri FR, Sun SY. Death receptor regulation and celecoxib-induced apoptosis in human lung cancer cells. *J Natl Cancer Inst* 2004;96:1769-80.
18. Liu X, Yue P, Schonthal AH, Khuri FR, Sun SY. Cellular FLICE-inhibitory protein down-regulation contributes to celecoxib-induced apoptosis in human lung cancer cells. *Cancer Res* 2006;66:11115-9.
19. Chen S, Liu X, Yue P, Schonthal AH, Khuri FR, Sun SY. CHOP-dependent DR5 induction and ubiquitin/proteasome-mediated c-FLIP downregulation contribute to enhancement of TRAIL-induced apoptosis by dimethyl-celecoxib in human non-small cell lung cancer cells. *Mol Pharmacol* 2007;72:1269-79.
20. Raja SM, Chen S, Yue P, Acker TM, Lefkove B, Arbiser JL, et al. The natural product honokiol preferentially inhibits cellular FLICE-inhibitory protein and augments death receptor-induced apoptosis. *Mol Cancer Ther* 2008;7:2212-23.
21. Sun SY, Yue P, Wu GS, El-Deiry WS, Shroot B, Hong WK, et al. Mechanisms of apoptosis induced by the synthetic retinoid CD437 in human non-small cell lung carcinoma cells. *Oncogene* 1999;18:2357-65.
22. Sun SY, Yue P, Dawson MI, Shroot B, Michel S, Lamph WW, et al. Differential effects of synthetic nuclear retinoid receptor-selective retinoids on the growth of human non-small cell lung carcinoma cells. *Cancer Res* 1997;57:4931-9.
23. Chen C, Sun X, Ran Q, Wilkinson KD, Murphy TJ, Simons JW, et al. Ubiquitin-proteasome degradation of KLF5 transcription factor in cancer and untransformed epithelial cells. *Oncogene* 2005;24:3319-27.
24. Nam SY, Jung GA, Hur GC, Chung HY, Kim WH, Seol DW, et al. Upregulation of FLIP(S) by Akt, a possible inhibition mechanism of TRAIL-induced apoptosis in human gastric cancers. *Cancer Sci* 2003;94:1066-73.
25. Panka DJ, Mano T, Suhara T, Walsh K, Mier JW. Phosphatidylinositol 3-kinase/Akt activity regulates c-FLIP expression in tumor cells. *J Biol Chem* 2001;276:6893-6.
26. Hsu AL, Ching TT, Wang DS, Song X, Rangnekar VM, Chen CS. The cyclooxygenase-2 inhibitor celecoxib induces apoptosis by blocking Akt activation in human prostate cancer cells independently of Bcl-2. *J Biol Chem* 2000;275:11397-403.
27. Kulp SK, Yang YT, Hung CC, Chen KF, Lai JP, Tseng PH, et al. 3-Phosphoinositide-dependent protein kinase-1/Akt signaling represents a major cyclooxygenase-2-independent target for celecoxib in prostate cancer cells. *Cancer Res* 2004;64:1444-51.
28. Arico S, Pattingre S, Bauvy C, Gane P, Barbat A, Codogno P, et al. Celecoxib induces apoptosis by inhibiting 3-phosphoinositide-dependent protein kinase-1 activity in the human colon cancer HT-29 cell line. *J Biol Chem* 2002;277:27613-21.
29. Leng J, Han C, Demetris AJ, Michalopoulos GK, Wu T. Cyclooxygenase-2 promotes hepatocellular carcinoma cell growth through Akt activation: evidence for Akt inhibition in celecoxib-induced apoptosis. *Hepatology* 2003;38:756-68.
30. Kardosh A, Wang W, Uddin J, Petasis NA, Hofman FM, Chen TC, et al. Dimethyl-celecoxib (DMC), a derivative of celecoxib that lacks cyclooxygenase-2-inhibitory function, potently mimics the anti-tumor effects of celecoxib on Burkitt's lymphoma *in vitro* and *in vivo*. *Cancer Biol Ther* 2005;4:571-82.
31. Vivanco I, Sawyers CL. The phosphatidylinositol 3-kinase AKT pathway in human cancer. *Nat Rev Cancer* 2002;2:489-501.
32. Zhang HH, Lipovsky AI, Dibble CC, Sahin M, Manning BD. S6K1 regulates GSK3 under conditions of mTOR-dependent feedback inhibition of Akt. *Mol Cell* 2006;24:185-97.
33. Armstrong JL, Bonavaud SM, Toole BJ, Yeaman SJ. Regulation of glycogen synthesis by amino acids in cultured human muscle cells. *J Biol Chem* 2001;276:952-6.
34. Sawyers CL. Will mTOR inhibitors make it as cancer drugs? *Cancer Cell* 2003;4:343-8.
35. Sun SY, Rosenberg LM, Wang X, Zhou Z, Yue P, Fu H, et al. Activation of Akt and eIF4E survival pathways by rapamycin-mediated mammalian target of rapamycin inhibition. *Cancer Res* 2005;65:7052-8.
36. Wang Q, Zhou Y, Evers BM. Neurotensin phosphorylates GSK-3 α /beta through the activation of PKC in human colon cancer cells. *Neoplasia* 2006;8:781-7.

37. Vilimek D, Duronio V. Cytokine-stimulated phosphorylation of GSK-3 is primarily dependent upon PKCs, not PKB. *Biochem Cell Biol* 2006;84:20–9.
38. Cook D, Fry MJ, Hughes K, Sumathipala R, Woodgett JR, Dale TC. Wingless inactivates glycogen synthase kinase-3 via an intracellular signalling pathway which involves a protein kinase C. *EMBO J* 1996;15:4526–36.
39. Fang X, Yu S, Tanyi JL, Lu Y, Woodgett JR, Mills GB. Convergence of multiple signaling cascades at glycogen synthase kinase 3: Edg receptor-mediated phosphorylation and inactivation by lysophosphatidic acid through a protein kinase C-dependent intracellular pathway. *Mol Cell Biol* 2002;22:2099–110.
40. Shin SY, Yoon SC, Kim YH, Kim YS, Lee YH. Phosphorylation of glycogen synthase kinase-3 β at serine-9 by phospholipase C γ 1 through protein kinase C in rat 3Y1 fibroblasts. *Exp Mol Med* 2002;34:444–50.
41. De Servi B, Hermani A, Medunjanin S, Mayer D. Impact of PKC δ on estrogen receptor localization and activity in breast cancer cells. *Oncogene* 2005;24:4946–55.
42. Kardosh A, Soriano N, Liu YT, Uddin J, Petasis NA, Hofman FM, et al. Multitarget inhibition of drug-resistant multiple myeloma cell lines by dimethyl-celecoxib (DMC), a non-COX-2 inhibitory analog of celecoxib. *Blood* 2005;106:4330–8.
43. Chen Z, Zhang X, Li M, Wang Z, Wieand HS, Grandis JR, et al. Simultaneously targeting epidermal growth factor receptor tyrosine kinase and cyclooxygenase-2, an efficient approach to inhibition of squamous cell carcinoma of the head and neck. *Clin Cancer Res* 2004;10:5930–9.
44. Hennessy BT, Smith DL, Ram PT, Lu Y, Mills GB. Exploiting the PI3K/AKT pathway for cancer drug discovery. *Nat Rev Drug Discov* 2005;4:988–1004.
45. Mackay HJ, Twelves CJ. Targeting the protein kinase C family: are we there yet? *Nat Rev Cancer* 2007;7:554–62.