

Activation of WNT/ β -catenin signaling results in resistance to a dual PI3K/mTOR inhibitor in colorectal cancer cells harboring *PIK3CA* mutations

Ye-Lim Park^{1,2*}, Hwang-Phill Kim^{1,2*}, Young-Won Cho^{1,2}, Dong-Wook Min^{1,2}, Seul-Ki Cheon^{1,2}, Yoo Joo Lim³, Sang-Hyun Song², Sung Jin Kim⁴, Sae-Won Han^{2,3}, Kyu Joo Park⁵ and Tae-You Kim^{1,2,3}

¹Department of Molecular Medicine and Biopharmaceutical Sciences, Graduate School of Convergence Science and Technology, Seoul National University, Seoul, South Korea

²Cancer Research Institute, Seoul National University, Seoul, South Korea

³Department of Internal Medicine, Seoul National University Hospital, Seoul, South Korea

⁴Department of Biochemistry and Molecular Biology, Pennsylvania State University, University Park, PA

⁵Department of Surgery, Seoul National University College of Medicine, Seoul, South Korea

PIK3CA is a frequently mutated gene in cancer, including about ~15 to 20% of colorectal cancers (CRC). *PIK3CA* mutations lead to activation of the PI3K/AKT/mTOR signaling pathway, which plays pivotal roles in tumorigenesis. Here, we investigated the mechanism of resistance of *PIK3CA*-mutant CRC cell lines to gedatolisib, a dual PI3K/mTOR inhibitor. Out of a panel of 29 CRC cell lines, we identified 7 harboring one or more *PIK3CA* mutations; of these, 5 and 2 were found to be sensitive and resistant to gedatolisib, respectively. Both of the gedatolisib-resistant cell lines expressed high levels of active glycogen synthase kinase 3-beta (GSK3 β) and harbored the same frameshift mutation (c.465_466insC; H155fs*) in *TCF7*, which encodes a positive transcriptional regulator of the WNT/ β -catenin signaling pathway. Inhibition of GSK3 β activity in gedatolisib-resistant cells by siRNA-mediated knockdown or treatment with a GSK3 β -specific inhibitor effectively reduced the activity of molecules downstream of mTOR and also decreased signaling through the WNT/ β -catenin pathway. Notably, GSK3 β inhibition rendered the resistant cell lines sensitive to gedatolisib cytotoxicity, both *in vitro* and in a mouse xenograft model. Taken together, these data demonstrate that aberrant regulation of WNT/ β -catenin signaling and active GSK3 β induced by the *TCF7* frameshift mutation cause resistance to the dual PI3K/mTOR inhibitor gedatolisib. Cotreatment with GSK3 β inhibitors may be a strategy to overcome the resistance of *PIK3CA*- and *TCF7*-mutant CRC to PI3K/mTOR-targeted therapies.

Introduction

The phosphatidylinositol 3-kinase (PI3K), AKT serine/threonine kinase, mammalian target of rapamycin (PI3K/AKT/mTOR) signaling pathway is aberrantly activated in many

types of cancer.¹ For example, activating *PIK3CA* gene mutations affecting the PI3K α catalytic subunit, such as H1047R, P449T, E545K and R88Q are detected in about ~15–20% of colorectal cancers (CRC).^{2,3} Therefore, components of the

Key words: colorectal cancer, PI3K/mTOR dual inhibitor, *TCF7* frameshift mutation, WNT/ β -catenin signaling pathway, GSK3 β , patient-derived colorectal cancer, organoids

Abbreviations: CRC: colorectal cancers; GSK3 β : Glycogen synthase kinase 3 beta; mTOR: mammalian target of rapamycin; PI3K: The phosphatidylinositol 3-kinase

Additional Supporting Information may be found in the online version of this article.

*Y-L.P. and H-P.K. contributed equally to this work.

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Correspondence to: Tae-You Kim, Professor, Department of Internal Medicine, Seoul National University Hospital, 101 Daehak-ro, Jongno-Gu Seoul, 03080, South Korea, E-mail: kimty@snu.ac.kr; Tel.: 82-2-2072-7200; Fax: 82-2-762-9662

What's new?

Mutations in the PI3K/mTOR and WNT/ β -catenin pathways are common in colorectal cancer. Here, in colorectal cancer cells, the authors show that the frameshift mutation H155fs* in transcription factor *TCF7* serves a critical role in mediating resistance to the PI3K/mTOR dual inhibitor gedatolisib. *TCF7* H155fs* maintained mTOR signaling by inducing the active form of glycogen synthase kinase 3-beta (GSK3 β) and aberrant WNT/ β -catenin signaling. The latter conferred resistance to gedatolisib. Co-treatment with a GSK3 β inhibitor increased sensitivity to gedatolisib, a combination that synergistically inhibited colorectal tumor growth in mice. The findings shed light on gedatolisib resistance mechanisms and potential resistance biomarkers.

PI3K/mTOR pathway have been considered attractive drug targets.^{4,5} Most PI3K/mTOR inhibitors, such as dactolisib (BEZ235),⁶ Voxelisib (XL765),⁷ and gedatolisib,⁸ are generally effective against solid tumors harboring *PIK3CA* mutations,⁹ whereas *KRAS*-mutant tumors tend to be resistant.¹⁰ These inhibitors are often used as targeted therapies, but their mechanisms of action are uncertain and their effects are often limited by other mutations or compensatory mechanisms.^{11,12} For these reasons, combination therapies that target other pathways are being investigated in an effort to overcome tumor resistance to dual PI3K/mTOR inhibitors.^{13,14}

Glycogen synthase kinase 3 beta (GSK3 β) is a critical component of a degradative multiprotein destruction complex that includes adenomatous polyposis coli (APC), casein kinase 1 (CKI), AXIN2, and the key transcriptional coactivator for the WNT signaling pathway, β -catenin.¹⁵ This complex acts as a negative regulator of WNT/ β -catenin signaling, which is frequently elevated in cancer.¹⁶ In addition to this role, GSK3 β functions as a link between the PI3K/mTOR and WNT/ β -catenin pathways through AMPK. Although GSK3 β is well known as a tumor suppressor, recent work suggests that it also functions in promoting the proliferation of many types of cancer cells.^{17,18} In particular, GSK3 β is thought to play both positive and negative roles in WNT/ β -catenin signaling, but the precise mechanisms have not yet been established.¹⁹

TCF7 is a transcription factor that mediates and positively regulates WNT/ β -catenin signaling by inducing transcription of target genes such as *CCND1*, *AXIN2*, *TCF7*, *LEF1*, *MET*, and *WNT3A*.^{20,21} About 1–2% of CRC harbor a mutation in *TCF7* that results in loss of the β -catenin binding domain, which reduces its transcriptional activity and weakens WNT/ β -catenin signaling.²² However, several *TCF7* frameshift mutations have also been reported in cancer, and current work suggests that they can increase or decrease transcription of WNT target genes.²³

In our study, we explored the mechanism of resistance of CRC cells to the dual PI3K/mTOR inhibitor gedatolisib, focusing on the relationship between PI3K/mTOR, GSK3 β , and WNT/ β -catenin signaling. We demonstrate a pivotal role for *TCF7* H155fs* in increasing GSK3 β activity and WNT/ β -catenin signaling and reveal their contribution to gedatolisib resistance in *PIK3CA*- and *TCF7*-mutant CRC.

Material and Methods**Reagents**

Gedatolisib (also known as PKI-587 or PF-05212384) was kindly provided by Pfizer (New York, NY). CHIR-99021 (HCl) and SB216763 were purchased from Selleck Chemicals (Houston, TX). LiCl was purchased from Sigma Aldrich (St Louis, MO). Stock solutions were prepared in dimethyl sulfoxide or distilled water and stored at -20°C .

Cell lines and cell culture

Human CRC cell lines, including HCT-116, WiDr, HT-29, SNU-C5, DLD-1, HCT-15, and LS174T, were obtained from the Korean Cell Line Bank (Seoul, Korea).²⁴ Cells were cultured in RPMI 1640 media containing 10% heat-inactivated fetal bovine serum (Gibco, Carlsbad, CA) and gentamicin (10 $\mu\text{g}/\text{mL}$) at 37°C in a 5% CO_2 atmosphere.

Cell viability assay

The viability of cells was assessed using MTT assays (Sigma-Aldrich, St Louis, MO). A total of 2×10^3 cells were seed in 96-well plates, incubated for 24 h, and treated for 72 h with indicated drugs at 37°C . After treatment, MTT solution was added to each well and incubated for 4 h at 37°C . The medium was then removed, and dimethyl sulfoxide (DMSO) was added and mixed thoroughly for 10 min at room temperature. Cell viability was determined by measuring absorbance at 540 nm using a VersaMax microplate reader (Molecular Devices, Sunnyvale, CA). The concentration of drug required to inhibit cell growth by 50% was determined *via* interpolation from dose–response curves using Calcsyn software (Biosoft, Ferguson, MO). Six replicate wells were utilized for each analysis, and at least three independent experiments were conducted. The data from replicate wells are presented at the mean number of the remaining cells with 95% confidence intervals.

Western blotting antibodies

Cultured cells were washed with phosphate buffered saline (PBS) and lysed with lysis buffer (50 mM Tris-HCl, pH 7.5 1% NP-40, 0.1% sodium deoxycholate, 150 mM NaCl, 50 mM NaF, 1 mM sodium pyrophosphate, 1 mM EDTA, and protease/phosphatase inhibitors). Lysates were clearly by centrifugation at 13,000 rpm for 20 min. Protein

concentrations were quantified with a Bicinchoninic Acid Protein Assay Reagent (Pierce, Rockford, IL), according to manufacturer's instructions. Samples containing equal quantities of total proteins were resolved in SDS-polyacrylamide denaturing gel, transferred to nitrocellulose membranes. The membranes were incubated in blocking buffer containing 1% skim milk and 1% bovine serum albumin (BSA) for an hour at room temperature and probed overnight at 4 °C with primary antibodies. Antibodies against PI3K p110, phosphorylated (p)-AKT (pS473), p-AKT (pThr308), p-TSC2 (pThr1462), p-mTOR (pS2481), p-p70S6K (pThr389), p-p70S6K (pS371), p-S6K (pS240/244), p-4E-BP1 (pS65), p-LRP6 (pS1490), p-GSK3 β (pS9/21), p- β -catenin, p-GS, AXIN2, AKT, TSC1, TSC2, mTOR, Raptor, p70S6K, S6K, 4E-BP1, GSK3 β , TCF7, caspase-3 and caspase-7 were purchased from Cell Signaling Technology (Beverly, MA). Antibodies to p-GSK3 β (pTyr216), β -tubulin, β -catenin, and active β -catenin were obtained from Santa Cruz Biotechnology (Dallas, TX). Quantitation of band intensity on western blots was performed with ImageJ software (National Institutes of Health, Bethesda, MD).

Cell cycle analysis and annexin V staining

Cells were treated for 24 h with drugs, washed twice in PBS, fixed in 70% ethanol, and then stored at -20 °C until analysis. Before analysis, the cell suspensions were washed with PBS, digested for 15 min with RNase A (50 μ g/mL) at 37 °C, and stained with propidium iodide (50 μ g/mL). The cell DNA contents (10,000 cells per experimental group) were determined with a flow cytometer (FACSCaliber, Becton Dickinson Biosciences) equipped with a ModFit LT program (Verity Software House, Inc.). Cells were left untreated or treated for 48 h with two different concentrations of gedatolisib and siRNA mediated GSK3 β (50 nM) or CHIR-99021 (0.1 and 1 μ M/L of gedatolisib and 10 and 20 μ M/L of CHIR-99021), at which point the cells were collected and stained with Annexin V-Fluorescein IsoThioCyanate (V-FITC). Apoptotic cell death was assessed by counting the numbers of cells that stained positive for Annexin V-FITC and negative for propidium iodide using an Apoptosis Detection Kit 2 (BD Pharmingen), coupled with fluorescence-activated cell sorting analysis.

Colony-forming assay

Colony-forming assays were performed in 6-well plates. Cells were plated in triplicate at 4×10^3 or 1×10^4 cells/well and incubated for 2 days before addition of vehicle, gedatolisib, and/or CHIR-99021 at the concentrations indicated in the figures. Seven days later, the medium was removed and the cells were stained with Coomassie Brilliant Blue. Cells were washed five times in phosphate-buffered saline and once in distilled water and then air dried. Colonies were enumerated using a GelCount colony counter (Oxford

Optronix, Abingdon, UK) according to the manufacturer's instructions.

Immunoprecipitation

For immunoprecipitation of mTOR-Raptor and TSC1-TSC2 complexes, HCT-116, HCT-15, and LS174T cells were seeded in 100 mm \times 20 mm dishes and incubated for 24 h. Vehicle or gedatolisib was added to a final concentration of 1 μ M and the cells were incubated for an additional 12 h and then harvested. Whole cell extracts were prepared and immunoprecipitations were performed as previously described.²⁵

Small interfering RNA-mediated knockdown

Small interfering RNAs (siRNAs) against GSK3 β and TCF7 were purchased from mbiotech (Seoul, Korea). Cells were transfected with siRNAs (50 nM) according to the manufacturer's instructions, as previously reported. Cell lysates were prepared at 48 h after transfection. siRNA sequences were: negative control: sense 5'-CCUCGUGCCGUCCAUCAGG UAGUU-3' and anti-sense 5'-CUACCUGAUGGAACGGC ACGAGGUU-3'; GSK3 β : sense 5'-CAGCUAUACAGACAC UAAAUU-3' and anti-sense 5'-UUUAGUGUCUGUAUAGC UGUU-3'; TCF7: sense 5'-GACAACUACGGGAAGAAGA UU-3' and anti-sense 5'-UCUUCUCCCGUAGUUGU CUU-3'.

Site-directed mutagenesis

Plasmid pcDNA3.1 encoding TCF7 was purchased from Addgene (Cambridge, MA), and pOTB7 encoding GSK3 β was purchased from KHGB (Korea Human Gene Bank, Seoul, Korea). The TCF7 H155 frameshift and GSK3 β S9A mutations were introduced using a QuikChange Site-Directed Mutagenesis kit (Stratagene, La Jolla, CA) with full-length *TCF7* and *GSK3 β* as the templates. The primers to introduce the TCF7 H155fs mutation were: forward 5'-TTGGGGGACA CCGGTGGGGGGGCTG-3' and reverse 5'-CAGCCCCCCCCA CCGGTGTCCCCCAA-3', and the primers to introduce the GSK3 β S9A mutation were: forward 5'-TTGGGGGACA CCGGTGGGGGGGCTG-3' and reverse 5'-CAGCCCCCCCCA CCGGTGTCCCCCAA-3'. Mutagenesis reactions were performed according to the manufacturer's instructions. The mutated sequences were confirmed by Sanger sequencing (Macrogen, Seoul, Korea).

BrdU incorporation and cell growth analysis

HCT-15 and LS174T cell lines were transfected with plasmids encoding GSK3 β wild-type and GSK3 β -S9A mutant proteins using Lipofectamine 2000 (Invitrogen, Carlsbad, CA). BrdU staining was performed as previously described.¹⁸

Establishment of patients-derived organoids and organoid culture

Human Colon cancer tissues were obtained from Seoul National University Hospital (Seoul, Korea). The study

protocol was approved by the Institute Review Board (IRB) of Seoul National University Hospital with informed consent (1608-054-784). Tumor cell isolation and organoid culture were processed as previously described.²⁶

Organoid electroporation and 3D drug response assay

Organoids were transfected with TCF7 H155fs mutation using NEPA21 Electroporation (Nepagene, Japan) as previously described.²⁷ After 24 h, drug was treated and cell viability was assessed using Cell-titer Glo 3D (Promega, US) after 6 days. Organoids images were obtained using ImageXpress Nano system (Molecular Devices, US).

Mouse xenograft model

All animal experiments were approved by the Institute Laboratory Animal Resources and Use Committee of Seoul National University. Four-week-old female BALB/c nude 20 mice were purchased from Central Lab Animal Inc. (Seoul, Korea) and weighed 18–20 g on arrival. Mice were allowed to acclimatize for 1 week before experiments. HCT-15 cells (1×10^7 /mouse) were injected subcutaneously into the right flank of the mouse. When the mice had developed a tumor volume of 100–200 mm³, they were randomized into the treatment groups ($n = 5$ per group) and treatment with vehicle, gedatolisib, LiCl, or gedatolisib plus LiCl was initiated. Mice treated with LiCl were also given additional NaCl to prevent electrolyte imbalance, as previously reported.²⁸ Gedatolisib (10 mg/kg) and LiCl (80 mg/kg) were injected intravenously and intraperitoneally, respectively, twice a week for 3 weeks. The tumors were then measured with calipers three times per week, and the tumor volume in mm³ was calculated in accordance with the after formula: $\{(width)^2 \times (height)\}/2$. After the final treatment on day 21, all of the mice were euthanized.

Immunohistochemistry (IHC) staining

Xenograft tumor specimens were harvested at the end of an efficacy study and fixed for 24 h in 10% buffered formalin. After embedding in paraffin and sectioning, general tissue morphology was visualized *via* H&E staining. Ki67 procedure has previously been described.²⁹

Statistical analysis

All experiments were conducted in duplicate or triplicate, with at least two biological replicates. Data are expressed as the mean \pm standard deviation (s.d.). Statistical significance was calculated using unpaired Student's *t*-test, except for the differences between tumor sizes in xenograft-bearing mice, which was analyzed by ANOVA. $p < 0.05$ was considered statistically significant. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$.

Results

PIK3CA-mutant CRC cell lines with elevated phosphorylated mTOR and reduced phosphorylated TSC2 expression are sensitive to gedatolisib

To explore the effects of the dual PI3K/mTOR inhibitor gedatolisib in CRC cells, we first determined the concentration of gedatolisib that reduced cell viability by 50% (IC₅₀) in a panel of 29 CRC cell lines (Fig. 1a). The cells were then divided into two groups, designated sensitive or resistant to gedatolisib, based on IC₅₀ values of less than or greater than 0.5 μ M, respectively. Seven of the 29 CRC cells harbored at least one *PIK3CA* mutation (Supporting Information Table S1); of these cells, 5 were gedatolisib sensitive (HCT-116, WiDr, HT-29, SNU-C5 and DLD-1; IC₅₀ < 0.5 μ M) and 2 were resistant (HCT15 and LS174T; IC₅₀ > 7 μ M). Next, we investigated the basal expression of additional PI3K/AKT/mTOR pathway molecules and observed that the levels of p-mTOR, which phosphorylated ser2481 ensues from an auto-phosphorylation mechanism and is found in both mTORC1 and 2 complexes,³⁰ were higher and the levels of p-TSC2 were lower in the gedatolisib-sensitive than the -resistant cell lines (Fig. 1b). In addition, since all seven cell lines have a *PIK3CA* hotspot mutation, it can be concluded that the PI3K/AKT/mTOR pathway is activated, the levels of p-AKT Thr308, readout of PI3K activation, and the levels of p-AKT ser473, readout of mTORC2 activation, are both high.

Furthermore, we evaluated the cell lines for their colony-forming ability. As expected, gedatolisib treatment markedly decreased both the number and the size of colonies formed by gedatolisib-sensitive cell lines, but had no effect on the resistant cell lines (Fig. 1c). To confirm these findings, we examined the cell cycle status by flow cytometry of cells, stained with the DNA-intercalating dye propidium iodide or by western blot analysis of cell cycle regulatory proteins. In the sensitive cell lines, gedatolisib dose-dependently increased the proportion of cells in sub-G1 phase. However, the resistant cell lines showed no significant change in sub-G1 phase (data not shown). So, we evaluated apoptosis induction by Annexin V-FITC staining and the number of Annexin V stained cells were increased in five sensitive cell lines, showing increased expression of active caspase-3 and active caspase-7, but had no effect on the resistant cell lines (Fig. 1d). In addition, we used patient-derived colorectal cancer organoids to determine whether they show similar tendencies to cell line. O29T organoids with *PIK3CA* R88Q mutation were more sensitive compared to O28T with *PIK3CA* wild-type. Next, phosphorylation of several components of the PI3K/mTOR signaling pathway, indicative of their activation, was also inhibited by gedatolisib treatment in the sensitive cell lines, whereas it was either maintained or upregulated in the resistant cell lines (Fig. 1f). These results showed that gedatolisib effectively suppressed activation of the PI3K/mTOR pathway, reduced cell growth and proliferation, and induced apoptosis in sensitive *PIK3CA*-mutant

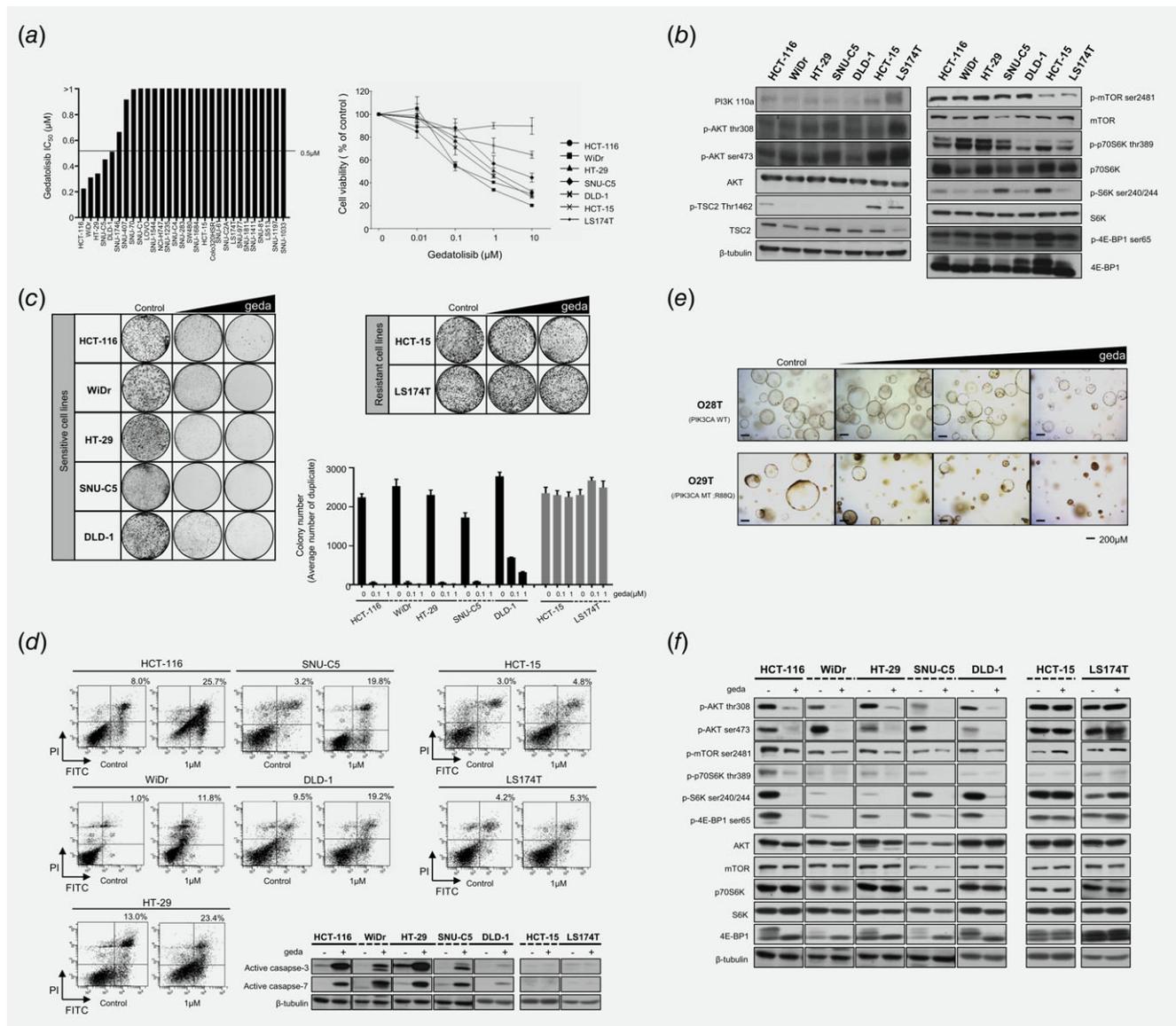


Figure 1. Gedatolisib sensitivity and resistance of CRC cell lines harboring *PIK3CA* mutations. (a) A total of 29 CRC cell lines were treated with vehicle or gedatolisib at the indicated concentrations for 72 h, and IC_{50} values were determined using the MTT viability assay. Untreated cells were considered 100% viable. (b) Western blot analysis of basal levels of the total or phosphorylated PI3K/mTOR pathway-associated proteins in CRC cell lines. Data are representative of three independent experiments. β -Tubulin was probed as a loading control in all western blots. (c) Colony-forming assay of CRC cell lines treated with vehicle or gedatolisib at 0.1 or 1 μM for 10 days. (d) Annexin V-FITC staining and western blotting of CRC cell lines treated with vehicle or gedatolisib at 1 μM for 72 h. (e) Human organoids, O28T (PIK3CA wild-type) and O29T (PIK3CA R88Q), were treated gedatolisib at 0.01 or 0.1 or 1 μM for 6 days measured cell viability using the cell titler glo. (f) CRC cell lines were treated with vehicle or gedatolisib at 1 μM for 24 h, and cell lysates were subjected to western blotting with antibodies specific for total or phosphorylated proteins as indicated. [Color figure can be viewed at wileyonlinelibrary.com]

CRC cell lines and this sensitivity was demonstrated also in human organoid samples.

Gedatolisib-resistant cell lines exhibit sustained mTOR signaling which is dependent on GSK3 β

HCT-15 and LS174T cell lines are resistant to gedatolisib despite carrying activating *PIK3CA* mutations. Moreover, gedatolisib treatment increased the levels of p-mTOR in these

cells (Fig. 1f), suggesting that a molecule upstream of mTOR might be involved in the mechanism of gedatolisib resistance. Tuberosclerosis proteins 1 and 2 (TSC1/TSC2) are upstream inhibitors of mTOR and function as integrators between mTOR and other signaling pathways. AKT negatively regulates the formation of TSC1-2 complexes by phosphorylating TSC2.³¹ Thus, based on our results and previous data,³¹ we hypothesized that TSC1-2 complex levels would be higher

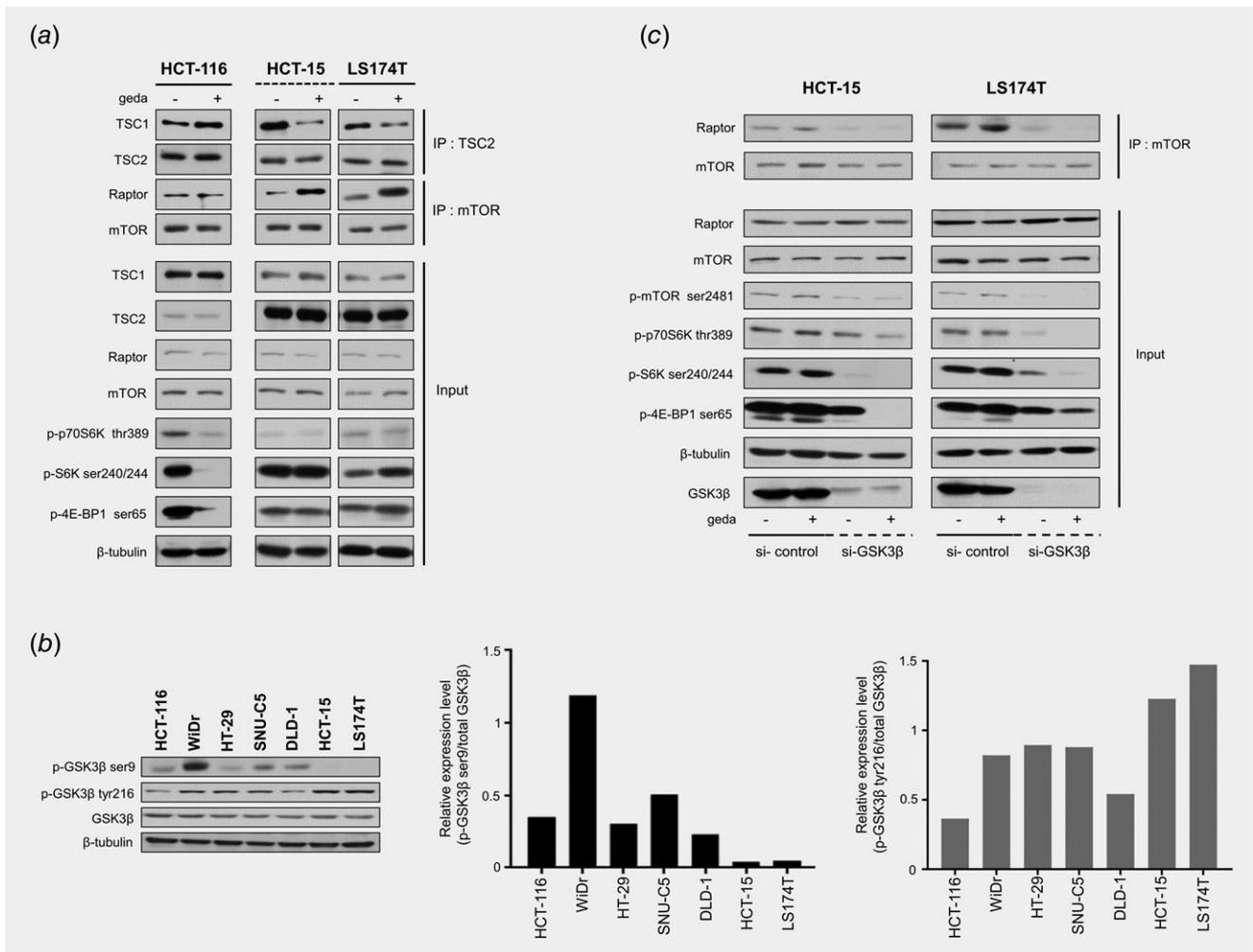


Figure 2. GSK3 β sustains mTOR signaling in gedatolisib-resistant cell lines. (a) HCT-116 (sensitive) and HCT-15 and LS174T (resistant) cells were treated with vehicle or gedatolisib at 1 μ M for 12 h. TSC2 and Raptor were immunoprecipitated from cell lysates and subjected to western blotting with antibodies to TSC1 or TSC2 and mTOR or Raptor (upper rows). 30% of input cell lysate was blotted with antibodies to total or phosphorylated forms of the indicated proteins (lower rows). (b) Western blot analysis and phosphorylated and total GSK3 β protein expression in the indicated CRC lines. The graph shows the ratio of p-GSK3 β (ser9)/p-GSK3 β (tyr216) to total GSK3 β expression levels. (c) HCT-15 and LS174T cells were transfected with control or GSK3 β -targeting siRNA for 48 h and then treated with vehicle or gedatolisib 1 μ M for 12 h. mTOR was immunoprecipitated from cell lysates and subjected to western blotting with antibodies to Raptor or mTOR (Upper rows). 30% of input lysate was blotted with antibodies to total or phosphorylated forms of the indicated proteins. β -Tubulin was probed as a loading control in all western blots. Data are representative of three independent experiments.

in gedatolisib-treated than -untreated sensitive cell lines, due to the inhibition of AKT activity by gedatolisib. Conversely, TSC1-2 complex formation was expected to be unaffected by gedatolisib in the resistant cell lines. To test this hypothesis, we examined TSC1-2 complex formation by immunoprecipitating TSC2 and western blotting to detect associated TSC1. As expected, increased amounts of TSC1 were complexed to TSC2 after gedatolisib treatment in the sensitive cell line HCT-116, and, accordingly, the levels of p-p70S6K, p-S6K, and p-4E-BP1 were decreased (Fig. 2a). However, formation of TSC1-2 complexes was relatively unaffected by gedatolisib treatment in the resistant cell lines, and, in fact, was slightly decreased.

Because mTOR-Raptor complexes have also been reported to be affected by GSK3 β ,³² we examined mTOR-Raptor complexes formation by immunoprecipitating mTOR and western blotting to detect associated Raptor. Immunoprecipitation of Raptor and western blotting for mTOR indicated that the formation of mTOR-Raptor complexes was decreased by gedatolisib treatment in sensitive cell lines due to the reduction in the activity of mTOR signaling, whereas complex formation was unchanged in the resistant cell lines (Fig. 2a).

Because GSK3 β is a well-known regulator of TSC1-2 and mTOR signaling,^{33,34} we examined its involvement in gedatolisib sensitivity in more detail. Interestingly, the gedatolisib-resistant cell lines expressed much lower levels of the inactive,

phosphorylated form of GSK3 β (ser9) and higher levels of the active, phosphorylated form of GSK3 β (tyr216) compared to the sensitive cell lines (Fig. 2b), suggesting that GSK3 β is more active in the resistant cells. Previous studies have shown that low p-GSK3 β levels and high GSK3 β activity can promote the proliferation of human ovarian and pancreatic cancer cells.^{25,35} To determine whether this is also observed in the gedatolisib-resistant cell lines, we transfected HCT-15 and LS174T cells with GSK3 β -S9A, a constitutively active form of GSK3 β , and examined cell proliferation (5-bromo-2'-deoxyuridine [BrdU] incorporation) and colony formation. Indeed, GSK3 β -S9A expression significantly increased BrdU incorporation and colony formation by the gedatolisib-resistant cells (Supporting Information Fig. S2). This confirms that the constitutive activation of GSK3 β does not affect cell proliferation in the sensitive cell line. These data suggested that active form of GSK3 β might enhance the proliferation in gedatolisib-resistant cell lines.

However, transfection of the resistant cells with GSK3 β -targeting siRNA led to a reduction in mTOR–Raptor complex formation upon treatment with gedatolisib, which was accompanied by a reduction in p-mTOR, p-P70S6K, p-S6K, and p-4E-BP1 levels (Fig. 2c). These data indicate that the gedatolisib-induced reduction in AKT activity increases the formation of TSC1-2 complexes, which induce separation of mTOR–Raptor complexes and subsequently decrease mTOR signaling. Furthermore, siRNA-mediated GSK3 β knockdown (KD) promoted formation of TSC1-2 complexes in gedatolisib-treated resistant cell lines (Supporting Information Fig. S3).

Furthermore, it appears that active form of GSK3 β is critically involved in the mechanism of resistance to PI3K/mTOR inhibition through its ability to sustain mTOR signaling.

Increased WNT signaling induced by TCF7 H155fs* mutation confers gedatolisib resistance

The gedatolisib-resistant cell lines HCT-15 and LS174T both carry a frameshift mutation in *TCF7* (c.465_466insC; Supporting Information Fig. S1), a critical transcription factor in the WNT/ β -catenin pathway. The frameshift results in loss of a corepressor binding domain and increases TCF7 transcriptional activity. Therefore, we hypothesized that the mutant TCF7 protein may increase the levels of active GSK3 β and the WNT/ β -catenin signaling pathway. Consistent with this, basal levels of p-LRP6, AXIN2 and active β -catenin, critical components in WNT/ β -catenin signaling, were much higher in gedatolisib-resistant HCT-15 and LS174T cells than in the sensitive cell lines (Fig. 3a). To investigate if resistant cell lines have WNT/ β -catenin signaling activation, we analyzed WNT/ β -catenin activity in 7 cell lines using TOP-flash reporter assay and also checked FOP-flash activity as a negative control. The basal activity of WNT/ β -catenin was enhanced in resistant cell lines, HCT-15 and LS174T (Fig. 3a). Therefore, we thought that the TCF7 mutation was responsible for the resistance of the

resistant cell line to gedatolisib. In the resistant cells, siRNA-mediated KD of TCF7 reduced the levels of p-LRP6, AXIN2, active β -catenin and active p-GSK3 β -tyr216 and specifically increased the levels of inactive p-GSK3 β -ser9 (Supporting Information Fig. S4). In addition, KD of TCF7 in combination with gedatolisib treatment inhibited both mTOR and WNT/ β -catenin signaling in the resistant cells (Fig. 3b). These results support the possibility that the *TCF7* H155fs* mutation is responsible for the elevated the levels of active GSK3 β and WNT/ β -catenin pathway signaling. In addition, we identified that in the presence of AES protein, which functions as transcription repressor,²³ TCF7 frameshift mutation increased transcription activity in WNT/ β -catenin signaling compared to Mock(empty vector) or TCF7 wild-type, using luciferase assay (Supporting Information Fig. S5). Also, TCF7 transcription activity was measured in seven cell lines, and it was confirmed that the activity was further increased in resistant cell lines (Supporting Information Fig. S5). To confirm these results, we examined the effect of TCF7 H155fs* expression on the gedatolisib-sensitive cell lines HCT-116 and HT-29. Interestingly, expression of TCF7 H155fs* rendered the cells more resistant to gedatolisib (>1 μ M IC₅₀) compared to the parental cell lines harboring Mock (empty vector) (Fig. 3c). In addition, the levels of p-AKT, p-mTOR, p-S6K, p-P70S6K, p-4E-BP1, p-LRP6, AXIN2 and active β -catenin in these cells was either unaffected or upregulated by gedatolisib treatment (Fig. 3d). p-GSK3 β also showed an increase in its active form through low phosphorylation expression under *TCF7* H155fs* mutation. In other words, these results showed the relationship between GSK3 β activation and TCF7 mutation. Similar results were obtained with a second gedatolisib-sensitive cell line, WiDr (Supporting Information Fig. S6) and patient-derived colorectal cancer organoids (Fig. 3e).

Collectively, these results indicate that the TCF7 H155fs* mutation decrease the levels of inactive GSK3 β suggesting an increase of the its active form. In addition, they also demonstrate that TCF7 H155fs* mutation leads WNT/ β -catenin signaling and induction of gedatolisib resistance.

Inhibition of GSK3 β confers sensitivity to gedatolisib

Because GSK3 β cross-regulates both the PI3K/mTOR and WNT/ β -catenin signaling pathways, we hypothesized that inhibition of GSK3 β might attenuate gedatolisib resistance caused by the mutant TCF7-induced increase in WNT/ β -catenin signaling. To test this, we cotreated the resistant cell lines with gedatolisib and si-GSK3 β . Indeed, the combination treatment of si-GSK3 β and gedatolisib inhibited not only colony formation ability but also cell viability of HCT-15 and LS174T cells (Fig. 4a). The combination treatment induced apoptosis, as shown by Annexin V-FITC staining and increased expression of active caspase-3 and active caspase-7 (Fig. 4b). Furthermore, it decreased the levels of p-mTOR, p-P70S6K, p-S6K, and p-4E-BP1 (Fig. 4c) compared to the effects of either agent alone. Interestingly, the levels of p-

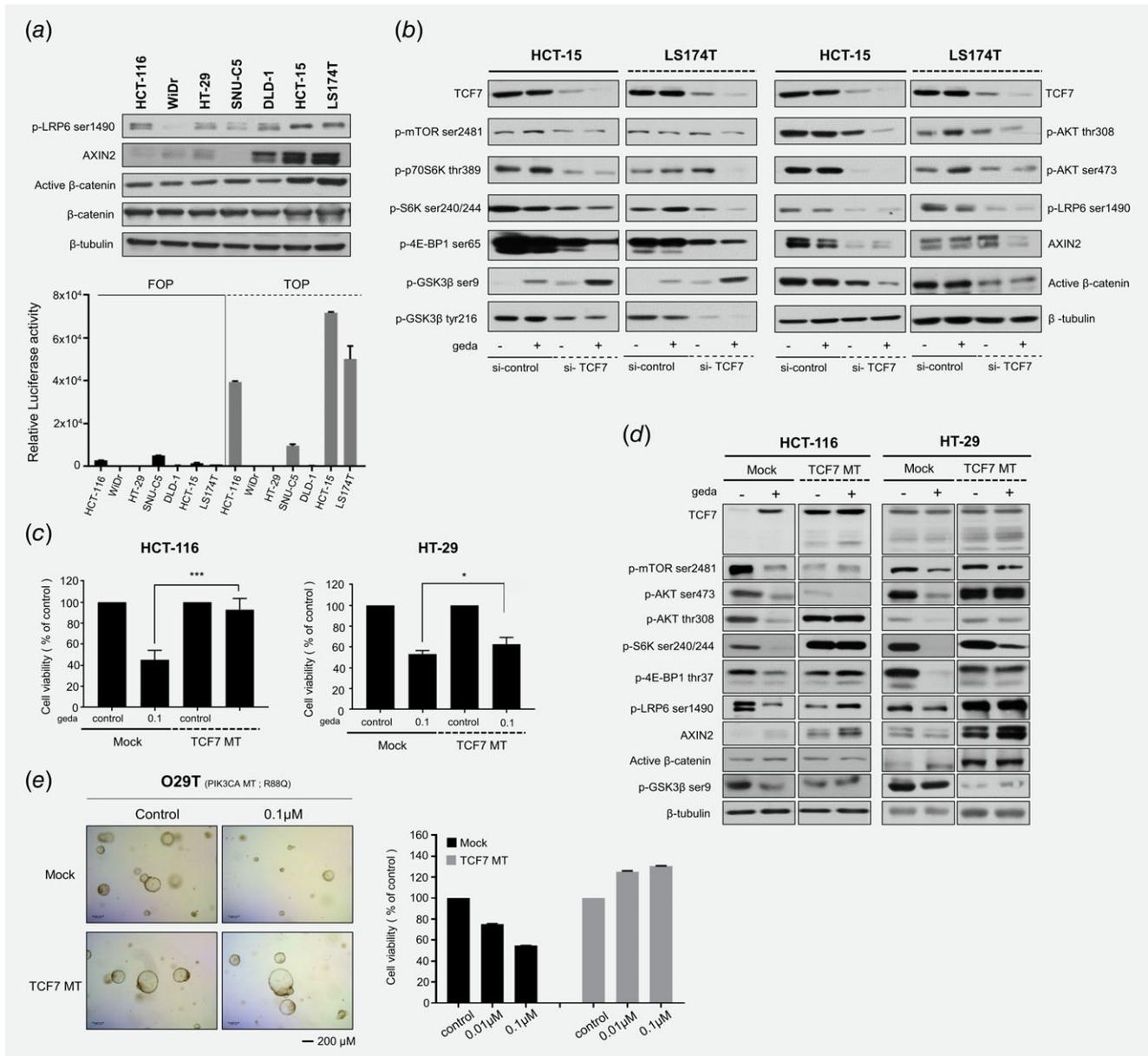


Figure 3. The WNT/β-catenin pathway is hyperactivated in CRC cell lines rendered resistant to gedatolisib by the *TCF7* H155 frameshift mutation. (a) Western blotting of basal levels of total and phosphorylated/active forms of the indicated WNT/β-catenin pathway proteins. TOP/FOP flash luciferase assay was conducted in 7 cell lines under the Wnt3a stimulation. (b) Western blot analysis of total and phosphorylated/active forms of the indicated proteins in HCT-15 and LS174T cells transfected with control or GSK3β-targeting siRNAs and treated with vehicle or gedatolisib 1 μM for 24 h. (c) Viability of HCT-116 and HT-29 cells after transfection with Mock (empty vector) or *TCF7* H155fs* (*TCF7* MT) followed by treatment for 72 h with vehicle or 0.1 μM gedatolisib. The percentage of viable cells is shown relative to the untreated controls (**p* < 0.05, ****p* < 0.001). (d) HCT-116 and HT-29 parental and *TCF7* MT cell lines were treated with vehicle or 0.1 μM gedatolisib for 24 h, and cell lysates were subjected to western blotting of total and phosphorylated/active forms of the indicated proteins. β-Tubulin was probed as a loading control in all western blots. Data are representative of three independent experiments. (e) Viability of O29T organoids after transfection with Mock (empty vector) or *TCF7* H155fs* (*TCF7* MT) followed by treatment for 6 days with vehicle or 0.01 μM or 0.1 μM gedatolisib using cell filter glo. The percentage of viable cells is shown relative to the untreated controls. [Color figure can be viewed at wileyonlinelibrary.com]

LRP6, AXIN2, and active β-catenin were also decreased by the combination treatment (Fig. 4c). Similarly, treatment of the resistant cells with gedatolisib plus CHIR-99021 (CHIR), a selective GSK3β inhibitor, significantly inhibited cell viability and colony-forming ability (Figs. 4d and 4e), induced

apoptosis showing increased active caspase-3/7 (Fig. 4f). The combination treatment of gedatolisib and CHIR-99021 decreased the levels of p-mTOR, p-P70S6K, p-S6K, p-4E-BP1, p-LRP6, p-GS, AXIN2, and active β-catenin (Fig. 4g). Similar results were obtained when the resistant cell lines were treated

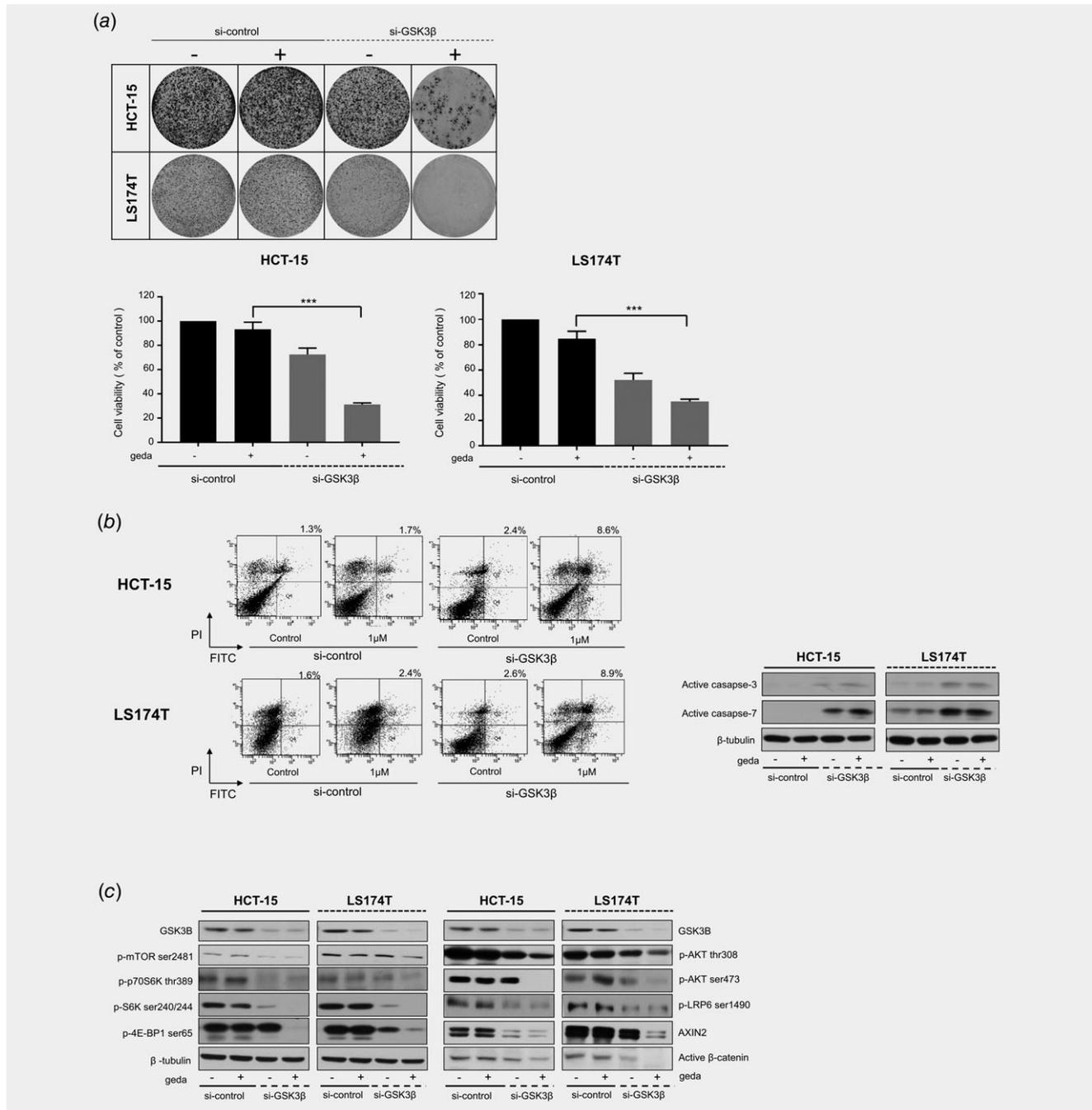


Figure 4. PI3K/mTOR and GSK3β inhibitors have synergistic effects on gedatolisib-resistant CRC cell lines. (a) Upper: Colony-forming assay in HCT-15 and LS174T cells transfected with control or GSK3β-targeting siRNAs and treated with vehicle or gedatolisib 1 μM for 10 days. Lower: Viability of HCT-15 and LS174T cells transfected with control or GSK3β-targeting siRNAs and treated with vehicle or gedatolisib 1 μM for 48 h. (***) $p < 0.001$ (b) Annexin V-FITC staining and western blotting of HCT-15 and LS174T cells transfected with control or GSK3β-targeting siRNAs and treated with vehicle or gedatolisib 1 μM for 48 h. (c) Western blot analysis of total and phosphorylated/active forms of the indicated proteins in HCT-15 and LS174T cells transfected with control or GSK3β-targeting siRNAs and treated with vehicle or gedatolisib 1 μM for 24 h. (d) Viability of HCT-15 and LS174T cells at 72 h after treatment with the indicated combinations of vehicle, gedatolisib (1 μM), and/or the GSK3β inhibitor CHIR-99021 (10 μM and/or 20 μM) (***) $p < 0.001$. (e) Colony-forming assay of HCT-15 and LS174T cells treated with the indicated concentrations of vehicle, gedatolisib, and/or CHIR for 10 days (***) $p < 0.001$ (f) Annexin V-FITC staining of HCT-15 and LS174T cells treated with indicated combinations of vehicle, gedatolisib (1 μM), and/or the GSK3β inhibitor CHIR-99021 (10 μM and/or 20 μM) for 48 h. (g) Western blot analysis of basal levels of total and phosphorylated/active forms of the indicated proteins in HCT-15 and LS174T cells. β-Tubulin was probed as a loading control in all western blots. Data are representative of three independent experiments.

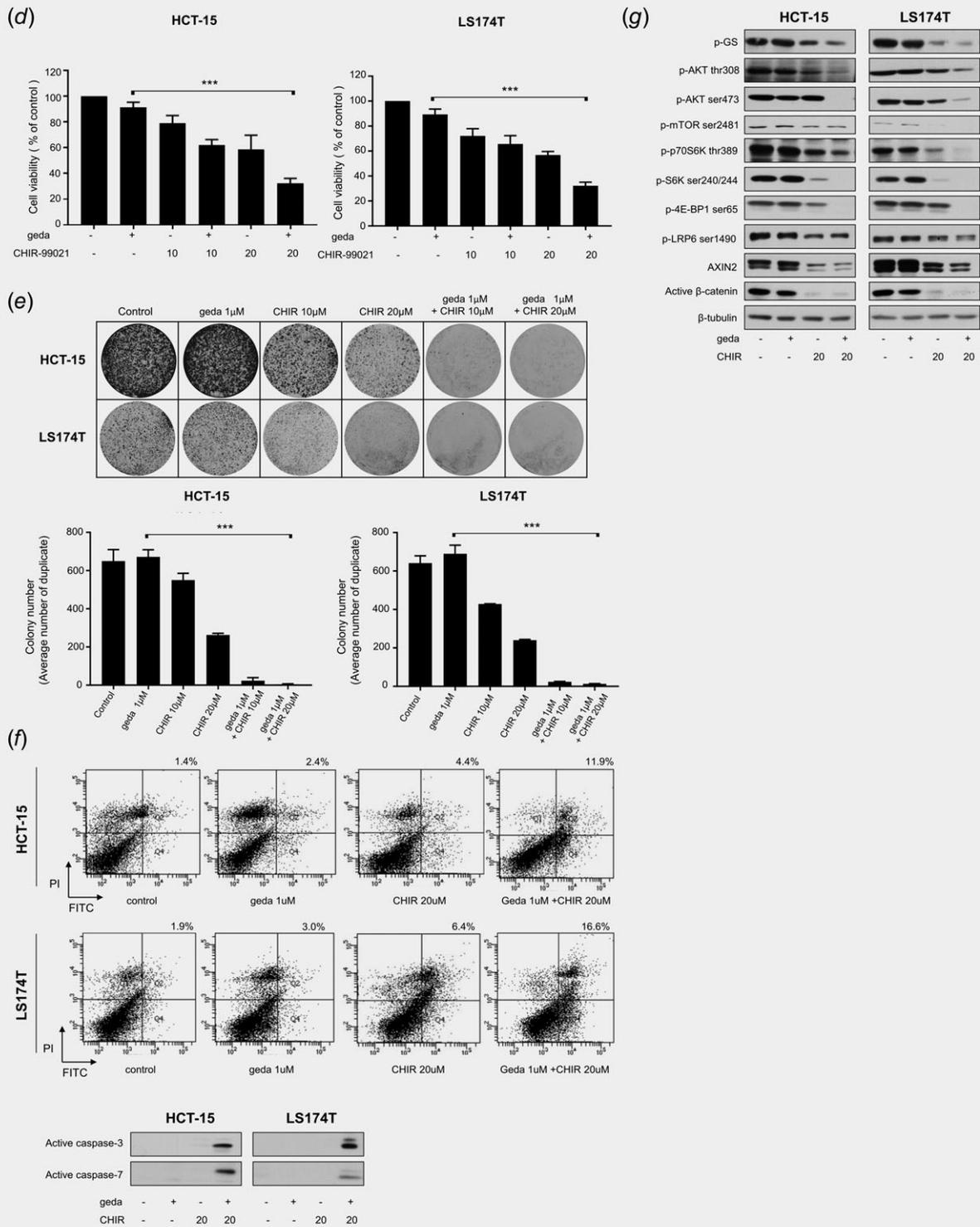


Figure 4. Continued.

with gedatolisib plus SB216763(SB) or LiCl, two additional GSK3β inhibitors (Supporting Information Figs. S7 and S8). In contrast, treatment of the gedatolisib-sensitive cell lines WiDr and HT-29 with gedatolisib and CHIR induced mTOR

and WNT signaling by increasing the levels of the phosphorylated and total S6 and 4E-BP1 and AXIN2 and active β-catenin (Supporting Information Fig. S9). Taken together, these results suggest that inhibition of GSK3β restores the

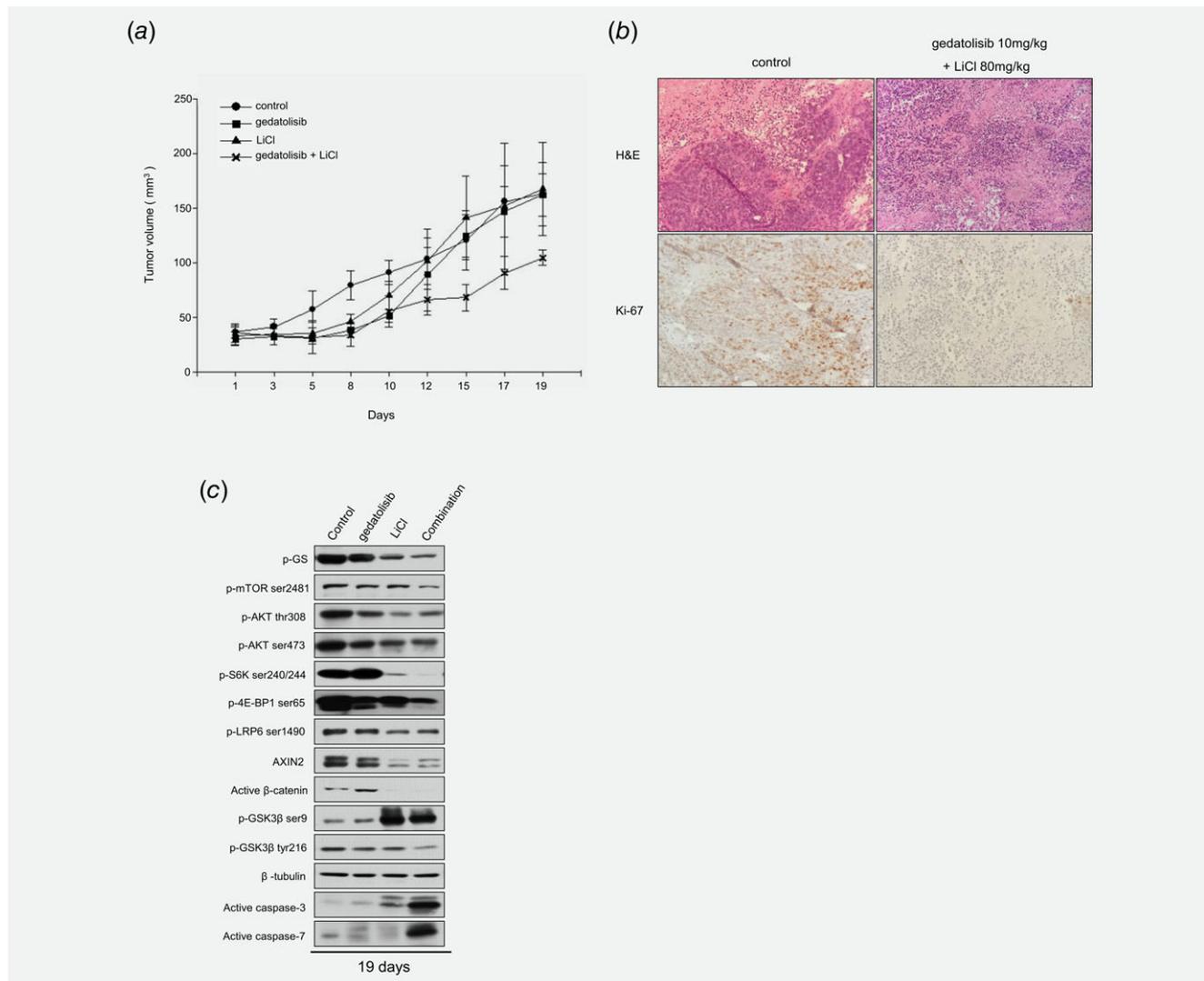


Figure 5. Co-treatment with PI3K/mTOR and GSK3 β inhibitors decreases CRC tumor growth in a xenograft mouse model. (a) Groups of 4-week-old female BALB/c nude mice were subcutaneously injected with HCT-15 cells (1×10^7 /mouse) and administered vehicle, gedatolisib (10 mg/kg), and/or LiCl (80 mg/kg) twice weekly for 3 weeks. Tumor size was measured every other day. Data are the mean \pm s.d. of 5 mice per group. Representative pictures of HCT-15 tumors excised from mice on day 19 of the experiment shown in (a). (b) Representative images of IHC staining of tumor tissues of mice with control and gedatolisib plus LiCl groups. H&E staining (upper) and Ki-67 staining (lower). (c) Western blot analysis of the total and phosphorylated/active forms of the indicated proteins extracted from HCT-15 tumors excised on day 19. β -Tubulin was probed as a loading control.

sensitivity of HCT-15 and LS174T cells to PI3K and mTOR inhibition.

Gedatolisib and the GSK3 β inhibitor LiCl synergistically inhibit the growth of CRC tumors in a xenograft mouse model

To determine whether GSK3 β inhibition restores sensitivity to gedatolisib, we examined xenograft tumor growth. HCT-15 cells were injected subcutaneously into BALB/c nude mouse, and groups of mice were treated for 3 weeks with vehicle, gedatolisib alone, LiCl alone, or both gedatolisib and LiCl. Mice treated with LiCl were administered

additional NaCl to prevent electrolyte imbalance, as previously reported.²⁸ While monotherapy with gedatolisib or LiCl alone had no effect on (or marginally increased) the tumor size compared to vehicle treatment, the combination of gedatolisib plus LiCl markedly decreased tumor growth (Fig. 5a). In addition, We also analyzed the effects of the treatments on PI3K/mTOR and WNT/ β -catenin pathway activation in tumor tissue excised at the end of the experiment (day 19). In Figure 5b, ki67 staining, which marks cell proliferation, decreased in combination therapy treated tumor tissue compared to control tumor tissue. We found that the levels of p-GS, p-AKT, p-S6K, p-4E-BP1, p-LRP6,

AXIN2, active β -catenin, and p-GSK3 β (tyr216) were decreased and level of p-GSK3 β (ser9) was increased by treatment with both gedatolisib and LiCl, but not with the single agents (Fig. 5c). Taken together, these results indicate that treatment of CRC cells with both gedatolisib and LiCl inhibits oncogenic signaling through PI3K/AKT/mTOR and WNT signaling pathways, resulting in apoptosis.

Discussion

In our study, we identified a novel mechanism of resistance to the dual PI3K and mTOR inhibitor gedatolisib in CRC. We confirmed that most cell lines carrying *PIK3CA* mutations are sensitive to gedatolisib and demonstrated that increased WNT/ β -catenin signaling induces resistance to gedatolisib in the presence of mutant *PIK3CA*. Our data demonstrate that the TCF7 H155fs* mutation reduces levels of inactive GSK3B suggesting its possible activation and, as a result, elevated WNT/ β -catenin signaling. This particular mechanism of resistance to a dual PI3K and mTOR inhibitor has not been previously described in CRC. There are evidences to date, suggesting that resistance of CRC to dual PI3K/mTOR-targeted therapy can occur *via* several routes; dysregulation of the KRAS-RAF-MEK pathway,^{10,36} activation of ERBB2 and ERBB3 through FOXO3a,³⁷ c-Myc induction by the Notch pathway,³⁸ and/or activation of the IGF1R/AKT pathway.³⁹ The present study is the first to suggest a WNT/ β -catenin signaling-related mechanism of gedatolisib resistance in *PIK3CA*-mutant CRC.

It has been well characterized that GSK3 β has multifunctional roles in cell death pathways, cancer-associated signaling pathways, cell proliferation and growth. It is notable that phosphorylation of GSK3 β by AKT is considered as inactive form. Through this, GSK3 β acts as a negative regulator by inducing up-regulation of WNT/ β -catenin signaling.^{34,40} On the other hand, as a positive regulator, GSK3 β modulates mTOR activity by regulating formation of TSC1-2 complexes,³³ which, in turn, influence mTOR/Raptor complex formation and the activity of downstream effectors such as ribosomal protein S6 kinase (S6K).^{32,41,42} Our study identified that mTOR/Raptor complex was affected by GSK3 β and it also contribute to drug response through decreased the levels of active mTOR (Fig. 2c). Furthermore, active GSK3 β was noted to be significantly higher in tumor cells than normal cells in 63.6% of CRC patients.²⁸ These data suggested that active GSK3 β is associated with cancer progression and poor response to anticancer therapy.⁴³ Thus, GSK3 β is emerging as a potential therapeutic target. When targeted, it results in induction of cell death through the after mechanisms; regulation of MDM2-p53 pathway,⁴⁴ JNK-dependent mechanisms²⁵, MYCN mRNA levels⁴⁵ and disruption of centrosome regulation in cancer cells.⁴² It is known that GSK3 β mediates LRP6 phosphorylation, which activates WNT/ β -catenin signaling.⁴⁶ However, a study showing the oncogenic role of GSK3 β in direct relationship with the

WNT/ β -catenin pathway, remains to be elucidated. In our study, we showed active form of GSK3 β in gedatolisib-resistant cell lines harboring TCF7 frameshift mutation and it resulted in increased WNT/ β -catenin signaling. These results conflict with previous studies stating that inhibition of GSK3 β induces WNT/ β -catenin signaling. Therefore, it seems likely that GSK3 β may have dual activity, acting as both negative and positive regulator of WNT/ β -catenin signaling.

Among the components of WNT/ β -catenin signaling, TCF/LEF family of transcription factors are essential molecules.²¹ Both *TCF* and *LEF* have β -catenin binding domains, which allow binding of β -catenin and initiate transcription process of WNT/ β -catenin signaling. Thus, lacking of β -catenin binding domain results in suppressed activity of WNT/ β -catenin signaling.⁴⁷ High expression of TCF/LEF and their truncated form that lacks a β -catenin binding domain were also found in many cancer types. In addition to these mutations, mutations in other TCF/LEF domains are also found. For example, a variant of TCF4 is highly expressed in CRC, and *TCF4* is mutated in about 40% of CRC patients with microsatellite instability and patients with leukemia.⁴⁸ Recent study suggested that TCF7 is also upregulated in CRC.^{49,50} Another *TCF7* frameshift mutation, c. 460delC, which also confers high transcriptional activity, has been reported to increase the expression of cyclin D1, a target of WNT/ β -catenin signaling, although the underlying mechanism was not clear.²³ We found novel TCF7 H155 frameshift mutation (c.465_466insC) which results in a truncated molecule that lacks a binding domain for the transcriptional repressor protein AES/Groucho.⁵¹ Therefore, AES/Groucho protein could not bind to the binding domain to suppress transcription of target genes in WNT/ β -catenin signaling. Thus, *TCF7* H155fs* has enhanced transcriptional activity, resulting in increased WNT/ β -catenin signaling activity. We demonstrated that TCF7 H155 frameshift mutation remarkably showed high transcription activity in either the absence or presence of AES/Groucho protein (Supporting Information Fig. 5). Although the *TCF7* H155 frameshift mutation has been reported only rarely (<1%) in CRC, our findings may be useful in guiding the current and future use of combination therapies for an n-of-1 trial.

In conclusion, our study demonstrates that *PIK3CA*-mutant CRC cells develop resistance to gedatolisib through activation of GSK3 β and WNT/ β -catenin signaling resulting from an activating *TCF7* mutation. We suggest that, although there is little clinical data on this particular *TCF7* mutation, it could be used as a biomarker in clinical trials to identify patients likely to be resistant to dual PI3K/mTOR inhibitors.

Acknowledgements

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