Pharmacological targeting of RAD6 enzyme-mediated translesion synthesis overcomes resistance to platinum-based drugs

Matthew A. Sanders, Brittany Haynes, Pratima Nangia-Makker, Lisa A. Polin, and Malathy P. Shekhar

From the Karmanos Cancer Institute and the Departments of Oncology and Pathology, Wayne State University School of Medicine, Detroit, Michigan 48201

Received for publication, April 20, 2017, and in revised form, May 8, 2017 Published, Papers in Press, May 10, 2017, DOI 10.1074/jbc.M117.792192

Volume 292, number 25 JBC (2017) 10347–10363

Platinum drug-induced cross-link repair requires the concerted activities of translesion synthesis (TLS), Fanconi anemia (FA), and homologous recombination repair pathways. The E2 ubiquitin-conjugating enzyme RAD6 is essential for TLS. Here, we show that RAD6 plays a universal role in platinum-based drug tolerance. Using a novel RAD6-selective small-molecule inhibitor (SMI#9) targeting the RAD6 catalytic site, we demonstrate that SMI#9 potentiates the sensitivities of cancer cells with innate or acquired cisplatin or oxaliplatin resistance. 5-Iodoodeoxyuridine/5-chlorodeoxyuridine pulse-labeling experiments showed that RAD6 is necessary for overcoming cisplatin-induced replication fork stalling, as replication restart was impaired in both SMI#9-pretreated and RAD6B-silenced cells. Consistent with the role of RAD6/TLS in late-S phase, SMI#9-induced DNA replication inhibition occurred preferentially in mid/late-S phase. The compromised DNA repair and chemosensitization induced by SMI#9 or RAD6B depletion were associated with decreased platinum drug-induced proliferating cell nuclear antigen (PCNA) and FANC(D2) monoubiquitinations (surrogate markers of TLS and FA pathway activation, respectively) and with attenuated FANC(D2), RAD6, γH2AX, and POL η foci formation and cisplatin-adduct removal. SMI#9 pretreatment synergistically increased cisplatin inhibition of MDA-MB-231 triple-negative breast cancer cell proliferation and tumor growth. Using an isogenic HCT116 colon cancer model of oxaliplatin resistance, we further show that γH2AX and monoubiquitinated PCNA and FANC(D2) are constitutively up-regulated in oxaliplatin-resistant HCT116-OxR cells and that γH2AX, PCNA, and FANC(D2) monoubiquitinations are induced by oxaliplatin in parental HCT116 cells. SMI#9 pretreatment sensitized HCT116-OxR cells to oxaliplatin. These data deepen insights into the vital role of RAD6/TLS in platinum drug tolerance and reveal clinical benefits of targeting RAD6 with SMI#9 for managing chemoresistant cancers.

DNA-damaging agents used in cancer therapy induce a variety of toxic DNA lesions. DNA interstrand cross-links (ICLs) are the most deleterious as they block DNA replication. If unrepaird, ICLs can lead to single-strand breaks, double-strand breaks (DSBs), and chromosomal rearrangements. ICL repair is significant because it is a determining factor in the response of tumor cells to platinum-based chemotherapies. The platinum-based drugs cisplatin, carboplatin, and oxaliplatin are widely used for treatment of colorectal, lung, head and neck, ovarian, and bladder cancers and more recently for triple-negative breast cancers. Processes that allow cancer cells to survive in the face of these damaging lesions such as up-regulation of DNA damage response and DNA damage tolerance (DDT) pathways are advantageous to cancer cells. Repair of platinum-induced ICLs requires activities of translesion synthesis (TLS), Fanconi anemia (FA), and homologous repair (HR) pathways. A major component of the DDT mechanism, constitutes a critical initial step in ICL repair as it prepares the leading template strand for repair by the HR pathway. It involves DNA synthesis over the damaged template, thus enabling cells to avoid the consequences of damage-induced replication stalling and stress.

RAD6 is a fundamental component of the TLS or postreplication repair pathway, and its E2 ubiquitin-conjugating activity is critical for TLS/PRR. Loss of RAD6 catalytic activity or mutations in the RAD6 pathway has been linked to hypersensitivity to ionizing and UV radiations, as well as alkylating and DNA cross-linking agents, supporting its important role in DNA damage tolerance. RAD6 participates in both error-prone TLS and error-free damage avoidance repair, and it plays an important role in balancing both processes.
RAD6 and chemoresistance

ivation is dependent upon RAD6 and its cognate RAD18 E3 ubiquitin ligase, and it is initiated upon replication fork stalling by RAD6/RAD18-mediated monoubiquitination of proliferating cell nuclear antigen (PCNA) at Lys-164 (11, 12). PCNA monoubiquitination triggers the recruitment of POL, η, POL κ, POL λ, or REV1 TLS polymerases (13, 14). Monoubiquityated PCNA facilitates recruitment of TLS polymerases via interactions with the ubiquitin-binding motifs in the TLS polymerases (15).

The human homologs of yeast RAD6, HHR6A (referred as UBE2A or RAD6A), and HHR6B (referred as UBE2B or RAD6B) proteins share 95% amino acid identity (16) and complement the PRR and mutagenesis defects of Saccharomyces cerevisiae mutant rad6 (17). RAD6A- and RAD6B-deficient mice are nonviable, indicating RAD6 requirement for cell survival (18). We have shown previously that RAD6B is weakly expressed in normal breast cells, and its overexpression correlates with breast cancer progression (19). Constitutive overexpression of RAD6B in normal breast cells induces transformation and resistance to doxorubicin and cisplatin (19–21), whereas RAD6B-silencing renders the cells PRR-compromised and chemosensitive (21).

The FA pathway is implicated in repair of ICL lesions as cells defective for the FA pathway exhibit hypersensitivity to ICL-inducing agents (14, 22–26). In response to DNA damage and replication stress, the FA core complex proteins FANCA, FANCB, FANCC, FANCE, FANCF, FANCG, and FANCM promote monoubiquitination of FANC2D and FANCI (surrogate markers of FA activation), which are then recruited to the damaged chromatin and coordinate DNA repair (14). Overexpression or down-regulation of RAD6 has been shown to increase or decrease FANC2D monoubiquitination, respectively (27). Similarly, DNA damage-induced FANC2D monoubiquitination is attenuated in RAD18-deficient cells (28). These data reveal RAD6/RAD18 as a common downstream link between the FA/BRCA and RAD6 pathways in DNA damage-induced response. However, it is not well established whether FA pathway activation is regulated concurrently, proximally, or distally to the TLS pathway. Our results described here show a direct role for the RAD6 ubiquitin-conjugating activity in coordinated activation of FA and TLS pathways in cisplatin- or oxaliplatin-treated triple-negative breast cancer (TNBC) or colon cancer cells, respectively. Inhibition of RAD6 ubiquitin-conjugating activity with our novel RAD6-selective small-molecule inhibitor SMI#9 (29) or RAD6B silencing suppresses FANC2D and PCNA monoubiquitinizations, as well as cisplatin-induced γH2AX, PCNA, POL η, FANC2D, and RAD6 foci formation. RAD6 is required for overcoming cisplatin-induced replication fork stalling as restart of cisplatin-stalled replication forks is impaired in SMI#9-pretreated and RAD6B-silenced cells. Consistent with these results, in vitro and in vivo assays show that SMI#9 treatment inhibits proliferation of MDA-MB-231 TNBC cells and enhances their in vitro and in vivo sensitivity to cisplatin. Our data from an isogenic colon cancer model of oxaliplatin resistance show that oxaliplatin induces PCNA and FANC2D monoubiquitinizations in the parental HCT116 colon cancer cells, whereas these proteins are constitutively monoubiquitinated in their oxaliplatin-resistant (HCT116-OxR) counterpart. SMI#9 treatment enhances sensitivity of HCT116-OxR cells to oxaliplatin. These data imply a general role for the RAD6-RAD18 ubiquitination pathway in repair or tolerance of ICLs induced by platinum drugs and indicate RAD6 inhibition as a potentially novel strategy for treatment of chemoresistant TNBC and colon cancer cells.

Results

RAD6 inhibition sensitizes platinum-resistant cancer cells

To determine whether RAD6 inhibition sensitizes cells to platinating agents, we examined the effect of our RAD6-selective inhibitor SMI#9 (29) on cell survival in two cancer cell models. MDA-MB-231 TNBC cells exhibit intrinsic resistance to cisplatin (IC50 12.2 μM) and pretreatment with SMI#9 decreased the IC50 of cisplatin to 2.4 μM (Fig. 1A). Similarly, RAD6B depletion by RAD6B siRNA transfection (Fig. 1B) decreased the IC50 of cisplatin to 4.0 μM as compared with 13 μM in nontarget siRNA-transfected cells (Fig. 1C). To determine whether the SMI#9-induced effects were selective to cisplatin, we generated oxaliplatin-resistant colon cancer cells by exposing HCT116 colon cancer cells to gradually increasing doses of oxaliplatin as described under “Experimental procedures.” Oxaliplatin-resistant HCT116 cells (HCT116-OxR) exhibited an 11-fold increase in oxaliplatin IC50, as compared with their parental HCT116 cells (13.2 μM for HCT116-OxR versus 1.2 μM for HCT116 parental; Fig. 1D). SMI#9 pretreatment of HCT116-OxR cells resulted in ~40% reduction in cell viability. Thus, when results were expressed relative to control cells, the oxaliplatin sensitization effects of SMI#9 were only marginally discernible potentially due to elimination of SMI#9-sensitive oxaliplatin-resistant HCT116 subpopulations (Fig. 1E). We confirmed by colony formation assay that pretreatment with SMI#9 decreases the clonogenic potentials of MDA-MB-231 cells grown in the presence of 0.5 (p = 0.0078) or 1 μM (p = 0.0011) cisplatin (Fig. 1F). Similarly, HCT116-OxR cells seeded in media containing 10 μM oxaliplatin showed inhibition of colony-forming potential when exposed to 1 μM SMI#9 regardless of the seeding densities (control versus SMI#9; p < 0.05; Fig. 1G). These data suggest a general role for RAD6 in survival and tolerance of platinum-induced damage.

SMI#9 attenuates cisplatin-induced increases in ubiquitinated PCNA and FANC2D protein levels

PCNA monoubiquitination by the RAD6-RAD18 pathway is essential for translesion synthesis of DNA (11–15). The RAD6-RAD18 pathway has also been implicated in FANC2D monoubiquitination, an essential event in repair of ICLs by the FA pathway (27, 28, 30). To determine the role of RAD6 in cisplatin-induced DNA damage response, vehicle or SMI#9 pretreated MDA-MB-231 cells and nontarget or RAD6B siRNA-transfected MDA-MB-231 cells were treated with cisplatin for 4 h and allowed to recover for 0–24 h after cisplatin washout (Fig. 2, A and B). The steady-state levels of RAD6, PCNA, FANC2D, and γH2AX were assessed by western blotting. Because our RAD6 antibody does not distinguish RAD6A and RAD6B proteins, the immunoreactive RAD6 detected in the immunoblots and immunostains is referred as RAD6 rather than RAD6A or RAD6B (see details under “Experimental procedures”). Although the steady-state levels of
RAD6, RAD18, and nascent PCNA were not notably altered by cisplatin, bands corresponding to mono- and polyubiquitinated PCNA were detectable starting at 2 h and intensified at 24 h post-cisplatin treatment. Pretreatment with SMI#9 abrogated the cisplatin-induced increases in monoubiquitinated PCNA and partially decreased PCNA polyubiquitination (Fig. 2A). A replicate blot of PCNA analysis provides unequivocal evidence of SMI#9 suppression of cisplatin-induced PCNA monoubiquitination (Fig. 2A). These data suggest that whereas RAD6 is completely responsible for PCNA monoubiquitination, it is only partially involved in PCNA polyubiquitination as the latter modification is also mediated by other E2 ubiquitin conjugases (Ubc13/Mms2) and E3 ligases (RAD5, SHPRF, and HLTF) (31–33). The authenticity of cisplatin-induced monoubiquitinated PCNA and its inhibition by SMI#9 was confirmed by immunoprecipitation of lysates with anti-PCNA antibody and western blotting with ubiquitin antibody (Fig. 2C). The membranes were stripped and probed with PCNA antibody to estimate the levels of monoubiquitinated PCNA relative to PCNA input. In Fig. 2C, the 39-kDa anti-ubiquitin reactive PCNA is located at the upper edge of the broad immunoprecipitated PCNA band and hence is not discernible as a distinct species when the blot is reprobed with anti-PCNA antibody. SMI#9 treatment caused an ~2-fold decrease in the levels of cisplatin-induced monou-
RAD6 and chemoresistance

**A**

Post-CDDP: recovery

+ SMI#9

Rad6
Rad18
pUb-PCNA
mUb-PCNA
PCNA
PCNA (short exposure)
mUb-PCNA
PCNA
mUb-FANCd2
FANCd2
γH2AX
α-tubulin

**B**

Post-CDDP: recovery

NT  Rad6B  Rad6B SMARTpool siRNA

Rad6
Rad18
PCNA
(PCNA (long exposure)
PCNA (short exposure)
FANCd2
γH2AX
α-tubulin

**C**

CDDP: + + + + + + + + + + + + + + + +

SMI#9:  + + + + + + + + + + + + + + + +

Con 0 24 0 24 0 24 0 24 0 24 0 24 0 24 0 24 0 24

nlgG IP: + + + + + + + + + + + + + + + +

anti-PCNA IP: + + + + + + + + + + + + + + + +

WB: anti-ubiquitin

WB: anti-PCNA

**D**

Post-CDDP: recovery

SMI#9

Rad6
Rad18
mUb-PCNA
PCNA (long exposure)
PCNA (short exposure)
γH2AX
mUb-FANCd2
FANCd2
GAPDH
Lamin A/C

Cytosolic  Nuclear

**E**

OxPt: 0 2 4 8 24 0 2 4 8 24 0 2 4 8 24

HCT16 Parental

Rad6
Rad18
pUb-PCNA
mUb-PCNA
PCNA
γH2AX
mUb-FANCd2
FANCd2
α-tubulin

HCT16-OxR

Rad6
Rad18
pUb-PCNA
mUb-PCNA
PCNA
γH2AX
mUb-FANCd2
FANCd2
α-tubulin

10350  J. Biol. Chem. (2017) 292(25) 10347–10363
biquitinated PCNA levels at 24 h (Fig. 2C and bar graph). Cisplatin-induced FANCD2 monoubiquitination mirrored PCNA ubiquitination, and although SMI#9 treatment did not affect FANCD2 ubiquitination, it did, however, decrease the steady-state levels of ubiquitinated FANCD2. Cisplatin-induced γH2AX paralleled PCNA and FANCD2 profiles, becoming noticeable at 2 h of recovery post-cisplatin treatment with continued increases at 24 h indicating the presence of double-strand breaks and DNA repair. Interestingly, γH2AX profiles, becoming noticeable at 2 h in SMI#9-pretreated cells as compared with the cisplatin-treated group (Fig. 2A). Similar analysis in two independent transfections of MDA-MB-231 cells with Smartpool RAD6B siRNAs showed dramatic declines in mono- and polyubiquitinated PCNA and γH2AX and FANCD2 steady-state levels as compared with nontarget siRNA control cells, whereas RAD18 and native (unmodified) PCNA levels were minimally affected (Fig. 2B). These data further support the involvement of RAD6 in the cisplatin-induced DNA damage response.

Next, we determined whether the SMI#9-induced effects on PCNA, FANCD2, and γH2AX were associated with changes in their subcellular distribution. MDA-MB-231 cells with or without SMI#9 pretreatment were treated with cisplatin for 4 h, washed, and allowed to recover for 0, 4, or 24 h, prior to preparation of cytoplasmic and nuclear subfractions. Interestingly, no differences in the levels of RAD6, RAD18, PCNA, FANCD2, and γH2AX proteins were observed in the cytoplasmic fractions prepared from cisplatin-treated cells with or without SMI#9 pretreatment (Fig. 2D). Cisplatin-induced increases in γH2AX, monoubiquitinated PCNA, and monoubiquitinated FANCD2 were observed in both the cytoplasmic and nuclear fractions; however, SMI#9 pretreatment only affected the cisplatin-induced responses in the nuclear compartment (Fig. 2D). The steady-state levels of RAD6 and RAD18 in the nuclear fractions were lower than those in the cytoplasm, and pretreatment with SMI#9 decreased the cisplatin-induced increases in nuclear RAD6 at 4 and 24 h. Whereas the cytoplasm contained a RAD18 immunoreactive band with a molecular mass of 55 kDa, the corresponding nuclear fractions contained RAD18 with molecular masses of ~52 and 60 kDa. Although the nucleusspecific RAD18 forms were present at very low levels, the cisplatin-mediated increase in the 52-kDa RAD18 band at 4 h was attenuated by SMI#9 pretreatment (Fig. 2D). Consistent with these data, SMI#9 pretreatment preferentially decreased the peak levels of nuclear monoubiquitinated PCNA relative to total PCNA at 24 h post-cisplatin treatment (ratio of monoubiquitinated PCNA/total PCNA, 0.117 (control) versus 0.0557 (SMI#9 pretreatment)) but had no effect on cytoplasmic PCNA. SMI#9 pretreatment similarly inhibited cisplatin-induced accumulation of γH2AX in the nucleus but not in the cytoplasm. Whereas both unmodified and monoubiquitinated forms of FANCD2 were detectable in the cytoplasmic fractions, only the upper band corresponding to monoubiquitinated FANCD2 was detected in the nuclear fractions, its levels peaking at 4 h post-cisplatin treatment. Consistent with the data in Fig. 2, A and B, SMI#9 pretreatment reduced the cisplatin-induced nuclear monoubiquitinated FANCD2 (Fig. 2D). The integrity of the subfractions was confirmed by cytoplasmic and nucleus-specific markers GAPDH and lamin A/C, respectively, and authenticated the validity of these data (Fig. 2D). Compared with the cisplatin group, lamin A/C levels were low at 0 and 4 h in SMI#9-pretreated fractions but showed delayed profound accumulation at 24 h with ~12-fold higher levels in SMI#9-pretreated cells as compared with the cisplatin group (Fig. 2D). However, despite the abundant levels of lamin A/C at 24 h in SMI#9-pretreated cells, the levels of monoubiquitinated PCNA, FANCD2, and γH2AX were clearly reduced in the 24-h SMI#9-pretreated cells as compared with cisplatin alone. If normalized for lamin A/C, this would only confirm further decreases in these proteins in the SMI#9-pretreated group. At this time, the reason for differential levels of lamin A/C in SMI#9-pretreated cells as compared with cisplatin alone is not clear but suggests potential alterations in the nuclear envelope.

To determine whether oxaliplatin resistance is also associated with RAD6- and RAD18-regulated PCNA and FANCD2 ubiquitinations, HCT116 and HCT116-OxR isogenic cells were exposed to 1 or 10 μM oxaliplatin, respectively, for 0–24 h, and whole-cell lysates were analyzed by western blotting. Whereas RAD18 was minimally expressed in parental (oxaliplatin-sensitiv) HCT116 cells and only marginal increases were induced by oxaliplatin treatment, RAD18 was constitutively overexpressed in HCT116-OxR cells. RAD6 was minimally induced by oxaliplatin in parental HCT116 cells, whereas RAD6 was expressed at ~2-fold higher levels in HCT116-OxR cells as compared with the parental cells (Fig. 2F). Consistent with RAD6 pathway activation, mono- and polyubiquitinated PCNA were detected at 4 h post-oxaliplatin treatment in parental HCT116 cells. FANCD2 and γH2AX levels were similarly induced by oxaliplatin in parental HCT116 cells. Mono- and polyubiquitinated PCNA were constitutively expressed in HCT-116-OxR cells and persisted throughout oxaliplatin exposure. γH2AX was also constitutively expressed in HCT116-OxR cells.

**Figure 2. RAD6 inhibition or depletion decreases PCNA ubiquitination and FANCD2 steady-state levels.** A and B, western blot analysis of the indicated proteins from MDA-MB-231 whole-cell lysates prepared from CDDP-treated cells with or without SMI#9 pretreatment (A) or two independent transfections with SMARTpool RAD6B siRNAs or nontarget (NT) siRNA (B). A replicate blot of PCNA analysis of SMI#9 suppression of CDDP-induced PCNA monoubiquitination is shown in A. C, indicated MDA-MB-231 lysates were immunoprecipitated with anti-PCNA antibody, and the immunoprecipitates and depleted supernatants were western-blotted (WB) with anti-ubiquitin antibody. Efficacy of PCNA immunoprecipitation was verified by reprobing the stripped blots with anti-PCNA antibody. Input lysates were analyzed for PCNA steady-state levels. Arrow indicates monoubiquitinated PCNA, and asterisks indicate the positions of heavy and light chains of IgG. Graph on the right shows the relative levels of monoubiquitinated PCNA in CDDP and SMI#9 + CDDP-treated samples. D, western blot analysis of the indicated proteins in cytoplasmic and nuclear subfractions of MDA-MB-231 cells. E, western blot analysis of the indicated proteins in whole-cell lysates prepared from HCT116 or HCT116-OxR cells exposed to 1 or 10 μM oxaliplatin, respectively. All protein profiles were captured by simultaneous analysis of the indicated proteins from 4 to 20%, 7 to 18% (PCNA blot in A), and 4 to 12% (PCNA immunoprecipitation) gradient gels.
OxR cells and persisted throughout oxaliplatin exposure, mirroring the PCNA ubiquitination profile. Monoubiquitinated FANCD2 was constitutively expressed in HCT116-OxR cells; however, prolonged oxaliplatin treatment caused a sharp decline in FANCD2 levels in HCT116-OxR cells (Fig. 2E). Induction of and constitutive up-regulation of RAD6/RAD18 pathway proteins in oxaliplatin-sensitive and -resistant HCT116 isogenic cells, respectively, imply a role for the RAD6-RAD18 ubiquitination pathway in oxaliplatin resistance and importantly a general role for the RAD6 pathway in tolerance to platinating agents.

**Nuclear localization of cisplatin-induced DNA repair proteins is impaired by RAD6 inhibitor**

Because the western blotting data in Fig. 2D showed that SMI#9 pretreatment selectively affects the regulation and levels of ubiquitinated FANCD2 and PCNA in the nucleus, we examined the intracellular localization of RAD6, PCNA, and FANCD2 in MDA-MB-231 cells treated with cisplatin with or without SMI#9 pretreatment. As shown in Fig. 3A, FANCD2 is present in both the nucleus and cytoplasm of untreated cells, and cisplatin treatment resulted in FANCD2 relocalization and foci formation in the nucleus. In SMI#9-pretreated cells, the majority of anti-FANCD2 immunoreactivity was localized to the cytoplasm, and SMI#9 pretreatment compromised cisplatin-induced FANCD2 nuclear localization and foci formation (Fig. 3, A and D). Treatment with cisplatin similarly promoted RAD6 nuclear localization compared with untreated control cells, and SMI#9 pretreatment hindered cisplatin-induced nuclear distribution as cells showed perinuclear localization of RAD6 as observed for FANCD2 (Fig. 3, B and D). SMI#9 induced similar decreases in PCNA nuclear localization (Fig. 3,
A and B). Immunofluorescence analysis of γH2AX showed a dramatic increase in γH2AX nuclear foci in cisplatin-treated MDA-MB-231 cells, which was strongly inhibited and replaced by diffuse cytoplasmic anti-γH2AX reactivity in SMI#9-treated cells (Fig. 3, C and D). These data are consistent with the results from subcellular fractionation analysis that showed SMI#9-induced selective decreases in nuclear γH2AX and monoubiquitinated FANCD2 and PCNA proteins (Fig. 2D), further corroborating a role for RAD6 in platinum-induced damage response.

**RAD6 is required for overcoming replication blocks induced by cisplatin**

Our data from analysis of cisplatin-induced DNA damage response indicated the involvement of RAD6 in PCNA and FANC2D monoubiquitinations that serve as surrogate markers of PRR/TLS and FA pathway activation, respectively. To confirm whether the tolerance or intrinsic resistance of MDA-MB-231 breast cancer cells to cisplatin involves RAD6-mediated PRR/TLS, we compared the restart of stalled replication forks in cells with and without SMI#9 pretreatments by using the DNA fiber assay (35). Vehicle or SMI#9-pretreated MDA-MB-231 cells were first pulse-labeled with IdU, treated with cisplatin, and then washed and pulse-labeled with CldU (Fig. 4A). Replication fork restart was quantified by determining the total number of IdU-labeled replication foci colabeled with CldU. Control and cisplatin-treated MDA-MB-231 cells showed fibers with similar numbers of contiguous IdU and CldU signals indicating resumption of DNA synthesis or replication fork restart (Fig. 4, B and C). In SMI#9-treated cells, CldU signals were reduced when compared with control (p < 0.05), and restart of cisplatin-stalled replication forks was also significantly impaired in SMI#9-pretreated cells (p < 0.01) (Fig. 4, B and C).

The impact of RAD6 inhibition or silencing with SMI#9 or RAD6B siRNA, respectively, on global DNA replication restart was assessed in MDA-MB-231 cells after release from cisplatin treatment. Cells pretreated with SMI#9 or transfected with Smartpool RAD6B siRNAs (or the corresponding controls) were pre-labeled with IdU, treated with cisplatin, and then post-labeled with CldU. Cells were fixed, and after denaturation the cells were immunostained with IdU and CldU antibodies. Whereas vehicle-treated or nontarget siRNA-transfected cells demonstrated strong IdU/CldU contiguous labeling, SMI#9-treated or RAD6B-depleted cells showed a significant reduction in CldU-labeled cells, which was exacerbated by cisplatin treatment (Fig. 4, D–F). In the majority of these cells, the stalled IdU-labeled replication forks were not restarted as they showed negligible incorporation of CldU. Consistent with robust repair capacity of MDA-MB-231 cells, cisplatin DNA adduct levels were greatly reduced by 24 h post-CDDP recovery in control cells. Pretreatment with SMI#9 prevented the decrease of DNA adducts out to 72 h post-CDDP recovery (Fig. 4G). These data provide mechanistic support for the role of RAD6 in repair of cisplatin-induced ICLs and the enhanced sensitization of cells to platinum drugs by RAD6 inhibition (Fig. 1).

**RAD6 inhibitor SMI#9 inhibits DNA synthesis in mid/late-S phase**

Our data from Fig. 4, C and F, show that RAD6 inhibition or deficiency reduces restart of replication forks in cisplatin-resistant MDA-MB-231 cells. As RAD6 is maximally expressed in late-S phase of the cell cycle (21), sequential IdU/CldU pulse-labeling experiments were performed on control or SMI#9-pretreated cells with or without cisplatin treatment as in Fig. 4A to determine whether RAD6 deficiency affected DNA synthesis in early- and/or mid/late-S phase cells. Cells were fixed and stained with antibodies to IdU and CldU and analyzed by fluorescence microscopy. Representative cells showing the different patterns associated with DNA synthesis in early- and mid-late-S phases are shown in Fig. 5A. In early-S phase cells, numerous replication foci are evenly distributed throughout the nucleus, whereas mid-S phase cells are characterized by distribution of replication foci around the periphery of the nucleus and in the nucleolar regions (36). Late-S phase cells have few large replication foci that correspond to the replication of heterochromatic regions (36). Because the MDA-MB-231 cells used here contained only a very small population of late-S phase cells, and because late-S- and mid-S-labeling patterns are sometimes difficult to distinguish, they were scored together. Incorporation of CldU and IdU nucleotides was measured by ImageJ as the ratio of mean pixel densities of CldU to IdU for each cell (indicated by the numbers on the right in Fig. 5A). No significant differences in CldU incorporation into early-S- or mid/late-S phase cells of cisplatin-treated cells were observed as these cells showed comparable labeling intensities for both nucleotides in the merged images (Fig. 5, A and B). Pretreatment with SMI#9 significantly decreased CldU incorporation into replication foci of mid/late-S phase cells as compared with the control cells (control versus SMI#9, p = 0.0331; control versus SMI#9 + cisplatin, p = 0.0025), whereas DNA synthesis in early-S phase cells was minimally affected by SMI#9 treatment (Fig. 5, A and B). The decreases in mid/late-S phase DNA synthesis in SMI#9-treated cells were not due to a decrease in the numbers of cells in mid/late-S phases as ~1.6–3.0-fold higher numbers of mid/late-S phase cells as compared with early-S phase cells were observed in the SMI#9 groups (Fig. 5C). Approximately 20 cells for each parameter were analyzed, and our data show that SMI#9 pretreatment preferentially impairs S phase progression in the mid/late-S phase.

**Cisplatin-induced FANC2D, PCNA, and POL η foci formation are weakened by RAD6B deficiency**

Our data from Fig. 2 showed that treatment with SMI#9 or RAD6B siRNA results in a decrease in cisplatin-induced PCNA and FANC2D monoubiquitinations and overall steady-state levels of FANC2D. During repair of ICLs, FANC2D, PCNA, and TLS DNA POL η play important roles in restart of replicating forks at the damage site (37). Formation of FANC2D foci is suggested to mark the site of cisplatin damage in the chromatin and in combination with PCNA and TLS polymerase POL η to play a role in resolution of stalled replication forks (38, 39). To verify whether the failure to restart stalled replication forks in RAD6B-deficient cells is related to defective FANC2D, PCNA,
and/or POL $\gamma$ foci formation, MDA-MB-231 cells were co-immunostained with FANCD2 and POL $\gamma$ or POL $\gamma$ and PCNA antibodies. PCNA and POL $\gamma$ foci formation and colocalization were strongly induced by cisplatin in nontarget siRNA control cells but exhibited diffuse immunoreactivities in RAD6B-depleted cells (Fig. 6, A and D). Similarly, untreated nontarget and RAD6B siRNA cells showed, albeit weakly, stained FANCD2 and POL $\gamma$ foci (Fig. 6, C and D). Cisplatin treatment induced robust FANCD2 and POL $\gamma$ foci formation and colocalization in nontarget siRNA control cells; however, this was impaired in RAD6B-deficient cells (Fig. 6, C and D). Immunofluorescence staining with anti-RAD6 antibody verified a decrease in RAD6B caused by Smartpool RAD6B siRNAs (Fig. 6B). Because phosphorylation of histone H2AX is required for FANCD2 recruitment to the site of DNA damage (35) and our data showed that RAD6 inhibition attenuates FANCD2 and $\gamma$H2AX foci formation (Fig. 3, A and C), we investigated the recruitment of $\gamma$H2AX to sites of replication fork restart marked by CldU.
incorporation. MDA-MB-231 cells exposed to cisplatin with or without SMI#9 pretreatments were post-labeled with ClDU, and cells were immunostained with anti-γH2AX and anti-ClDU antibodies followed by incubation with Texas Red- and FITC-conjugated secondary antibodies, respectively. Although control and cisplatin-treated cells effectively incorporated ClDU, only cisplatin-treated cells exhibited discrete γH2AX/ClDU foci colocalization (Fig. 6, E and F). SMI#9 pretreatment reduced γH2AX/ClDU foci formation and colocalization (Fig. 6, E and F). This effect was dramatically exacerbated with SMI#9 + cisplatin treatment, suggesting the involvement of RAD6 in cisplatin-induced recruitment of DNA damage-response proteins and repair.

**SMI#9 inhibits in vivo tumor growth and enhances cisplatin sensitivity**

We have previously shown that SMI#9 decreases colony formation and survival of MDA-MB-231 triple-negative breast cancer cells (Fig. 1) (29). To determine whether SMI#9 pretreatment influences the ability of cancer cells to develop tumors, MDA-MB-231 cells were treated overnight with SMI#9, cisplatin, a combination of SMI#9 and cisplatin, or vehicle. 5 × 10⁶ viable cells assayed by trypsin blue exclusion staining were implanted into the inguinal mammary fat pads of immunodeficient female nude mice. Tumors arising from SMI#9-treated cells were significantly growth-inhibited as compared with vehicle controls (p < 0.001) (Fig. 7, A and C). Cells treated with cisplatin prior to in vivo implantation were also significantly growth-inhibited, and cells exposed to SMI#9 + cisplatin combination showed dramatic inhibition of tumor development (Fig. 7, A and C). Consistent with these data, hematoxylin and eosin (H&E) staining showed increased angiogenesis (a factor causally associated with tumor progression) (Fig. 7B, control, arrow) and regional lymph node metastasis in control xenografts. H&E analysis of xenografts derived from SMI#9 and cisplatin-treated cells showed the presence of apoptotic (Fig. 7B, short arrow) and necrotic cells as well as cells with abnormal nuclear morphology that is characteristic of mitotic catastrophe (Fig. 7B, long arrow). Immunohistochemical analysis showed robust nuclear staining for PCNA in control xenografts (Fig. 7D, top right), whereas PCNA immunoreactivity was reduced and diffusely localized in the cytoplasm of SMI#9-treated xenografts (Fig. 7D, bottom right). RAD6 staining was detected in both the nuclear and cytoplasmic compartments of control xenografts (Fig. 7D, top left), although it was detected predominantly in the cytoplasm of SMI#9 xenografts (Fig. 7D, bottom left).

To evaluate the therapeutic responses of MDA-MB-231-derived tumors to SMI#9, cisplatin, or SMI#9 + cisplatin, 5 × 10⁶ MDA-MB-231 cells were implanted into the axillary or inguinal mammary fat pads of female nude mice, and treatments with vehicle, cisplatin, SMI#9, or SMI#9 + cisplatin were initiated when the lesions were ~150 mm³. Compared with controls, MDA-MB-231 tumors responded poorly to cisplatin or SMI#9 given individually; however, in mice that received a combination of SMI#9 and cisplatin, tumor growth was significantly inhibited as compared with cisplatin, SMI#9, or vehicle controls (p < 0.0001, one-way ANOVA) (Fig. 7, E and F). H&E staining showed vascularized tumors in control and cisplatin-treated animals. Tumors from the SMI#9-treated group showed the presence of apoptotic (Fig. 7H, long arrow) and multinucleated giant cells (Fig. 7H, short arrow), which were enriched in the tumors derived from SMI#9 and cisplatin combination treatments (Fig. 7H, long and short arrows). Lymph node metastasis seen in control and cisplatin-treated mice were inhibited in mice treated with SMI#9 and cisplatin combinations (supplemental Fig. 1). Consistent with our data in Figs. 1 and 2, western blot analysis of tumor lysates showed PCNA monoubiquitination in cisplatin-treated mice but not in control or SMI#9-treated mice (Fig. 7G). RAD6 protein levels were marginally induced in tumors from cisplatin-treated mice, but no consistent treatment-induced alterations in RAD18 and FANC D2 levels were observed (Fig. 7G). These in vivo data confirm in vitro data in Figs. 1 and 2, and the data provide further support for an important role for RAD6 in tumor growth and progression and the therapeutic potential of SMI#9 in sensitizing cisplatin-resistant tumors and inhibiting tumor growth.

**Discussion**

In this study, we present evidence to support a critical role for RAD6 in repair of platinum compound-induced DNA damage and the potential therapeutic impact of SMI#9, a RAD6-selective small-molecule inhibitor, in sensitization of triple-negative breast cancer cells to cisplatin. Using a mismatch repair deficient HCT116 colon cancer isogenic model of oxaliplatin resistance, we also show that the RAD6 pathway is constitutively up-regulated in oxaliplatin-resistant cells, and their oxaliplatin sensitivity is enhanced by RAD6 inhibition. This chemosensitization by SMI#9 or RAD6B siRNA is associated with attenuation of platinum-induced PCNA and FANC D2 ubiquitinations, surrogate markers of TLS and FA pathway activation, respectively, providing support for the importance of RAD6 catalytic activity in ICL repair and tolerance of platinum-based drugs. These data are in agreement with those of Song et al. (28) who showed regulation of these events by the ubiquitin ligase RAD18, an enzyme whose activity requires the ubiquitin-conjugating catalytic activity of RAD6.

γH2AX and FANC D2 participate in the same pathway in response to ICL damage (40), and γH2AX foci and FANC D2 activation provide an indirect measurement of DSBs (41). Our data show that γH2AX, FANC D2, and PCNA ubiquitinations are concurrently activated during cisplatin-induced damage response, and RAD6 inhibition attenuates all these events. This
RAD6 and chemoresistance

A

B

C

D

E

F

J. Biol. Chem.
(2017) 292(25) 10347–10363
RAD6 and chemoresistance

is further corroborated by not only the failure to form γH2AX and FANCD2 nuclear foci in SMI#9-treated cells but also by the localization of γH2AX, FANCD2, and RAD6 immunoreactivities in the nuclear periphery and cytoplasm of RAD6-inhibited cells. γH2AX is regarded as a canonical marker for DSBs; however, the decrease in γH2AX levels and foci noted in our study corresponds to a collapse rather than a completion of repair as evidenced by decreased repair of cisplatin-DNA adducts in SMI#9-treated cells. This is further supported by our data that show RAD6 inhibition hampers cisplatin-induced colocalization of γH2AX with CldU-labeled replication fork restart sites, implicating a central role for RAD6 in assembly/recruitment of repair factors and ICL processing. Subcellular fractionation analysis showed that cisplatin-induced monoubiquitinated FANC2 peaks before monoubiquitinated PCNA, a key event for the recruitment of TLS polymerases (11, 12, 27, 28). SMI#9 decreased the levels of both cisplatin-induced FANC2 and PCNA ubiquitinations, indicating that RAD6 catalytic activity is required for these modifications but that RAD6-induced PCNA monoubiquitination is not a prerequisite for FANC2 activation. These findings are in agreement with Fu et al. (39) who demonstrated a rise in monoubiquitinated FANC2 prior to monoubiquitinated PCNA. Studies using replication-competent Xenopus cell-free extracts similarly showed that TLS events occur distal to FANC2-FANCI activation when replication forks converge on ICLs (42), but they differ from others that showed PCNA ubiquitination is a prerequisite for RAD18-induced FANC2 monoubiquitination (28).

We have taken advantage of IdU and CldU thymidine analogs to analyze the effects of RAD6 inhibition or depletion on DNA replication during and after replication stalling with cisplatin (43). Consistent with the intrinsic cisplatin resistance of BRCA1 wild-type MDA-MB-231 cells, these cells efficiently resume DNA replication after exposure to cisplatin; however, this is strongly impeded by RAD6 inhibition or silencing. IdU and CldU incorporation experiments also revealed that the DNA replication inhibition by SMI#9 or RAD6B siRNA is selective to mid-S/late-S phase cells. Replication of heterochromatin segments occurs in late-S phase (44), and inhibition of heterochromatin replication may be critical due to their temporal proximity to mitosis and increased susceptibility to mutagenic damage because of their location at the nuclear periphery (45). The proportion of HR decreases as cells progress to late-S and G2 phases (46), whereas RAD6 is maximally expressed in late-S/G2 phases of the cell cycle (21), and SMI#9 induces G2/M arrest in human breast cancer cells (29), providing further support for an important role for RAD6 in synthesis of DNA in late-S phase. Although IdU was efficiently incorporated in initial DNA labeling, continuation of DNA synthesis as measured by CldU incorporation was reduced in both SMI#9 and RAD6B siRNA cells, albeit more severely in RAD6B siRNA cells. These data suggest that RAD6 is not required for initiation of DNA synthesis but is necessary for replication progression in heterochromatin. Because the impact on DNA replication progression was more severe with RAD6B siRNA as compared with SMI#9, it is plausible that RAD6 may have other roles that are independent of its catalytic activity. Our data also suggest that the intra-S checkpoint activated by cisplatin-induced DNA damage in BRCA1 wild-type breast cancer cells is not robust but is strengthened in mid/late-S phase by the RAD6 inhibitor. TLS occurs both in S and G2 phases of the cell cycle (47) and is dependent upon the activity of damage-induced PCNA monoubiquitination by RAD6/RAD18 (11, 12). Monoubiquitination of PCNA plays a critical role in switching replicative polymerases with Y family TLS polymerases POL η, POL ι, POL κ, or POL ζ (48). We analyzed POL η recruitment into foci formation as it has the highest affinity for PCNA and is the first TLS polymerase that is recruited to stalled replication forks to initiate nucleotide insertion at the damaged template (49). Consistent with the decreases in cisplatin-induced PCNA and FANC2 ubiquitinations in RAD6-inhibited or RAD6B-depleted cells, the robust PCNA, FANC2, and POL η foci signals induced by cisplatin in control cells were dramatically reduced in RAD6B siRNA cells. RAD18 is the primary RAD6-regulated E3 ligase implicated in TLS. However, recently Hung et al. (50) reported that monoubiquitination of histone H2B facilitates lesion bypass and fork recovery. Because RAD6 catalytic activity is also required for Bre1 E3 ligase-regulated histone H2B monoubiquitination, these data further substantiate the importance of RAD6 in fork recovery and gap-filling repair following damage.

Clinical data suggest that patients with defects in BRCA1 and BRCA2 have better overall responses to platinum-based therapies because of the critical roles these proteins play in homologous recombination repair of cisplatin-induced DNA damage (51–54). The augmentation of BRCA1 wild-type breast cancer cell cisplatin sensitivity by RAD6 inhibition not only supports the important role of RAD6/TLS in platinum-induced ICL repair and tumor survival but also brings to light the potential therapeutic advantage of leveraging this target for treatment of HR-competent triple-negative breast cancers (supplemental Fig. 2).

Experimental procedures

Cell culture

MDA-MB-231 human triple-negative breast cancer cells and HCT116 human colon cancer cells were purchased from American Type Culture Collection and maintained in Dulbecco's minimal essential medium/F-12 (Invitrogen) supplemented with 5% fetal bovine serum. Both cell lines were authenticated by ATCC by short tandem repeat DNA profiling. Cells were expanded, and multiple aliquots were cryopre-
served. Cells were used within 10–15 passages or within 3 months. To generate the isogenic model of acquired oxaliplatin resistance, HCT116 cells were exposed to gradually increasing concentrations of oxaliplatin to select HCT116 cells capable of tolerating 10 μM oxaliplatin (HCT116-OxR cells).

**Cell survival assays**

MDA-MB-231, parental HCT116, or HCT116-OxR cells were seeded at 2500–5000 cells/well in 96-well dishes, and the sensitivities of MDA-MB-231 cells to cisplatin or HCT116 and HCT116-OxR cells to oxaliplatin were measured at the indi-
cated doses. To determine effect of the RAD6-selective small-molecule inhibitor SMI#9 (29) on cisplatin or oxaliplatin responses, cells were pretreated overnight with 5 μM SMI#9 (29) or vehicle prior to addition of cisplatin (MDA-MB-231) or oxaliplatin (HCT116-OxR). For depletion of RAD6B, cells were transfected with 20 pmol of SMARTpool containing four RAD6B-specific siRNAs or nontarget siRNA (described below) and treated with cisplatin. Cell viability was assessed at 72 h by MTT assay and RAD6 expression by western blot analysis. Experiments were done in quadruplicates and results expressed from at least two independent experiments.

**Colony formation assays**

MDA-MB-231 cells were treated with vehicle, SMI#9, cisplatin, or a combination of SMI#9 and cisplatin for 24 h and replated at 100 cells per well in 24-well plates for colony formation. HCT116-OxR cells maintained in 10 μM oxaliplatin were treated with vehicle or SMI#9 and reseeded at 400, 200, 100, or 10 cells per well in 24-well plates. Colony formation was detected by crystal violet staining and assessed with GelCount™ Oxford Optronix and CHARM algorithm with a minimum diameter of 100 μm set as the threshold for colony classification. Colony-forming efficiency was expressed relative to control cells.

**DNA damage-response assays**

MDA-MB-231 cells were treated with cisplatin (10 μM) for 4 h, washed, and allowed to recover in drug-free media for 0–24 h. To assess the involvement of RAD6 in cisplatin-induced damage response, cells were either pretreated with 5 μM SMI#9 (or vehicle) or transfected with 20 pmol of SMARTpool RAD6B siRNAs or nontarget siRNA prior to cisplatin treatment. Cells were lysed at various times of recovery for immunoblot analysis.

**RAD6B knockdown with siRNA**

siRNA-targeting RAD6B/UBE2B (On-Target Plus SMARTpool containing four siRNAs: GGAAUGCAGUAAUUAUGG; GAACGGAUCUAAACAGUC; GAGUUCGCCAUUGUUGA; and UAGAAAUCCUUCAGAUGC) was obtained from Dharmacon (Lafayette, CO). Control siRNA (UUCUCGGAACGUGUCAGCU) was obtained from Qiagen, Inc. (Valencia, CA). Transfections were performed with 20 pmol of siRNA and Metafectene SI+ (Bionteck Laboratories GmbH, Germany).

**Immunoblot analysis**

Cisplatin- or oxaliplatin-treated cells were lysed in Triton X-100 lysis buffer (1% Triton X-100, 150 mM NaCl, 50 mM Tris-HCl, pH 7.4) supplemented with protease inhibitor mixture (Roche Applied Science) and 1 mM PMSF, sodium orthovanadate, and sodium pyrophosphate. Protein-matched aliquots were subjected to SDS-PAGE and immunoblot analysis of RAD6 (19), PCNA (Dako, CA), FANCID2 (Novus Biologicals, CO), α-tubulin (Sigma), β-actin (Sigma), γH2AX (BioLegend, CA), and RAD18 (Imgenex Corp., CA). We previously demonstrated that the RAD6B gene is overexpressed in breast cancer by transcript sequencing and RAD6B shRNA transfections (19, 55). However, because the peptide we used to generate RAD6B antibody is conserved 91% in human RAD6A, the RAD6 proteins detected by our antibody will not distinguish RAD6A and RAD6B proteins (19). Hence, the immunoreactive RAD6 detected from immunoblot and immunostaining is referred to as RAD6 rather than RAD6A or RAD6B. To isolate the observed changes to RAD6B, transfections were performed with RAD6B-specific siRNAs and analyzed by western blotting or immunostaining with the anti-RAD6 antibody. For analysis of cell subfractions, cells were lysed using a cytosolic/nuclear protein fractionation kit (MBL International). For PCNA ubiquitination analysis, lysates were subjected to immunoprecipitation with anti-PCNA or corresponding normal IgG, and captured immune complexes were analyzed by immunoblotting with anti-ubiquitin antibody. The membranes were stripped and reprobed with PCNA antibody to verify the PCNA inputs in the samples.

**Immunofluorescence staining**

MDA-MB-231 cells transfected with SMARTpool RAD6B siRNAs (or nontarget siRNA) or treated overnight with SMI#9 prior to cisplatin treatment were fixed in 10% buffered formalin and permeabilized with methanol/acetone (1:1, v/v). Slides were incubated with RAD6/PCNA, FANCID2/PCNA, PCNA/POL η (Abcam, MA), FANCID2/PCNA, or γH2AX antibodies followed by FITC- and Texas Red-conjugated rabbit and mouse secondary antibodies, respectively (Molecular Probes). Nuclei were counterstained with 4’,6-di-amidino-2-phenylindole (DAPI). Slides were also stained in the absence of primary antibody or with isotype-matched nonimmune IgG to assess nonspecific reactions. Images were collected on an Olympus BX60 microscope equipped with a Sony high resolution/sensitivity CCD video camera and SlideBook software. The results shown are representative of data collected from ~30–75 cells in three to five fields and two independent experiments.

**Figure 7. SMI#9 treatment enhances sensitivity to cisplatin and inhibits tumor growth.** A–D, MDA-MB-231 cells were treated overnight with vehicle, SMI#9, CDDP, or SMI#9 + CDDP, and 5 × 10^6 viable cells were implanted into the mammary fat pads of female nude mice. A, tumor volumes (mean ± S.E.); C, vertical scatter plots of mass of excised tumors at sacrifice. B, H&E analysis of tumors. Inset panels show enlarged images. Arrow in control shows angiogenesis; long and short arrows in SMI#9 and SMI#9 + CDDP indicate mitotic catastrophe and apoptosis, respectively. D, immunohistochemical staining of RAD6 in control (top left panel) and SMI#9 (bottom left panel) groups and PCNA in control (top right panel) and SMI#9 (bottom right panel) groups. Inset panels show enlarged images of RAD6 and PCNA distribution in the control and SMI#9-treated groups. Original magnification ×40. E–H, MDA-MB-231 cells (5 × 10^6) were implanted orthotopically and when the tumors reached ~150 mm^3, mice were randomly assigned to the following groups: vehicle control, CDDP (4 mg/kg body weight, once/week, intraperitoneal); SMI#9 (2.5 mg/kg body weight, twice/week, intratumoral); or a combination of SMI#9 and CDDP. E, tumor volumes (mean ± S.E.); F, vertical scatter plots of mass of excised tumors at time of sacrifice at 24 days. G, western blot analysis of the indicated proteins in representative tumor lysates from each treatment group. The position of monoubiquitinated PCNA in CDDP-treated tumors is indicated by an arrow and verified in the adjacent blot. *Indicates nonspecific band recognized by anti-mouse antibodies. H, H&E analysis. Arrow in control shows angiogenesis; long and short arrows in SMI#9 and SMI#9 + CDDP indicate apoptosis and multinucleated giant cells, respectively. Original magnification ×40. Data are analyzed by one-way ANOVA and two-tailed Student’s t test.
DNA fiber assay for DNA replication restart studies

Exponentially growing MDA-MB-231 cells were pulsed with 200 μM IdU for 45 min, washed with PBS, and pulsed with 200 μM CldU for 45 min (56). To analyze the effect of cisplatin on replication restart, IdU-labeled cells were treated with 5 μM cisplatin (or vehicle) for 1 h, washed, and pulsed with CldU. To determine the role of RAD6 in replication fork-restart following cisplatin-induced damage, cells were pretreated with 1 μM SMI#9 or vehicle for 12 h prior to IdU/CldU labeling and cisplatin treatment. To prepare DNA fiber spreads, cell suspensions were spotted onto glass slides and mixed with lysis buffer (100 mM Tris-HCl, pH 8, 50 mM EDTA, 1% SDS) for 5 min (56). Slides were tilted to spread the suspension, and the air-dried DNA spreads were fixed in methanol/acetic acid (3:1, v/v). For immunostaining, slides were rinsed with PBS, and DNA was denatured with 1.5 N HCl for 45 min at room temperature. Slides were washed and incubated in 5% BSA blocking buffer prior to incubation with mouse anti-idU (Pierce) and rat anti-CldU (Pierce) antibodies and corresponding Texas Red- or FITC-conjugated secondary antibodies. Images were taken from randomly selected fields of untangled DNA fibers and analyzed by ImageJ software. Approximately 35–75 individual fibers were analyzed for each experiment, and the average of three independent experiments was presented.

Immunofluorescence staining of replication foci

MDA-MB-231 cells were labeled with 100 μM IdU and CldU as described above for DNA fiber analysis. Cells were transfected with SMARTpool RAD6B siRNAs (or nontarget siRNA) or treated with SMI#9 prior to IdU/CldU labeling and cisplatin treatment. Cells were fixed in methanol/acetone (1:1, v/v), and DNA denaturation and immunostaining were performed as described for DNA fiber analysis. Approximately 35–75 individual cells were analyzed for each experiment, and the average of three independent experiments was presented. For localization of γH2AX with CldU nucleotide, CldU-labeled cells were fixed in methanol/acetone, blocked with 5% BSA, and incubated with anti-γH2AX antibody. Slides were washed and incubated with Texas Red-conjugated anti-rabbit antibody. To stain CldU nucleotide, slides were fixed in 4% paraformaldehyde, 0.2% Triton X-100, DNA-denatured, and stained with anti-CldU antibody as described above. Approximately 30 cells from five to nine fields were analyzed for CldU/γH2AX foci colocalization by ImageJ.

Measurement of cisplatin DNA adduct removal

The repair kinetics of cisplatin DNA adducts was assessed by ELISA using an anti-cisplatin modified DNA antibody (clone CP9/19, Abcam) (57). Genomic DNA isolated at various times of recovery from MDA-MB-231 cells exposed to cisplatin with or without SMI#9 pretreatment was coated on 96-well DNA-BIND ELISA plates in triplicate and blocked with 2% rabbit serum prior to incubation with CP9/19 antibody. Plates were washed, incubated with HRP-conjugated goat anti-rat antibody, and developed with ultra-3,3′,5,5′-tetramethylbenzidine (Pierce). Absorbance was measured at 450 nm. The percentages of DNA adducts were calculated relative to the 0-h time point that was set at 100% (58).

Orthotopic tumor growth assays

MDA-MB-231 cells were treated overnight with vehicle, SMI#9 (5 μM), cisplatin (10 μM), or SMI#9 + cisplatin, and 5 × 10⁶ cells suspended in 100 μl of Matrigel were injected orthotopically into the fat pads of the inguinal mammary glands of female nude mice. Mice were sacrificed at 41 days postimplantation. To assess the therapeutic responses of tumors to SMI#9, cisplatin, or a combination of SMI#9 and cisplatin, 5 × 10⁶ MDA-MB-231 cells were orthotopically implanted into the axillary or inguinal mammary gland fat pads. When the tumors reached ~150 mm³, mice were randomly assigned to the following groups: vehicle control, cisplatin (4 mg/kg body weight, once/week, intraperitoneal), SMI#9 (2.5 mg/kg body weight, twice/week, intratumoral), or a combination of SMI#9 and cisplatin. Tumors were measured with calipers twice a week and tumor volumes calculated using the formula (length × width²)/2. Mice were sacrificed at 24 days postimplantation 2 days after the last administration, and tumor growth inhibition (a measure of anti-tumor effectiveness) was calculated using the mean tumor burden mass for each group. The in vivo studies were conducted in accordance with the institutional animal care and use committee (IACUC) guidelines of Wayne State University. Excised tumors were either sonicated in lysis buffer supplemented with protease and phosphatase inhibitors for western blot analysis of RAD6, RAD18, PCNA, FANCD2, and β-actin, or fixed and paraffin-embedded for histological analysis. Four-micron sections were stained with hematoxylin and eosin (H&E) or incubated with anti-RAD6 (19) or anti-PCNA antibodies followed by the appropriate biotinylated secondary antibody and HRP-conjugated streptavidin (Vector Laboratories). Nuclei were counterstained with hematoxylin. Control sections were stained with corresponding normal IgG or secondary antibody only.

Statistical analysis

Statistical analysis was performed with GraphPad Prism. Results are presented as the mean ± S.D. or S.E. and were analyzed by two-tailed Student’s t-test and one-way analysis of variance (ANOVA). Statistical significance was accepted at a p value of < 0.05.

Author contributions—M. P. S. conceived, designed, and coordinated the project and wrote the paper with M. A. S. and B. H. M. A. S. and B. H. conducted most of the experiments and analyzed the results. M. A. S., P. N. M., L. A. P., and M. P. S. conducted the in vivo experiments and analyzed the data. All authors reviewed the results and approved the final version of the manuscript.

References

1. Aguilería, A., and Gómez-González, B. (2008) Genome instability: a mechanistic view of its causes and consequences. Nat. Rev. Genet. 9, 204–217
2. Helleday, T., Petermann, E., Lundin, C., Hodgson, B., and Sharma, R. A. (2008) DNA repair pathways as targets for cancer therapy. Nat. Rev. Cancer 8, 193–204
3. Kartalou, M., and Essigmann, J. M. (2001) Mechanisms of resistance to cisplatin. Mutat. Res. 478, 23–43
4. Zamble, D. B., and Lippard, S. J. (1995) Cisplatin and DNA repair in cancer chemotherapy. Trends Biochem. Sci. 20, 435–439
RAD6 and chemoresistance

5. Kelland, L. (2007) The resurgence of platinum-based cancer chemotherapy. *Nat. Rev. Cancer* 7, 573–584

6. Salehan, M. R., and Morse, H. R. (2013) DNA damage repair and tolerance: a role in chemotherapeutic drug resistance. *Br. J. Biomed. Sci.* 70, 31–40

7. Dronkert, M. L., and Kanaar, R. (2001) Repair of DNA interstrand cross-links. *Mutat. Res.* 486, 217–247

8. Haynes, B., Saadat, N., Myung, B., and Shekhar, M. P. (2015) Crosstalk between translation synthesis, Fanconi anemia network, and homologous recombination repair pathways in interstrand DNA cross-link repair and development of chemoresistance. *Mutat. Res. Rev. Mutat. Res.* 763, 258–266

9. Ulrich, H. D. (2005) The RAD6 pathway: control of DNA damage bypass and mutagenesis by ubiquitin and SUMO. *Chembiochem.* 6, 1735–1743

10. Lawrence, C. (1994) The RAD6 DNA repair pathway in *Saccharomyces cerevisiae*: what does it do, and how does it do it? *BioEssays* 16, 253–258

11. Kannoouche, P. L., and Lehmann, A. R. (2004) Ubiquitination of PCNA and the polymerase switch in human cells. *Cell Cycle* 3, 1011–1013

12. Kannoouche, P. L., Wing, J., and Lehmann, A. R. (2004) Interaction of human DNA polymerase η with monoubiquitinated PCNA: a possible mechanism for the polymerase switch in response to DNA damage. *Mol. Cell* 14, 491–500

13. Ohmori, H., Friedberg, E. C., Fuchs, R. P., Goodman, M. F., Hanaoka, F., Hinkle, D., Kunkel, T. A., Lawrence, C. W., Livneh, Z., Nohmi, T., Prakash, L., Prakash, S., Todo, T., Walker, G. C., Wang, Z., and Woodgate, R. (2001) The Υ-family of DNA polymerases. *Mol. Cell* 8, 7–8

14. Moldovan, G. L., and D’Andrea, A. D. (2009) How the fanconi anemia pathway guards the genome. *Annu. Rev. Genet.* 43, 223–249

15. Bienko, M., Green, C. M., Crosseto, N., Rudolf, F., Zapat, G., Coull, B., Kannoouche, P., Wider, G., Peter, M., Lehmann, A. R., Hofmann, K., and Dikic, I. (2005) Ubiquitin-binding domains in Υ-family polymerases regulate translation synthesis. *Science* 310, 1821–1824

16. Koken, M. H., Smit, E. M., Jaspers-Dekker, I., Oostra, B. A., Hagemeijer, A., Hoogerbrugge, J. W., van Cappellen, W. A., Hoeijmakers, J. H., and Kanaar, R. (2001) Preservation of two human homologs of the yeast DNA repair gene RAD6. *Genomics* 8865–8869

17. Koken, M. H., Smit, E. M., Jaspers-Dekker, I., Hoogerbrugge, J. W., van Cappellen, W. A., Hoeijmakers, J. H., and Kanaar, R. (1992) Localization of two human homologs, HR6A and HR6B, of the yeast DNA repair gene RAD6 to chromosomes Xq24-q25 and 5q23-q31.

18. Koken, M. H., Smit, E. M., Jaspers-Dekker, I., Hoogerbrugge, J. W., van Cappellen, W. A., Hoeijmakers, J. H. (1991) Structural and functional conservation of two human homologs of the yeast DNA repair gene RAD6. *Proc. Natl. Acad. Sci. U.S.A.* 88, 8685–8689

19. Roest, H. P., Baarends, W. M., de Wit, J., van Klaeveren, J. W., Wassenaar, E., Hoogerbrugge, J. W., van Cappellen, W. A., Hoeijmakers, J. H., and Grootegeest, J. A. (2004) The ubiquitin-conjugating DNA repair enzyme HR6A is a maternal factor essential for early embryonic development in *mice*. *Cell Mol. Life Sci.* 54, 5485–5495

20. Shekhar, M. P., Lyakhovich, A., Visscher, D. W., Heng, H., and Kondrat, N. (2002) RAD6 overexpression induces multineucleation, centrosome amplification, abnormal mitosis, aneuploidy, and transformation. *Cancer Res.* 62, 2115–2124

21. Lyakhovich, A., and Shekhar, M. P. (2003) Supramolecular complex formation between Rad6 and proteins of the p53 pathway during DNA damage-induced response. *Mol. Cell. Biol.* 23, 2463–2475

22. Lyakhovich, A., and Shekhar, M. P. (2004) RAD6B overexpression confers chemoresistance: RAD6 expression during cell cycle and its redistribution to chromatin during DNA damage-induced response. *Oncogene* 23, 3097–3106

23. Kennedy, R. D., and D’Andrea, A. D. (2005) The Fanconi Anemia/BRCA pathway: new faces in the crowd. *Genes Dev.* 19, 2925–2940

24. Niednerhofer, L. J., Lalai, A. S., and Hoeijmakers, J. H. (2005) Fanconi anemia (cross)linked to DNA repair. *Cell* 123, 1191–1198

25. Patel, K. J., and Joenje, H. (2007) Fanconi anemia and DNA replication repair. *DNA Repair* 6, 885–890

26. Thompson, L. H., and Hinz, J. M. (2009) Cellular and molecular consequences of defective Fanconi anemia proteins in replication-coupled DNA repair: mechanistic insights. *Mutat. Res.* 668, 54–72

27. Wang, W. (2007) Emergence of a DNA-damage response network consisting of Fanconi anaemia and BRCA protein. *Nat. Rev. Genet.* 8, 735–748
46. Karanam, K., Kafri, R., Loewer, A., and Lahav, G. (2012) Quantitative live cell imaging reveals a gradual shift between DNA repair mechanisms and a maximal use of HR in mid S phase. *Mol. Cell* **47**, 320–329

47. Diamant, N., Hendel, A., Vered, I., Carell, T., Reissner, T., de Wind, N., Geacinov, N., and Livneh, Z. (2012) DNA damage bypass operates in the S and G2 phases of the cell cycle and exhibits differential mutagenicity. *Nucleic Acids Res.* **40**, 170–180

48. Lehmann, A. R., Niimi, A., Ogi, T., Brown, S., Sabbioneda, S., Wing, J. F., Kannouche, P. L., and Green, C. M. (2007) Translesion synthesis: Y-family polymerases and the polymerase switch. *DNA Repair* **6**, 891–899

49. Knipscheer, P., Räschle, M., Smogorzewska, A., Enoiu, M., Ho, T. V., Schärer, O. D., Elledge, S. J., and Walter, J. C. (2009) The Fanconi anemia pathway promotes replication-dependent DNA interstrand cross-link repair. *Science* **326**, 1698–1701

50. Hung, S.-H., Wong, R. P., Ulrich, H. D., and Kao, C.-F. (2017) Monoubiquitylation of histone H2B contributes to the bypass of DNA damage during and after DNA replication. *Proc. Natl. Acad. Sci. U.S.A.* **114**: E2205–E2214

51. Foulkes, W. D. (2006) BRCA1 and BRCA2: chemosensitivity, treatment outcomes and prognosis. *Fam. Cancer* **5**, 135–142

52. Sakai, W., Swisher, E. M., Karlan, B. Y., Agarwal, M. K., Higgins, J., Friedman, C., Villegas, E., Jacquemont, C., Farrugia, D. J., Couch, F. J., Urban, N., and Taniguchi, T. (2008) Secondary mutations as a mechanism of cisplatin resistance in BRCA2-mutated cancers. *Nature* **451**, 1116–1120

53. Silver, D. P., Richardson, A. L., Eklund, A. C., Wang, Z. C., Szallasi, Z., Li, Q., Juul, N., Leong, C. O., Calogrias, D., Buraimoh, A., Fatima, A., Gelman, R. S., Ryan, P. D., Tung, N. M., De Nicolo, A., et al. (2010) Efficacy of neoadjuvant cisplatin in triple-negative breast cancer. *J. Clin. Oncol.* **28**, 1145–1153

54. Liu, M., Mo, Q. G., Wei, C. Y., Qin, Q. H., Huang, Z., and He, J. (2013) Platinum-based chemotherapy in triple-negative breast cancer: A meta-analysis. *Oncol. Lett.* **5**, 983–991

55. Shekhar, M. P., Gerard, B., Pauley, R. J., Williams, B. O., and Tait, L. (2008) Rad6B is a positive regulator of β-catenin stabilization. *Cancer Res.* **68**, 1741–1750

56. Yokochi, T., and Gilbert, D. M. (2007) Replication labeling with halogenated thymidine analogs. *Curr. Protoc. Cell Biol.* Chapter 22, Unit 22.10

57. Jazaeri, A. A., Shibata, E., Park, J., Bryant, J. L., Conaway, M. R., Modesitt, S. C., Smith, P. G., Milhollen, M. A., Berger, A. J., and Dutta, A. (2013) Overcoming platinum resistance in preclinical models of ovarian cancer using the neddylation inhibitor MLN4924. * Mol. Cancer Ther.* **12**, 1958–1967

58. Arora, S., Kothandapani, A., Tillison, K., Kalman-Maltese, V., and Patrick, S. M. (2010) Downregulation of XPF-ERCC1 enhances cisplatin efficacy in cancer cells. *DNA Repair* **9**, 745–753