14-3-3σ-Dependent Resistance to Cisplatin

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Abstract. *Background: A major factor that impedes the clinical success of cisplatin-based chemotherapy for cancer is cisplatin resistance by cancer cells. Materials and Methods: The sensitivity of parental HCT116 human colon cancer cell line and its isogenic gene-knockout sub-lines to cisplatin was determined by clonogenicity assay; furthermore, p53 activation, p21 expression, cell cycle arrest and senescence in these cells after cisplatin treatment were investigated. Results: Parental cells were six times more resistant than 14-3-3σ-knockout (σ-KO) cells to cisplatin. Moreover, activation of p53, p53-dependent expression of p21 and p21-dependent senescence were observed in σ-KO, but not parental cells after a treatment with a low cisplatin dose. Conclusion: A 14-3-3σ-dependent mechanism inhibits p53 activation in parental cells treated with a low cisplatin dose, thereby blocking p21 expression that is essential for senescence and consequently conferring to the parental cells a significant degree of resistance to cisplatin.*

Cisplatin is an important anticancer drug and its anticancer activity is due to its ability to react, after it is activated inside the cell, with DNA to form covalent platinum (Pt)-DNA adducts (Pt adducts), which, if not repaired, trigger a DNA damage response that results in cell cycle arrest and/or apoptosis (1). It should be noted that cisplatin damages DNA non-discriminatorily in cancerous and normal cells, and hence, it has a very narrow therapeutic index – that is, cisplatin overdose in cancer patients kills not only a large number of cancerous cells, but also many normal cells, resulting in severe toxicity (2). The narrow therapeutic index of cisplatin also explains why a small increase in cisplatin resistance by cancer cells can impede the success of cisplatin-based chemotherapy (1). Thus, it is of clinical importance to identify cellular factors that affect the sensitivity of cancer cells to cisplatin and then develop strategies and agents to increase the sensitivity of cancer cells to cisplatin (1).

The mammalian *14-3-3* gene family includes seven members, termed β, ε, γ, θ, τ, σ, and ζ (3). *14-3-3σ* is unique in that it can be regulated by p53 (4). It was initially suggested that 14-3-3σ acts synergistically with p21 to stably arrest cells at the G_2-check point after DNA damage (5), but it was subsequently demonstrated that 14-3-3σ was not essential at this check point (6). In addition, because ectopic over-expression of 14-3-3σ in cells resulted in cell cycle arrest, 14-3-3σ was considered as a tumor suppressor (4). However, high expression levels of 14-3-3σ are found in many pancreatic and colorectal cancers and are correlative to poor prognosis for patients with these cancers, suggesting an important role for 14-3-3σ in the progression of these cancers (7-9). Thus, the role of 14-3-3σ in tumorigenesis remains unclear. Nevertheless, the fact is that 14-3-3σ can interact with over 100 cellular proteins (10). Therefore, 14-3-3σ appears to regulate a plethora of diverse cellular activities. The challenge now is to identify the specific cellular activities that are regulated by 14-3-3σ.

The parental HCT116 human colon cancer cell line and its various gene knockout sub-lines provide an important

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experimental system for the identification of gene-specific cellular activities (5, 6, 11). During the course of the investigation on how colorectal cancer cells respond to a short-term treatment with low concentrations of cisplatin, it was found that the clonogenicity (*i.e.* the dividing capability) of σ-KO HCT116 cells was much more sensitive than that of parental HCT116 cells to cisplatin. This finding indicated the presence of a hitherto unknown 14-3-3σ-dependent mechanism of cisplatin resistance and prompted the investigation on how 14-3-3σ affects cisplatin resistance.

Materials and Methods

Cells. Dr. Bert Vogelstein (Johns Hopkins University, Baltimore, MD) kindly provided parental, *p53* knockout (*p53*-KO), σ-KO and *14-3-3σ/p21* double knockout (*σ/p21*-DKO) HCT116 cell lines (5, 11, 12). The cell lines were cultured in growth medium (McCoy's 5A medium supplemented with 10% FBS and antibiotics) as previously described (5, 11, 12). In all experiments, cells in stock cultures were trypsinized, seeded into 100-mm dishes containing 10 ml of growth medium and cultured for 24 h. Then the cells were left untreated or treated for 60 min with cisplatin, washed gently with serum-free medium and incubated in 10 ml of fresh growth medium for additional hours.

Materials. Cisplatin, cycloheximide, x-gal, antibody to β-tubulin and cocktails of protease and phosphatase inhibitors were obtained from Sigma-Aldrich, Inc. (St. Louis, MO, USA). Cisplatin was dissolved at 1 mg/ml in phosphate-buffered saline (PBS), pH 7.4, and stored at room temperature in the dark. The widely used antibody to human 14-3-3σ (#1433S01) was from NeoMarkers, Inc. (Fremont, CA, USA); the antibodies to p21 (C-19), p53 (DO-1), 14-3-3β (4E1), 14-3-3γ (3F8), 14-3-3ε (8C3), 14-3-3θ (3B9) and 14-3-3ζ (C-16) and ECL Western blot reagents were from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA); and the antibodies to acetyl-Lys382 p53 (#2525), phospho-Ser15 p53 (#9284) and histone H3 (#9715) from Cell Signaling Technology, Inc. (Danvers, MA, USA). High performance (HP)-validated human p53 siRNA (#SI02655170), control siRNA (#1022076) and HiPerFect transfection reagent were obtained from Qiagen, Inc. (Germantown, MD, USA).

Clonogenicity assay. Cells were seeded at 250, 500 and 1,000 cells per 100-mm dish containing 10 ml growth medium and incubated for 24 h. Then, the cells were either left untreated or treated for 60 min with various concentrations of cisplatin in triplicate, gently washed once with 10 ml serum-free medium and cultured in 10 ml growth medium for 7-10 days. Cell colonies were fixed with methanol and visualized after staining with 0.1% crystal violet blue in water. Colonies consisting of at least 50 cells were scored and the clonogenicity was calculated as previously described (13).

Determination of Pt adducts in the genome. RNA-free genomic DNA was isolated from cells after a treatment with cisplatin, dissolved in ultra-pure water, and aliquots (80-100 μg) of DNA were analyzed by inductively coupled plasma mass spectrometry to determine the amount of DNA-bound Pt (pg Pt/μg DNA) to represent the amount of Pt adducts (14). Consistently, it was determined that the rate of Pt adduct formation in the genome of cisplatin-treated HCT116 cells was linearly dependent on both cisplatin concentration and treatment duration [This study and (14)].

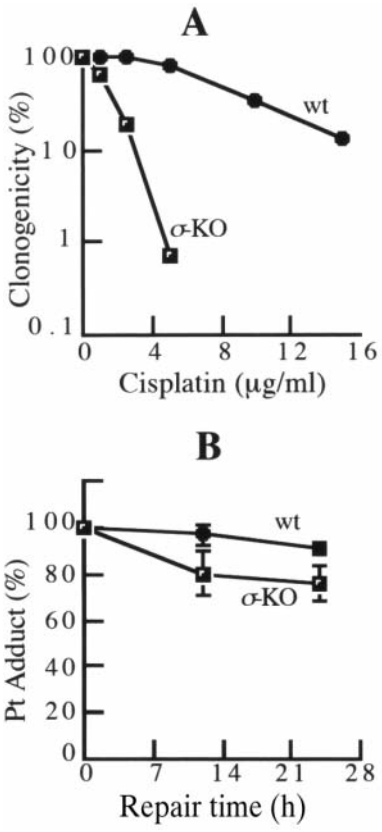

Figure 1. *Cisplatin-induced inhibition of clonogenicity, Pt adducts in the genome and repair of Pt adducts in HCT116 cells. (A) Clonogenicity of parental and σ-KO HCT116 after a 60-min treatment with various cisplatin concentrations. The results represent the average of three independent experiments. (B) Immediately after the cells were treated for 60 min with 5 μg/ml cisplatin, the amounts of Pt adducts (pg Pt/μg DNA) in some cells were determined and defined as the 100% base line. Other cells were then washed and incubated in cisplatin-free medium and relative quantitative changes in Pt adducts were monitored at various time points. The results were the average of two independent experiments.*

Flow cytometric analysis of apoptosis and cell cycle. Non-adherent and trypsin-detached cells in the same dish were pooled and fixed in 95% methanol, washed in PBS, stained in propidium iodide/RNase solution (50 μg/ml propidium iodide, 500 μg/ml RNase A) for 10 min at 37˚C, and analyzed in a FACSCalibur™ flow cytometer (Becton Dickinson) to determine the percentages of G_1, S and G_2/M cells and apoptotic cells with a sub-G_1 DNA content.

Enrichment of chromatin-bound proteins. Cells were harvested, washed in PBS, and gently lysed in 20 volumes of lysis buffer (10 mM Tris-HCl, pH 7.2, 2.5 mM EDTA, 1X protease inhibitor cocktail, 1X phosphatase inhibitor cocktail, 0.2% Triton® X-100). The lysate was centrifuged for 5 min at 10,000 ×*g* to pellet the chromatin. The pellet was suspended in 3 volumes of water and sonicated. The sonicated sample was mixed with three volumes of acetone, and stored at –20˚C

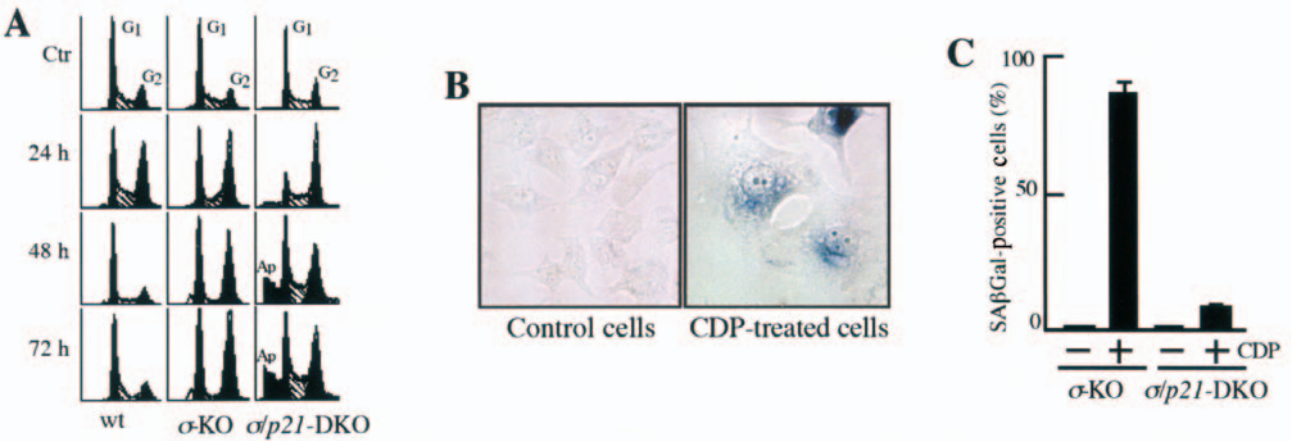

Figure 2. *p21-dependent cell cycle arrest and premature senescence in σ-KO HCT116 cells. (A) Histograms of flow cytometric analyses of cells with no treatment (Ctr) and treated for 60 min with 5 μg/ml cisplatin, and washed and incubated in cisplatin-free medium for 24, 48 and 72 h. G_1, G_0 + G_1 cells; G_2, G_2 + M cells; Ap, apoptotic cells. (B) Untreated (control) SAβGal-negative σ-KO HCT116 cells and SAβGal-positive σ-KO HCT116 cells 72 h after a standard cisplatin treatment. (C) Expression of SAβGal in σ-KO and σ/p21-DKO HCT116 cells receiving no treatment (–) and 72 h after a standard cisplatin treatment (+). CDP, cisplatin.*

to precipitate proteins. The precipitated proteins were pelleted by centrifugation, dissolved in SDS-PAGE sample buffer and subjected to Western blot analysis.

Western blotting analysis. Whole cell protein extracts or fractionated protein extracts were prepared and aliquots of the extracts (50 μg proteins) were subsequently subjected to SDS-PAGE and Western blotting analysis as previously described (15).

RNAi. Cells were seeded into 100-mm dishes at 20% confluence in 10 ml of growth medium and incubated for 24 h. The cells were treated for 36 h with 50 nM control siRNA or *p53*-specific siRNA with the aid of HiPerFect transfection reagents according to the protocol provided by the manufacturer (Qiagen, Inc., Germantown, MD, USA). The cells either received no further treatment or were subjected to a standard cisplatin treatment, washed once with 10 ml of serum-free medium, and incubated for another 24 h in 10 ml of cisplatin-free growth medium before they were processed to obtain whole cell protein extracts for Western blot analysis.

Detection of senescence-associated β-galactosidase (SAβGal). Cells were washed in PBS, fixed with 0.5% glutaraldehyde in PBS, pH 7.4 at room temperature for 15 min, incubated three times in pH 6.0 PBS (5 min per incubation), and then incubated for 20 h at 37˚C in X-gal/PBS solution, pH 6, to detect SAβGal activity under an anaerobic condition (16).

Results

Differential sensitivity of parental and σ-KO HCT116 cells to cisplatin. It was determined that a 60-min treatment with approximately 10.9 μg/ml and 1.8 μg/ml of cisplatin induced 50% inhibition of the clonogenicity of parental HCT116 cells and σ-KO HCT116 cells, respectively (Figure 1A). Thus, the parental HCT116 cells were six times more resistant than the σ-KO HCT116 cells to cisplatin. Because a 60-min treatment with 5 μg/ml of cisplatin had very little effect on the clonogenicity of parental HCT116 cells but blocked the clonogenicity of most σ-KO HCT116 cells (Figure 1A), this treatment was applied as a standard cisplatin treatment in the experiments described below to investigate why the parental HCT116 cells were more resistant than the σ-KO HCT116 cells to cisplatin.

The results from two independent experiments showed that the amounts of Pt adducts in the genome of σ-KO and parental HCT116 cells immediately after a standard cisplatin treatment were 2.035 pg Pt/μg DNA and 2.062 pg Pt/μg DNA, respectively, indicating that the cells incurred a similar amount of Pt adducts. Moreover, approximately 90% and 80% of the Pt adducts in the genome of parental HCT116 and σ-KO HCT116 cells, respectively, were not repaired 24 h after the termination of the cisplatin treatment (Figure 1B). These findings demonstrated that the repair of Pt adducts in the genomes of both parental and σ-KO HCT116 cells was gradual and slow and the parental and σ-KO HCT116 cells had a similar capacity to repair Pt adduct. These findings strongly suggested that the parental HCT116 cells were more resistant than the σ-KO HCT116 cells to cisplatin because the parental HCT116 cells were less sensitive than σ-KO HCT116 cells to the presence of Pt adducts in the genome.

p21-Dependent senescence of σ-KO HCT116 cells after a standard cisplatin treatment. The parental HCT116 cells exhibited temporary G_1 and G_2 arrests after a standard cisplatin treatment, but subsequently resumed cycling

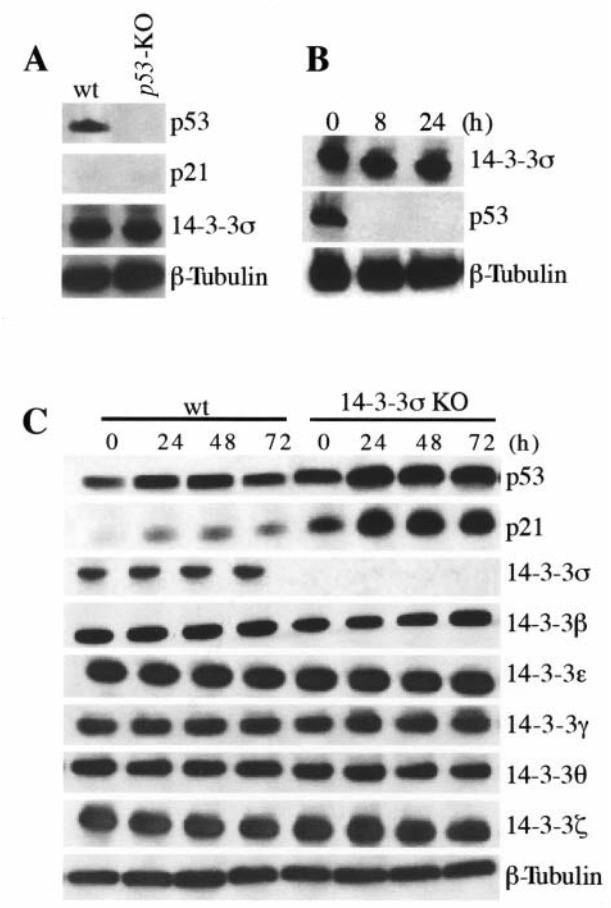

Figure 3. *Expression of 14-3-3, p53 and p21 proteins in wt and σ-KO HCT116 cells. (A) Western blot showing the indicated proteins in whole cell extracts from parental and p53-KO HCT116 cells. (B) Western blot showing the expression levels of 14-3-3σ and p53 in whole cell extracts from parental HCT116 cells untreated (0 h) and treated with 100 μM cycloheximide for 8 h and 24 h. β-Tubulin served as an internal indicator of equal protein loading. (C) Western blot showing the indicated proteins in whole extracts from parental and σ-KO HCT116 cells untreated (0 h), and at 24, 48 and 72 h after the termination of a standard cisplatin treatment.*

(Figure 2A), whereas the σ-KO HCT116 cells were viable and stably arrested at G_1 and G_2 checkpoints after the same treatment (Figure 2A). Furthermore, the cisplatin-treated σ-KO HCT116 cells became senescent because they were not only stably arrested but also irregularly enlarged and expressed the SAβGal activity (Figure 2B) (16-18). In striking contrast, the *σ/p21*-DKO HCT116 cells, which lack both *14-3-3σ* and *p21* genes (5), displayed a temporary G_2 arrest after a standard cisplatin treatment and then resumed cycling, which was accompanied by a high degree of apoptosis (Figure 2A). Moreover, very few of the viable, cisplatin-treated *σ/p21*-DKO HCT116 cells expressed SAβGal (Figure 2C). These findings provided direct genetic evidence indicating that p21 was essential for σ-KO HCT116 cells to remain viable and become senescent after a standard cisplatin treatment.

Expression of 14-3-3 proteins in parental and σ-KO HCT116 cells. Although HCT116 cells are known to contain a barely detectable level of *14-3-3σ* mRNA in the absence of active p53 (4, 6, 19), they contain a steady-state level of 14-3-3σ that is readily detected by Western blotting (6). Both parental and *p53*-KO HCT116 cells expressed 14-3-3σ (Figure 3A), and it was found that treatment of the cells for up to 24 h with 100 μM of the translation inhibitor, cycloheximide, did not alter the expression level of 14-3-3σ (Figure 3B). These findings indicated that 14-3-3σ was very stable and thus constant expression of the *14-3-3σ* mRNA was not required for HCT116 cells to maintain a steady-state level of 14-3-3σ. Both wild-type (wt) and σ-KO HCT116 cells expressed similar levels of the other 14-3-3 family proteins (Figure 3C), and it was determined that a standard cisplatin treatment neither altered the level of 14-3-3σ in parental HCT116 cells nor had any effect on the expression levels of the other 14-3-3 family proteins in both parental and σ-KO HCT16 cells (Figure 3C). These data demonstrated that parental HCT116 cells possessed a 14-3-3σ-dependent mechanism for cisplatin resistance.

Inhibitory effect of 14-3-3σ on p53 activation and p21 expression. The parental HCT116 cells expressed a very low level of p53 and a barely detectable level of p21, whereas σ-KO HCT116 cells expressed a higher level of p53 and a basal level of p21 (Figure 3C). A standard cisplatin treatment induced (i) approximately 50% increase in the amount of p53 in both parental and σ-KO HCT116 cells (Figure 3C), (ii) very little p21 expression in parental HCT116 cells (Figure 3C), and (iii) stable and robust expression of p21 in σ-KO HCT116 cells (Figure 3C). These data indicated that the presence of 14-3-3σ suppressed expression of p21 in parental HCT116 cells. It was also found that RNAi-mediated knock-down of p53 in the σ-KO HCT116 cells impaired their ability to express p21 after a standard cisplatin treatment (Figure 4A). Thus, p21 was expressed by a p53-dependent mechanism in the cisplatin-treated σ-KO HCT116 cells. Next, investigation was performed on whether parental HCT116 and σ-KO HCT116 cells differed in their ability to activate p53 and consequently differed in their ability to express p21 because of the fact that *p21* is a p53-responsive gene (11). Since Lys382 acetylation and Ser15 phosphorylation of p53 are characteristics of p53 activation after DNA damage (20), these two types of p53 modifications in both parental and σ-KO HCT116 cells untreated and 24 h after a standard cisplatin treatment were investigated. Acetylated or phosphorylated p53 was barely detected in whole cell protein extracts from either cells with or without the cisplatin

treatment (data not shown), but after analysis of samples enriched with chromatin-associated proteins, p53 that was acetylated and phosphorylated at Lys382 and Ser15, respectively, were easily detected in the σ-KO but not the parental HCT116 cells only after a standard cisplatin treatment (Figure 4B). These findings collectively indicated that 14-3-3σ exerted an inhibitory effect on the process of p53 activation, thereby preventing p21 expression in parental HCT116 cells after a standard cisplatin treatment.

Discussion

In this report, it has been demonstrated that although cisplatin treatment lacked long-term effect on the survival and proliferation of most parental HCT116 cells, it induced (i) activation of p53 (Figure 4B), (ii) p53-dependent expression of p21 (Figure 4A), and (iii) p21-dependent senescence in nearly all σ-KO HCT116 cells (Figure 2) without altering the levels of other 14-3-3 proteins (Figure 3C). These data and the isogenic relationship between parental HCT116 and σ-KO HCT116 cells indicate the presence of a hitherto unknown 14-3-3σ-dependent mechanism that can inhibit p53 activation, thereby preventing expression of p21 to induce senescence and consequently conferring to parental HCT116 cells a significant degree of cisplatin resistance (Figure 1A). Currently, investigations on how exactly 14-3-3σ inhibits p53 activation are being performed.

Cisplatin-based chemotherapy for metastatic testicular germ-cell cancer (TGCC) achieves a cure rate of over 80% (21). This success rate is explained by a high sensitivity of TGCC cells to cisplatin: it has been demonstrated that a 60-min treatment with 1.3-1.9 μg/ml of cisplatin induces 50% inhibition of the clonogenicity of TGCC cells, while a 60-min treatment with >4 μg/ml of cisplatin is required to induce 50% inhibition of the clonogenicity of various breast, bladder, colon and prostate cancer cells (1, 13, 22). In this study, it was found that a 60-min treatment with approximately 10.9 μg/ml of cisplatin and 1.8 μg/ml of cisplatin induced 50% inhibition of the clonogenicity of wt HCT116 cells and σ-KO HCT116 cells, respectively (Figure 1A). Thus, loss of 14-3-3σ increased the sensitivity of HCT116 to cisplatin to a level that is compatible to that of TGCC cells to cisplatin. Although several studies have linked an insufficiency of nucleotide excision repair (NER) to the sensitivity of TGCC cells to cisplatin (1), many human TGCCs lack 14-3-3σ (23). Thus, it may be presumed that both NER insufficiency and 14-3-3σ deficiency are important determinants for the high sensitivity of TGCC cells to cisplatin. In this context, it is also noteworthy that HCT116 cells are defective in certain NER activity (24). Thus, it is plausible that the observed sensitivity of σ-KO HCT116 cells to cisplatin results from both the loss of 14-3-3σ and NER insufficiency.

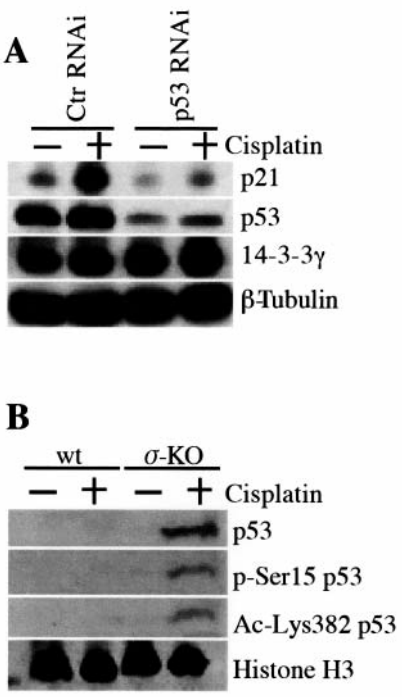

Figure 4. *p53-dependent expression of p21 and activation of p53. (A) p53 RNAi induced reduction of the p53 level and inhibition of p21 expression in σ-KO HCT116 cells untreated with cisplatin (–) and 24 h after a standard cisplatin treatment (+). β-Tubulin and 14-3-3γ proteins served as internal controls of equal total protein loading. (B) Western blot showing the absence and presence of activated p53 in the samples enriched with chromatin-associated proteins. Histone 3 (H3) served as an internal control of equal loading of chromatin proteins.*

Because high expression levels of 14-3-3σ correlate with poor prognosis for patients with pancreatic and colorectal cancers (7-9), it will be important to investigate whether selective inhibition of the expression or function of 14-3-3σ in pancreatic and colorectal cancer cells would significantly slow down cancer progression and increase their sensitivity to standard cisplatin-based chemotherapy. It should be noted that the relevance of 14-3-3σ to treatment of non-small cell lung cancer (NSCLC) patients with cisplatin is factually supported by a study showing that 14-3-3σ is an independent prognostic factor for the poor outcome of these patients after they receive clinically standard treatment with cisplatin (25). Specifically, that study demonstrated that those patients with 14-3-3σ-negative NSCLC survived longer than the patients with 14-3-3σ-positive NSCLC after they had been treated with cisplatin (25). The findings in this study suggest that it is likely that 14-3-3σ-negative NSCLC cells are more able than 14-3-3σ-positive NSCLC cells to activate p53 in order to express p21 and become arrested (or senescent) in the presence of Pt adducts in the genome, and hence, the 14-3-3σ-negative NSCLC cells were more sensitive than the 14-3-3σ-positive NSCLC cells to cisplatin.

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