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

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Running Title: GSK3B and drug-resistance of p53-null carcinomas

Key words: drug resistance, colon cancer, GSK-3, necroptosis, p53

Grant Support

This work was supported by F.A.R. grants from the University of Milano-Bicocca and PON 01_02782/7 grant to ML; from the European Union Framework 6 programme (INTACT) and from the Italian Cancer Society (AIRC) grants to K.H.

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Disclosure of Potential Conflict of Interests

The authors declare that no potential conflicts of interests exist.

Word count: 4850
Total number of figures and tables: 6

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/anti-apoptotic mechanisms. By studying a cohort of stage II colon carcinoma patients we found that GSK3B is activated in almost half of all colon carcinomas and in 2/3 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. Since p53 function is compromised in the vast majority of human cancers and that caspase-dependent apoptosis is frequently impaired in tumors, GSK3B inhibition in combination with chemotherapy may represent a molecularly targeted approach to treat resistant tumors.

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 shRNA 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 performed to confirm re-sensitization 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 by 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 vs responsive patients.
Conclusions: Targeting GSK3B, in combination with chemotherapy, may represent a novel strategy for the treatment of chemotherapy-resistant tumors.

Introduction

Two main problems affect the outcome of cancer therapy: 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, i.e. 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, i.e. to find specific molecules to target, also in the case of drug resistance. Kinases are the best candidates for this approach for at least two reasons: 1) It is known that several kinases are usually co-activated by redundant inputs and participates 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); 2) Kinases are thought to be “druggable” targets. Based on these premises we performed a phenotype screen using the kinase pools of the NKI 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 since 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 down-regulation of 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.

Methods

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

Cell lines and cell culture. DLD-1 and SW480 colon carcinoma cell lines were from the ATCC. Isogenic p53 wild type and p53 knockout HCT116 colon carcinoma cell lines were a kind gift of Dr. Bert 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 5
All cell lines were maintained in McCoy medium (Invitrogen) supplemented with 10% fetal bovine serum (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. 72 hrs later dead cells were counted - triplicate wells in each experiment - after Trypan blue staining. Graphs shown throughout the paper represent the average of three to five independent experiments. Average ± STDs is plotted in the graphs.

Colony assay. 3 x 10^5 cells/well were seeded in 6-well plate, let adhere overnight and treated with 200 μM 5FU for 12 hs. Cells were then trypsinized, counted, and reseeded at a low density (1000 cells/well in 6-well plate) in triplicate. In experiments without drug treatment 1000 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. 4 x 10^4 cells/well were seeded in triplicate in 96-well plate, let adhere overnight and treated with 200 μM 5FU for 72 hrs before evaluating active caspase-3/7 by the Caspase-Glo3/7 Assay System (Promega) according to the manufacturer's instructions.
**Cell proliferation.** 1 x 10⁴ cells/well were seeded in triplicate in 96-well plate and starting the following day (day 0) proliferation was evaluated each 24 hrs 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 x 10⁴ 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 x 10⁴ cells/well seeded in triplicate in a 96-well plate and reporter activity was evaluated 48 hrs 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 50mM, pH 7.5, NaCl 500 mM, DTT 1mM, EDTA 1mM, 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-1766), anti-caspase-3 (total) (sc-6549) were from SantaCruz Biotechnology.
**Immunofluorescence.** Cells were fixed with 4% paraformaldehyde in phosphate-buffered saline. Permeabilization and staining with anti-PAR (clone mAb 10H, Alexis), anti-AIF (sc-13116, SantaCruz Biotechnology), anti-γH2AX (Ab 22551, Abcam), anti-RPA70 (clone 2H10, Sigma-Aldrich) was performed 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 31 December 2010 or death, whichever came first. The median duration of overall follow up was 9.2 years (25th-75th percentile = 3.8-12.7 yrs). Overall survival (OS) 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 performed with the Stata/SE 12 package (Stata, College Station, TX).

**Immunohistochemistry.** Sample triplicates were arrayed using BioRep Tissue MicroArray System and antibody against pTyr216-GSK3B (sc-135653, Santacruz Biotechnology) was used as described (13). pTyr216-GSK3B staining was graded accordingly to an increasing intensity by blind reading by two experienced operators.
**In vivo xenograft studies.** All the experiments involving animals were carried out and 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 performed). Tumors were established by subcutaneously injecting $1 \times 10^6$ cells (in 100 $\mu$l of a 50% PBS and 50% Matrigel solution), HCT116p53KO cells into the left flanks and HCT116 into the right flanks, of 5-7-weeks-old female CD-1 nude mice (Charles River Laboratories, Calco, Italy). When HCT116p53KO tumors reached the average volume of 100 mm$^3$ (day 7 post-engraftment), animals were randomized and given vehicle, 5FU (via intraperitoneal injection, 75mg/Kg, twice a week), LiCl (via intraperitoneal injection, 80 mg/Kg, twice a day for 5 days a week) or a combination thereof. 5FU treatment started at day 8 post-engraftment, while 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-Damico-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 hystological and immunohistochemistry analysis. Tumor biopsies were removed from formalin, dehydrated, diafanized with xylene, put in paraffin, sectioned with microtome, put on slides and stained with Hematoxylin/Eosin following standard 9...
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 performed a phenotype screen using the kinase pools of the NKI shRNA library (7) and 5FU-resistant HCT116p53KO cells as a model system (Supplementary Fig. 1, A). After having validated several of the hits (Supplementary Fig. 1, B-D) 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/βcatenin pathway which support both invasive and metastatic processes (14, 15). However, HCT116p53KO cells express mutated, non-phosphorylatable 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 anti-proliferative 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 1) that prevents GSK3B-mediated regulation. Next, we analyzed several parameters related to cell cycle and proliferation in cells stably silenced for GSK3B. Notably, down-regulation of GSK3B does not 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 demonstrate 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 re-sensitize cells to drug-induced cytotoxicity (Fig. 2B-D). In order to further confirm the role of GSK3B in drug resistance we inhibited its function by two more different means (17). Transient GSK3B protein depletion by use of siRNA restored cell death in response to 5FU (Supplementary Fig.2). We confirmed these findings by treating HCT116p53KO, DLD-1 and SW480 cells with 5FU in the presence of LiCl (a GSK3B inhibitor 14 approved by FDA for the treatment of bipolar disorder) (Fig. 2E). We further tested the role of GSK3B in drug resistance by treating colon cancer cells with oxaliplatin (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. 3).

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 GSK3 inhibition influence 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 demonstrated 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 appears to be dependent on GSK3B activity, since 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, 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. 4) 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). Poly(ADP-ribose) polymerase 1 (PARP1) is an important activator of caspase-independent necroptosis: 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 performed several experiments to assess a possible role of GSK3B as a modulator of PARP1 and AIF in drug-induced caspase-independent necroptosis. First, we found that polymers of ADP-ribose (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.5A) and re-localized to cell nuclei upon 5FU exposure of GSK3B-depleted cells (Fig. 4C). Third, to test whether AIF re-localization was dependent on tBid and PARP-1 we pre-treated 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 4E). Accordingly, we also showed that silencing AIF in GSK3B-depleted HCT116p53KO cells reduced 5FU cytotoxicity (Supplementary Fig.5B). Finally, since TNFalpha-mediated necroptosis is dependent on the activation of RIP1 kinase (24) we tested its involvement in our model by pre-incubating GSK3B-depleted cells with the RIP1 specific inhibitor necrostatin-1 (25) before adding 5FU. Cell death was not prevented by necrostatin-1 (Fig. 4, panel D) indicating that, at variance with TNFalpha, DNA damage does not require RIP1 activity to trigger necroptosis.

Therefore our data indicate that, in the absence of functional p53, GSK3B regulates 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 performed 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. 5, panels A and B). As expected, in HCT116 control tumors, 5FU alone significantly decreased the tumor burden. Histology of tumor masses is shown in Figure 5C, where murine stromal cells were identified as positive to p53 staining while HCT116p53KO tumor cells tested negative (upper row). Different morphology and tissue organization, are evident in untreated, 5FU- and LiCl-treated- vs 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 re-sensitizes drug-resistant tumors to chemotherapy also *in vivo*.

**GSK3B is activated in colon carcinoma samples from patients.** Preliminary data from two different laboratories reported high expression of active GSK3B in cell lysates from small groups of colon carcinoma samples (26, 27). Since 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 2). As a control we analyzed also a TMA of 24 colonic biopsies taken from patients undergoing surgery for pathologies different than cancer.
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.6 and Table 2 and 3). Notably, the percentage of active GSK3B was significantly higher in samples from patients that relapsed after 5FU treatment than in patient that responded to therapy being 63.6% (7/11) vs 48.7% (19/50) (p=0.002599, 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 sub-set of MDM2 positive patients (Fig.6B-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 anti-proliferative role in cancer cells.
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 demonstrated that
GSK3B inhibition leads to apoptosis via p53 activation (38, 39). Here we present a novel
role for GSK3B in colon carcinomas demonstrating that its inhibition re-sensitizes 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, non-
phosphorylatable β–catenin or mutated APC (see Supplementary Table 1), thus
rendering β–catenin activation constitutive and GSK3B-independent (16). Consistently,
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 three
pathways of DNA repair that are differently affected by p53 absence (39) and directly or
indirectly activated by 5FU treatment (40): base excision repair (BER), non-homologous
end joining (NHEJ) and homologous recombination (HR). In fact, in absence of wild type
p53, activation of BER is suppressed whereas NHEJ and HR 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 (41). 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 demonstrated 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 (42), radiations (43), a few drugs (44) and so far
its activation is thought to be central for the modulation of the necroptotic response.
Intriguingly, in our model, RIP1 kinase, is not involved in mediating the necroptotic
response as demonstrated by a lack of protection when using necrostatin-1 (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 demonstrated 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 2 and 3, and supplementary Figure 6). 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 sub-set of MDM2-positive patients (Fig. 6B-C). These findings are particularly relevant taking into account that the treatment for stage II primary colon cancer remains controversial: while chemotherapy is often recommended for high-risk stage II disease, many tumors with similar histopathologic features will relapse, even after chemotherapy (45). 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 (46). 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 two 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 non-responsive 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 necroptotic 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.

Acknowledgments
We thank: Leda Dalprà helpful discussion and critically reading of the manuscript; Bert Vogelstein for the gift of HCT116p53KO cells; R. Bernards for the pRS-shRNA library; Andrea Affuso and Dario Consolante for the precious assistance in the xenograft experiments. We are grateful to: the Department of Experimental Oncology, European Research.
Institute of Oncology for the use of its laboratory resources for the infections and screen phases of this work; BIOGEM Institute for the use of its facilities for the xenograft experiments.

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LEGENDS

Figure 1. GSK3B silencing in p53-null colon carcinoma cell lines does not affect proliferation or cell cycle. (A) Decreased expression of GSK3B in HCT116p53KO, SW480 and DLD-1 cells stably infected with empty (pRS) and GSK3B shRNA-encoding vector (pRSGSK3B). An antibody recognizing both GSK3A and GSK3 was used: GSK3A levels served as an internal loading control. (B) The indicated cell lines were seeded at low density and grown for 2 weeks before staining. (C) Growth curve of HCT116p53KO stably infected with pRS and pRSGSK3B. (D) DNA content of HCT116p53KO stably infected with pRS and pRSGSK3B in the logarithmic phase of growth was evaluated after PI staining and flow cytometric analysis; percentage of cells in sub-G1, G1, S phase and G2/M are indicated. (E) β-catenin activity in HCT116p53KO-pRS and –pRSGSK3B was evaluated 48 hrs after co-transfection of firefly (FF) luciferase under the control responsive reporter together with constitutively expressed renilla (R) luciferase used as a normalizator. RLU = Relative Light Units.

Figure 2. GSK3B silencing or inhibition abolishes drug resistance of p53-null colon carcinoma cell lines. (A) The indicated cell lines were trypsinized and re-seeded at low density 12 hrs after 5FU treatment. Colony formation was assessed 2 weeks after the re-seeding.
seeding. Cell death 72 hrs after 200 μM 5FU treatment of (B) HCT116p53KO (C) SW480 and (D) DLD-1 cells stably infected with empty (pRS) and GSK3B shRNA-encoding vector (pRSGSK3B). HCT116 cells were used as a positive control. (E) Cell death 72 hrs after 200 μM 5FU treatment of HCT116p53KO, SW480 and DLD-1 cells in the presence or absence of 10mM LiCl. In the inset: increased levels of inhibitory pSer-GSK3B (but not of pSer21-GSK3A) upon LiCl treatment of HCT116p53KO cells were assessed by incubating the blot with a mix of pSer9-GSK3B and pSer21-GSK3A antibodies. (F) Cell death of HCT116p53KO, DLD-1 and SW480 cells stably infected with empty pRS and pRSGSK3B 72 hs after treatment with 50 μM OxPt. (G) As in (F) after treatment with 200 μM 5FU + 50 μM OxPt.

**Figure 3.** GSK3B inhibition abolishes drug resistance of p53-null colon carcinoma cells by affecting the response to DNA damage. HCT116p53KO cells stably infected with empty (pRS) and GSK3B shRNA-encoding vector (pRSGSK3B) untreated (cnt) or treated for 18 hrs with 200 μM 5FU (5FU) and stained with anti-γH2AX antibody (A) or anti-RPA70 antibody (B) and counterstained with DAPI. HCT116p53KO untreated (cnt), treated for 18 hrs with 200 μM 5FU (5FU) or with 200 μM 5FU + 10 mM LiCl and stained with anti-γH2AX antibody (C) or anti-RPA70 antibody (D) and counterstained with DAPI. White arrows indicate cells with very few or no RPA70 foci.
Figure 4. p53-null, GSK3B-silenced colon carcinoma cells treated with 5FU die by RIP1-independent necroptosis. (A) HCT116p53KO-pRS and HCT116p53KO-pRSGSK3B were treated for the indicated times with 200 μM 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 hrs) 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 indicate intermediate forms produced during proteolytic activation; an aliquot of the same samples at 72 hrs after treatment was used for a luminometric caspase-3/-7 assay (lower); (B) HCT116p53KO-pRS and HCT116p53KO-pRSGSK3B untreated (cnt) and treated for 18 hrs with 200 μM 5FU and stained with anti-PAR antibody as well as DAPI; (C) HCT116p53KO-pRS and HCT116p53KO-pRSGSK3B untreated (cnt) and treated for 30 hrs (when 40-50% cells are dead) with 200 μM 5FU and stained with anti-AIF antibody as well as DAPI; > 80% 5FU-treated HCT116p53KO-pRSGSK3B showed nuclear AIF; (D) HCT116p53KO-pRSGSK3B were pre-incubated for 2 hrs with pan-caspase inhibitor QVD-OPh (10μM), Bid inhibitor (20μM Bi6C9), PARP1 inhibitor (100μM DiQ), Bi6C9+DiQ or Necrostatin-1 (20 μM Nec1) before adding 200 μM 5FU and counted 72 hrs later; (E) HCT116p53KO-pRSGSK3B were treated for 30 hrs with 200 μM 5FU in presence of 100μM DiQ and stained with anti-AIF antibody as well as DAPI.
Figure 5. GSK3 activation influences the therapeutic response to 5FU in vivo

Sentative pictures taken at the moment of sacrifice of mice, and relative tumoral masses, treated with vehicle only (CNT), 5FU, LiCl and 5FU+LiCl as described in Material and Methods. (B) Graph representing the average Relative Tumor Volume (in mm³) of xenografted tumours in the different treatment groups. Statistical analysis of the results was performed using the Kruskal Wallis test, followed by Nemenyi-Damico-Wolfe-Dunn test for multiple pairwise comparisons between groups. A p value >0.05 was considered as significant. (C) Immunohistochemical staining for p53 on slides of HCT116p53KO xenograft tumors, showing positivity only for stromal murine cells (upper row) and hematoxylin-eosin staining (central and lower rows) of representative sections of xenografted tumors taken from vehicle only (CNT), 5FU, LiCl and 5FU+LiCl-treated mice at the moment of sacrifice.

Figure 6. GSK3 activation correlates with poorer outcome after adjuvant chemotherapy

(A) Kaplan-Meier plot of the survival probability of 5FU-treated patients stratified by pTyr216-GSK3B positivity; (B) Kaplan-Meier plot representing overall survival of 5FU-treated p53-positive patients stratified by pTyr216-GSK3B positivity; (C) Kaplan-Meier plot representing overall survival of 5FU-treated MDM2-positive patients stratified by pTyr216-GSK3B positivity.
Figure 1. GSK3B silencing in p53-null colon carcinoma cell lines does not affect proliferation or cell cycle
Figure 2. GSK3B silencing or inhibition abolishes drug resistance of p53-null colon carcinoma cell lines

A

B

C

D

E

F

G

Author Manuscript Published Online First on May 31, 2013; DOI: 10.1158/1078-0432.CCR-12-3289
Author manuscripts have been peer reviewed and accepted for publication but have not yet been edited.

Research.
Figure 3. GSK3B inhibition abolishes drug resistance of p53-null colon carcinoma cells by affecting the response to DNA damage.
Figure 4. p53-null, GSK3B-depleted colon carcinoma cells treated with 5FU die by RIP1-independent necroptosis.
Figure 5. GSK3 inhibition restores the therapeutic response of p53-null cells to 5FU in vivo

A

B

C

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Figure 6. GSK3B activation correlates with poorer outcome after adjuvant chemotherapy.

Figure 6. GSK3B activation influences overall survival in 5FU-treated patients.
Clinical Cancer Research

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Clin Cancer Res  Published OnlineFirst May 31, 2013.

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