Inhibition of GSK3B Bypass Drug Resistance of p53-Null Colon Carcinomas by Enabling Necroptosis in Response to Chemotherapy

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Abstract

Purpose: Evasion from chemotherapy-induced apoptosis due to p53 loss strongly contributes to drug resistance. Identification of specific targets for the treatment of drug-resistant p53-null tumors would therefore increase the effectiveness of cancer therapy.

Experimental Design: By using a kinase-directed short hairpin RNA library and HCT116p53KO drug-resistant colon carcinoma cells, glycogen synthase kinase 3 beta (GSK3B) was identified as a target whose silencing bypasses drug resistance due to loss of p53. p53-null colon cancer cell lines with different sets of mutations were used to validate the role of GSK3B in sustaining resistance and to characterize cell death mechanisms triggered by chemotherapy when GSK3B is silenced. In vivo xenograft studies were conducted to confirm resensitization of drug-resistant cells to chemotherapy upon GSK3 inhibition. Colon cancer samples from a cohort of 50 chemotherapy-treated stage II patients were analyzed for active GSK3B expression.

Results: Downregulation of GSK3B in various drug-resistant p53-null colon cancer cell lines abolished cell viability and colony growth after drug addition without affecting cell proliferation or cell cycle in untreated cells. Cell death of 5-fluorouracil (5FU)-treated p53-null GSK3B-silenced colon carcinoma cells occurred via PARP1-dependent and AIF-mediated but RIP1-independent necroptosis. In vivo studies showed that drug-resistant xenograft tumor mass was significantly reduced only when 5FU was given after GSK3B inhibition. Tissue microarray analysis of colon carcinoma samples from 5FU-treated patients revealed that GSK3B is significantly more activated in drug-resistant versus responsive patients.


Introduction

Two main problems that affect the outcome of cancer therapy are the use of "poorly specific" drugs and, in a high percentage of patients, the lack of response due to drug resistance. Poor specificity is due to the fact that "classical" chemotherapeutic drugs act by inducing a generic damage (either to the DNA or the microtubuli) that cells recognize as an apoptotic trigger (1). However, several apoptotic mechanisms, or their regulation, are disabled during oncogenic transformation and progression, thus rendering a consistent percentage of tumors resistant to chemotherapy-induced cell death (2). To bypass the "poor specificity" issue, more rational approaches have been pursued by applying a molecularly targeted approach, that is developing new drugs acting specifically by targeting a single molecule crucial for the survival of tumor cells. In the last decade, several kinases hyperactivated in different types of cancers have been successfully targeted and the corresponding specific inhibitors have entered therapy (3).

To increase the effectiveness of cancer therapy it could be appropriate to apply the molecular therapy approach, that is to find specific molecules to target, also in the case of drug resistance. Kinases are the best candidates for this approach for at least 2 reasons: (i) It is known that several kinases are usually coactivated by redundant inputs and participate in the pathogenesis of most solid tumors (4). Moreover, they often directly or indirectly contribute to render cancer cells more resistant to different types of stress (5, 6). (ii) Kinases are thought to be "druggable" targets.
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**Translational Relevance**

DNA-damaging agents are among the most used drugs in the treatment of carcinomas. However, their efficacy is often hindered by development of drug resistance, usually derived from the alteration or misregulation of one or more apoptotic/antiapoptotic mechanisms. By studying a cohort of stage II colon carcinoma patients we found that glycogen synthase kinase 3 beta (GSK3B) is activated in almost half of all colon carcinomas and in two thirds of drug-resistant ones. Moreover, we show that upon GSK3B inhibition, DNA-damaging drugs bypass the need of p53 to induce cell death and tumor cells die by caspase-independent necroptotic death. Because p53 function is compromised in the vast majority of human cancers and caspase-dependent apoptosis is frequently impaired in tumors, GSK3B inhibition in combination with chemotherapy may represent a molecularly targeted approach to treat resistant tumors.

Based on these premises, we conducted a phenotype screen using the kinase pools of the NKI short hairpin RNA (shRNA) library (7) and 5-fluorouracil (5FU)-resistant HCT116p53KO cells (8) as a model. We decided to use as a model a p53-null background because p53 activity is either lost or compromised in most tumors (9), which abolishes the apoptotic response to many anticancer agents (10). Here we report that the downregulation of glycogen synthase kinase 3 beta (GSK3B) abolishes growth after treatment with DNA-damaging drugs in the absence of p53 in resistant cells. Moreover, we show that GSK3B-depleted colon carcinoma cells undergo PARP1-dependent and AIF-mediated necroptosis. Accordingly, GSK3 inhibition by LiCl restores sensitivity to 5FU in xenograft experiments. Finally, studying a cohort of 50 colon carcinoma stage II patients we found that GSK3B is activated in 47% of all samples studied and in 63.6% of those from drug-resistant patients. Based on these results we propose that GSK3B is an interesting candidate target for the treatment of patients with 5FU-resistant tumors.

**Materials and Methods**

**Drugs and reagents**

5FU (Teva), oxaliplatin (OxPt; Sanofi-Aventis) were from San Gerardo Hospital (Monza, Italy). LiCl and necrostatin-1 were from Sigma-Aldrich.

**Cell lines and cell culture**

DLD-1 and SW480 colon carcinoma cell lines were from the American Type Culture Collection (Manassas, VA). Isogenic p53 wild-type and p53 knockout HCT116 colon carcinoma cell lines were a kind gift of Dr. B. Vogelstein (Johns Hopkins University, Baltimore, MD). Upon arrival, cells were expanded and frozen as a seed stocks of first or second passage. All cells were passaged for a maximum of 6 weeks, after which new seed stocks were thawed for experimental use. All cell lines were maintained in McCoy medium (Invitrogen) supplemented with 10% FBS (Invitrogen) and 1% penicillin–streptomycin at 37°C in 5% CO2. Cell lines stably interfered for each gene identified in the screen were obtained by retroviral infection and selection with the appropriate antibiotic as previously described (11). shRNAGSK3B target sequence GATGAGGTCTATCTTAATC (nt:1353-1371).

**Cell viability**

Cells were seeded overnight at 70% confluency and the next morning treated or not with the indicated drugs and inhibitors. Seventy-two hours later dead cells were counted—triplicate wells in each experiment—after Trypan blue staining. Graphs shown throughout the article represent the average of 3 to 5 independent experiments. Average ± SDs is plotted in the graphs.

**Colony assay**

A total of 3 × 10^5 cells/well were seeded in 6-well plate, let adhere overnight, and treated with 200 μmol/L 5FU for 12 hours. Cells were then trypsinized, counted, and reseeded at a low density (1,000 cells/well in 6-well plate) in triplicate. In experiments without drug treatment, 1,000 cells/well were directly seeded in 6-well plates. In both cases, medium was replaced every 3 days, and after 2 weeks colonies were fixed and stained in 1% crystal violet, 35% ethanol.

**Caspase assay**

A total of 4 × 10^4 cells/well were seeded in triplicate in 96-well plate, let adhere overnight, and treated with 200 μmol/L 5FU for 72 hours before evaluating active caspase-3/7 by the Caspase-Glo3/7 Assay System (Promega) according to the manufacturer’s instructions.

**Cell proliferation**

A total of 1 × 10^4 cells/well were seeded in triplicate in 96-well plate and starting the following day (day 0) proliferation was evaluated each 24 hours by CellTiter 96 AQueous Non-Radioactive Cell Proliferation Assay (Promega) according to the manufacturer’s instructions.

**Flow cytometric analysis**

Exponentially growing cells were trypsinized, washed twice with cold PBS, fixed in ice-cold 96% ethanol, washed twice with cold PBS, and incubated overnight at 4°C with propidium iodide (10 μg/mL) and RNase A (12.5 μg/mL) in PBS. Fluorescence intensity of 1 × 10^5 cells/sample was determined with a FACSCalibur instrument and data analyzed using Modfit Cell Cycle Analysis (Becton Dickinson) as previously described (12).

**Reporter assay**

0.2 μg TopFlash + 0.2 μg pGL4.75 reporters were transfected in 5 × 10^4 cells/well seeded in triplicate in a 96-well plate and reporter activity was evaluated 48 hours later by Dual-Glo Luciferase Assay (Promega) according to the manufacturer’s instructions. For a detailed description see Supplementary Data.
Western blot analysis

Cells were lysed in high-salt lysis buffer (Hepes 50 mmol/L, pH 7.5, NaCl 500 mmol/L, DTT 1 mmol/L, EDTA 1 mmol/L, 0.1% NP40) supplemented with 1% protease inhibitor cocktail (PIC; Sigma-Aldrich) and Western blots performed as described previously (11) using the following antibodies: anti-actin (A1978; Sigma-Aldrich), anti-cleaved Caspase-3 (#9661), anti-pSer9-GSK3B (clone D85E12), anti-pSer21-GSK3A (clone 36E9) were from Cell Signaling; anti-GSK3A/B sc-56913), anti–caspase-3 (total; sc-6549) were from Santa Cruz Biotechnology.

Immunofluorescence

Cells were fixed with 4% paraformaldehyde in PBS. Permeabilization and staining with anti-PAR (clone mAb 10H, Alexis), anti-AIF (sc-13116; Santa Cruz Biotechnology), anti-H2AX (Ab 22551; Abcam), anti-RPA70 (clone 2H10; Sigma-Aldrich) was conducted as described (11). Cells were counterstained with DAPI before microscopic examination using 60× magnification and a Nikon Eclipse 80i microscope. Images were acquired using Genikon (Nikon) software and processed with Adobe Photoshop.

Patients

The case study was composed of 50 patients with a clinical diagnosis of colon cancer who received 5FU adjuvant chemotherapy after surgery. All samples were classified by a pathologist as stage II. At the first diagnosis of colon cancer, patients had no other cancers and they were followed up until December 31, 2010 or death, whichever came first. The median duration of overall follow-up was 9.2 years (25th–75th percentile = 3.8–12.7 years). Overall survival was defined as the time from surgery to colon cancer–specific death. Log-rank test was used to check the dependence of patients’ survival on single variables or on combinations of variables. All P values are two-sided with values <0.05 regarded as statistically significant. Statistical analyses were conducted with the Stata/SE 12 package (Stata).

Immunohistochemistry

Sample triplicates were arrayed using BioRep Tissue MicroArray System and antibody against pTyr216-GSK3B (sc-135653, Santa Cruz Biotechnology) was used as described (13). pTyr216-GSK3B staining was graded accordingly to an increasing intensity by blind reading by 2 experienced operators.

In vivo xenograft studies

All the experiments involving animals were carried out in accordance with Italian law (DDL 116/92) and European Guidelines for use and care of laboratory animals, according to a protocol approved by the local ethical committee of BIOGEM Institute (where the experiments have been conducted). Tumors were started by s.c. injecting 1 × 10⁶ cells (in 100 μL of a 50% PBS and 50% Matrigel solution), HCT116 cells into the left flanks and HCT116 into the right flanks, of 5 to 7 weeks old female CD-1 nude mice (Charles River Laboratories). When HCT116p53KO tumors reached the average volume of 100 mm³ (day 7 post-engraftment), animals were randomized and given vehicle, 5FU [via intraperitoneal (i.p.) injection, 75 mg/kg, twice a week], LiCl (via i.p. injection, 80 mg/kg, twice a day for 5 days a week), or a combination thereof. 5FU treatment started at day 8 post-engraftment, whereas LiCl treatment started at day 7 post-engraftment. Mice that received LiCl were also given additional NaCl to prevent electrolyte imbalance. Control mice received i.p. injections of vehicle (0.9% NaCl solution) with the same schedule of the other groups. Tumors were measured with caliper twice/week. Statistical significance was determined with a Kruskal–Wallis non parametric test (normal distribution not assumable), followed by Nemenyi–Damic–Wolfe–Dunn test for multiple pairwise comparisons between groups. In all cases, a P value < 0.05 was considered as significant. Resected tumors were weighed then fixed in 4% paraformaldehyde and processed for histological and immunohistochemistry analysis. Tumor biopsies were removed from formalin, dehydrated, deaffanized with xylene, put in paraffin, sectioned with microtome, put on slides, and stained with hematoxylin/eosin following standard procedures. Anti-p53 [mouse monoclonal (DO-7); Ventana Medical] was used at a 1:500 dilution. Hystological and immunohistochemistry slides were then digitally acquired using ScanScope (Aperio) system.

Results

GSK3B silencing abolishes drug resistance of p53-null colon carcinoma cell lines

HCT116p53KO colon carcinoma cell line is resistant to many genotoxic drugs due to lack of p53 (8). To identify kinases whose activation sustain resistance to DNA-damaging chemotherapy, we conducted a phenotype screen using the kinase pools of the NIKI shRNA library (7) and 5FU-resistant HCT116p53KO cells as a model system (Supplementary Fig. S1A). After having validated several of the hits (Supplementary Fig. S1B–S1D), we focused on one of these, GSK3B. In epithelial cells, this kinase, by phosphorylating β-catenin, negatively regulates proliferation (14); in addition, it is widely accepted that GSK3B suppresses cancer-associated signaling pathways via negative regulation of the Wnt/β-catenin pathway that support both invasive and metastatic processes (14, 15). However, HCT116p53KO cells express mutated, nonphosphorylatable β-catenin, that is constitutively active and not regulatable by GSK3B (16) suggesting that in our model system the effect of GSK3B inhibition is independent of its known antiproliferative role. To test this hypothesis, we first established stable cell lines expressing low-to-undetectable levels of GSK3B by transducing HCT116p53KO, as well as DLD-1 and SW480, with retroviruses expressing shRNAs to GSK3B (Fig. 1A). DLD-1 and SW480 express mutated p53 and constitutively active β-catenin due to an APC truncation (16 and Supplementary Table S1) that prevents GSK3B-mediated regulation. Next, we analyzed several parameters related to cell cycle and proliferation in cells stably silenced for GSK3B. Notably, downregulation of GSK3B does not

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change the proliferation of these cells as assessed either by colony assay (Fig. 1B) or by growth curve (Fig. 1C). Also cell-cycle distribution is not affected by lack of GSK3B (Fig. 1D). Finally, as expected, the decrease in GSK3B expression does not alter β-catenin activity, as shown by the reporter assay experiment in Fig. 1E.

Altogether these data show that GSK3B silencing in colon carcinoma cells does not affect cell cycle or proliferation.

Next, we studied the role of GSK3B in the response to chemotherapy and found that GSK3B stable silencing in p53-null drug-resistant colon carcinoma cells abolishes growth of colonies after drug exposure (Fig. 2A) and resensitize cells to drug-induced cytotoxicity (Fig. 2B–D). To further confirm the role of GSK3B in drug resistance, we inhibited its function by 2 more different means (17). Transient GSK3B protein depletion by use of siRNA restored cell death in response to 5FU (Supplementary Fig. S2). We confirmed these findings by treating HCT116p53KO, DLD-1, and SW480 cells with 5FU in the presence of LiCl (a GSK3B inhibitor approved by FDA for the treatment of bipolar disorder; ref. 14; Fig. 2E). We further tested the role of GSK3B in drug resistance by treating colon cancer cells with OxPt, another DNA-damaging drug commonly used in colon carcinoma therapy, usually given in combination with 5FU and found that inhibition of GSK3B expression reverts resistance to OxPt treatment (Fig. 2F). In particular genetic settings, inhibition of GSK3B expression is also able to lower resistance to the concomitant addition of 5FU and OxPt, which is significant in DLD-1 cells (Fig. 2G). Finally, we investigated whether GSK3B inhibition might also abolish the resistance to targeted drugs currently used for colon carcinomas and found that GSK3B inactivation did not sensitize resistant cells to cetuximab, panitumumab, and bevacizumab (Supplementary Fig. S3).

Thus, our findings indicate that, in the absence of p53, GSK3B depletion or inhibition restores the response of colon carcinoma drug-resistant cells only to DNA-damaging chemotherapy.

GSK3B inhibition abolishes drug resistance of p53-null colon carcinoma cell lines by affecting the response to DNA damage

To investigate whether GSK3B inhibition influences DNA damage response/repair systems, we analyzed γH2AX foci formation as markers of the DNA damage response and RPA70 foci formation as markers of DNA repair (18, 19). To
this end, we immunostained cells stably silenced for GSK3B and control cells, in presence and absence of 5FU (Fig. 3A): DNA damage is sensed upon 5FU treatment, even in absence of p53, as showed by γH2AX foci formation and this step is not impaired by GSK3B silencing. Also RPA70 foci are formed in p53-null cells (Fig. 3B), indicating that...
DNA repair is initiated: this process seems to be dependent on GSK3B activity, because silenced cell have very few or no RPA70 foci. We further confirmed these findings by inhibiting GSK3B activity in HCT116p53KO cells with LiCl (Fig. 3C and D).

Taken together these results suggest that, in the absence of p53, GSK3B activity allows cells to survive despite treatment with DNA-damaging drugs by sustaining DNA repair.

**GSK3B silencing enables RIP1-independent necroptosis in response to 5FU in p53-null colon carcinoma cells**

To investigate the mechanisms of cell death induced by 5FU when GSK3B is silenced, we measured typical hallmarks of apoptosis, such as caspase activation. 5FU-treated GSK3B-silenced HCT116p53KO cells did not show appreciable levels of processed caspases (Supplementary Fig. S4) and only showed minor caspase-3 activation (Fig. 4A); moreover, QVD-OPh addition did not prevent cell death (Fig. 4D). These data collectively suggest that GSK3B participates in the regulation of caspase-independent cell death (20). PARP1 is an important activator of caspase-independent necrosis: DNA damage-induced PARP1 activation leads to Calpain activation which in turn, via BID cleavage, activates BAX, thus facilitating the release from the mitochondria of a truncated form of AIF (tAIF) produced by Calpain (21). Once liberated in the cytosol tAIF translocates to the nucleus, where it promotes large-scale fragmentation of DNA, peripheral chromatin condensation, and, ultimately, cytotoxicity (22). We conducted several experiments to assess a possible role of GSK3B as a modulator of PARP1 and AIF in drug-induced caspase-independent necrosis. First, we found that polymers of PAR, whose formation depends on PARP activation (23), accumulated only when 5FU was added to GSK3B-depleted cells and not to controls (Fig. 4B). Second, we showed that tAIF was released into the cytosol (Supplementary Fig. S5A) and relocalized to cell nuclei upon 5FU exposure of GSK3B-depleted cells (Fig. 4C). Third, to test whether AIF relocalization was dependent on tBID and PARP-1, we pretreated cells with tBID and PARP1-specific inhibitors (Bi6C9 and DiQ, respectively) before drug addition and we showed that both inhibitors prevented 5FU cytotoxicity as well as tAIF nuclear translocation (Fig. 4D and E). Accordingly, we also showed that silencing AIF in GSK3B-depleted HCT116p53KO cells reduced 5FU cytotoxicity (Supplementary Fig. S5B). Finally, because TNF-α–mediated necroptosis is dependent on the activation of RIP1 kinase (24), we tested its involvement in our model by preincubating GSK3B-depleted cells with the RIP1 specific inhibitor necrostatin-1 (25) before adding 5FU. Cell death was not prevented by necrostatin-1 (Fig. 4D) indicating that, at variance with TNF-α, DNA damage does not require RIP1 activity to trigger necroptosis.

Therefore, our data indicate that, in the absence of functional p53, GSK3B regulates a necroptotic response to DNA-damaging chemotherapy.

**GSK3 inhibition restores the therapeutic response to 5FU in a xenograft model**

To test whether GSK3B inhibition restores sensitivity to chemotherapy of p53-null colon carcinoma cells also in vivo, we conducted xenograft experiments. HCT116p53KO cells (left flank), and HCT116 cells as a control (right flank), were subcutaneously inoculated into CD-1 nude mice and treated with vehicle, 5FU only, LiCl only, and LiCl + 5FU. We observed that, 5FU, as well as LiCl given alone, had little or no effect on xenografted HCT116p53KO tumors, whereas GSK3B inhibition by LiCl prior 5FU administration significantly decreased the tumor burden (Fig. 5A and B). As expected, in HCT116 control tumors, 5FU alone...
Figure 4. p53-null, GSK3B-silenced colon carcinoma cells treated with 5FU die via RIP1-independent necroptosis. A, HCT116p53KO-pRS and HCT116p53KO-pRSGSK3B were treated for the indicated times with 200 μmol/L 5FU and total cell lysates blotted with antibodies recognizing pro-caspase-3 (upper) or the cleaved forms of caspase-3 (central); lysates from 5FU-treated HCT116 cells (72 hours) were also loaded on the same gel as a control; the lane after the control (containing the molecular weight marker) has been cut out; solid arrows indicate active forms of caspase-3, asterisk indicates intermediate forms produced during proteolytic activation; an aliquot of the same samples at 72 hours after treatment was used for a luminometric caspase-3/-7 assay (lower). B, HCT116p53KO-pRS and HCT116p53KO-pRSGSK3B untreated (cnt) and treated for 18 hours with 200 μmol/L 5FU and stained with anti-PAR antibody as well as DAPI. C, HCT116p53KO-pRS and HCT116p53KO-pRSGSK3B untreated (cnt) and treated for 30 hours (when 40–50% cells are dead) with 200 μmol/L 5FU and stained with anti-AIF antibody as well as DAPI. D, HCT116p53KO-pRSGSK3B were preincubated for 2 hours with pan-caspase inhibitor QVD-OPh (10 μmol/L), BID inhibitor (20 μmol/L Bi6C9), PARP1 inhibitor (100 μmol/L DiQ), Bi6C9 + DiQ, or Necrostatin-1 (20 μmol/L Nec1) before adding 200 μmol/L 5FU and counted 72 hours later. E, HCT116p53KO-pRSGSK3B were treated for 30 hours with 200 μmol/L 5FU in the presence of 100 μmol/L DiQ and stained with anti-AIF antibody as well as DAPI.
significantly decreased the tumor burden. Histology of tumor masses is shown in Fig. 5C, where murine stromal cells were identified as positive to p53 staining whereas HCT116p53KO tumor cells tested negative (upper row). Different morphology and tissue organization, are evident in untreated, 5FU- and LiCl-treated versus LiCl + 5FU-treated HCT116p53KO xenografts (central row). In the latter, the significant decrease of the tumor masses is not paralleled by the massive appearance of hyperchromatic, pyknotic nuclei (consistent with massive apoptosis) that are at variance visible in regressing 5FU- and LiCl + 5FU-treated HCT116 xenografts (lower row).

On the whole, our results confirm that GSK3B inhibition resensitizes drug-resistant tumors to chemotherapy also in vivo.

GSK3B is activated in colon carcinoma samples from patients

Preliminary data from 2 different laboratories reported high expression of active GSK3B in cell lysates from small groups of colon carcinoma samples (26, 27). Because our data indicated an important functional role for GSK3B in restoring sensitivity to 5FU both in vitro and in vivo, we decided to assess whether GSK3B was activated in a case
study of colon carcinoma stage II 5FU-treated patients (n = 50) with long follow up. By the end of the follow-up, 11 5FU-treated patients (22%) relapsed. All tumor samples were also characterized for MLH1, p53, p21, MDM2, pTyr216-GSK3B expression by IHC on tissue microarrays (TMA; Supplementary Table S2). As a control, we analyzed also a TMA of 24 colonic biopsies taken from patients undergoing surgery for pathologies different than cancer (i.e., diverticulosis). Phosphorylation on Tyr216 allows the activation of GSK3B (14). Using a commercial phospho-specific antibody, we observed no or very low anti-pTyr216 reactivity in peritumoral samples or diverticulosis colonic mucosa, whereas 52% (26/50) of cancer patients samples were positive (Supplementary Fig. S6 and Tables S2 and S3). Notably, the percentage of active GSK3B was significantly higher in samples from patients who relapsed after 5FU treatment than in patient who responded to therapy being 63.6% (7/11) versus 48.7% (19/50; McNemar test). Moreover, the log-rank test confirmed that active GSK3B is associated with cancer progression, poor response to therapy and worse overall survival. In particular, survival probability is significantly higher in colon cancers with inactive GSK3B, tested as immunohistochemically negative to pTyr216 (Fig. 6A). When patients are stratified for p53, again inactive GSK3B correlate with better survival probability, which is significant in the subset of MDM2 positive patients (Fig. 6B and C).

Altogether, our data indicate that colon cancers with active GSK3B, compared to those where GSK3B is not activated, have a worse outcome and are more prone to develop drug resistance.

Discussion

So far GSK3B has been described to be involved in modulating biological processes as opposite as proliferation or apoptosis, depending on the cellular, molecular, and developmental context (28–37). In fact, GSK3B is known to play an antiproliferative role by promoting APC-dependent phosphorylation—and hence proteosome-mediated degradation—of β-catenin, a transcription factor positively regulating Myc and cyclin D1 expression (14). In HCT116 colon carcinoma cell line it has been shown that GSK3B inhibition leads to apoptosis via p53 activation (38, 39). Here we present a novel role for GSK3B in colon carcinomas showing that its inhibition resensitizes drug-resistant p53-null colon cancer cells to chemotherapy both in vitro and in vivo and that GSK3B negatively regulates RIP1-independent necroptosis in response to chemotherapy. Moreover, in accordance with in vitro and in vivo data, we showed that GSK3B is activated in a high percentage (63.6%) of samples from 5FU-treated stage II colon carcinoma patients relapsed after 5FU-based therapy and that positivity for active form of GSK3B correlates with worse outcome and survival probability after adjuvant chemotherapy.

All the cell lines we used for the experiments express either mutated, nonphosphorylatable β-catenin or mutated APC (see Supplementary Table S1), thus rendering β-catenin activation constitutive and GSK3B-independent (16). Consequently, no proliferative defects were evident in cell lines stably silenced for GSK3B (Fig. 1). Being all the cell lines p53-null, we found particularly intriguing that GSK3B silencing had such a dramatic effect on the response to chemotherapy and reasoned that in this setting hitherto unrecognized pathways are likely to be crucially regulated by GSK3B-mediated phosphorylation. Our data suggest
that GSK3B plays a relevant role in drug resistance. In fact, we have shown that when GSK3B is expressed, p53-null colon carcinoma cells survive and proliferate despite chemotherapy and its silencing or inhibition abolishes cell growth after anticancer therapy both in vitro (Fig. 2A) and in vivo (Fig. 5). Therefore, GSK3B inhibition is sufficient to allow a cell death response to DNA-damaging drugs in resistant cells even in absence of p53 (Fig. 2). In particular, our results suggest that GSK3B modulates the response to DNA damage by affecting DNA repair (Fig. 3B) and negatively regulating PARP1 activity (Fig. 4B). Notably, PARP1 is involved in 3 pathways of DNA repair that are differently affected by p53 absence (40) and directly or indirectly activated by 5FU treatment (41): base excision repair (BER), nonhomologous end joining (NHEJ), and homologous recombination. In fact, in absence of wild type p53, activation of BER is suppressed whereas NHEJ and homologous recombination are active leading to aberrant double strand breaks repair. Accordingly, it has been reported that after severe genotoxic damage, p53 mutant cells can recover from a G2 arrest and resume proliferation following aberrant DNA repair (42). Our data indicates that GSK3B-regulated PARP1 activity is important for modulating DNA repair and tilting the balance toward cell death when too much damage occurs. Moreover, we showed that in p53 null cells, where drug-induced apoptosis is defective, the inhibition of GSK3B enables necroptosis as a response to chemotherapy (Fig. 4). RIP1 has been shown to mediate necroptosis in response to TNF receptor engagement (43), radiations (44), a few drugs (45) and so far its activation is thought to be central for the modulation of the necrotic response. Intriguingly, in our model, RIP1 kinase is not involved in mediating the necrotic response as showed by a lack of protection when using necrostatin-I (Fig. 4D). In addition, our results suggest that DNA damage triggered a RIP1-independent pathway negatively regulated by GSK3B.

Consistently with the role of GSK3B in drug resistance showed in in vitro (Fig. 2) and in vivo in xenograft experiments (Fig. 5), we observed expression of active GSK3B in 63.6% of biopsies from colon carcinoma stage II patients not responsive to 5FU-based therapy (Supplementary Tables S2 and S3 and Fig. S6). Accordingly, survival probability is significantly higher in colon cancers with inactive GSK3B (Fig. 6A). When patients are stratified for p53, again, inactive GSK3B correlate with better survival probability, which is significant in the subset of MDM2-positive patients (Fig. 6B and C). These findings are particularly relevant taking into account that the treatment for stage II primary colon cancer remains controversial. Although chemotherapy is often recommended for high-risk stage II disease, many tumors with similar histopathologic features will relapse, even after chemotherapy (46). Finding molecular markers with predictive value for the response to therapy in stage II colon cancer would therefore help clinicians with information to decide whether and how to treat these patients with adjuvant chemotherapy (47). The strong correlation of GSK3B activation with drug resistance, worse survival probability, and clinical outcome in 5FU-treated patients (Fig. 6A) suggests that GSK3B may be considered a candidate prognostic/predictive biomarker. Further studies on larger cohorts, stratified for both p53 and MDM2 are awaited to confirm these findings.

On the whole, our results add further support to previous data suggesting that GSK3B is an good candidate target for anticancer therapy and are particularly relevant for 2 main reasons. First, when GSK3B is inhibited DNA-damaging drugs bypass the need of p53 to induce cell death: triggering p53-independent cell death mechanisms is therefore an effective way to bypass one of most relevant causes of drug resistance. Therefore, the addition of GSK3B inhibitors to standard chemotherapy might be beneficial to a large number of colon carcinomas. Second, in a large number of tumors, classical apoptotic mechanisms are altered and such defects render treatment with traditional chemotherapeutic agents ineffective. Our findings that GSK3B inhibition in combination with chemotherapy unleashes a necroptotic response would therefore represent an alternative strategy to selectively treat otherwise nonresponsive tumors.

In conclusion, our study showed that GSK3B: (i) is a target whose inhibition restores the sensitivity to DNA-damaging agents in p53-null tumors; (ii) in vitro modulates a necrotic response to chemotherapy; (iii) its inhibition bypasses drug resistance in in vivo tumor xenografts; (iv) its activation correlates with worse survival probability and clinical outcome in colon cancer stage II patients treated with adjuvant therapy. Altogether our findings suggest that GSK3B may be a potential teranostic marker in colon cancer.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

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References


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Inhibition of GSK3B Bypass Drug Resistance of p53-Null Colon Carcinomas by Enabling Necroptosis in Response to Chemotherapy

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