DNA-Dependent Protein Kinase (DNA-PK)–Dependent Cisplatin-Induced Loss of Nucleolar Facilitator of Chromatin Transcription (FACT) and Regulation of Cisplatin Sensitivity by DNA-PK and FACT

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Abstract

Both the Ku subunit of the DNA-dependent protein kinase (DNA-PK) and the facilitator of chromatin transcription (FACT) complex reportedly bind cisplatin-DNA adducts. For this study, we developed an immunocytochemical assay based on detergent extraction allowing unveiling nucleolar subpopulations of proteins present in both the nucleoplasm and the nucleolus. Immunofluorescence analysis in various human cancer cell lines and immunoblotting of isolated nucleoli show that DNA-PK catalytic subunit (DNA-PKcs), Ku86, the Werner syndrome protein (WRN), and the structure-specific recognition protein 1 (SSRP1) subunit of FACT colocalize in the nucleolus and exit the nucleolus after cisplatin treatment. Nucleolar localization of Ku is also lost after γ or UV irradiation and exposure to DNA-damaging drugs, such as actinomycin D, mitomycin C, hydroxyurea, and doxorubicin. Ku86 and WRN leave the nucleolus after exposure to low (>1 μg/mL) doses of cisplatin. In contrast, the SSRP1 association with the nucleolus was disrupted only by high (50-100 μg/mL) doses of cisplatin. Both cisplatin-induced loss of nucleolar SSRP1 and DNA-PK activation are suppressed by pretreatment of the cells with wortmannin or the DNA-PK inhibitor NU7026 but not by the phosphatidylinositol 3-kinase inhibitor LY294002. In the same conditions, kinase inhibitors did not alter the exit of DNA-PKcs and WRN, suggesting that different mechanisms regulate the exit of DNA-PK/WRN and FACT from the nucleolus. Furthermore, RNA silencing of DNA-PKcs blocked the cisplatin-induced exit of nucleolar SSRP1. Finally, silencing of DNA-PKcs or SSRP1 by short hairpin RNA significantly increased the sensitivity of cancer cells to cisplatin. (Mol Cancer Res 2009;7(4):581–91)

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Introduction

Cisplatin is a powerful antitumor agent developed nearly 3 decades ago. Platinum compounds are widely used in the treatment of solid tumors. They have revolutionized the treatment of germ cell tumors, producing high cure rates, and are standard therapy for ovarian, bladder, cervical, head and neck, and both small cell and non–small cell lung cancers (1). Clinical trials are evaluating platinum as first-line treatment for patients with triple-negative breast cancer, a tumor subtype that expresses neither the estrogen receptor, the progesterone receptor, nor the receptor-like HER2 protein (2).

The chemotherapeutic effect of cisplatin results from its interaction with DNA. Platinum binds to guanine and adenine in DNA, thereby generating interstrand and intrastrand cross-links, DNA-protein cross-links, and monoadducts (3, 4). Cisplatin is usually active in previously untreated patients; however, in patients previously exposed to chemotherapy, the response rates to cisplatin decline markedly (5). In ovarian cancer, cisplatin typically induces rapid tumor regression, which is temporary and leads to resistance within months. Resistance to cisplatin chemotherapy is thus a major obstacle to effect treatment of ovarian cancer and predictably triple-negative breast cancer.

The resistance of tumor cells to cisplatin is believed to be multifactorial and includes cellular pumps that decrease intracellular drug accumulation, increased metabolic inactivation, changes in sensitivity to apoptosis, and increased repair of cisplatin-induced DNA damage (1). Tumor cells may overcome the effect of cisplatin by alterations of cisplatin/DNA damage recognition by such proteins as mismatch repair and high mobility group domain-containing proteins. The structure-specific recognition protein 1 (SSRP1) is a high mobility group–containing protein that exists in a complex called the facilitator of chromatin transcription (FACT) complex, composed of suppressor of Ty (Spt16) and SSRP1 (6). FACT binds cisplatin-damaged DNA through SSRP1 (4, 7). FACT also seems to stimulate RNA polymerase II transcription by removing H2A/H2B dimers from the nucleosome, thus facilitating access to chromatin (6). Recently, increased levels of SSRP1 protein were detected in tumor samples from ovarian as well as breast and lung cancer patients, indicating that up-regulation of SSRP1 might be a general cancer-related event (8).

Ku, an autoimmune antigen in patients with scleroderma and polymyositis, is involved in telomere maintenance, apoptosis, and gene transcription but has primarily been associated with

DNA damage repair (9). The Ku heterodimer of 70 kDa (Ku70) and 86 kDa (Ku86) associates with the 400-kDa catalytic subunit, DNA-PKcs, to form the DNA-dependent protein kinase (DNA-PK). It was previously shown that Ku is capable of binding to cisplatin-modified DNA (7, 10). During purification of the Ku86 protein complex, we found that the FACT and the Ku/DNA-PK protein complexes physically interact in a large macromolecular complex formed mostly by nucleolar proteins. Furthermore, nucleolar Ku and DNA-PKcs exit the nucleolus after UV irradiation.[3] Another Ku86-associated protein, the Werner syndrome protein (WRN) was shown to leave the nucleolus after DNA damage (11, 12). Moreover, a nucleolar p53 protein pool undergoes exit after UV irradiation, implicating a role for nucleolar exit of proteins in response to DNA damage (13). Thus, the nucleolus, a nuclear body responsible for rRNA transcription and preribosomal particle assembly, is implicated in DNA damage response through mechanisms yet not fully elucidated.

In the present study, we found SSRP1 to be nucleolar in several epithelial tumor cell lines and that Ku86, WRN, and SSRP1 leave the nucleolus after cisplatin treatment. In contrast to Ku86 and WRN, the exit of SSRP1 from the nucleolus can be specifically regulated by DNA-PK kinase activity. Finally, both DNA-PK and FACT regulate the sensitivity of cancer cells to cisplatin.

Results

Nucleolar Subpopulations of Specific Nuclear Proteins Are Unveiled by a Detergent-Based Immunocytochemical Assay

Most of the proteins found in the Ku complex are present in the nucleolus[3] (14) and nucleolar localization of Ku86 was previously reported (15-17). The nucleolus is a typically round granular body composed of proteins, DNA, and RNA in the nucleus of the cell. We designed an immunocytochemical assay to visualize the nucleolar localization of proteins present in both the nucleoplasmic and the nucleolar compartments of adherent human cells in culture.

When MDA-MB-231 cells were fixed with paraformaldehyde (PFA) and then extracted with methanol, anti-Ku86 immunofluorescence showed reactivity in the whole nucleus (Fig. 1A, *top left*). The weaker staining in the nucleoli compared with the nucleoplasm is likely due to the small amount of Ku86 proteins present in the nucleolus. This observation is supported by the biochemical analysis of Ku86 levels in subnuclear fractions (Fig. 3A). Triton X-100 is a nonionic detergent that can be used to disrupt weak protein-protein interactions (18). We hypothesized that nucleoplasm-associated proteins would be extracted by Triton X-100 before the more stably associated nucleolar proteins. To monitor differential extractability of the nucleoplasmic versus nucleolar Ku86, MDA-MB-231 cells were extracted for different lengths of time (Fig. 1A) before fixation with PFA and immunofluorescence analysis. Indeed, after extraction for 30 seconds with Triton X-100 before fixation, most of the diffuse nucleoplasmic staining obtained with the PFA-methanol fixation-extraction method was lost, revealing a nucleolar subpopulation of Ku86 (Fig. 1A). The effect was time dependent because extraction for 15 seconds was insufficient to reveal the Ku86 nucleolar subpopulation, whereas exposure to Triton X-100 for 1 or 2 minutes led to a complete loss of nuclear staining (Fig. 1A). The nucleolar marker B23/nucleophosmin, a protein present in the granular component of the nucleolus (19), was confined to the nucleolus when either PFA-methanol or Triton X-100–PFA protocols were done (Fig. 1A, *middle*). The specificity of this detergent-based extraction assay was further established by doing immunocytochemistry for the nonnucleolar p300 coactivator. Immunofluorescence analysis of p300 showed general distribution of the antigen in the nucleoplasm and was devoid of any nucleolar subpopulation with or without Triton X-100 extraction (Fig. 1A). Similar results were obtained with the E2F1 transcription factor and the cyclin-dependent kinase 2 (data not shown). A schematic of the proposed method is delineated in Fig. 1B. As shown in Fig. 1C, the nucleolar staining pattern obtain with the Triton X-100–PFA protocol is general, with nearly all cells displaying nucleolar Ku86.

In support of our findings, Ku was previously detected in the nucleoli of human cells, fixed with acetone or PFA before incubation with Triton X-100 (16, 20). Electrophoretic mobility shift assay with Ku antibodies at the rDNA promoter (21) and proteomic analysis of purified nucleoli (15) further established the presence of Ku in the nucleolus. Interestingly, Higashiura and colleagues (16) reported that flat-shaped human fibroblasts exhibited readily detectable levels of nucleolar Ku, whereas confocal analysis was necessary to unveil nucleolar Ku in more spherical-shaped HeLa cells. In similar experiments, we were able to readily detect Ku86 in the nucleolus of BJ1 fibroblasts using the PFA-methanol protocol (data not shown). Taken together our data and works from others, it is unlikely that Triton X-100 extraction per se is responsible for the presence of Ku in the nucleolus.

Cisplatin Induces Loss of Nucleolar Ku86, WRN, and SSRP1

Increased expression of the FACT subunit SSRP1 was recently shown in breast and ovarian cancers (8). Ku and SSRP1 have the ability to bind to cisplatin-DNA adducts. We recently found that SSRP1 is part of the Ku/DNA-PK protein complex and that nucleolar Ku staining is lost after UV irradiation of M059K glioma cells.[3] Furthermore, the Ku-associated Werner syndrome helicase (WRN) and B23/nucleophosmin exit the nucleolus after UV-induced DNA damage (22, 23). Thus, we wondered if SSRP1 could be detected in the nucleolus and if cisplatin had an effect on the nucleolar localization of Ku, WRN, and SSRP1.

When the PFA-methanol fixation method was done on growing MDA-MB-231 cells, SSRP1 reactivity was clearly visible in the nucleus (Fig. 2A), whereas the Triton X-100–PFA method unveiled a nucleolar subpopulation of SSRP1 (Fig. 2B). After cisplatin treatment, this subpopulation of SSRP1 leaves the nucleolus (Fig. 2B). The same staining

[3] G. Adelmant et al. Quantitative proteomic analysis of the nuclear Ku complex reveals a link between DNA damage, ribosome biosynthesis and translation, in preparation.

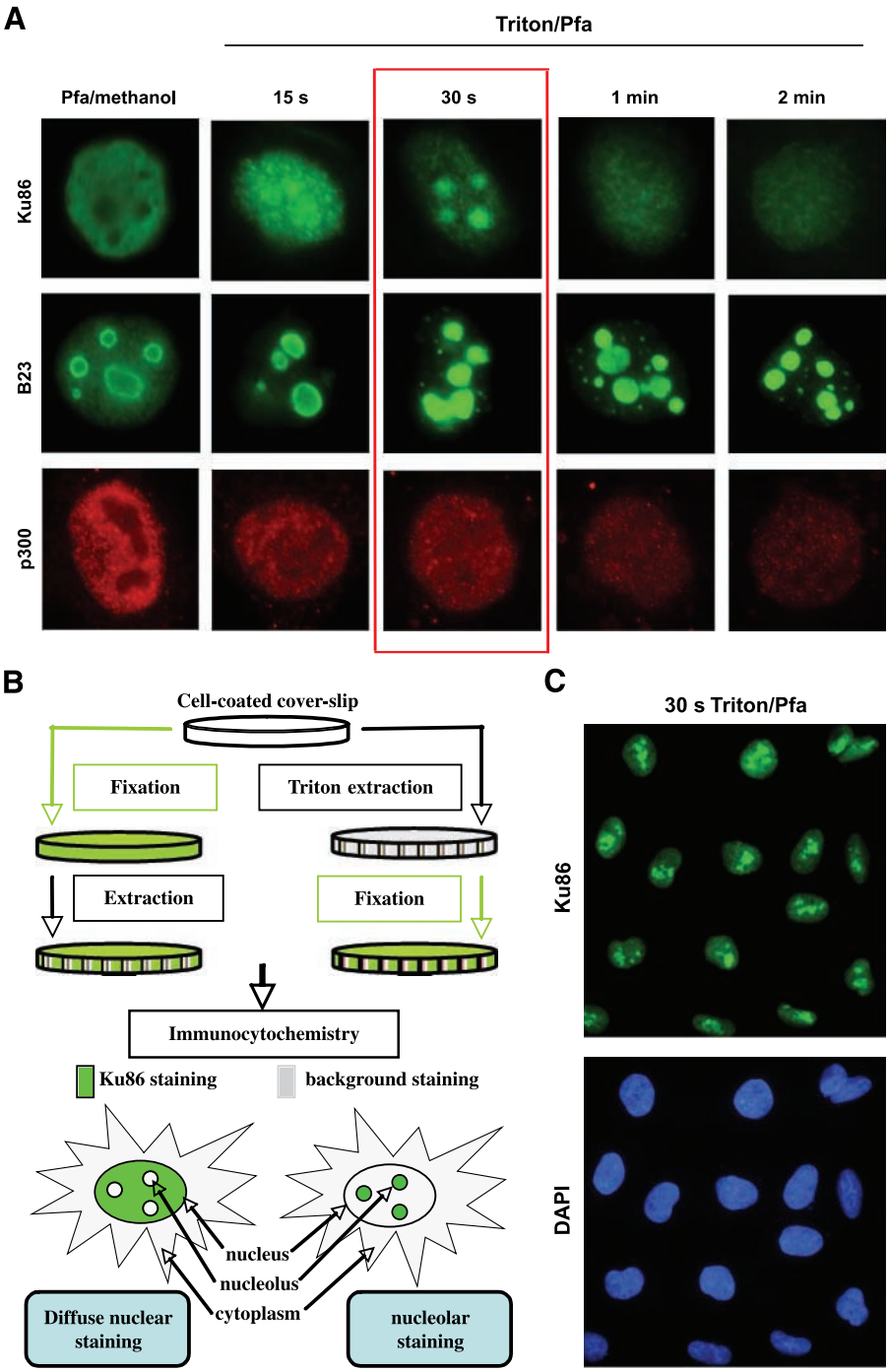

FIGURE 1. An assay to reveal nucleolar pools of nucleolar/nucleoplasmic proteins. **A.** Ku86, B23, and p300 reactivity was monitored in PFA-fixed/methanol-extracted (PFA/M) or Triton X-100–extracted/PFA-fixed (T/PFA) MDA-MB-231 breast cancer cells at intervals after Triton X-100 extraction (15 s to 2 min). One representative nucleus is shown in each panel and the red box emphasizes the optimal time after extraction (30 s). **B.** Schematic depicting the PFA/M and T/PFA assays. **C.** Lower magnification picture showing uniform nucleolar localization of Ku86 unveiled by T/PFA assay in MDA-MB-231 cells. Nuclei were stained with DAPI.

pattern was seen for Ku86, WRN (Fig. 2C), Ku70, and DNA-PKcs (data not shown). Low-power examination of a field of cells shows that virtually every nucleus behaves in the same fashion as shown for one nucleus in Fig. 2B and C (data not shown). Nucleolar markers B23/nucleophosmin (Fig. 2C), Nol-I, and nucleolin (data not shown) were still seen after cisplatin treatment, excluding disintegration of the entire nucleolus. To confirm that the loss of nucleolar reactivity was not due to protein degradation, Western blots before and after cisplatin treatment were done. No changes in nuclear protein levels of SSRP1, Ku86, or WRN were seen (Fig. 2D). Western blot for SSRP1 in nuclear extracts of MDA-MB-231 cells showed a single 80-kDa band (Fig. 2E). Furthermore, Triton X-100/PFA–extracted cells transfected with short hairpin RNA (shRNA) against SSRP1 did not show any nucleolar SSRP1 reactivity, whereas nucleolar SSRP1 was still detected in control cells (Fig. 2G). These experiments show the specificity of the SSRP1 antibody used in this study.

To biochemically confirm the results presented above, we isolated nucleoli from HeLa S3 cells before and after cisplatin treatment. Nuclei isolated from HeLa S3 cells were separated into nucleoplasmic and nucleolar fractions (24) and analyzed by immunoblotting for the nucleoplasmic marker lamin B and the nucleolar marker fibrillarin to assess the purity of the respective fractions (Fig. 3A). DNA-PKcs, WRN, SSRP1, and Ku86 were detected in each fraction, confirming the presence of these proteins in the nucleolus (Fig. 3A). The amounts of nucleolar and nucleoplasmic WRN were similar (Fig. 3A), suggesting the presence of an important reservoir of WRN proteins in the nucleolus. However, due to the small volume of the nucleolus in comparison with the nucleoplasm, the amount of SSRP1 and Ku86 in the nucleoplasm represented >95% of

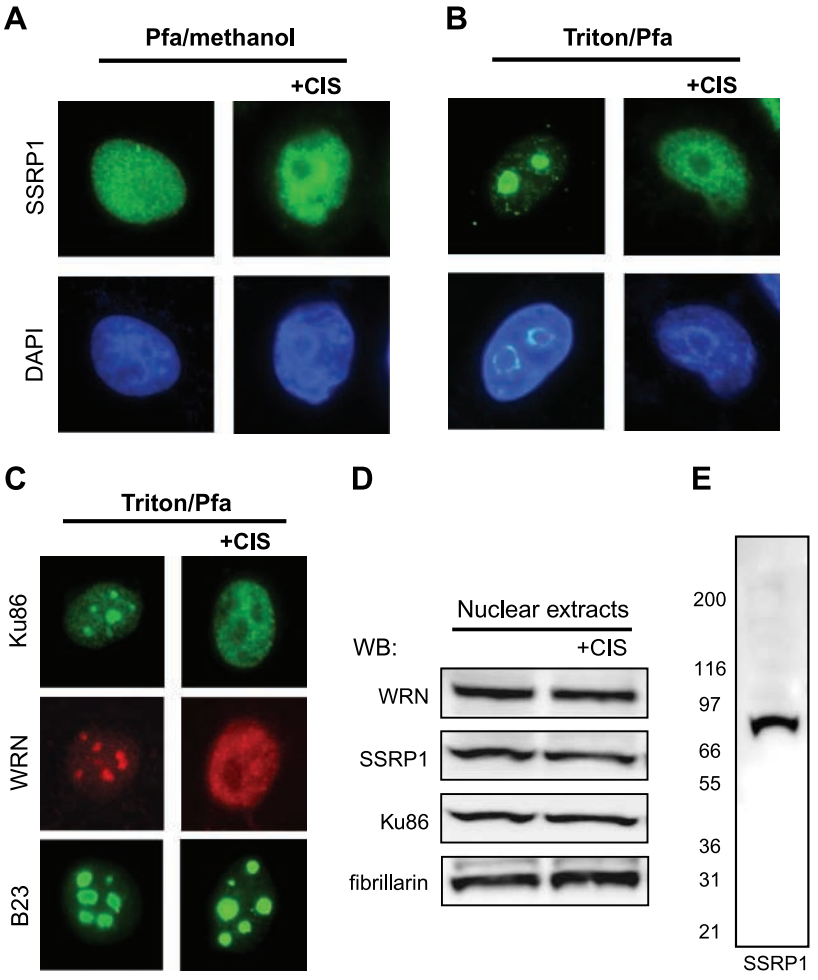

FIGURE 2. Cisplatin alters the nuclear localization of SSRP1, Ku86, and WRN. **A.** SSRP1 reactivity after PFA/M in MDA-MB-231 cells before and 2.5 h after treatment with 100 μg/mL cisplatin. Nuclei are stained with DAPI. **B.** SSRP1 reactivity after T/PFA treatment as in Fig. 1, 30 s after Triton X-100 extraction, clearly showing cisplatin-induced loss of nucleolar SSRP1. **C.** Ku86, WRN, and B23 reactivity in cells treated as in **B**. Ku86 and WRN leave the nucleolus after cisplatin. B23 is a nucleolar protein, and its staining confirms the presence of nucleoli after cisplatin treatment. **D.** Immunoblot of WRN, Ku86, SSRP1, and fibrillarin in nuclear extracts 2.5 h after treatment of the cells with 100 μg/mL cisplatin. **E.** Immunoblot of SSRP1 in nuclear extracts of MDA-MB-231 cells showing the specificity of the SSRP1 antibody. **F.** SSRP1 reactivity in MDA-MB-231 cells expressing SSRP1 or control shRNAs after PFA/M or T/PFA.

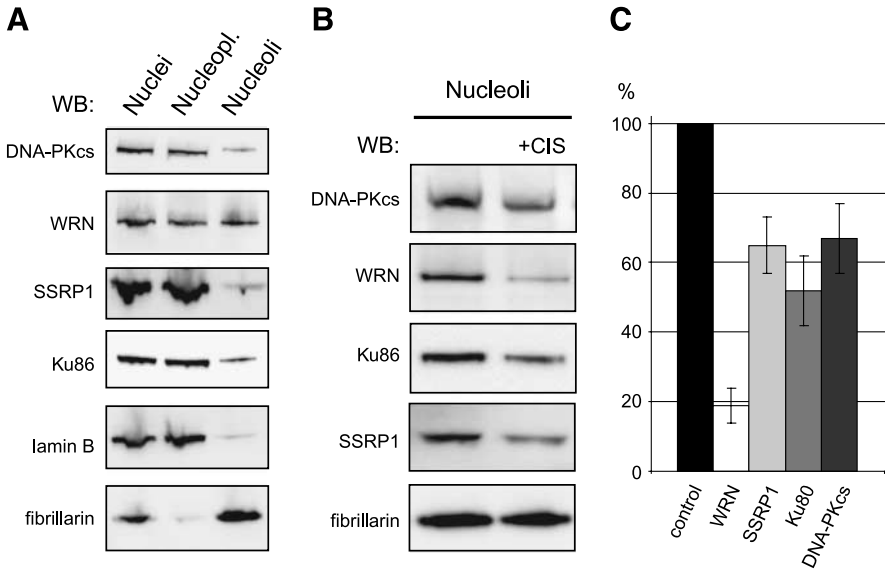

FIGURE 3. Purified nucleoli exhibit loss of WRN, SSRP1, and Ku after cisplatin treatment. **A.** Immunoblots of DNA-PKcs, WRN, Ku86, SSRP1, lamin B, and fibrillarin in nuclear extracts (*Nuclei*), nucleoplasmic (*Nucleopl.*), and nucleolar (*Nucleoli*) fractions isolated from HeLa S3 cells. **B.** Immunoblots for DNA-PKcs, WRN, Ku86, SSRP1, and fibrillarin in nucleolar fractions before and 2.5 h after treatment with 100 μg/mL cisplatin. **C.** Graph showing a significant decrease in the nucleolar WRN/SSRP1/Ku86/DNA-PKcs ratio to fibrillarin after cisplatin treatment. Columns, mean of four independent experiments; bars, SD.

the total amount detected in the nucleus (data not shown), ruling out the possibility that moving the nucleolar reservoir of FACT and Ku/DNA-PK into the nucleoplasm can significantly alter the overall amounts of these proteins in the nucleoplasm. As expected, nucleoli purified 2.5 hours after cisplatin treatment exhibited a loss of DNA-PKcs, WRN, SSRP1, and Ku that was not observed for fibrillarin (Fig. 3B and C). Taken together, these results establish that FACT, Ku/DNA-PK, and WRN leave the nucleolus after cisplatin treatment.

Loss of Nucleolar Proteins Is a General Response to DNA Damage

To investigate whether the exit of specific nucleolar proteins is a general mechanism induced by DNA damage, we exposed MDA-MB-231 cells to different kinds of DNA-damaging agents. UVC, γ irradiation, actinomycin D, mitomycin C, doxorubicin, and hydroxyurea all induced the exit of Ku86 from the nucleolus of >90% of the examined cells after 2.5 hours (Table 1). Furthermore, nucleolar loss of Ku86, WRN, and SSRP1 occurred in several breast cancer cell lines (Table 2) as well as in A2780 ovarian carcinoma cells, M059K glioma cells, and HeLa S3 cells (data not shown). As presented in Table 2, nucleolar loss of SSRP1 occurred between 1 and 2.5 hours. The exit of nucleolar proteins from the nucleolus was neither confined to a single cell line nor specific to only one type of DNA damage. This finding supports the notion that DNA damage–induced exit of specific proteins from the nucleolus is a general event and may be an important part of the cellular response to DNA damage.

The Effect of Cisplatin on the Nucleolar Loss of SSRP1 and Ku86/WRN Is Concentration but not Time Dependent

To investigate whether the cisplatin-induced loss of nucleolar proteins was concentration dependent, we titrated the dose of cisplatin. Whereas Ku86 and WRN reactivity disappeared from the nucleolus at the lowest (1 μg/mL) concentration of cisplatin, the nucleolar localization of SSRP1 was disrupted only at concentrations higher than 25 μg/mL (Fig. 4A). Prolonged exposure (24 hours) to cisplatin did not affect the concentration at which loss of nucleolar SSRP1 occurred (Fig. 4B). For simplicity of presentation, only one nucleus is shown in the dose response; however, the conclusions reflect >90% of the population of cells examined. These findings suggest that the associations of SSRP1, Ku86, or WRN with the nucleolus are regulated by separate mechanisms.

Loss of Nucleolar SSRP1 Is DNA-PK Dependent

The observed differences between nucleolar trafficking of SSRP1, Ku86, or WRN suggest that different mechanisms of signal transduction may regulate the association of these proteins with the nucleolus. The transduction of signals from DNA damage recognition to the engagement of a cellular response is often controlled by the phosphatidylinositol 3-kinase (PI3K)–related kinases (PIKK), such as the ataxia-telangiectasia mutated gene product, the ataxia-telangiectasia mutated–related protein kinase, or DNA-PKcs (25). To evaluate whether PIKKs are involved in regulating the subnuclear localization of Ku86, WRN, and SSRP1, MDA-MB-231 cells were pretreated with wortmannin, a broad inhibitor of PIKKs (26). Immunofluorescence for Ku86, WRN, and SSRP1 done after 2.5 hours of incubation with 100 μg/mL cisplatin revealed nucleolar loss of Ku86/WRN (Fig. 5A). In contrast, SSRP1 remained localized

Table 1. Nucleolar Loss of Ku86 after DNA Damage

DNA Damage	Dose	Nucleolar Exit of Ku86*
Cisplatin	1-100 μg/mL	Yes
UVC	300 J/m^2	Yes
γ-Irradiation	10 Gy	Yes
Doxorubicin	5 μmol/L	Yes
Actinomycin D	3 μg/mL	Yes
Hydroxyurea	30 mmol/L	Yes
Mitomycin C	50 μg/mL	Yes

*Nucleolar loss of Ku86 in >90% of the cells examined.

Table 2. Percentage of Cells with Nucleolar SSRP1 after Cisplatin Treatment

Cell line	Cisplatin (h)				*P*
	0	1	2.5	4	
MDA-MB-231	62.7 (47-74)	67.3 (53-89)	5.3 (3-9)	3.7 (0-11)	<0.001
MDA-MB-468	43.3 (32-41)	44.0 (34-58)	2.3 (0-7)	3.0 (0-6)	<0.001
SkBr3	66.7 (41-92)	58.7 (42-76)	4.7 (0-14)	0.3 (0-1)	<0.001
BT474	57.3 (49-63)	60.0 (45-71)	9.7 (4-15)	9.0 (2-13)	<0.001

to the nucleolus and its exit prevented by wortmannin (Fig. 5A). Because FACT was identified as a subunit of the Ku86 complex,[3] we wondered if DNA-PK was the kinase responsible for the exit of SSRP1 from the nucleolus. To dissect this signaling further, we pretreated the cells with either the DNA-PK–specific inhibitor NU7026 (27) or the PI3K inhibitor LY294002 (28). As with wortmannin, neither NU7026 nor LY294002 prevented the nucleolar exit of Ku86 and WRN (Fig. 5A). However, whereas inhibition of PI3K by LY294002 did not have any effect, inhibition of DNA-PK by NU7026 prevented SSRP1 from leaving the nucleolus after cisplatin (Fig. 5A).

On DNA damage, DNA-PK is activated and DNA-PKcs is autophosphorylated at Ser^{2056} (29). After cisplatin treatment, we detected activated DNA-PK by immunoblotting with an antibody directed against DNA-PKcs phospho-Ser^{2056}. However, pretreatment with NU7026 prevented cisplatin-induced DNA-PK activation (Fig. 5B). Consistent with the absence of effect on SSRP1 localization, LY294002 at the concentrations used had no effect on the cisplatin-induced autophosphorylation of

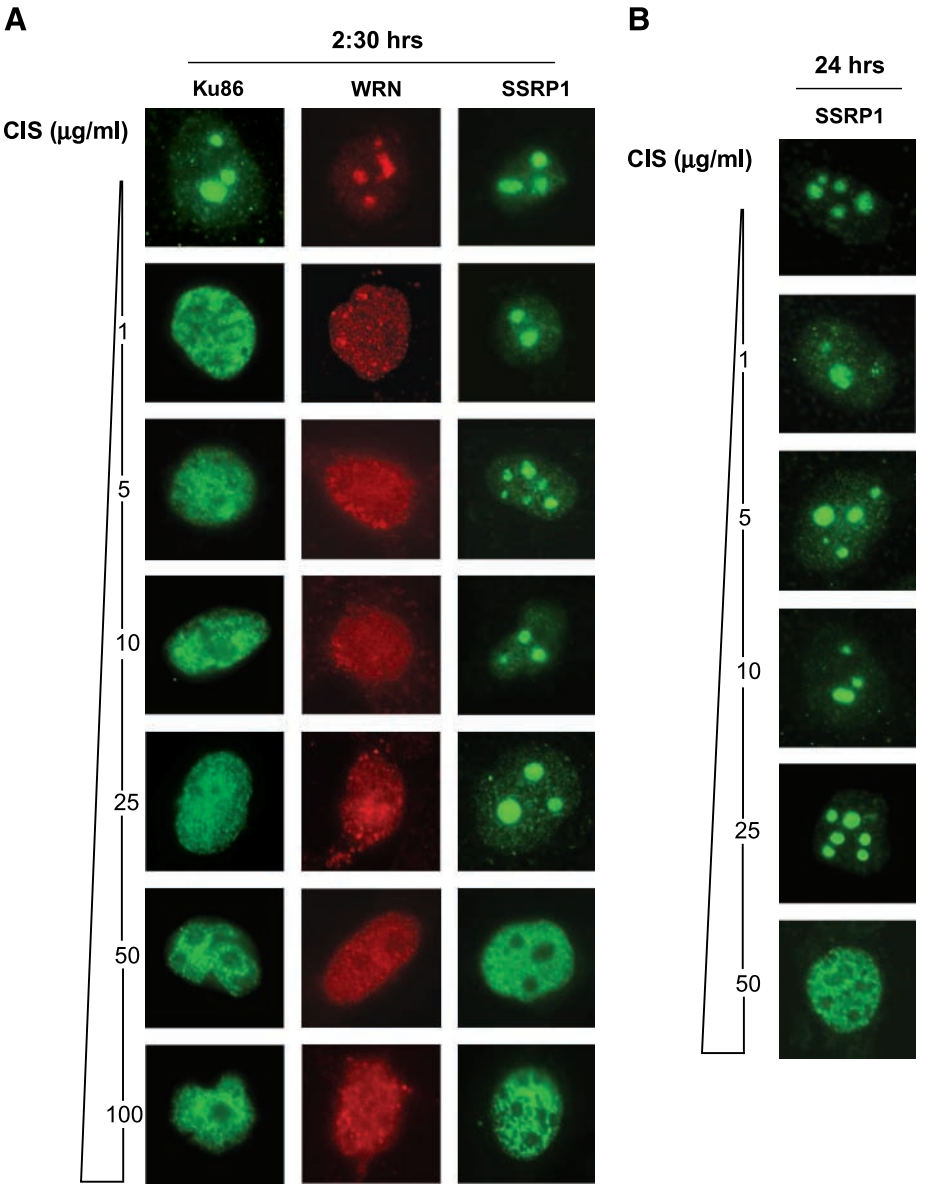

FIGURE 4. Cisplatin differentially alters the subnuclear localization of Ku86, WRN, and SSRP1. **A.** Immunofluorescence analysis of Ku86, WRN, and SSRP1 after T/PFA with Triton X-100 extraction for 30 s in MDA-MB-231 cells before and 2.5 h after treatment of the cells with 1 to 100 μg/mL of cisplatin as indicated. **B.** Immunofluorescence analysis of SSRP1 in cells treated for 24 h with 1 to 50 μg/mL of cisplatin.

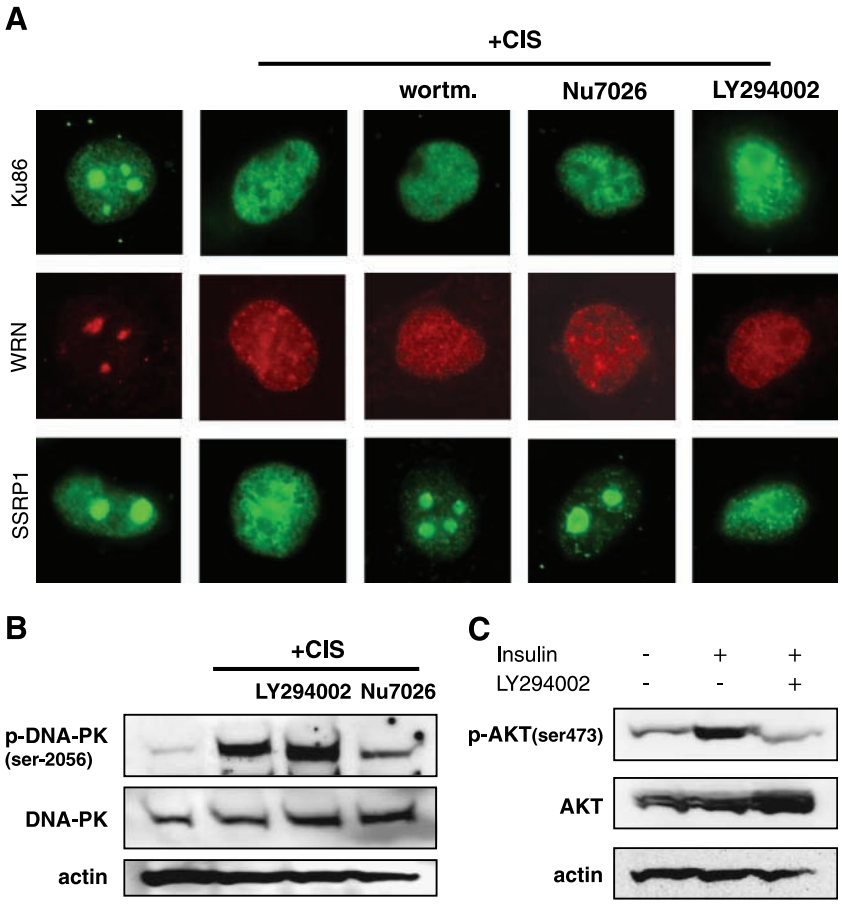

FIGURE 5. Kinase inhibitors wortmannin and NU7026 prevent selectively the cisplatin-induced exit of SSRP1 from the nucleolus. **A.** Immunofluorescence analysis of Ku86, WRN, and SSRP1 after T/PFA with Triton X-100 extraction for 30 s in MDA-MB-231 cells untreated or pretreated 30 min before cisplatin with wortmannin (100 nmol/L), NU7026 (10 μmol/L), or LY294002 (5 μmol/L). **B.** NU7026 but not LY294002 inhibits DNA-PKcs. Immunoblots of DNA-PK autophosphorylation are shown before and 2.5 after cisplatin with or without NU7026 (10 μmol/L) or LY294002 (5 μmol/L). **C.** LY294002 inhibits PI3K. Immunoblots for phospho-AKT, total AKT, and β-actin in MDA-MB-231 cells before (–) and after (+) 10 μg/mL insulin for 10 min, either untreated (–) or treated (+) with 5 μmol/L LY294002.

DNA-PK (Fig. 5B) but prevented the previously reported insulin-induced activation of AKT by PI3K (Fig. 5C; ref. 30).

To further assess for specific dependence on DNA-PK, we produced MDA-MB-231 cell lines from single colonies stably expressing a control shRNA or shRNA for DNA-PKcs. Immunoblotting shows an effective silencing of DNA-PKcs expression in cells stably expressing the DNA-PKcs shRNA but not in the control cells, and β-actin was expressed at the same levels in both cell lines (Fig. 7A). Immunofluorescence against DNA-PKcs verified the presence or absence of the DNA-PKcs protein in the control shRNA and the DNA-PKcs shRNA cell lines (Fig. 6A). Immunofluorescence done after 2.5 hours of incubation with 100 μg/mL cisplatin revealed nucleolar loss of SSRP1 in the shRNA control cells but not in the DNA-PKcs shRNA cells (Fig. 6B). Contrastingly, Ku86 and WRN leave the nucleolus in both cell lines (data not shown). Our results suggest that cisplatin at high doses induces activation of DNA-PK that subsequently leads to the exit of SSRP1 from the nucleolus, whereas Ku86 and WRN leave by DNA-PK– independent mechanisms and at lower doses of cisplatin (Fig. 6C).

Loss of DNA-PKcs or SSRP1 Increases Sensitivity of Tumor Cells to Cisplatin

Nucleolar exit of key DNA repair proteins has previously been shown to occur in response to DNA damage. Thus, relocalization of nucleolar SSRP1 and DNA-PK after cisplatin treatment suggests that SSRP1 and DNA-PK may be important factors of the intrinsic resistance of the cell to the cisplatin insult. To investigate the role of DNA-PK and SSRP1 in the response to cisplatin, we used cells stably expressing shRNA against DNA-PKcs (Fig. 7A) or SSRP1 (Fig. 7B) and monitored their sensitivity to cisplatin. The different cell lines were treated with varying concentrations of cisplatin for 24 hours. After 24 hours, a 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2*H*-tetrazolium, inner salt (MTS) cytotoxicity assay was used to monitor the cell viability. As shown in Fig. 7C and D, both knockdown of DNA-PKcs (Fig. 7C) and SSRP1 (Fig. 7D) significantly increased the sensitivity of the cells to cisplatin. Taken together, the results of our studies establish a role for DNA-PK and FACT in the response to cisplatin-induced DNA damage and in determining the sensitivity of cells to cisplatin.

Discussion

Cisplatin is a clinically useful chemotherapeutic agent that damages DNA and induces a cascade of signaling events that trigger cell cycle arrest, apoptosis, and repair processes (3). Using a mild timely dosed extraction of proteins from the nucleoplasm by the use of a detergent, we were able to unveil a nucleolar subpopulation of specific proteins (i.e., Ku70,

Ku86, DNA-PKcs, WRN, and SSRP1) that exit the nucleolus after DNA damage, suggesting the involvement of the nucleolus in the cellular response to genotoxic stress. These observations were confirmed biochemically through the analysis of purified nucleoli (Fig. 3). Nucleolar loss of Ku86 was induced by different kinds of DNA damage and was shown in several different tumor cells, indicating that the exit of DNA repair proteins from the nucleolus after DNA damage is a general mechanism. Indeed, DNA damage–induced relocalization of nucleolar WRN, p53, B23/nucleophosmin, and nucleolin has been reported (22, 31, 32). However, we discovered specificity in the cisplatin-induced nucleolar trafficking of these credentialed DNA repair proteins. Nucleolar pools of Ku/WRN and SSRP1 react differently to low or high cisplatin concentrations, respectively, and to chemical kinase inhibitors (Fig. 6C). Abundant literature describes the functions of Ku and WRN in the DNA damage response (9, 33, 34), and interestingly, Ku and WRN are physically and functionally interacting and leave the nucleolus after UV damage (34).

Ku proteins bind to sites of cisplatin-DNA adducts and to DNA strand breaks. The FACT complex (SSRP1 and Spt16) also binds to cisplatin-modified DNA, and SSRP1 leaves the nucleolus in response to high, but not low, cisplatin doses. We were able to link the egress of SSRP1 with DNA-PK by showing that the release of SSRP1 from the nucleolus is dependent on DNA-PK and can be prevented by the kinase inhibitor NU7026 or by RNA silencing of the catalytic subunit DNA-PKcs. Interestingly, Cheng et al. (12) reported that bleomycin-induced exit of WRN from the nucleolus is controlled by the c-Abl protein kinase and inhibited by the Abl kinase inhibitor STI-571.

H2AX is a histone variant critical for DNA repair. Phosphorylation of H2AX by DNA-PK facilitates the FACT-induced exchange of H2AX at sites of damaged chromatin (35). These results predict that disruption of DNA-PK or FACT function will affect DNA repair and subsequently cell survival. Indeed, we found that depletion of DNA-PKcs or SSRP1 by specific shRNAs enhanced cisplatin killing, perhaps by interfering with histone H2A exchange, a function for FACT suggested by Heo et al. (35). Because both FACT and DNA-PK are present on H2AX-containing nucleosomes (35), it is likely that the control of FACT interaction with the nucleolus occurs at the level of chromatin.

Depletion of cellular stores of DNA-PKcs and SSRP1 by shRNA clearly augments cell killing by cisplatin most likely through inhibition of DNA repair. It is not possible to say with certainty that the nucleolar DNA-PK and SSRP1 per se are responsible for the role of these proteins in the regulation of the cell sensitivity to cisplatin. However, the importance of the nucleolus in the cellular response to DNA damage was emphasized by Rubbi and Milner (32), who showed that targeted UV irradiation of the nucleolus induces apoptosis, whereas UV irradiation of the nucleoplasm that excludes the nucleolus does not induce apoptosis. In the light of this work and the results presented here, it is probable that key nucleolar proteins regulate the cellular fate after DNA damage. Furthermore, we showed that the FACT and Ku/DNA-PK nucleolar but not nucleoplasmic fractions were significantly altered by exposure to cisplatin. From these results, two nonexclusive hypotheses emerge: (*a*) the FACT and Ku/DNA-PK complexes leaving the nucleolus are different from their nucleoplasmic counterparts and do a specific function in the nucleoplasm or (*b*) the

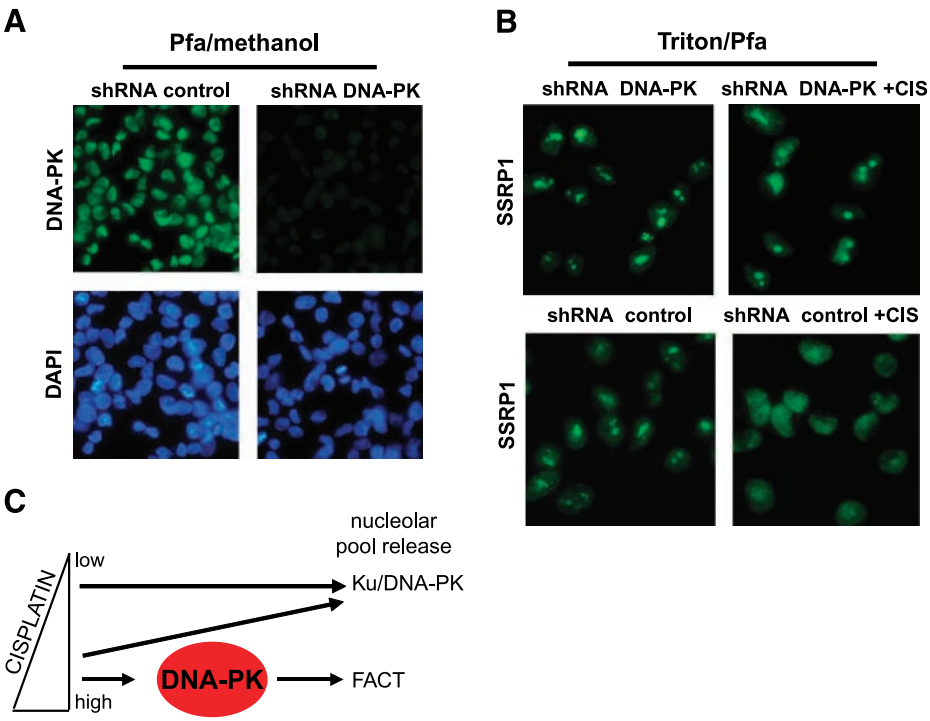

FIGURE 6. Down-regulation of DNA-PKcs prevents the cisplatin-induced exit of SSRP1 from the nucleolus. **A.** PFA/M immunofluorescence analysis of DNA-PKcs in MDA-MB-231 cells expressing DNA-PKcs or control shRNAs. Nuclei are stained with DAPI. **B.** T/PFA immunofluorescence analysis of SSRP1 in control and DNA-PKcs–silenced MDA-MB-231 cells before and after treatment with 100 μg/mL cisplatin. **C.** Schematic of the proposed mechanism of DNA-PK–dependent, DNA-PK–independent, cisplatin-induced exit of SSRP1 and Ku/WRN.

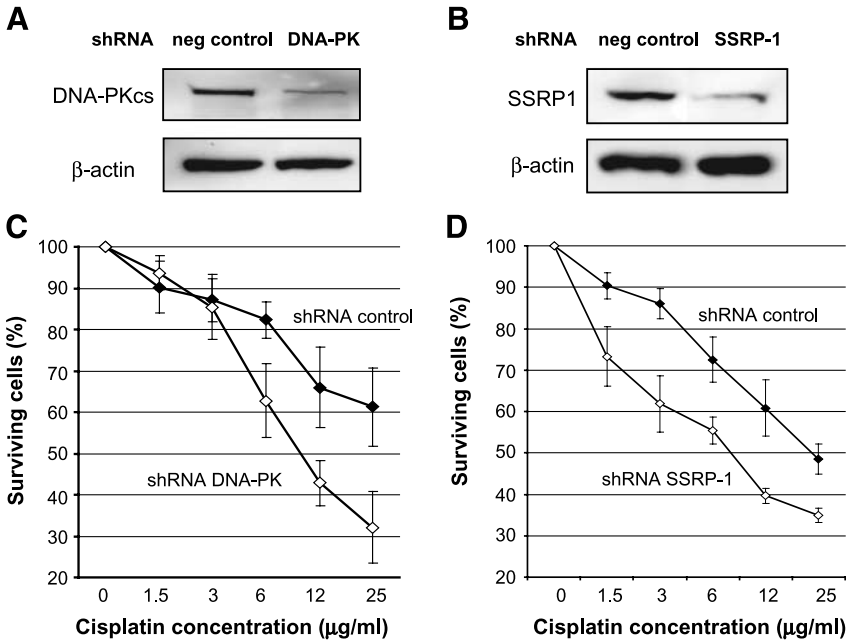

FIGURE 7. Down-regulation of DNA-PKcs or SSRP1 increases sensitivity to cisplatin. **A.** DNA-PKcs and β-actin protein levels determined by immunoblotting in MDA-MB-231 cells stably expressing a DNA-PKcs or control shRNA. **B.** SSRP1 and β-actin protein levels determined by immunoblotting in 293T cells stably expressing a SSRP1 or control shRNA. **C.** MTS assay of DNA-PK knockdown or control cells treated with different concentrations of cisplatin for 24 h. Points, mean of six independent experiments; bars, SE. $P = 0.0063$. **D.** MTS assay of SSRP1 knockdown or control cells treated as in **C**. Points, mean of four independent experiments; bars, SE. $P = 0.0082$.

cisplatin-induced exit of these proteins from the nucleolus induces the loss of a specific function in the nucleolus itself.

In summary, we showed that cisplatin treatment causes nucleolar SSRP1 to leave the nucleolus and that this exit is DNA-PK dependent. Furthermore, the sensitivity of tumor cells to cisplatin is enhanced by inhibition of DNA-PKcs and by depletion of either DNA-PKcs or SSRP1. DNA-PK inhibitors seem to increase cisplatin sensitivity *in vitro* (36) and these inhibitors are candidates for future cancer therapy (33). Thus, deciphering the function of cisplatin-induced release of key nucleolar proteins may reveal additional ways to understand the mechanisms of cisplatin sensitivity, and DNA-PK and FACT may be relevant molecular targets whose inhibition will improve the results of cisplatin therapy.

Materials and Methods

Cell Lines

MDA-MB-231 human breast adenocarcinoma cells and A2780 ovarian adenocarcinoma cells (American Type Culture Collection) were cultured in RPMI 1640 containing 10% (v/v) fetal bovine serum, 100 units/mL penicillin, and 100 μg/mL streptomycin (Invitrogen). HeLa S3 cells (American Type Culture Collection) were cultured in DMEM containing 10% (v/v) newborn calf serum (Invitrogen) and antibiotics. Linx (Open Biosystems), a packaging cell line derived from the HEK293 cell line with the viral gag, pol, and env genes stably integrated into the genome, was cultured in DMEM containing 10% (v/v) newborn calf serum (Invitrogen) and antibiotics. To maintain gag, pol, and env expression, the medium was supplemented with 100 μg/mL hygromycin.

Immunofluorescence

Cells were grown on 12-mm glass coverslips. Semiconfluent growing cells were incubated with cisplatin, actinomycin D, hydroxyurea, doxorubicin (Sigma), LY294002 (Calbiochem), wortmannin (Sigma), NU7026 (Calbiochem), or DMSO (Sigma) alone. UVC light was applied using a UV Stratalinker 2400 (Stratagene). For immunofluorescence analysis, cells were fixed with 3.7% PFA. The cells were either permeabilized after fixation with −20°C methanol for 10 min or extracted before fixation with 0.1% Triton X-100 as indicated. The cells were incubated with the following primary antibodies: mouse anti-Ku86 (NeoMarkers), mouse anti-SSRP1 (Biolegends), rabbit anti-WRN, mouse anti–DNA-PKcs (NeoMarkers), rabbit anti-p300, and mouse anti-B23 (Santa Cruz Biotechnology), all at a 1:300 dilution for 1 h at 37°C, and subsequently incubated for 30 min at 37°C with goat anti-rabbit and anti-mouse secondary antibodies conjugated to Alexa Fluor 488 or Alexa Fluor 594 (Molecular Probes) fluorochromes, respectively, at a 1:300 dilution. DNA was visualized by 4′,6-diamidino-2-phenylindole (DAPI) staining (Sigma). Fluorochromes were visualized with a Zeiss Axioskop II microscope and imaged with AxioVision 4.5 software (Zeiss).

Plasmids and Transfection

For DNA-PK, retroviral particles were produced by transfecting a retroviral pSM2 expression vector (Open Biosystems) containing a puromycin resistance marker and either a control shRNA (5′-TCTCGCTTGGGCGAGAGTAAG) or a shRNA to DNA-PKcs (5′-GGAGCTTACATGCTAATGTAT) into the Linx packaging cell line according to the manufacturer's instructions. Briefly, cells were grown in 100-mm tissue culture plates to 30% to 40% confluency and transfection mixes containing 10 μg of pSM2 DNA and 75 μg of Arrest-In transfection reagent were added to the cells. On day 3, virus-containing supernatants were added to MDA-MB-231 cells (seeded in 100-mm dishes the previous day at 70% confluence) and incubated at 37°C in the presence of 5 μg/mL polybrene. The medium containing viruses was replaced with fresh medium the

next day. Selection with puromycin was started 24 h later. Ten days after infection, puromycin-resistant colonies were expanded. Six different colonies were assayed for DNA-PK protein expression by Western blot and immunofluorescence.

To generate the pcDNA6.2-SSRP1 plasmid for expression knockdown, a small hairpin sequence specific to SSRP1 (5′-CACCACAGTACTGCGTCTGTT) was cloned into the pcDNA6.2 vector (Invitrogen). A control shRNA sequence (5′-GTCTCCACGCGCACTACATTT) was used to generate a nonsilencing control plasmid. 293T or MDA-MB-231 cells were transfected with either plasmid using the Fugene (Roche) transfection reagent according to the instructions of the manufacturer. Selection with blasticidin was started 48 h after transfection. After 10 d, blasticidin-resistant colonies were expanded and assayed for SSRP1 protein expression by Western blot and immunofluorescence.

Immunoblotting

Cells untreated or treated with cisplatin were lysed with 40 mmol/L Tris-HCl (pH 8), 200 mmol/L NaCl, 10% glycerol, 2 mmol/L EDTA, and 0.5% NP40 supplemented with a protease inhibitor mix (Roche) for 30 min at 4°C. Lysates were centrifuged at 14,000 × *g* for 10 min at 4°C and the supernatants were analyzed. Protein concentrations were determined by the Bradford protein assay (Bio-Rad). Samples were heated at 70°C for 10 min in reducing sample buffer. Subsequently, samples were separated by NuPAGE electrophoresis (Invitrogen) and transferred to nitrocellulose membranes (Bio-Rad) before immunoblotting. We used mouse antibodies directed against β-actin (Sigma), Ku86 (NeoMarkers), and SSRP1 (Biolegends) and rabbit antibodies directed against WRN (Santa Cruz Biotechnology), AKT, AKT phospho-Ser^{473} (Cell Signaling), DNA-PKcs phospho-Ser^{2056} (a kind gift from Dr. B. Chen, University of Texas Southwestern Medical Center at Dallas, Dallas, Texas; ref. 29), lamin B, and fibrillarin (Abcam). Anti-mouse and anti-rabbit secondary antibodies were from Amersham. Immunoreactive bands were visualized with SuperSignal West Pico chemiluminescent reagent (Pierce) and a Fujifilm luminescent image analyzer LAS-1000 and LAS-3000 (Fuji). Quantifications were done using the Multi Gauge V3.1 software, directly from the LAS-3000 digital acquisitions (Fuji).

Nucleolar Isolation

Nucleoli were prepared from HeLa S3 cell nuclei using the method developed by Andersen et al. (24) based on the method described by Muramatsu and Busch (37). Briefly, nuclei were resuspended in hypotonic buffer [10 mmol/L Tris (pH 8), 1.5 mmol/L $MgCl_2$, 10 mmol/L KCl] and Dounce homogenized 10 times using a tight pestle followed by centrifugation at 228 × *g* for 5 min at 4°C. The pelleted nuclei were resuspended in 3 mL of 0.35 mol/L sucrose and 10 mmol/L $MgCl_2$, layered over 3 mL of 0.35 mol/L sucrose with 0.5 mmol/L $MgCl_2$, and centrifuged at 1,430 × *g* for 5 min at 4°C. The clean pelleted nuclei were resuspended in 3 mL of 0.35 mol/L sucrose and 0.5 mmol/L $MgCl_2$ and sonicated for 2 × 30 s. The sonicated samples were layered over 3 mL of 0.88 mol/L sucrose and 0.5 mmol/L $MgCl_2$ and centrifuged at 2,800 × *g* for 10 min at 4°C. The resulting pellet contained the nucleoli, whereas the supernatant contained the nucleoplasm. Finally, the nucleoli were washed by resuspension in 0.35 mol/L sucrose and 0.5 mmol/L $MgCl_2$ followed by centrifugation at 2,000 × *g* for 2 min at 4°C. Finally, the nucleolar pellets were resuspended in 0.35 mol/L sucrose and 0.5 mmol/L $MgCl_2$. Nucleoplasmic and nucleolar fractions and nuclei were analyzed by immunoblotting.

Cytotoxicity Assay

In vitro cytotoxicity was assessed by the MTS assay using the CellTiter 96 Aqueous One Solution Proliferation Assay (Promega) according to the manufacturer's protocol. Briefly, cells expressing shRNA against DNA-PK, SSRP1, or a nonsilencing control sequence were seeded in 96-well plates at a density of 5,000 per well. After overnight incubation, cisplatin was added at the concentrations indicated. After 24 h, 20 μL of MTS were added to each well and the plates were incubated for 2 h at 37°C. The absorbance of each well was measured using an automated plate reader at 490 nm and the resulting absorbance was proportional to the number of viable cells. Values for untreated cells were considered as 100% viability. The dose-response curves were plotted as percentage absorbance of untreated cells.

Statistical Analysis

One-way ANOVA was used to analyze for statistically significant differences in nucleolar staining after treatment in the different cell lines. For the MTS assays, all values were expressed as mean ± SE. Differences between groups were tested for statistical significance using Student's *t* test. $P < 0.05$ denotes the presence of a significant difference.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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