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| メタデータ | 言語: eng                                                                           |
|       | 出版者:                                                                              |
|       | 公開日: 2017-10-05                                                                   |
|       | キーワード (Ja):                                                                       |
|       | キーワード (En):                                                                       |
|       | 作成者:                                                                              |
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|       | 所属:                                                                               |
| URL   | <a href="https://doi.org/10.24517/00027533">https://doi.org/10.24517/00027533</a> |

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# **Deregulated Glycogen Synthase Kinase-3 $\beta$ Sustains Gastrointestinal Cancer Cells Survival by Modulating Human Telomerase Reverse Transcriptase and Telomerase**

**Short Title: GSK3 $\beta$  in gastrointestinal cancers**

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**Key words:** GSK3 $\beta$ , gastrointestinal cancer, cell survival, hTERT, telomerase

## Abbreviations

ABC, avidine-biotine-peroxidase complex; APC, adenomatous polyposis coli;  $\beta$ -gal,  $\beta$ -galactosidase; BrdU, bromodeoxyuridine; CDK, cyclin-dependent kinase; COX-2, cyclo-oxygenase-2; DMSO, dimethyl sulfoxide; ELISA, enzyme-linked immunosorbent assay; ERK, extracellular signal-regulated kinase; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; GSK3 $\beta$ , glycogen synthase kinase-3 $\beta$ ; HE, hematoxylin and eosin; hTERT, human telomerase reverse transcriptase; JNK, c-Jun NH<sub>2</sub>-terminal kinase; NF- $\kappa$ B: nuclear factor-kappaB; NRIKA, nonradioisotopic *in vitro* kinase assay; PCNA, proliferating cell nuclear antigen; PI3K, phosphatidylinositol 3-kinase; PTEN, phosphatase and tensin homologue deleted on chromosome 10; RNAi, RNA interference; RT-PCR, reverse transcription-PCR; siRNA, small interfering RNA; TRAIL, tumor necrosis factor-related apoptosis-inducing ligand; TRAP, telomeric repeat amplification protocol; TUNEL, terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling; WST-8; 4-[3-(4-iodophenyl)-2-(4-nitrophenyl)-2H-5-tetrazolio]-1,3-benzene disulfonate

## Translational Relevance

We showed that survival of gastrointestinal, pancreatic and liver cancer cells depends on aberrant glycogen synthase kinase-3 $\beta$  (GSK3 $\beta$ ) expression and activity. We demonstrated therapeutic effect of GSK3 $\beta$  inhibition against these cancer cells and colon cancer xenografts in rodents. No detrimental effects of GSK3 $\beta$  inhibition were observed in normal cells or vital organs. These results may enable applying a therapeutic strategy targeting GSK3 $\beta$  to treatment of cancer patients. Molecular target-directed therapy holds great promise for cancer patients who are refractory to conventional chemotherapy and radiation. Current paradigms include the targeting of protein tyrosine kinases and angiogenesis factors. Since GSK3 $\beta$  is a serine/threonine protein kinase, targeting of GSK3 $\beta$  has the potential to treat a broad spectrum of cancers. Because none of the available inhibitors has found clinical use for treatment of diseases, it is important to develop a new class of inhibitors that selectively controls deregulated GSK3 $\beta$  expression and activity in cancer.

## Abstract

**Purpose:** Glycogen synthase kinase-3 $\beta$  (GSK3 $\beta$ ) regulates multiple cell signaling pathways and has been implicated in glucose intolerance, neurodegenerative disorders and inflammation. We investigated the expression, activity and putative pathological role of GSK3 $\beta$  in gastrointestinal, pancreatic and liver cancers.

**Experimental Design:** Colon, stomach, pancreatic and liver cancer cell lines, non-neoplastic HEK293 cells and matched pairs of normal and tumor tissues of stomach and colon cancer patients were examined for GSK3 $\beta$  expression and its phosphorylation at serine 9 (S9, inactive form) and tyrosine 216 (Y216, active form) by Western immunoblotting and for GSK3 $\beta$  activity by *in vitro* kinase assay. The effects of small-molecule GSK3 $\beta$  inhibitors and of RNA interference on cell survival, proliferation and apoptosis were examined *in vitro* and on human colon cancer cell xenografts in athymic mice. The effects of GSK3 $\beta$  inhibition on human telomerase reverse transcriptase (hTERT) expression and telomerase activity were compared between colon cancer and HEK293 cells.

**Results:** Cancer cell lines and most cancer tissues showed increased GSK3 $\beta$  expression, increased Y216 phosphorylation and activity, but decreased S9 phosphorylation compared with HEK293 cells and nonneoplastic tissues. Inhibition of GSK3 $\beta$  resulted in attenuated cell survival and proliferation and increased apoptosis in most cancer cell lines and in HT-29 xenografts in rodents, but not in HEK293 cells. GSK3 $\beta$  inhibition in colon cancer cells was associated with decreased hTERT expression and telomerase activity.

**Conclusion:** The results indicate that deregulated GSK3 $\beta$  sustains gastrointestinal cancer cells survival via modulation of hTERT and telomerase.

## Introduction

A large proportion of malignant tumors are cancers of the digestive organs including stomach, large bowel, pancreas and liver (1). Progress has been made in understanding the molecular characteristics of these tumors and in developing methods for early diagnosis and modalities for surgical and systemic treatment. Nevertheless, gastrointestinal cancers remain a leading cause of cancer deaths worldwide (2-4). In particular, only 20% of patients with resectable pancreatic cancer survive for 5 years (4). There is clearly a need to develop new treatment strategies other than surgery, chemotherapy and radiation. In this respect, molecular target-directed therapy holds great promise. Current paradigms include the targeting of protein tyrosine kinases and angiogenesis factors because of the known roles of these oncogene products in cancer (5). Recently, pharmacological inhibitors and humanized monoclonal antibodies targeting the epidermal growth factor receptor, HER2/neu and vascular endothelial growth factor have been combined with chemotherapeutic agents for metastatic colorectal cancer and pancreatic cancer (3, 4). These protocols are at the stage of clinical trials and there is a need for more promising therapeutic targets that cover a broader spectrum of gastrointestinal cancers.

Glycogen synthase kinase-3 $\beta$  (GSK3 $\beta$ ) is a serine/threonine protein kinase that regulates fundamental cellular pathways depending on the substrates it phosphorylates (6, 7). GSK3 $\beta$  has recently emerged as a promising target for development of drugs against chronic diseases because of causative associations with diabetes mellitus and neuropsychiatric disorders (8-11). Under physiological conditions GSK3 $\beta$  phosphorylates and triggers degradation of several transcription factors and proto-oncoproteins such as  $\beta$ -catenin. It is therefore hypothesized to suppress tumor development by interfering with oncogenic signaling (12). However, there are no reports of decreased expression or inactivation of GSK3 $\beta$  in tumors, nor of induction of tumors following loss of expression or inactivation of GSK3 $\beta$ . Although this kinase is thought to negatively regulate Wnt signaling (12), we have demonstrated that deregulated expression and activity of GSK3 $\beta$  are distinct features of colorectal cancer and contribute to cancer cell survival and proliferation in a manner unrelated to Wnt/ $\beta$ -catenin signaling and Akt activation

(13). This novel pathological property of GSK3 $\beta$  is supported by observations that inhibition of kinase activity attenuates survival and proliferation of human colon cancer cells, thus predisposing them to undergo apoptosis *in vitro* and in tumor xenografts. We have therefore proposed GSK3 $\beta$  as a potential therapeutic target in colorectal cancer (13-15).

In the same time and following our studies of the anti-tumor effects of GSK3 $\beta$  inhibition (13-16), similar observations were reported in colon, pancreatic, ovarian, esophageal and thyroid cancer cells and melanoma (17-22). Deregulated expression and activity of GSK3 $\beta$  may therefore be a common and fundamental characteristic of various gastrointestinal cancer types. In the present study we investigated expression, activity and putative pathological properties of GSK3 $\beta$  in gastrointestinal cancer cell lines and primary tumors. To understand the mechanism underlying pathological roles for GSK3 $\beta$  in cancer, we examined its influence on telomerase, one of the hallmarks of cancer cells (23). Finally, we evaluated the therapeutic effects of GSK3 $\beta$  inhibitors against colon cancer xenografts in immunodeficient mice to ascertain possible therapeutic benefits.

## Materials and Methods

**Cell lines and tissue specimens.** Human embryonic kidney cells (HEK-293) and colon (SW480, HCT116, HT-29), pancreatic (MIAPaCa-2, Capan-1, BxPC-3) and liver (HepG2) cancer cell lines were obtained from the American Type Culture Collection (ATCC; Manassas, VA). Human stomach cancer cell lines (MKN-28, NKPS, TMK-1, NUGC-4) were obtained from the Human Science Research Resources Bank (Osaka, Japan). Matched pairs of non-neoplastic (normal) mucosa and tumor tissues were collected from fresh surgical specimens of 10 patients with stomach cancer and 4 with colorectal cancer ([Supplementary Table 1](#)). After sampling, the surgical specimens were fixed in neutral-buffered formalin, embedded in paraffin and processed for histopathologic diagnosis and immunohistochemical examination. This study was approved by the Institutional Review Board of Kanazawa University.

**Western immunoblotting.** Cellular protein was extracted from cultured cells and fresh

surgical specimens using lysis buffer (CellLytic-MT) containing a mixture of protease and phosphatase inhibitors (Sigma-Aldrich, St. Louis, MO). An aliquot of protein extract was analyzed by Western immunoblotting (13) for the proteins of interest. Amount of protein in each sample was monitored by expression of  $\beta$ -actin. The following primary antibodies were used at the dilutions shown against both GSK3 isoforms (GSK3 $\alpha$  and  $\beta$ ) (1:1000; Upstate Biotechnology, Lake Placid, NY), GSK3 $\beta$  (1:1000; BD Biosciences, Lexington, KY) and its fractions that are phosphorylated at the serine (S) 9 residue (pGSK3 $\beta$ <sup>S9</sup>) (1:1000; Cell Signaling Technology, Beverly, MA) and the tyrosine (Y) 216 residue (pGSK3 $\beta$ <sup>Y216</sup>) (1:1000; BD Biosciences),  $\beta$ -catenin (1:1000; BD Biosciences) and its fractions phosphorylated at S33, S37 and/or threonine (T) 41 residues (p- $\beta$ -catenin<sup>S33/37/T41</sup>), p53, p21, cyclin D1, cyclin-dependent kinase (CDK) 6, Rb and its fraction phosphorylated at the S780 (p-Rb<sup>S780</sup>), S795 (p-Rb<sup>S795</sup>) and S807/811 residues (p-Rb<sup>S807/811</sup>) (all of them in 1:1000; Cell Signaling Technology), c-Jun NH<sub>2</sub>-terminal kinase (JNK) 1 (1:1000; BD Biosciences), and  $\beta$ -actin (1:4000; Ambion, Austin, TX). Immunoblotting signals were measured by CS analyzer (ATTO, Tokyo, Japan).

***Nonradioisotopic in vitro kinase assay (NRIKA).*** The NRIKA developed in our laboratory (24) was used to detect GSK3 $\beta$  activity in cultured cells and colorectal cancer tissues. It uses a sequential combination of immunoprecipitations to isolate GSK3 $\beta$  in protein samples extracted from cultured cells or tissue specimens, an *in vitro* kinase reaction that uses recombinant  $\beta$ -catenin protein (substrate) and non-radioisotopic ATP, followed by immunoblotting to detect p- $\beta$ -catenin<sup>S33/37/T41</sup>. To test the inhibitory effects of small molecule inhibitors against GSK3 $\beta$  activity by NRIKA in cancer cells, the kinase reaction was carried out in the presence of dimethyl sulfoxide (DMSO; Sigma-Aldrich), a solvent for the inhibitors, or 25  $\mu$ M of the inhibitors SB-216763 (Sigma-Aldrich) or AR-A014418 (Calbiochem, La Jolla, CA).

***Effects of GSK3 $\beta$  inhibitors on cell survival, proliferation and apoptosis.*** Cells seeded in 96-well culture plates were treated with DMSO or with GSK3 $\beta$  inhibitor (SB-216763 or AR-A014418) dissolved in DMSO at the indicated final concentrations in the

medium. The concentrations of GSK3 $\beta$  inhibitors used in this study (e.g. 25  $\mu$ M) are within a range of the pharmacologically relevant doses as previously reported (9, 25, 26). At designated time points, the relative numbers of viable cells were determined by using a WST-8 (4-[3-(4-iodophenyl)-2-(4-nitrophenyl)-2H-5-tetrazolio]-1,3-benzene disulfonate) assay kit (Wako, Osaka, Japan). After treatment with DMSO or GSK3 $\beta$  inhibitor, relative numbers of proliferating cells and apoptosis were determined using Cell Proliferation ELISA BrdU kit and Cellular DNA Fragmentation ELISA kit (both from Roche Diagnostics, Indianapolis, IN), respectively. Occurrence of apoptosis was further demonstrated by observing fragmented DNA ladders and changes in cell-cycle fractions.

**RNA interference (RNAi).** Small interfering RNA (siRNA) specific to human GSK3 $\beta$  (target sequence: 5'-GCUCCAGAUCAUGAGAAAGCUAGAU-3'; GSK3 $\beta$  Validated Stealth RNAi) and negative control siRNA (Stealth RNAi Negative Control Low GC duplex) were purchased from Invitrogen (Carlsbad, CA). Cells were transfected with 20 nM of either GSK3 $\beta$ -specific or negative control siRNA by using Lipofectamine RNAiMAX (Invitrogen). Effects of RNAi on expression of GSK3 $\beta$  mRNA and protein were determined by semi-quantitative reverse transcription (RT)-PCR and Western immunoblotting, respectively. GAPDH (glyceraldehyde-3-phosphate dehydrogenase) was used as an internal control to monitor the efficiency of RT-PCR. The sequences of primers and thermal cycling programs for RT-PCR are available upon request. At 72 hrs after siRNA transfection, relative numbers of viable cells, proliferating cells and apoptosis were determined as described above.

**Cell senescence, hTERT expression and telomerase activity.** Cell senescence was examined by scoring  $\beta$ -galactosidase ( $\beta$ -gal)-positive cells (27) after treatment with DMSO or the GSK3 $\beta$  inhibitors. Expression of hTERT mRNA was determined by semi-quantitative RT-PCR (28). Details of the RT-PCR including the sequences of primers are available upon request. Telomerase activity was measured by telomeric repeat amplification protocol (TRAP) assay using the TRAP-eze Telomerase Detection Kit (Intergen, Purchase, NY) according to the manufacturer's protocol. The human cervical cancer cell line C33A (ATCC) was analyzed as a positive control for detection of hTERT mRNA expression and telomerase activity.

**Animal study.** Effect of GSK3 $\beta$  inhibition on tumor proliferation was examined for colon cancer HT-29 cells inoculated in rodents. Design and protocol of animal experiment and body weights of animals monitored during treatment were shown in [Supplementary Fig. 1](#). The study was conducted according to the Guidelines for the Care and Use of Laboratory Animals at Kanazawa University and to the National Guidelines for Animal Usage in Japan ([http://www.lifescience.mext.go.jp/policies/pdf/an\\_material011.pdf](http://www.lifescience.mext.go.jp/policies/pdf/an_material011.pdf)).

**Immunohistochemical and histochemical examinations.** Expression and localization of GSK3 $\beta$  in colorectal cancer were examined using rabbit monoclonal antibody to GSK3 $\beta$  (diluted 1:100; Epitomics, Burlingame, CA) by the avidine-biotin-peroxidase complex (ABC) method (29). Expressions of proliferating cell nuclear antigen (PCNA) and hTERT in tumor xenografts in mice were examined using rabbit polyclonal antibody to PCNA (diluted 1:100; Santa Cruz Biotechnology, Santa Cruz, CA) and rat monoclonal antibody to hTERT (diluted 1:1,000; ref. 30), respectively, by the ABC method with modifications (14). For the negative control, primary antibodies were replaced by non-immune rabbit and rat IgG (DakoCytomation). Apoptosis was detected in tumor xenografts by TUNEL (terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling) method using an *in situ* apoptosis detection TUNEL kit (Takara). Frequency of proliferating cells and of apoptosis in the tumors was calculated as described previously (29).

**Statistical analysis.** The Student's *t* test was used to determine statistical differences in the incidence of cell senescence, survival, proliferation and apoptosis between cells treated with DMSO and GSK3 $\beta$  inhibitor, or between cells treated with non-specific and GSK3 $\beta$ -specific siRNA. Statistical differences in body weight of rodents, tumor volume and PCNA- and TUNEL-positive cells between groups of mice treated with DMSO and GSK3 $\beta$  inhibitor were determined using one way ANOVA test followed by the Tukey-Kramer and Bonferroni/Dunn tests. All statistical tests using Statcel version 2 (Tokyo, Japan) were two-sided and *P* values of <0.05 was considered to be statistically significant.

## Results

**Expression, phosphorylation and activity of GSK3 $\beta$ .** Most cancer cells showed higher levels of GSK3 $\beta$  and pGSK3 $\beta$ <sup>Y216</sup> (active) and lower levels of pGSK3 $\beta$ <sup>S9</sup> (inactive) compared to HEK 293 cells expressing both phosphorylated forms (Fig. 1A). NRIKA demonstrated that cancer cell-derived GSK3 $\beta$  was active for phosphorylation of its substrate ( $\beta$ -catenin) (Fig. 1B). These results indicate that most colon, stomach, pancreatic and liver cancer cells contain active GSK3 $\beta$  and that its activity appears uncontrolled by differential phosphorylation at S9 and Y216. Immunoblotting analysis of gastric and colorectal cancer tissues showed higher expression of GSK3 $\beta$ , more pGSK3 $\beta$ <sup>Y216</sup> and less pGSK3 $\beta$ <sup>S9</sup> compared to matching non-neoplastic tissues (Fig. 2A, B). In the colorectal cancer patients, increased levels of GSK3 $\beta$  and pGSK3 $\beta$ <sup>Y216</sup> and decreased pGSK3 $\beta$ <sup>S9</sup> were associated with higher GSK3 $\beta$  activity in tumors compared to non-neoplastic tissues (Fig. 2B). Immunohistochemistry showed overexpression of GSK3 $\beta$  in the cytoplasm of colorectal cancer cells (Fig. 2C).

**Effects of GSK3 $\beta$  inhibition on cell survival, proliferation and apoptosis.** We demonstrated by NRIKA that SB-216763 or AR-A014418 inhibited the activity of cancer cell-derived GSK3 $\beta$  (Fig. 1C). The same effect was observed in other cancer cell types (data not shown). Inhibition of GSK3 $\beta$  activity was associated with attenuated cell survival, a significant decrease in proliferating cells and an increase in apoptosis (Fig. 3; Supplementary Fig. 2). Influence of GSK3 $\beta$  expression on the biological properties of cancer cells was investigated using RNAi. Depletion of GSK3 $\beta$  significantly attenuated cell viability and proliferation and increased apoptosis in all cancer cell lines (Fig. 3C). Induction of apoptosis following GSK3 $\beta$  inhibition was demonstrated by DNA fragmentation and an increase in the sub-G0 cell-cycle fraction compared to cells treated with DMSO, and confirmed by an increase in Annexin V-positive cells (Supplementary Fig. 3).

Importantly, inhibition of GSK3 $\beta$  activity or expression had no significant effects on survival, growth or apoptosis in HEK293 cells (Fig. 3) in which both GSK3 $\beta$  S9 and Y216 are phosphorylated (Fig. 1A). pGSK3 $\beta$ <sup>S9</sup> and pGSK3 $\beta$ <sup>Y216</sup> were also detected in most non-neoplastic tissues from stomach and colorectal cancer patients (Fig. 2; ref. 13). These

results indicate that active GSK3 $\beta$  exerts a pathological role in cancers of the digestive organs by promoting tumor cell survival and proliferation and by inhibiting apoptosis.

***Effects of GSK3 $\beta$  inhibition on telomerase activity.*** Understanding the molecular mechanism behind a pathological role for GSK3 $\beta$  in cancer is important for the development of treatment strategies that target this kinase. To evaluate changes associated with GSK3 $\beta$  inhibition, colon (SW480 and HCT116), stomach (NUGC-4) and pancreatic (BxPC-3) cancer cells were examined for expression of the molecules responsible for cell-cycle regulation and apoptosis and known to be regulated by GSK3 $\beta$  (16-22). GSK3 $\beta$  knockdown increased the levels of p21 and JNK1 in both SW480 and HCT116 cells and of p53 in HCT116, but not of mutant and stable p53 expressed in SW480 cells. Levels of cyclin D1, CDK6 and the phosphorylation of Rb decreased in both cell types. The similar changes in expression of p21 and CDK6 and levels of Rb phosphorylation were observed in NUGC-4 and BxPC-3 cells (Fig. 4).

Since these molecules are involved in cell senescence (31), a possible role for GSK3 $\beta$  in regulating hTERT expression and telomerase activity in cancer cells was investigated. Following DMSO treatment, the incidence of  $\beta$ -gal-positive cells was significantly higher for HEK293 than colon cancer (SW480, HCT116) cells. No difference was seen between SW480 and HCT116 cells. GSK3 $\beta$  inhibitors significantly increased the incidence of  $\beta$ -gal-positivity in colon cancer cells, but not in HEK293 cells (Fig. 5A). Following depletion of GSK3 $\beta$  or inhibition of its activity, hTERT expression decreased in SW480 and HCT116 cells, but not in HEK293 cells (Fig. 5B, C). Telomerase activity was also attenuated in colon cancer cells by inhibiting expression or activity of GSK3 $\beta$ , whereas no such changes were observed in HEK293 cells (Fig. 5D). Neither GSK3 $\beta$  inhibitor SB-216763 nor AR-A014418 directly inhibited the activity of the cancer cells-derived telomerase in TRAP assay (Supplementary Fig. 4). Thus, decreases in hTERT expression and telomerase activity in colon cancer cells appear to be consequences of GSK3 $\beta$  inhibition.

***Effects of GSK3 $\beta$  inhibitors on proliferation of colon cancer xenografts.*** Our earlier study found that inhibition of GSK3 $\beta$  activity by treatment with SB-216763 or AR-A014418

for 5 weeks attenuates the proliferation of SW480 cells in rodents (14). In the present study, HT-29 cells were xenografted into athymic mice and treated for 8 weeks with DMSO or 10 weeks with GSK3 $\beta$  inhibitors ([Supplementary Fig. 1A](#)). This allowed evaluation of the therapeutic effect of GSK3 $\beta$  inhibition in different cancer cells and for a longer period.

Mice treated with DMSO were euthanized 8 weeks after treatment because of large tumors and cachexia. Mice treated with GSK3 $\beta$  inhibitors tolerated this well for the 10 week treatment period. Significant and dose-dependent decreases in tumor volume were observed in mice treated for 7 and 10 weeks with GSK3 $\beta$  inhibitors compared to DMSO treatment for 8 weeks ([Fig. 6A, B](#)). Three weeks after treatment (from week 5 in [Fig. 6A](#)) tumor growth decreased significantly in mice treated with GSK3 $\beta$  inhibitor. No difference was observed between mice treated with varying doses of either inhibitor. Following 8 weeks of treatment (from week 10 in [Fig. 6A](#)), a statistical difference in tumor volume was seen between mice treated with different doses of inhibitor. At necropsy, visible tumor was not seen in one mouse treated with 2 mg/kg SB-216763 and in two mice with 5mg/kg AR-A014418 ([Fig. 6B](#)).

No difference in tumor histology (medullary and tubular proliferation of cancer cells) was observed between mice treated with different protocols. A significant dose-dependent increase in TUNEL-positive (apoptotic) and decrease in PCNA-positive (proliferating) cancer cells was observed in tumors from mice treated with inhibitors ([Fig. 6C](#)).

Immunohistochemical examination showed that nuclear hTERT levels were decreased in most xenografts in the rodents treated with the GSK3 $\beta$  inhibitors for 10 weeks, compared to those treated with DMSO for 8 weeks ([Fig. 6D](#)).

No adverse events were observed in mice during treatment with inhibitors and there was no statistical difference in body weights between groups treated with different doses of SB-216763 or AR-A014418. Seven weeks after treatment (from week 9 in [Supplementary Fig. 1B](#)), the mean body weight of mice treated with DMSO was significantly lower than other groups of mice treated with GSK3 $\beta$  inhibitors. At necropsy, gross observation and histological examination failed to identify pathological features, primary cancers or metastatic tumors in the lungs, liver, gastrointestinal tract, pancreas, spleen or kidneys of any of the mice.

## Discussion

The present study demonstrated that active GSK3 $\beta$  in various gastrointestinal cancer types was associated with distinctive pathologic properties. Compared to non-neoplastic cells and tissues, increased expression and activity of GSK3 $\beta$  and deregulation of S9 and Y216 phosphorylation were common characteristics of gastrointestinal cancer cell lines and primary colorectal and stomach cancers. Inhibition of GSK3 $\beta$  attenuated cell survival and proliferation and induced apoptosis in all cancer cell lines *in vitro*. Consistent with our preliminary study (14), these effects were also observed in HT-29 colon cancer cell xenografts in mice with no adverse effects, indicating reproducibility of the therapeutic effect of GSK3 $\beta$  inhibition against cancer *in vivo*. Although the role of this kinase in cancer has until recently been controversial (8, 12, 32, 33), the present results, our earlier studies (13-16) and several subsequent reports (17-22) indicate that GSK3 $\beta$  is a potential therapeutic target for a broad spectrum of cancer types.

Importantly, there was little effect of GSK3 $\beta$  inhibition on cell survival, growth or apoptosis in non-neoplastic cells. This is consistent with earlier reports (13-16) and follow-up studies showing that GSK3 $\beta$  inhibition did not influence survival or growth of human mammary epithelial cells, embryonic lung fibroblasts (WI38) and mouse embryonic fibroblasts (NIH-3T3) (18, 21). These observations support the hypothesis that cancer cells depend upon active GSK3 $\beta$  for sustained survival and/or proliferation. This is in line with the concept of oncogene addiction, a recently proposed rationale for molecular targeting in cancer treatment (34, 35). The unique state of dependence that may arise in cancer cells is further highlighted by the fact that inactivation of the normal counterpart of such oncogene products in normal cells is tolerated without obvious consequence (36). The absence of detrimental effects of GSK3 $\beta$  inhibitors on non-neoplastic cells and on mice with tumor xenografts in the present study confirms the potential application of GSK3 $\beta$  inhibitors for cancer treatment in a clinical setting.

There is considerable interest in the molecular mechanism by which GSK3 $\beta$  exerts a putative pathological role in promotion of tumor cell survival and proliferation. In the same

time and shortly after the discovery of a novel pathological role for GSK3 $\beta$  in colorectal and prostatic cancers (13-15, 37), other groups have investigated the molecular mechanisms by which GSK3 $\beta$  may promote cancer. These include roles for GSK3 $\beta$  in tumor cell resistance to apoptosis induced by tumor necrosis factor-related apoptosis-inducing ligand (TRAIL), p53 and c-Myc (37-39), NF- $\kappa$ B-mediated gene transcription (18), induction of cyclin D1 expression (19), and phosphorylation and destabilization of PTEN (40). GSK3 $\beta$  has also been reported to facilitate cell migration by binding to h-prune and modulating focal adhesions (41) and to inhibit colonocyte differentiation by destabilizing the transcription factor Hath1 (42). In glioblastoma, inhibition of GSK3 $\beta$  increases the expression of p53 and p21 in cells with wild-type p53 and decreases Rb phosphorylation and CDK6 expression in all cell lines analyzed to date (16). By analyzing molecular changes in colon cancer cells transfected with GSK3 $\beta$ -specific siRNA, we showed here a decrease in the level of hTERT mRNA following GSK3 $\beta$  depletion. Inhibition of GSK3 $\beta$  attenuated telomerase activity and increased  $\beta$ -gal-positive colon cancer cells. These effects were associated with increased expression of p53, p21 and JNK1 and decrease in CDK6 expression and Rb phosphorylation, consistent with known relationships between such proteins and cell senescence (29) as well as GSK3 $\beta$  activity (16, 17, 38, 43, 44). The putative role of GSK3 $\beta$  for sustaining cancer cells survival may therefore be due in part to effects on hTERT expression and telomerase activity as well as on cellular senescence. The latter effect may be consistent with a recent report that enhanced glycogenesis is directly linked to cellular senescence via modulation of GSK3 $\alpha/\beta$  and glycogen synthase (45).

While many studies have suggested GSK3 $\beta$  as a promising target for drug development (8-11), none of the available inhibitors has found clinical use for treatment of diseases such as diabetes mellitus, Alzheimer's disease, inflammation and cancer. This is because of suspected risks for tumorigenesis through activation of Wnt signaling following GSK3 $\beta$  inhibition and the multiple functions of GSK3 $\beta$  in cellular metabolism and signaling (6-8, 12). Previous studies have suggested that GSK3 $\beta$  may have tumor suppressor-like functions based on its roles in Wnt/ $\beta$ -catenin signaling, expression of cyclin D1, c-Myc and COX-2, activity of

ERK1/2 and epithelial-mesenchymal transition (12, 46-48). However, none of these studies showed neoplastic transformation or tumor development following GSK3 $\beta$  inhibition. In the present study, GSK3 $\beta$  inhibition had no effect on survival and growth of HEK293 cells or on development of primary cancers in mice. GSK3 $\beta$  phosphorylation was detected in both S9 and Y216 in HEK293 cells, suggesting kinase activity in non-neoplastic cells is regulated by differential phosphorylation of these two key residues. In contrast, increased expression and activity of GSK3 $\beta$  and deregulation of S9 and Y216 phosphorylation were observed in most cancer cell lines and primary stomach and colorectal cancers, suggesting a selective and common effect of GSK3 $\beta$  inhibition. **In the present study, no detrimental effects were observed in mice treated with GSK3 $\beta$  inhibitors.** The differential effects of GSK3 $\beta$  inhibition on non-neoplastic and neoplastic cells may therefore depend on differences in biological properties and functions of the kinase and support the potential clinical application of GSK3 $\beta$  inhibitors for cancer treatment.

~~The use of GSK3 $\beta$  inhibitors for the treatment of chronic diseases requires increased awareness of safety issues. Because GSK3 $\beta$  is a multi-task kinase, systemic inhibition of GSK3 $\beta$  could lead to unexpected side effects due to disruption of normal metabolism and/or cellular signaling. In the present study, however, no detrimental effects were observed in mice treated with GSK3 $\beta$  inhibitors. Post-translational modification of this kinase by phosphorylation is thought to underlie the mechanism by which normal cells are protected from undesirable effects. Due to the role of GSK3 $\beta$  in regulating various proto-oncoproteins, there are concerns that long-term inhibition of GSK3 $\beta$  may increase the risk of cancer development (8, 12). However, a previous study reported that long-term administration of lithium, the classical GSK3 $\beta$  inhibitor, did not increase cancer mortality but was instead associated with a reduction in overall mortality of patients with bipolar disorder (45). Lithium is not thought to be mutagenic or carcinogenic (46) and treatment with this compound did not significantly increase the incidence of intestinal tumors in genetically predisposed APC-mutant mice (47). This is consistent with the absence of primary tumors in mice from the present study. Inhibition of GSK3 $\beta$  is not sufficient to stabilize  $\beta$ -catenin in normal cells and~~

this appears to occur only when one or more transforming events such as APC protein-truncation has already taken place (48). Furthermore, in normal cells the critical function of GSK3 $\beta$  in mediating Wnt/ $\beta$ -catenin signaling is performed by cell membrane-associated GSK3 $\beta$ . This antagonizes the phosphorylation of  $\beta$ -catenin by cytoplasmic GSK3 $\beta$  and thus its degradation (49). Therefore, the available evidence suggests that any increased risk of cancer associated with long-term GSK3 $\beta$  inhibition can be avoided by generating new compounds that spatially and temporally regulate GSK3 $\beta$  expression and activity. A number of drugs in clinical use have been recently found to inhibit GSK3 $\beta$  activity and include imetidine, hydroxychloroquine and gemifloxacin (50). Based on their effect against GSK3 $\beta$ , they are candidates for potent anti-cancer agents.

## Disclosure of Potential Conflicts of Interest

No potential conflicts of interests were disclosed.

## Acknowledgments

The authors would like to dedicate this article to Dr. Masayoshi Mai, who had continuously encouraged and supported us to conduct this project. We lost him on July 28, 2008. We thank Dr. Barry Iacopetta (School of Surgery, University of Western Australia) for critical reading of the manuscript.

**Grant Support:** Supported in part by Grants-in-Aid for Scientific Research from the Japanese Ministry of Education, Science, Sports, Technology and Culture, from the Ministry of Health, Labour and Welfare, and from the Japan Society for the Promotion of Science.

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## Figure Legends

**Figure 1.** Expression, phosphorylation and activity of GSK3 $\beta$  in cell lines. **A**, Protein extracts from HEK293 cells and colon (SW480, HCT116, HT-29), stomach (MKN-28, NKPS, TMK-1, NUGC-4), pancreatic (MIAPaCa-2, Capan-1, BxPC-3) and liver (HepG2) cancer cells were analyzed by Western immunoblotting for GSK3 $\beta$  expression and levels of pGSK3 $\beta$ <sup>S9</sup>, pGSK3 $\beta$ <sup>Y216</sup> and  $\beta$ -actin. **B**, GSK3 $\beta$  activity was detected by NRIKA (24) in HEK293 cells and the cancer cell lines indicated above. GSK3 $\beta$  was isolated by immunoprecipitation from 1 mg aliquot of cells lysate from each sample. An *in vitro* kinase reaction was then carried out in the presence of immunoprecipitated GSK3 $\beta$ , its substrate of recombinant human  $\beta$ -catenin protein ( $\beta$ -catenin<sup>His</sup>) and non-radioisotopic ATP in the kinase reaction buffer. The resultant products were analyzed by Western immunoblotting for phosphorylation of  $\beta$ -catenin<sup>His</sup> (p- $\beta$ -catenin<sup>S33/37/T41</sup>). As a negative control reaction (NC) for each cell line, the mouse monoclonal antibody to GSK3 $\beta$  was replaced by an equal amount of non-immune mouse IgG in the immunoprecipitation step. GSK3 $\beta$  activity was demonstrated in all cells by presence of p- $\beta$ -catenin<sup>S33/37/T41</sup> in the test lanes (P) and by the observation of little or no expression of p- $\beta$ -catenin<sup>S33/37/T41</sup> in NC. The amount of GSK3 $\beta$  immunoprecipitated from cell extracts and the presence of  $\beta$ -catenin<sup>His</sup> in the kinase reaction mixture were evaluated by immunoblotting with mouse monoclonal antibodies to GSK3 $\beta$  and  $\beta$ -catenin, respectively. **C-a, c**, Effects of GSK3 $\beta$  inhibitors (SB-216763, AR-A014418) on the activity of GSK3 $\beta$  derived from HT-29 and HCT116 colon cancer cells. In the NRIKA, the kinase reaction was carried out in the presence of DMSO or 25  $\mu$ M (final concentration) SB-216763 or AR-A014418 at 30°C for 30 min, followed by Western immunoblotting analysis for p- $\beta$ -catenin<sup>S33/37/T41</sup>,  $\beta$ -catenin<sup>His</sup> and GSK3 $\beta$ , respectively. **C-b, d**, The effects of SB-216763 and AR-A014418 on p- $\beta$ -catenin<sup>S33/37/T41</sup> were examined to address whether they affect the phospho-acceptor sites (S33, S37 and T41) of  $\beta$ -catenin<sup>His</sup>. The kinase mixture was separated from immunoprecipitated GSK3 $\beta$ -bound resins

following reaction in the absence of DMSO or GSK3 $\beta$  inhibitors. DMSO or 25  $\mu$ M (final concentration) of SB-216763 or AR-A014418 was then added to the kinase reaction mixture and incubated at 30°C for 30 min, followed by Western immunoblotting analysis for p- $\beta$ -catenin<sup>S33/37/T41</sup>,  $\beta$ -catenin<sup>His</sup> and GSK3 $\beta$ , respectively. Full length blots/gels for **A**, **B** and **C** are presented in [Supplementary Fig. 5](#).

**Figure 2.** Expression and phosphorylation of GSK3 $\beta$  and levels of  $\beta$ -actin analyzed by Western immunoblotting in matched pairs of normal (N) and tumor (T) tissues from stomach (**A**) and colorectal cancer patients (**B upper panels**). **B lower panels**, The activity of GSK3 $\beta$  in each tissue extract was detected by NRIKA (24). Full length blots/gels for **A** and **B** are presented in [Supplementary Fig. 5](#). **C**, Serial sections of a primary colon cancer were examined histologically (HE) and immunostained for GSK3 $\beta$ . A scale bar in each panel indicates 500  $\mu$ m in length.

**Figure 3.** Effects of GSK3 $\beta$  inhibitors and RNAi on cell survival, proliferation and apoptosis. **A**, Viable cells at each time point were measured for HEK293 and cancer cells (HT-29, NUGC-4, BxPC-3, Hep G2) treated with DMSO, SB-216763 or AR-A014418. **B**, Effect of 72 hrs treatment with DMSO, SB-216763 or AR-A014418 on cell survival, proliferation and apoptosis of HEK293 and the cancer cells. **C**, Effects of GSK3 $\beta$  RNAi on HEK293 and cancer cells. Relative numbers of viable cells, proliferating cells and apoptosis in HEK293 and the cancer cells were determined 72 hrs after transfection with non-specific (NS) or GSK3 $\beta$ -specific (S) siRNA. **A-C**, All assays were performed in triplicates and the values shown are mean with SDs. Asterisks indicate a statistically significant difference between cells treated with DMSO and either GSK3 $\beta$  inhibitor and between cells treated with non-specific and GSK3 $\beta$ -specific siRNA.

**Figure 4.** Changes in the expression of proteins in cancer cells by GSK3 $\beta$  depletion. The respective cells were transfected with non-specific (NS) and GSK3 $\beta$ -specific (S)

siRNA and examined by Western immunoblotting for expression and phosphorylation of proteins indicated. Relative levels of protein expression were compared between the cells transfected with the respective siRNA (*right panel*). Full length blots/gels are presented in [Supplementary Fig. 5](#).

**Figure 5.** Effects of GSK3 $\beta$  inhibition on  $\beta$ -gal-positive cells, hTERT expression and telomerase activity in HEK293 and colon cancer (SW480, HCT116) cells. **A**,  $\beta$ -gal-positive cells were scored in cells treated for 72 hrs with DMSO, SB-216763 or AR-A014418. Asterisks indicate a statistically significant difference in the number of  $\beta$ -gal-positive cells. **B**, Effects of GSK3 $\beta$  RNAi on the expression of GSK3 $\alpha$ , GSK3 $\beta$ ,  $\beta$ -actin (Western immunoblotting) and hTERT and GAPDH mRNA (RT-PCR) in HEK293 and colon cancer cells 72 hrs after transfection of non-specific (NS) or GSK3 $\beta$ -specific (S) siRNA. **Numbers below the lanes reflect the relative amounts of proteins and mRNA quantified by densitometry and normalized to those of  $\beta$ -actin and GAPDH, respectively.** **C**, Expression of hTERT and GAPDH mRNA were analyzed by RT-PCR in HEK293 and HCT116 cells treated with DMSO, 25  $\mu$ M SB-216763 or AR-A014418 for 0 to 72 hrs. P, positive control (C33A cells); N, negative control without cDNA sample. The intensity of hTERT was measured by densitometry and normalized to that of GAPDH. **D**, Telomerase activity in HEK293 and HCT116 cells treated as described above was analyzed by TRAP assay (*upper panels*). The products at 0 hr in cells treated with SB-216763 or AR-A014418 were normalized to that of cells treated with DMSO. As a negative control (**N**), the protein extract was not added to the assay. **Effect of RNA interference for GSK3 $\beta$  on telomerase activity in HEK293 cells and colon cancer cell lines (SW480, HCT116) (*lower panels*).** Cells were transfected with 20 nM of non-specific siRNA (NS) or GSK3 $\beta$ -specific siRNA (S). At 72 hrs after transfection, telomerase activity was detected by TRAP assay. As a negative control (NC), protein extract was deleted in the TRAP assay. The assay products were separated by 12% native PAGE and stained with SYBR Green (*lower left-hand panels*). Telomerase activity was compared between the cells transfected

with non-specific siRNA and those transfected with GSK3 $\beta$ -specific siRNA. The level of telomerase activity in each sample shown in the PAGE was determined by densitometry. All assays were performed in triplicates and the values shown are mean with SDs. Asterisks indicate a statistically significant difference ( $p < 0.05$ ) between cells treated with non-specific (NS) and GSK3 $\beta$ -specific (S) siRNA (**lower right-hand panel**). M, molecular size marker. Full length blots/gels for **B**, **C**, **D** and **E** are presented in [Supplementary Fig. 5](#).

**Figure 6.** Effects of GSK3 $\beta$  inhibitors on HT-29 colon cancer cells xenografts in athymic mice, as detailed in [Supplementary Fig. 1](#). **A**, Time course of the effects of DMSO and GSK3 $\beta$  inhibitors on HT-29 xenografts in rodents. **B**, Gross appearance of tumor xenografts removed at autopsy from the mice after 10 weeks of treatment with different doses of SB-216763 or AR-A014418 (**left hand panel**). At autopsy, one of the mice treated with 2 mg/kg of SB-216763 and two mice treated with 5 mg/kg of AR-A014418 showed no detectable tumor (**right hand panels**). **C**, Scores for TUNEL-positive (apoptotic) and PCNA-positive (proliferating) cells in tumor tissues removed from mice after 8 weeks treatment with DMSO and after 10 weeks treatment with SB-216763 or AR-A014418. Mean scores for TUNEL- and PCNA-positive cells were calculated with SDs. **D**, Expression and localization of hTERT in the HT-29 colon cancer cell xenografts in rodents treated with DMSO and AR-A014418 (5 mg/kg) were examined by immunohistochemical staining with rat monoclonal antibody to hTERT (30).

Figure 1

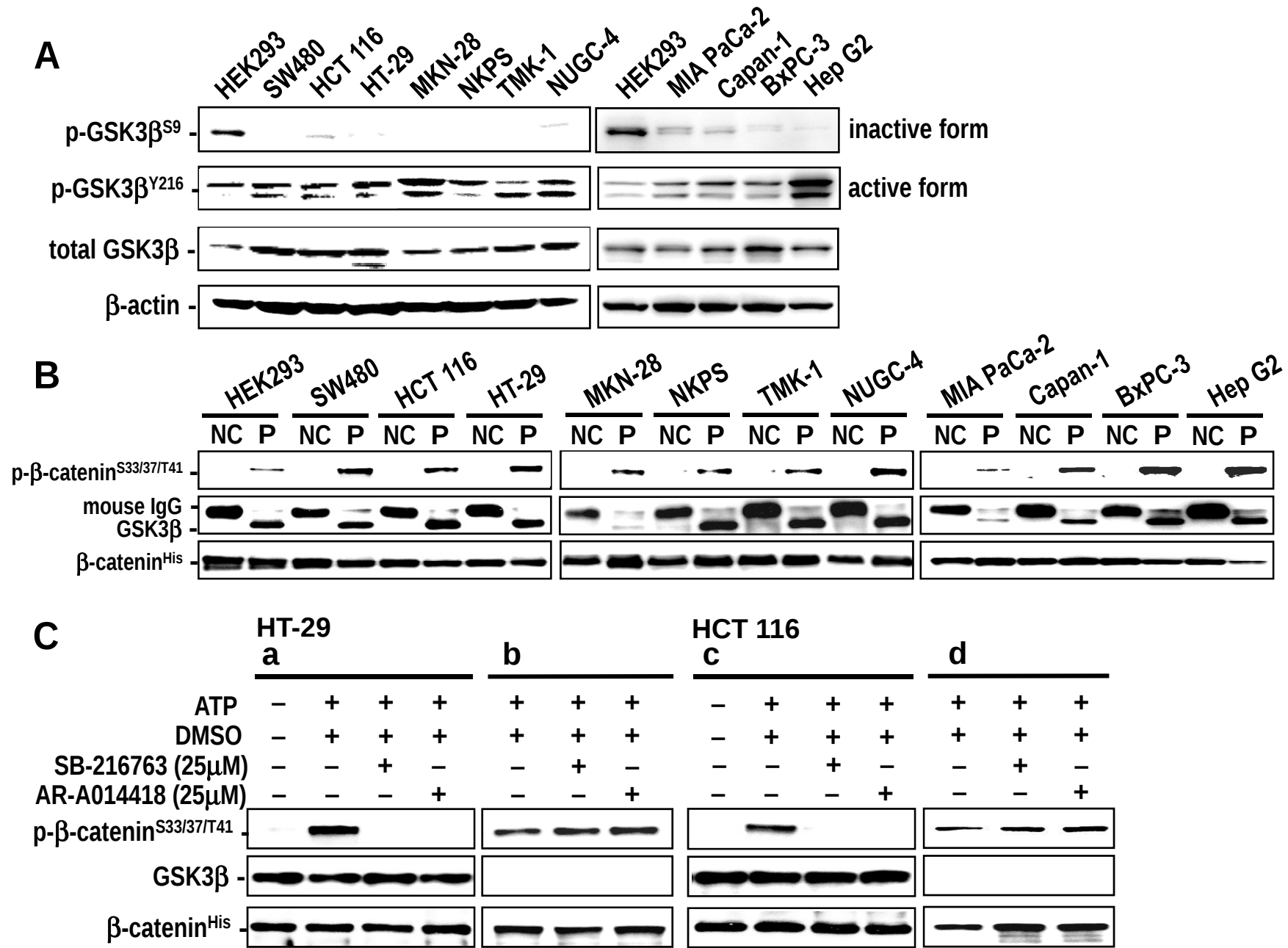


Figure 2

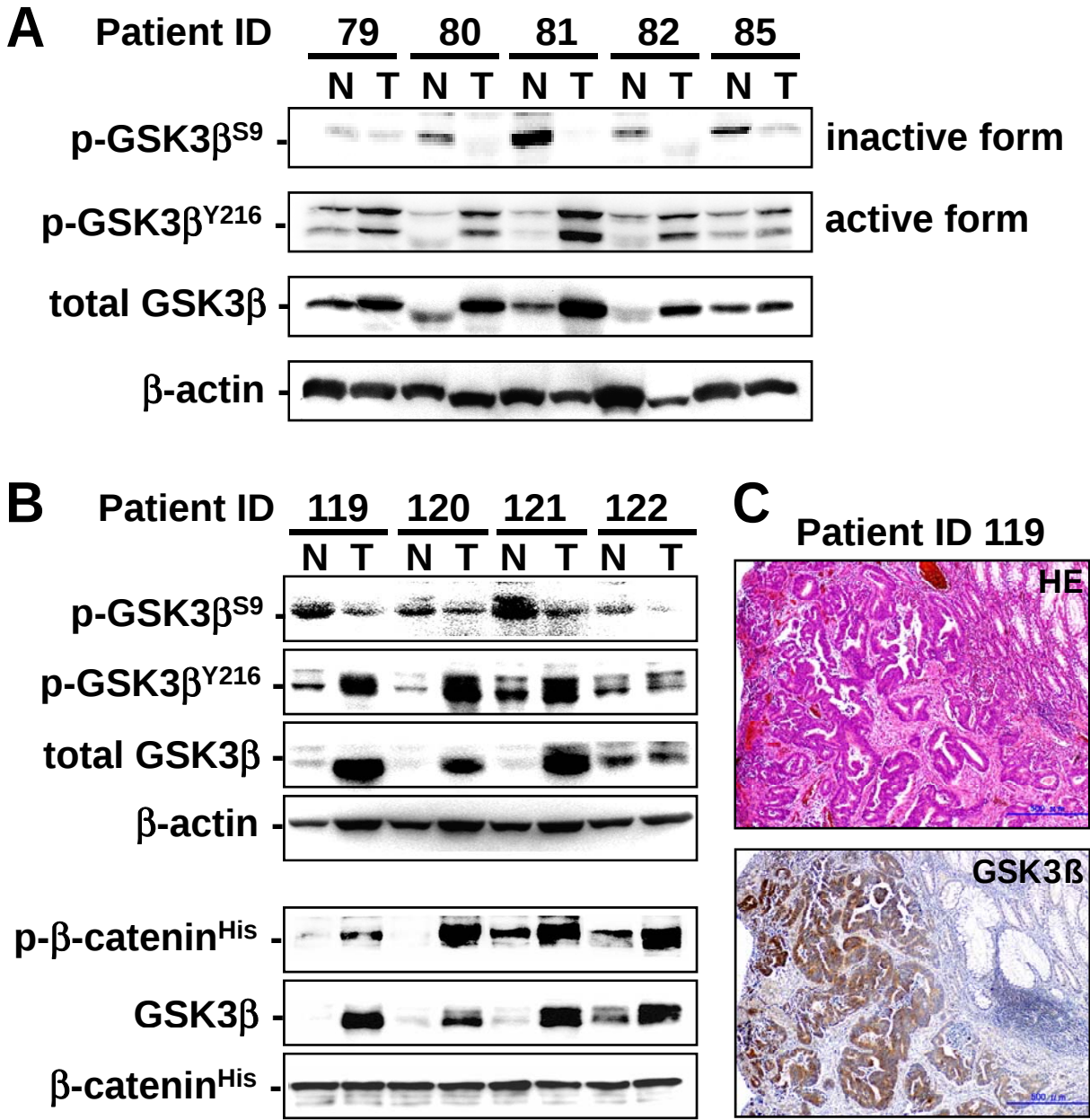


Figure 3

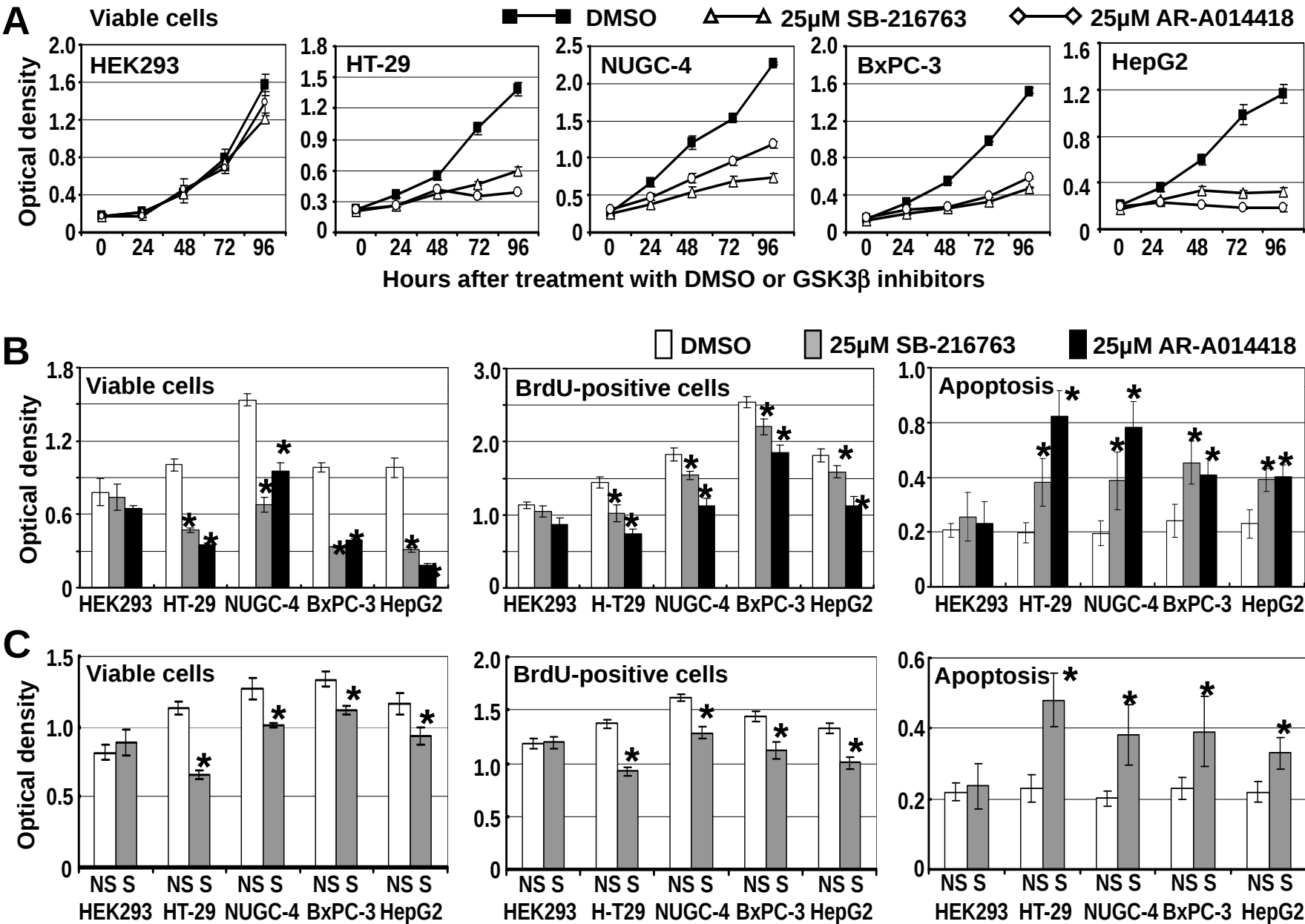


Figure 4

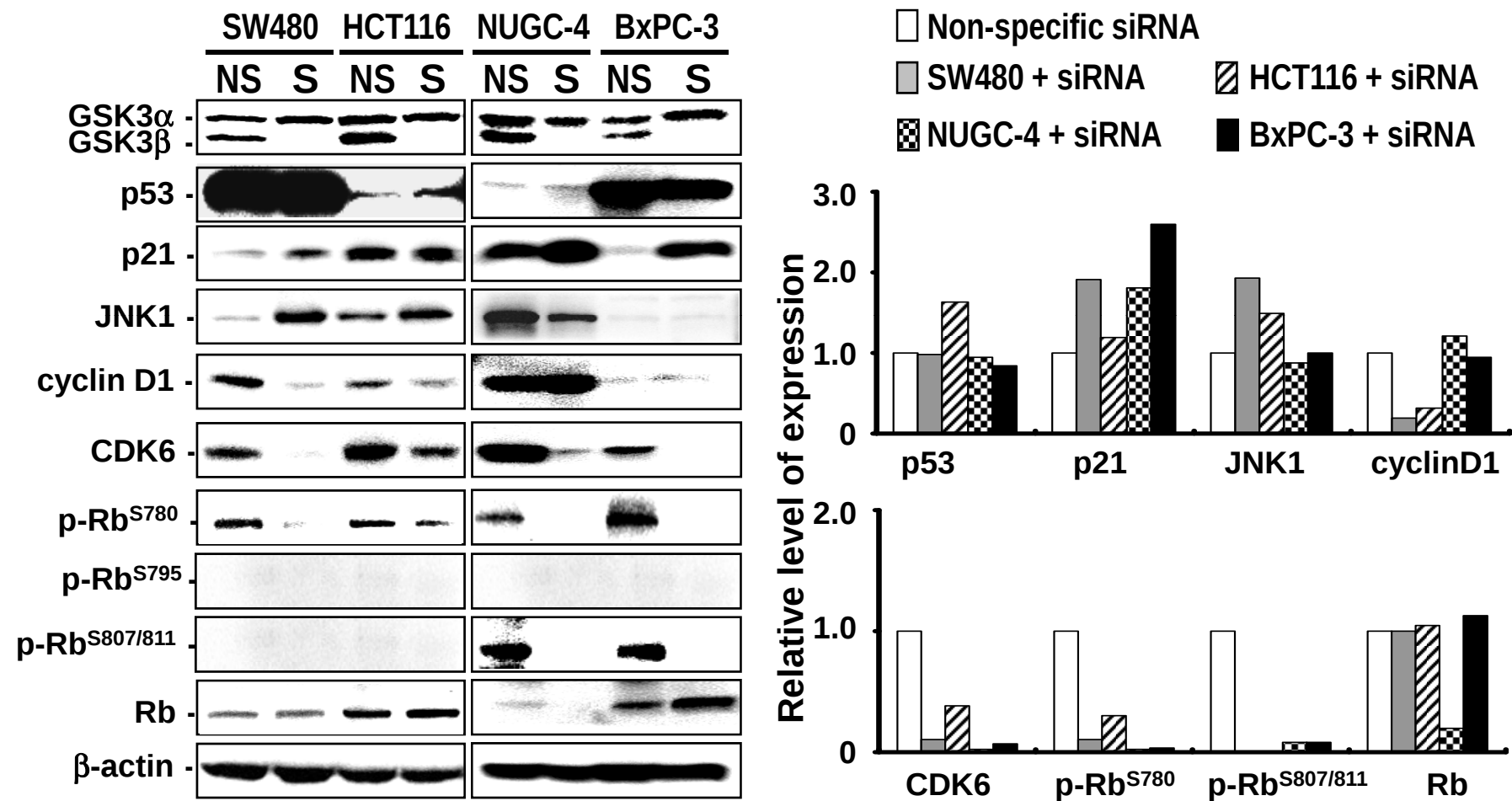


Figure 5

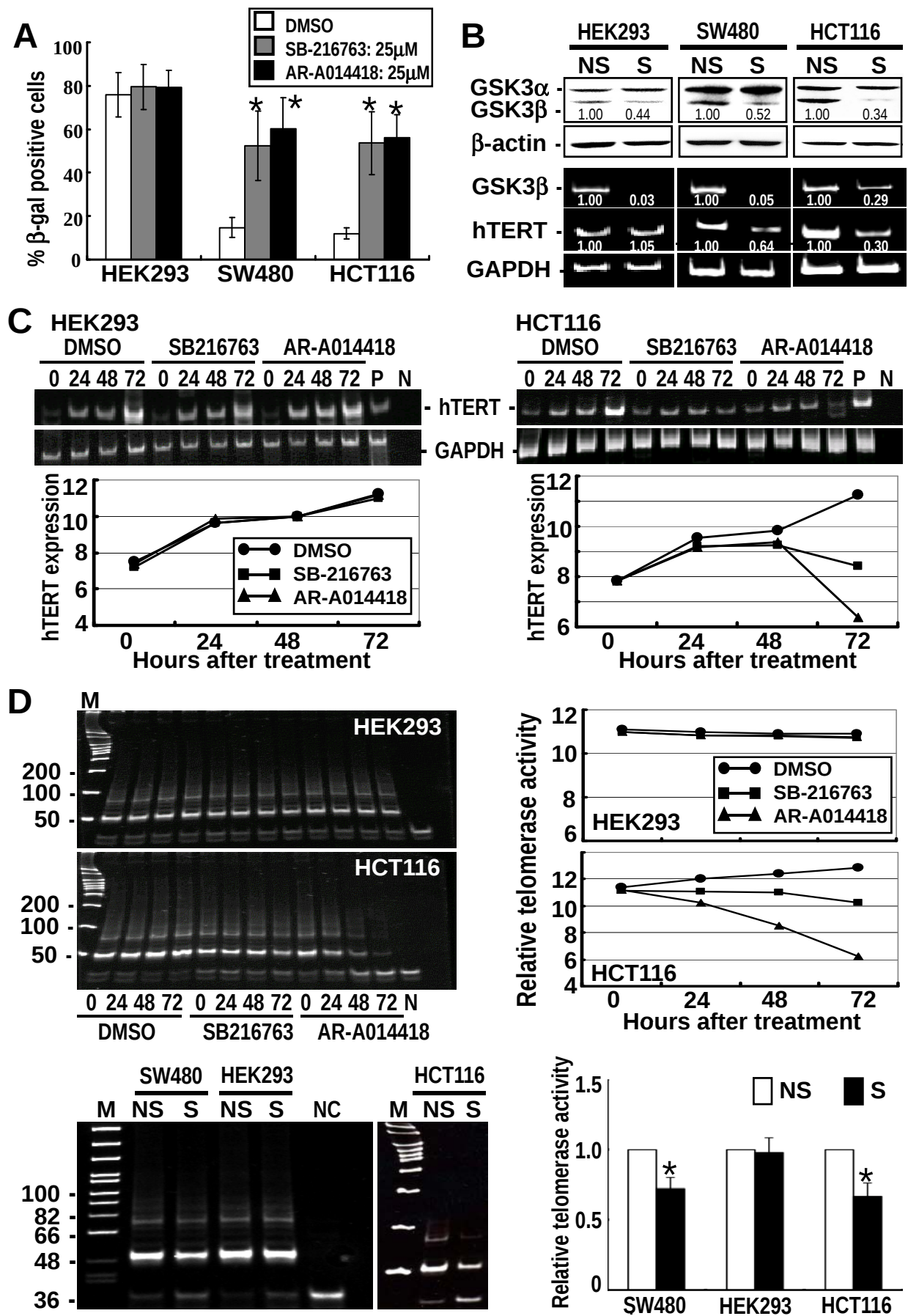
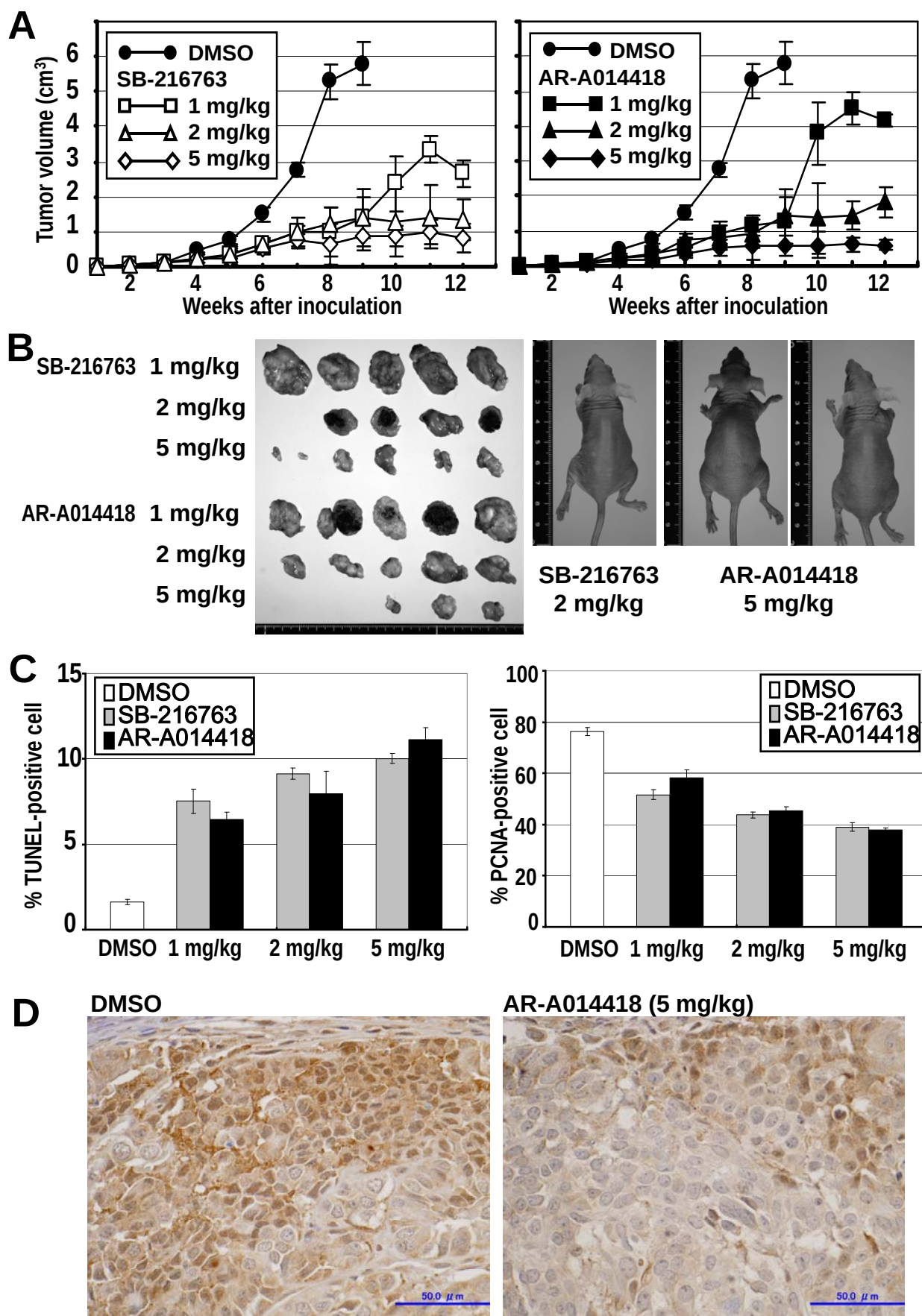


Figure 6

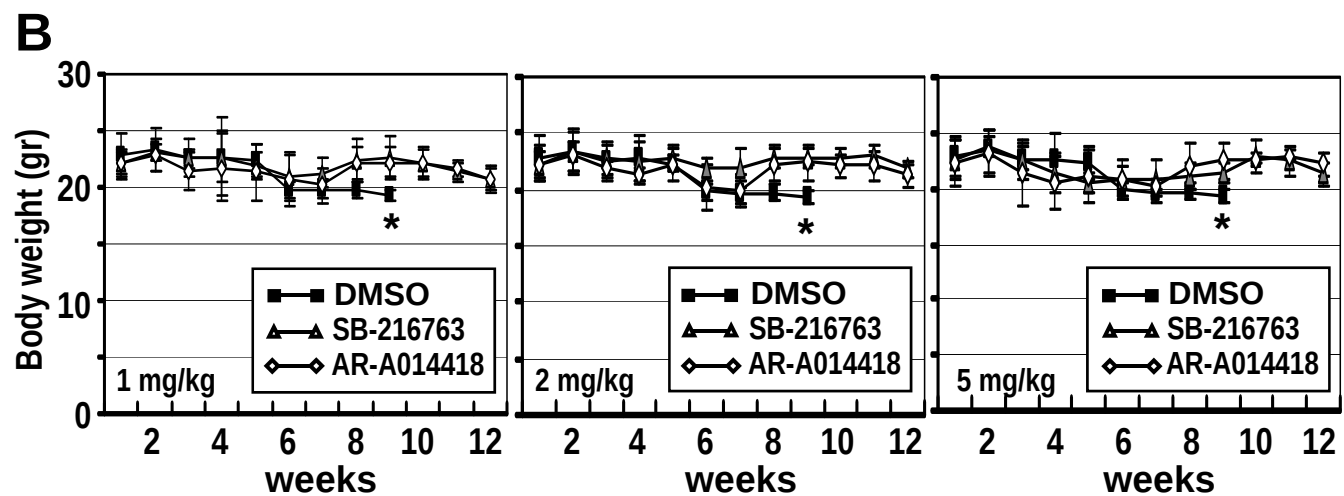
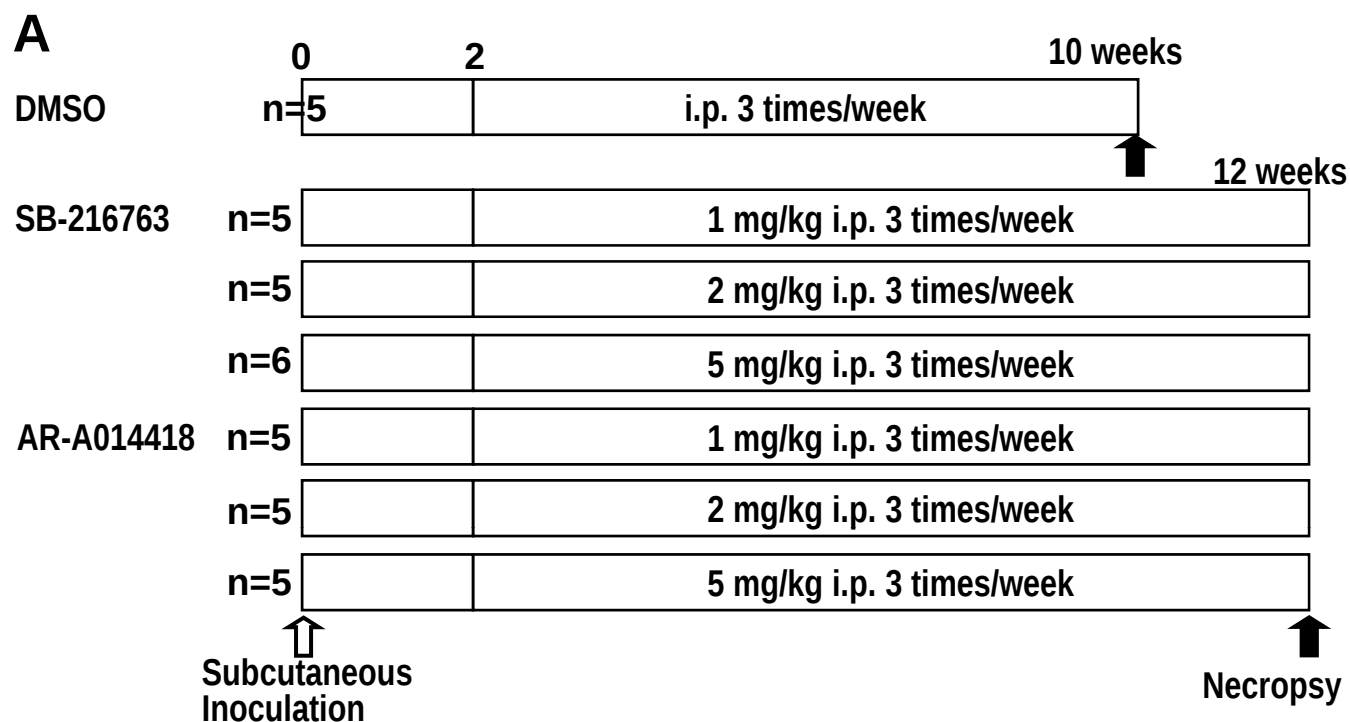


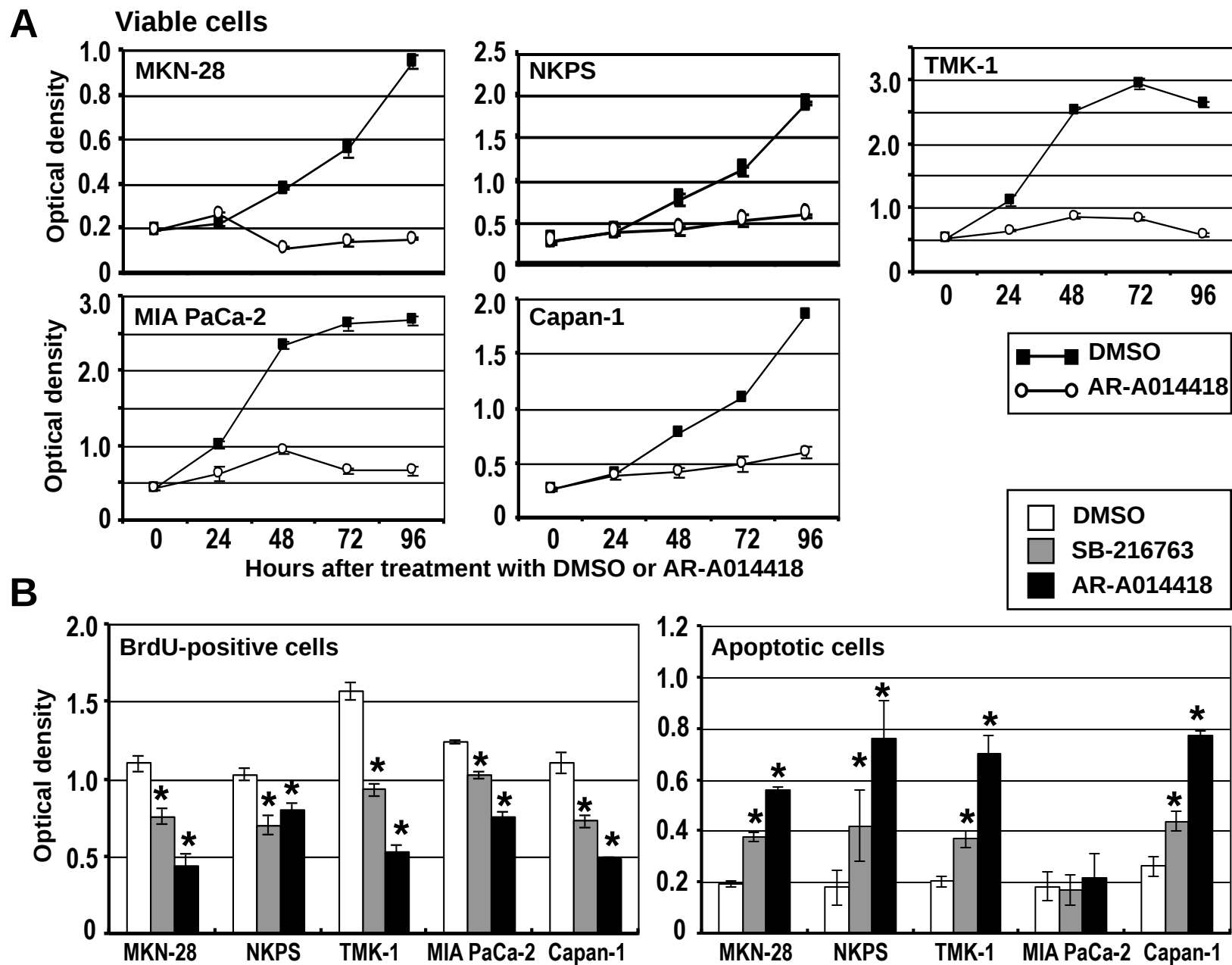
**Supplementary Table 1.** Clinical and histopathological findings of patients with stomach cancer and those with colorectal cancer

| Patient ID        | Age (yr)/ Sex | Site of tumor | Gross appearance       | Tumor size (cm) | Histological type                    | TNM stage          |
|-------------------|---------------|---------------|------------------------|-----------------|--------------------------------------|--------------------|
| Stomach cancer    |               |               |                        |                 |                                      |                    |
| 71                | 75/M          | cardia        | localized ulcerative   | 8 x 7.5         | papillary adenocarcinoma             | T2N1M0, stage II   |
| 74                | 72/M          | corpus        | localized ulcerative   | 6.5 x 5         | mucinous adenocarcinoma              | T2N0M0, stage IB   |
| 75                | 61/F          | corpus        | localized ulcerative   | 7 x 7           | medullary adenocarcinoma             | T2N2M0, stage IIIA |
| 76                | 72/M          | cardia        | protruding             | 5 x 4.5         | tubular adenocarcinoma               | T2N1M0, stage II   |
| 78                | 67/M          | corpus        | fungating              | 8.5 x 7         | papillary adenocarcinoma             | T1N2M0, stage II   |
| 79                | 61/M          | whole         | diffusely infiltrative | undetermined    | poorly differentiated adenocarcinoma | T2N3M0, stage IV   |
| 80                | 56/M          | corpus        | diffusely infiltrative | 7 x 6           | poorly differentiated adenocarcinoma | T2N3M0, stage IV   |
| 81                | 70/M          | corpus        | protruding             | 5 x 4           | papillary adenocarcinoma             | T2N1M0, stage II   |
|                   |               | antrum        | localized ulcerative   | 4 x 3           | tubular adenocarcinoma               |                    |
| 82                | 77/F          | corpus        | diffusely infiltrative | 11 x 6.5        | poorly differentiated adenocarcinoma | T2N3M0, stage IV   |
| 85                | 73/F          | corpus        | localized ulcerative   | 4.5 x 4         | tubular adenocarcinoma               | T2N1M0, stage II   |
| Colorectal cancer |               |               |                        |                 |                                      |                    |
| 119               | 80/F          | rectum        | localized ulcerative   | 5.5 x 4         | tubular adenocarcinoma               | T3N0M0, stage II   |
| 120               | 73/M          | sigmoid       | localized ulcerative   | 7 x 4.5         | papillary adenocarcinoma             | T3N1M0, stage III  |
| 121               | 75/M          | sigmoid       | localized ulcerative   | 5.5 x 5         | tubular adenocarcinoma               | T2N2M1, stage IV   |
| 122               | 57/M          | sigmoid       | localized ulcerative   | 5 x 3           | tubular adenocarcinoma               | T3N1M1, stage IV   |

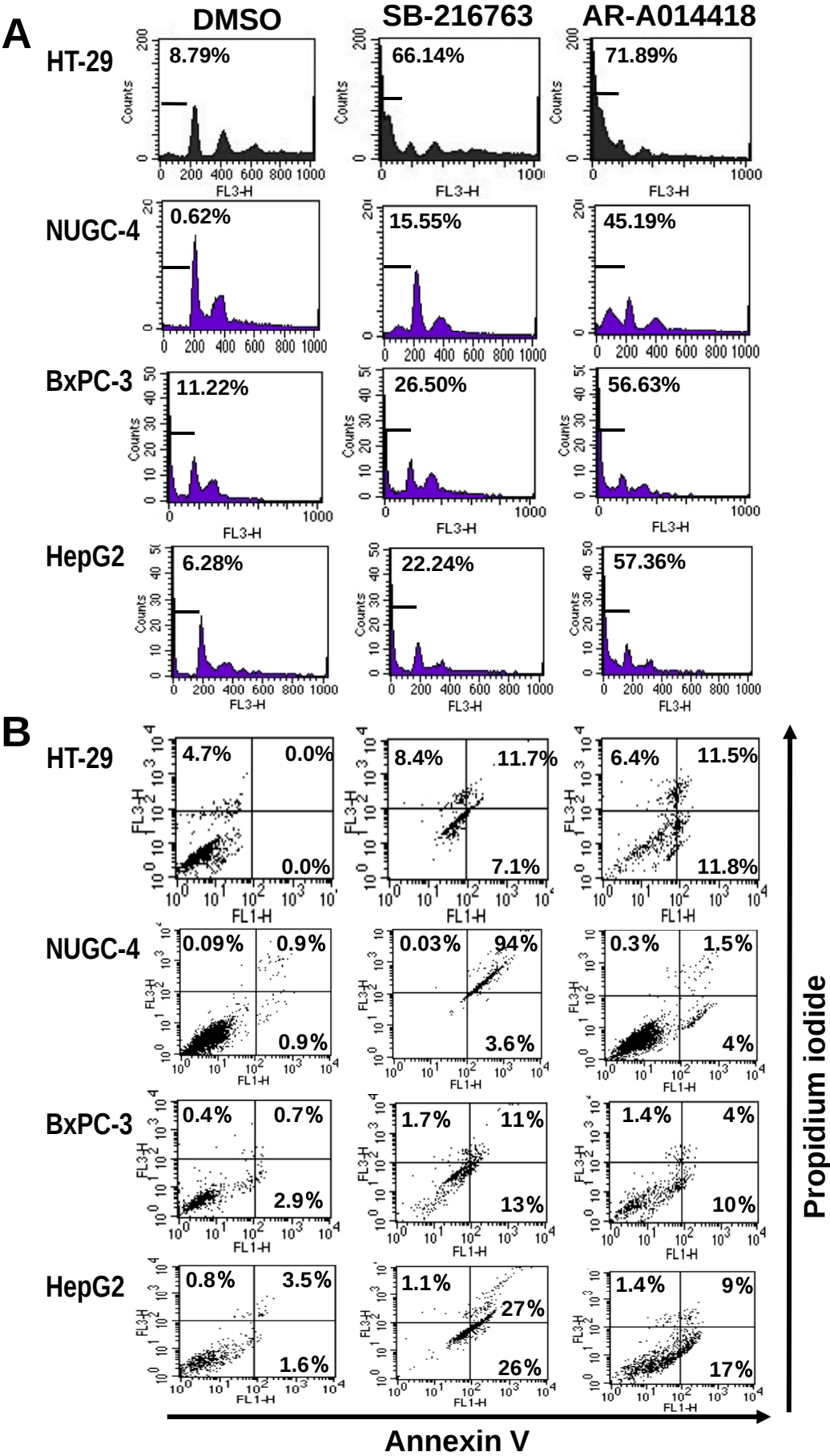
F, female; M, male; TNM, (primary) tumor, (lymph) node and (distant) metastasis

## Supplementary Figure 1

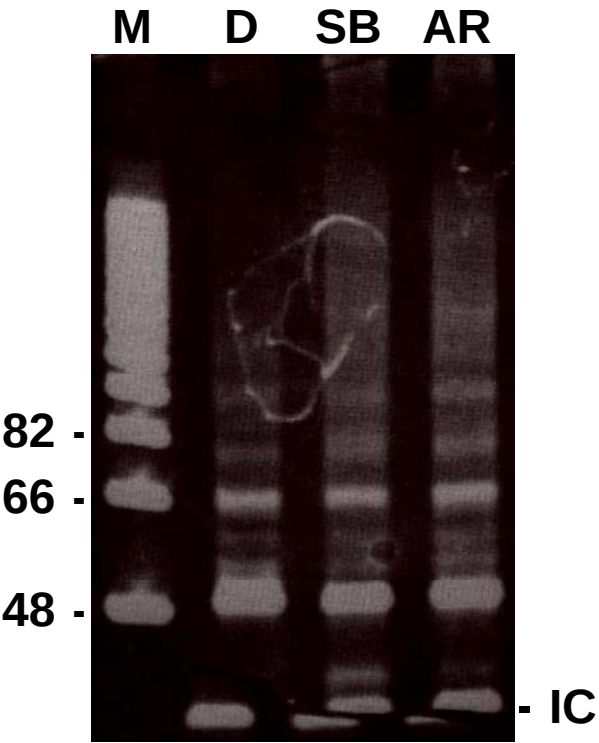




Supplementary Fig. 3

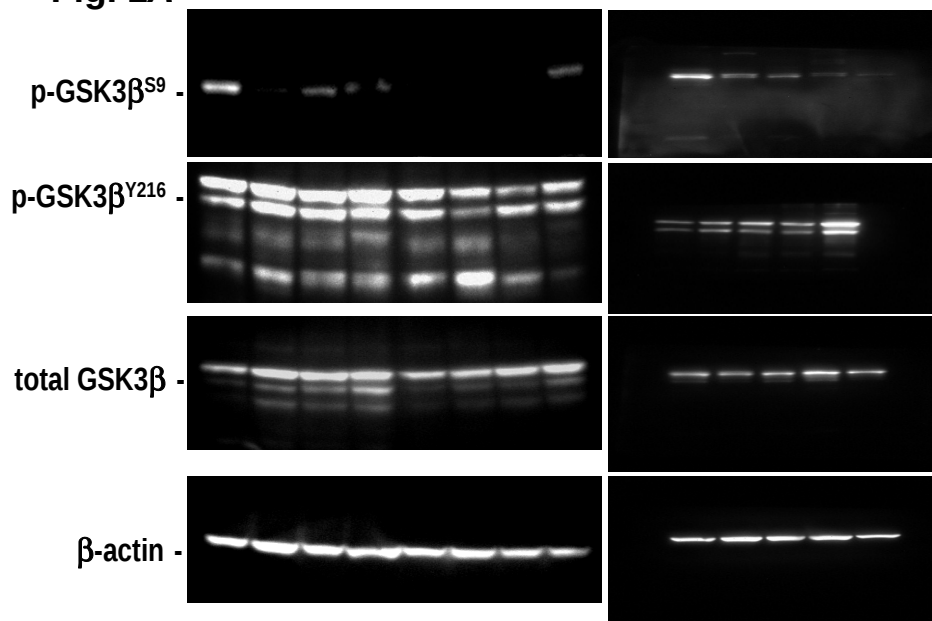


Supplementary Figure 4

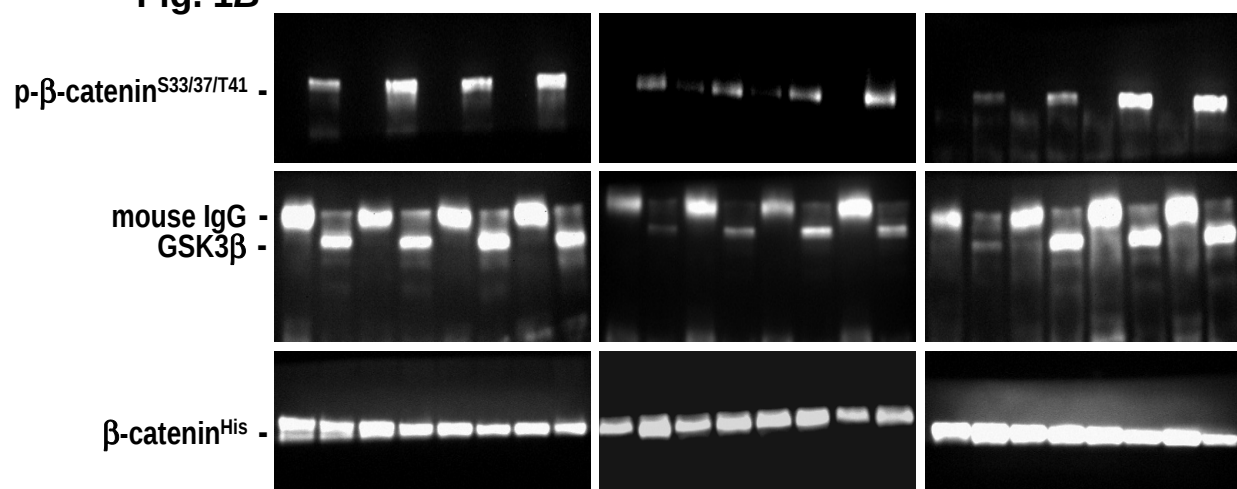


Supplementary Figure 5A

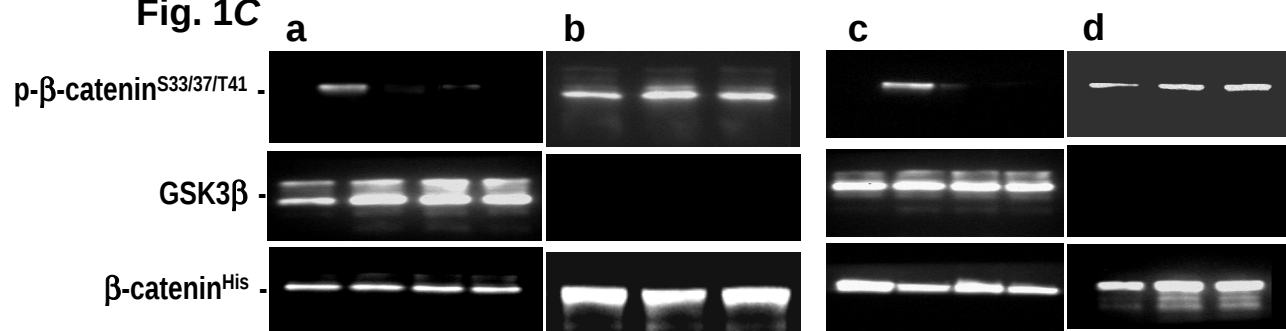
**Fig. 1A**

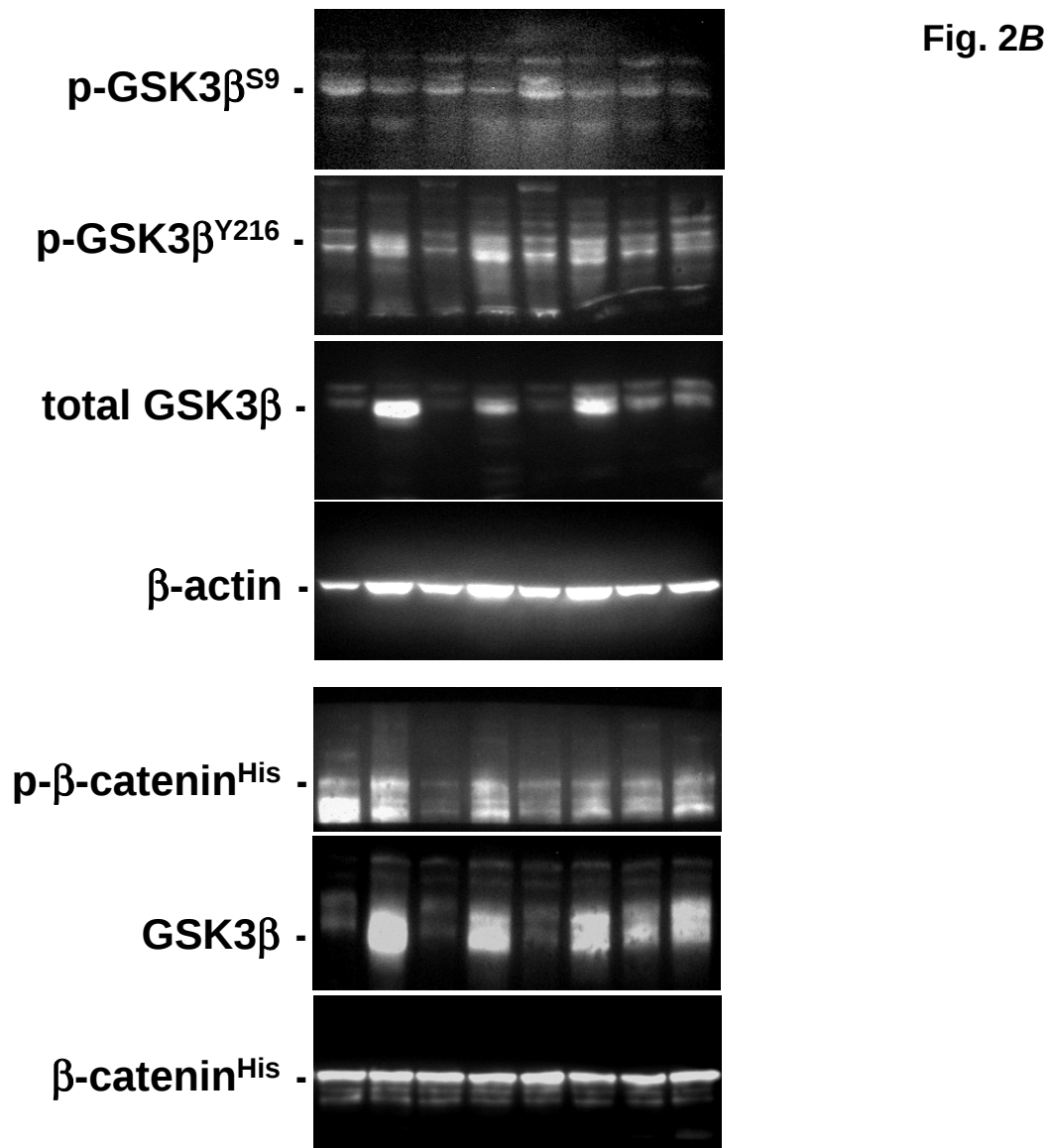
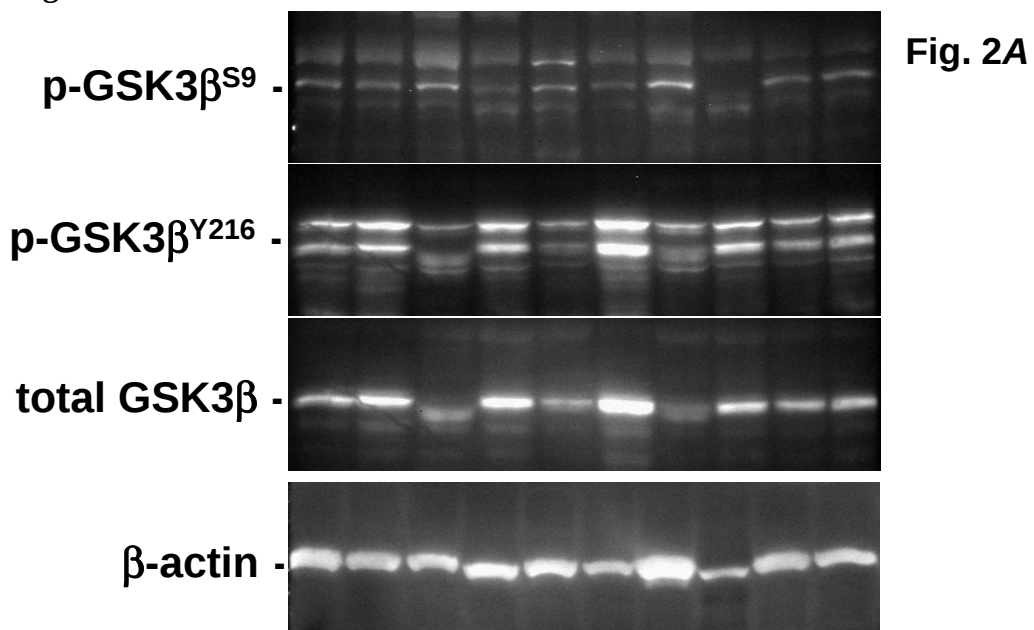


**Fig. 1B**

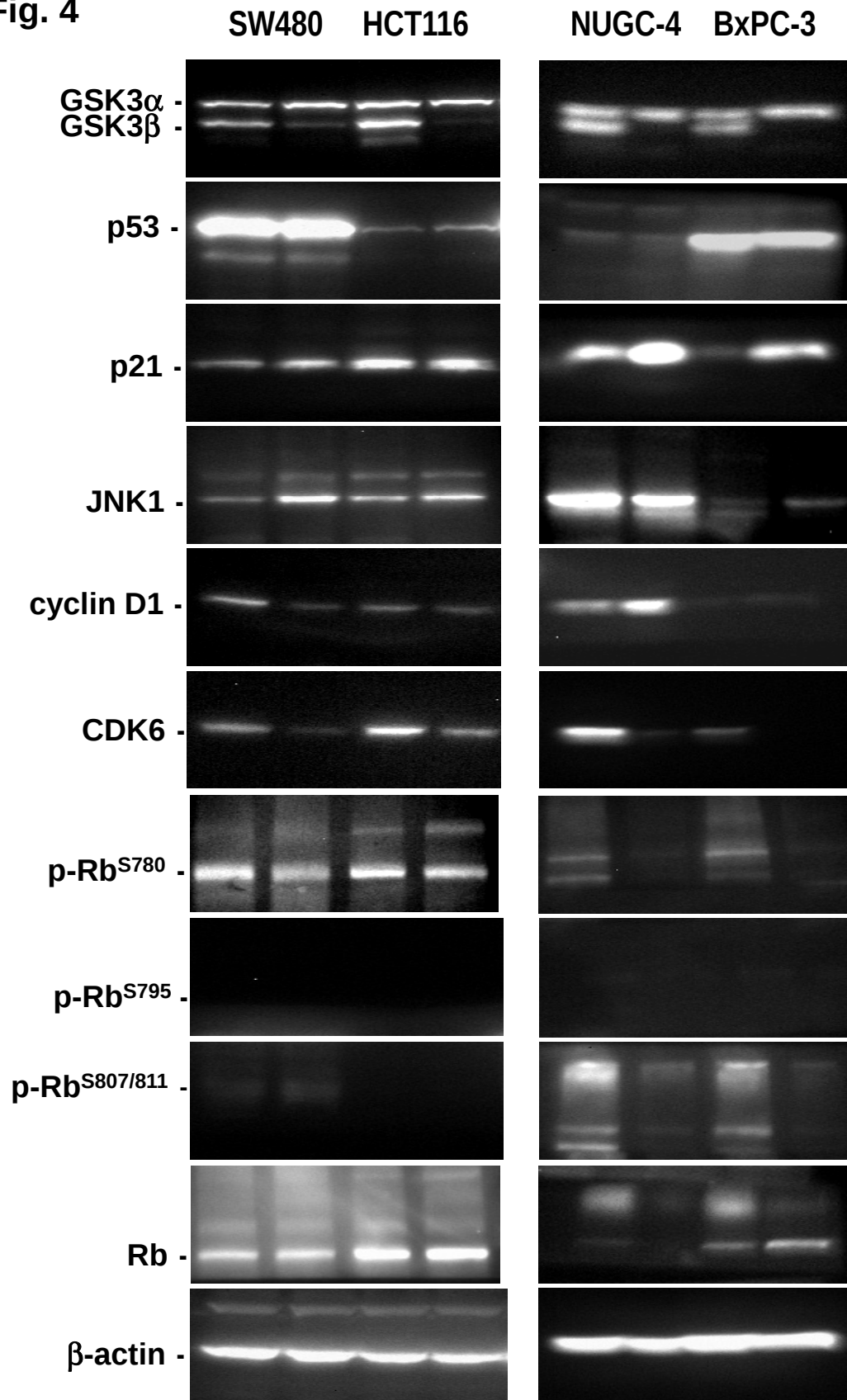


**Fig. 1C**

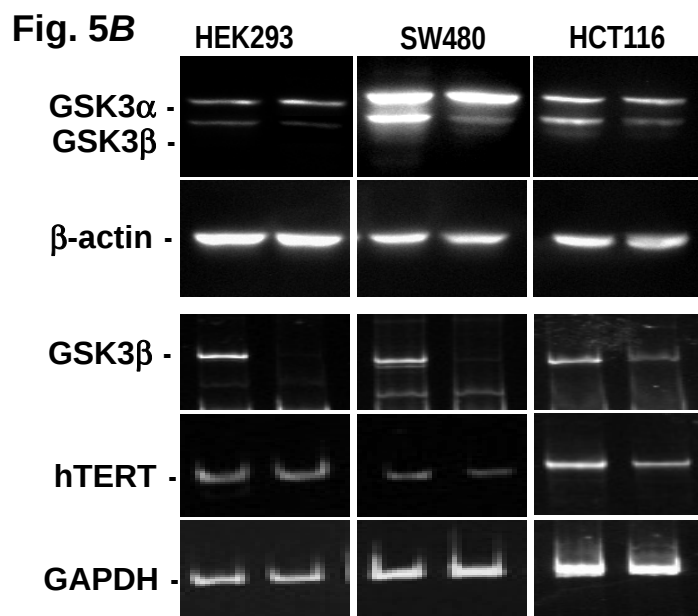




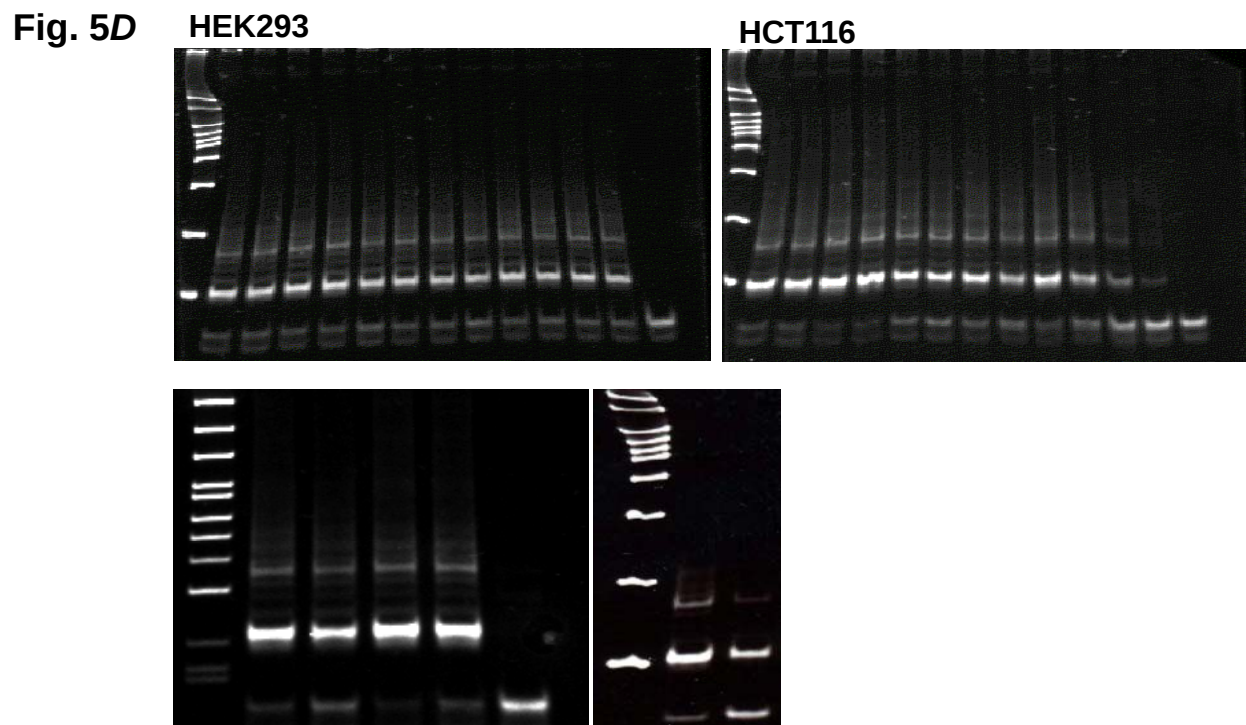
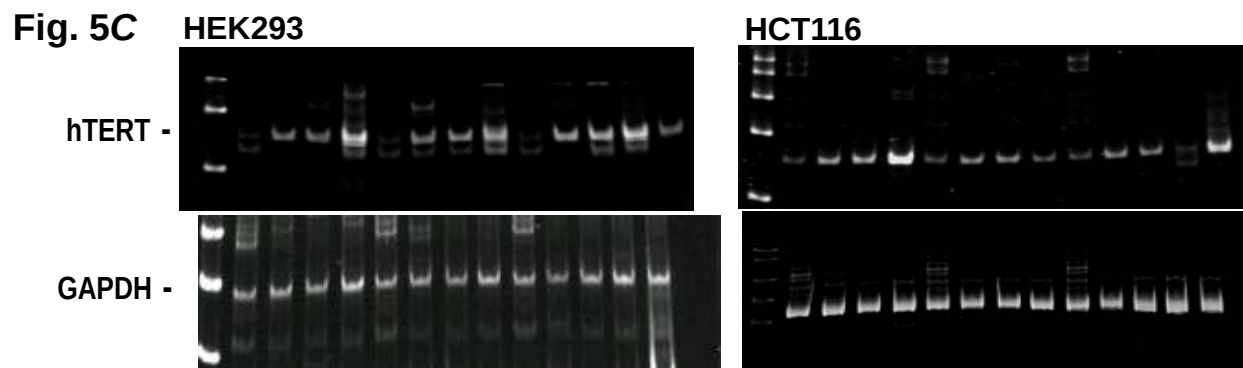
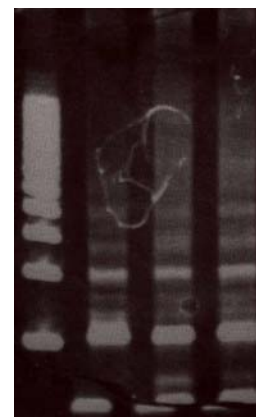
**Fig. 4**



Supplementary Figure 5D



**Suppl. Fig. 4**



## Legends to Supplementary Figures

**Supplementary Figure 1.** Design and protocol of animal experiments, gross appearance of tumor xenografts at autopsy and changes in body weights of animals during treatment. **A**, Pathogen-free 6-week-old female athymic mice (Balb/c, nu/nu) were quarantined for 3 weeks in pathogen-free conditions. At week 0, a total of  $1 \times 10^6$  HT-29 human colon cancer cells suspended in 50  $\mu$ L of phosphate buffered saline were subcutaneously inoculated into each of 36 mice. Two weeks after inoculation, visible subcutaneous tumors formed in all mice and were size-matched. The diameter of each tumor was approximately 2 mm. Mice were randomly assigned to seven groups for treatment with DMSO (a solvent for the GSK3 $\beta$  inhibitors) or the small-molecule GSK3 $\beta$  inhibitors SB-216763 and AR-A014418. Mice were given tri-weekly intraperitoneal (i.p.) injections of a 200  $\mu$ L aliquot of 75% DMSO or GSK3 $\beta$  inhibitor at different doses (1 mg/kg, 2 mg/kg and 5 mg/kg body weight) dissolved in 200  $\mu$ L DMSO. Five mice were treated with DMSO for 8 weeks to address an ethical issue and the remaining mice with GSK3 $\beta$  inhibitors for 10 weeks. A pilot study of SW480 colon cancer cell xenografts (14) has estimated the doses of GSK3 $\beta$  inhibitor corresponding to concentrations of inhibitors used previously in the treatment of cells *in vitro* (13, 15, 16). Assuming that total body fluid in mice accounts for about 60% of their body weight, inhibitor doses of 1, 2 and 5 mg/kg body weight correspond to concentrations of approximately 5, 10 and 25  $\mu$ M in culture media, respectively. These are known to be pharmacological doses for GSK3 $\beta$  inhibitors (9, 25, 26). Throughout the experiment all mice were carefully observed each day for adverse events and their body weight and tumors (in two dimensions) were measured weekly. Tumor volume ( $\text{cm}^3$ ) was calculated using the formula:  $0.5 \times S^2 \times L$ , where S is the smallest tumor diameter (cm) and L is the largest (cm) (14). At the end of treatment, all mice were euthanized under deep anesthesia by inhalation of ether. At necropsy, tumor xenografts and the major vital organs (lungs, liver, gastrointestinal tract, pancreas, kidneys, and spleen) were removed, fixed in 10% neutral buffered formalin and

embedded in paraffin for histopathologic, histochemical and immunohistochemical examination. **B**, Effects of i.p. injection of GSK3 $\beta$  inhibitors on the body weight of mice during the course of treatment. Results are shown for groups of mice treated with the indicated doses of GSK3 $\beta$  inhibitors. Asterisks indicate statistically significant differences in body weights at week 9 between mice treated with DMSO and those treated with the GSK3 $\beta$  inhibitors.

**Supplementary Figure 2.** Effects of small molecule GSK3 $\beta$  inhibitors on cell survival, proliferation and apoptosis of stomach (MKN-28, NKPS, TMK-1) and pancreatic (MIA PaCa-2, Capan-1) cancer cell lines. **A**, Cells were grown in 96-well culture plates and treated with DMSO or 25  $\mu$ M of SB-216763 or AR-A014418 for designated times. The relative number of viable cells at each time point was measured by WST-8 assay kit and spectrophotometry using a microtiter-plate reader. All assays were performed in triplicate and values shown as means with SDs. **B**, Effects of 72 hrs treatment with DMSO or 25  $\mu$ M of SB-216763 or AR-A014418 on cell survival, proliferation and apoptosis. Relative numbers of viable cells, proliferating cells (BrdU-positive) and apoptotic cells were determined by WST-8 assay, measurement of BrdU incorporation and measurement of fragmented DNA, respectively. All assays were performed in triplicate and the values shown are mean optical densities with SDs. Asterisks indicate statistically significant differences between cells treated with DMSO and those treated with GSK3 $\beta$  inhibitor.

**Supplementary Figure 3.** Cell-cycle fractions and apoptotic cells labeled with annexin V were evaluated by flow cytometry for colon (HT-29), stomach (NUGC-4), pancreatic (BxPC-3) and liver (HepG2) cancer cell lines treated for 48 hrs with DMSO or GSK3 $\beta$  inhibitors. **A**, The fraction of cell cycle and apoptotic cells were measured by flow cytometric analysis. Cancer cells treated for 48 hrs with DMSO or with GSK3 $\beta$  inhibitors (SB-216763, AR-A014418) were harvested by trypsinization and fixed in 70% ethanol at  $-20^{\circ}\text{C}$  for 30 min. Fixed cells were serially incubated with 100  $\mu\text{g/mL}$

RNase A (Takara, Tokyo, Japan) and 50 µg/mL propidium iodide (PI) (Molecular Probes, Eugene, OR) for 30 min at room temperature and then examined for DNA content in a FACS Calibur (Becton Dickinson Instrument, Bedford, MA). The distribution of cells in each cell-cycle phase was determined using cell ModFitLT Software (Becton Dickinson). The number and bar shown in each graph indicate the proportion of cells in sub-G0 fraction. **B**, The relative number of apoptotic cells was further assessed by staining the fixed cells with both PI and annexin V using human Annexin V-FITC Kit (Bender MedSystem, Inc., Burlingame, CA) according to the manufacturer's instruction. At least  $2 \times 10^4$  stained cells were analyzed for phosphatidyl serine exposure level on a FACS caliber system for each determination. The number in each quadrant indicates the proportion of cells present within it.

**Supplementary Figure 4.** Effect of the GSK3β inhibitors on the activity of telomerase derived from C33A human cervical cancer cells. DMSO (D) or either SB-216763 (SB) or AR-A014418 (AR) at the final concentration of 25 µM was directly added to the TRAP assay for C33A cell extract. M, molecular size marker; IC, internal control to normalize the efficiency of PCR amplification.

**Supplementary Figure 5.** The full length blots/gels of **(A)** Figure 1A, B and C, **(B)** Figure 2A and B, **(C)** Figure 4, and **(D)** Figure 5B, C and D, and **Supplementary Figure 4**.