Cell Cycle 12:13, 2033–2041; July 1, 2013; © 2013 Landes Bioscience

Zinc finger protein 668 interacts with Tip60 to promote H2AX acetylation after DNA damage

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Keywords: ZNF668, Tip60, H2AX acetylation, DNA damage repair

Many tumor suppressors play an important role in the DNA damage pathway. *Zinc finger protein 668 (ZNF668)* has recently been identified as one of the potential tumor suppressors in breast cancer, but its function in DNA damage response is unknown. Herein, we report that ZNF668 is a regulator of DNA repair. ZNF668 knockdown impairs cell survival after DNA damage without affecting the ATM/ATR DNA-damage signaling cascade. However, recruitment of repair proteins to DNA lesions is decreased. In response to IR, ZNF668 knockdown reduces Tip60-H2AX interaction and impairs IR-induced histone H2AX hyperacetylation, thus impairing chromatin relaxation. Impaired chromatin relaxation causes decreased recruitment of repair proteins to DNA lesions, defective homologous recombination (HR) repair and impaired cell survival after IR. In addition, ZNF668 knockdown decreased RPA phosphorylation and its recruitment to DNA damage foci in response to UV. In both IR and UV damage responses, chromatin relaxation counteracted the impaired loading of repair proteins and DNA repair defects in ZNF668-deficient U2OS cells, indicating that impeded chromatin accessibility at sites of DNA breaks caused the DNA repair defects observed in the absence of ZNF668. Our findings suggest that ZNF668 is a key molecule that links chromatin relaxation with DNA damage response in DNA repair control.

Introduction

DNA damage response (DDR) encompasses multiple signal transduction pathways that sense DNA damage, induce cell cycle arrest, repair DNA and, in the case of irreparable damage, activate cell death mechanisms.[1,2] Maintaining the fidelity of such pathways requires various tumor suppressor proteins such as *ATM, ATR, BRCA1, BRCA2, CHK2* and *p53*.[3-7] Loss-of-function mutations to these tumor suppressors cause defective DNA repair, invariably leading to genetic instability and increased susceptibility to tumor formation. Therefore, understanding DDR protein functions helps link specific mutations to their effects on genetic stability, ultimately improving tumor profiling and therapeutic treatment.

DNA damage in the form of double-strand breaks (DSBs) can arise from exogenous agents such as ionizing radiation (IR) and chemotherapeutic drugs or from naturally occurring cellular processes such as meiotic recombination.[2,7] In response to DSBs, either the homologous recombination (HR) or non-homologous end joining (NHEJ)-mediated repair pathway becomes activated, depending in part on cell cycle phase.[8-10] Both ATM and ATR of the phosphatidylinositol 3-kinase-related kinases (PIKK) family are important upstream regulators of HR. ATR is also activated by single-strand DNA breaks caused by UV radiation and stalled replication forks.[1,2] ATM- and ATR-mediated phosphorylation of several key effector molecules, including Chk1/2, p53 and RPA, serve to arrest cell cycle, allowing time for DNA repair. Therefore, effective DNA repair requires upstream repair proteins such as ATM or ATR to access DNA lesions. Because genomic DNA is packed with histones in a condensed chromatin structure,[11] accessing these lesions requires remodeling and relaxing chromatin structures. Therefore, regulating chromatin structures during the DNA damage response pathway is important for effective DNA repair and maintaining genomic stability.

Zinc finger protein 668 (ZNF668) was initially identified and validated as a highly mutated gene in breast cancer cells.[12,13] We previously found that ZNF668 functions as a tumor suppressor by promoting the DNA damage-induced activation and stabilization of p53.[14] Since p53 plays an important role in DDR, our findings suggested that in addition to regulating p53, ZNF668 might have other roles in the DDR pathway. Indeed, we show here that ZNF668 maintains genomic stability through DDR regulation. We investigated the role of ZNF668 in IR- and UV-induced DNA damage signaling, checkpoint activation and DNA repair. We report that ZNF668 function is dispensable for both ATM/Chk2 and ATR/Chk1 signaling after IR or UV treatment, respectively. More importantly, ZNF668 is critical for the upstream process of Tip60-mediated histone acetylation leading to chromatin relaxation to facilitate repair protein recruitment and HR-directed repair of DSBs caused by IR. Furthermore, ZNF668 promotes RPA phosphorylation and recruitment to

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Submitted: 04/01/13; Revised: 05/14/13; Accepted: 05/16/13
http://dx.doi.org/10.4161/cc.25064

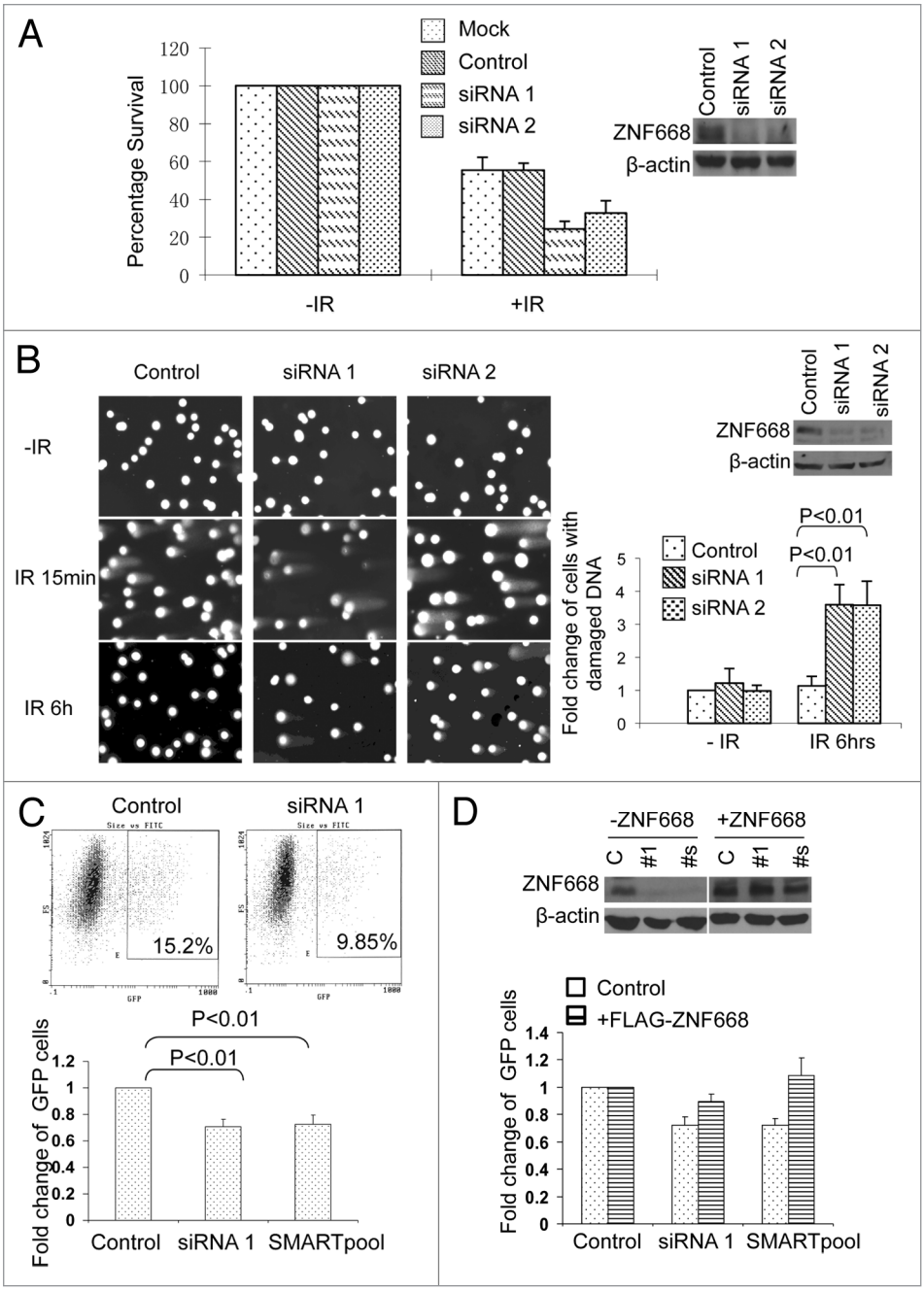

Figure 1. ZNF668 knockdown impairs DNA DSB repair after IR. (**A**) Control or ZNF668-deficient U2OS cells were seeded and exposed to IR. Viable cell colonies in three plates were counted. The graphs represent the mean ± SD of three independent experiments. A western blot demonstrating the effectiveness of ZNF668 knockdown is shown to the right of the graph. (**B**) Comet assay analyses at the indicated times after exposure of control or ZNF668-deficient U2OS cells to IR. Left, representative images of cells in comet assay. Right, quantitative analysis results from three independent experiments and representative western blot analysis showing ZNF668 knockdown. The percentage of control cells with damaged DNA that were not exposed to IR was set to 1. Each value represents the mean ± SEM from three independent experiments using Student's t-test. (**C and D**) Control and ZNF668-deficient cells (**C**) or FLAG-ZNF668 overexpression cells (**D**) were subjected to HR analysis. Each value represents the mean ± SD from three independent experiments using Student's t-test. A western blot analysis demonstrating the effectiveness of ZNF668 knockdown and FLAG-ZNF668 overexpression are shown above the graph in (**D**).

DNA damage foci in response to UV. Together, our findings indicate differential roles for ZNF668 in response to various DNA damage signals.

Results

ZNF668 is required for DSB repair and cell survival in response to IR. To understand the role of ZNF668 in DNA damage response, we first analyzed the impact of ZNF668 on cell survival following IR treatment. Our cell survival assay revealed that ZNF668-knockdown cells were more sensitive to IR than control cells (**Fig. 1A**), indicating that ZNF668-knockdown cells were sensitive to DNA damage-induced cell death. Effective DNA repair of damaged DNA is essential to cell survival. To test whether ZNF668 plays a role in DNA repair, we measured DNA repair efficiency in ZNF668-knockdown cells using the neutral comet assay that specifically measures DSBs. The intensity of the comet tails at 15 min post-IR treatment suggests similar levels of DSB induction for control and ZNF668-deficient cells. However, after 60 min, ZNF668-deficient cells displayed longer comet tails (**Fig. 1B**, left panel) and a significantly higher amount of damaged cells (**Fig. 1B**, right panel), indicating that ZNF668 knockdown compromised DSB repair efficiency. To further understand the role of ZNF668 in DSB repair, we analyzed ZNF668-deficient cells using an HR analysis system. In this system, U2OS cells containing a single copy of the HR reporter DR-GFP in a random locus were generated as described previously.[15] Introduction of I-SceI endonuclease generates DSBs within DR-GFP, which can be repaired via HR. Therefore, HR efficiency is directly proportional to the intensity of GFP-positive signal. HR analysis revealed that the rate of HR in ZNF668 knockdown cells was significantly lower (~30%) than that of control cells, indicating that HR in ZNF668-deficient cells was defective (**Fig. 1C**). Reintroducing ectopic FLAG-ZNF668 into the

ZNF668-knockdown cells restored HR efficiency, indicating that defective HR in ZNF668-knockdown cells resulted specifically from reduced ZNF668 expression (**Fig. 1D**). As controls, we show that decreased GFP signal in the ZNF668-deficient cells compared with ZNF668-containing cells was not caused by differences in I-SceI-induced cutting efficiency (**Fig. S1A**), nor did reduced ZNF668 expression alter the cell cycle profile during I-SceI-induced cleavage (**Fig. S1B**). Furthermore, we show that the lower number of GFP$^+$ cells among ZNF668-deficient cells was not due to differences in transfection efficiency (**Fig. S1C**). Taken together, these data reveal a novel tumor suppressor function for ZNF668 in promoting DSB repair and HR efficiency.

ZNF668 is dispensable for DSB-induced ATM signaling. IR-induced DSBs activate the ATM signaling pathway, resulting in increased phosphorylation and activation of H2AX and Chk2 DDR proteins.[16,17] To test whether reduced ZNF668 expression affects DNA damage detection and signaling, we exposed ZNF668-containing and -deficient cells to IR and assessed ATM signaling. We found that IR treatment induced similar levels of ATM and Chk2 phosphorylation between control and ZNF668-knockdown cells (**Fig. 2A**). In parallel, the amount of IR-induced p-ATM foci was indistinguishable between ZNF668-containing and ZNF668-defecient cells (**Fig. 2B**), indicating that ZNF668 has no significant effect on ATM autophosphorylation, foci formation and downstream targeting. In addition, western blotting revealed that IR treatment induced similar levels of H2AX phosphorylation in ZNF668-containing and -deficient cells (**Fig. 2C**). These results suggest that ZNF668 promotes DSB repair in a manner independent of the ATM and Chk2 pathway.

ZNF668 promotes Tip60-mediated H2AX acetylation. Recruitment of DDR proteins to DSBs requires histone post-translational modifications. Although ATM signaling and γ-H2AX formation occur in the absence of ZNF668 (**Fig. 2A–C**), we find that H2A acetylation at lys5 (H2AacK5) was reduced in ZNF668-deficient cells (**Fig. 3A**). This effect was reversed using sodium butyrate (NaB) and Trichostatin A (TSA) to induce histone hyperacetylation through HDAC inhibition (**Fig. 3B**). In addition to H2AacK5, the H2AX variant is also acetylated at lys5 (H2AXacK5) as part of the DDR response.[18] To determine whether ZNF668 also regulates H2AX acetylation, we transfected FLAG-H2AX into normal or ZNF668-deficient U2OS cells. After irradiation, the cells were collected over a 30 min period, and the lysate was probed for both H2AacK5 and H2AXacK5 using an antibody that recognizes both variants.[19] We find that IR-induced upregulation of H2AXacK5, like H2AacK5, is defective in ZNF668-deficient cells (**Fig. 3C**). These data indicate that ZNF668 is necessary for efficient H2AX lys5 acetylation after IR-induced DNA damage.

The Tip60 histone acetyltransferase is required for DSB-induced H2AX lys5 acetylation.[20] We suspected that ZNF668 promotes H2AX lys5 acetylation by regulating the Tip60-H2AX interaction. We test this by checking whether the IR-induced Tip60-H2AX interaction was compromised after ZNF668 knockdown. Immunoprecipitation results show that Tip60 binding to H2AX was elevated after IR as observed previously;[18]

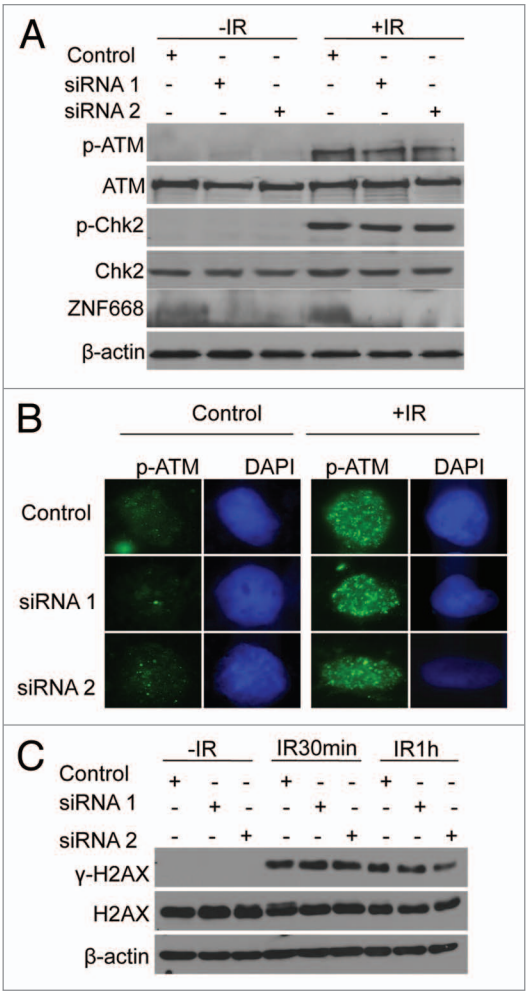

Figure 2. ZNF668 is not required for DSB-induced ATM signaling. (**A**) Control or ZNF668-deficient U2OS cells were exposed to IR (10 Gy), and cell lysates were prepared 1 h after treatment. Cell lysates were analyzed using western blotting and immunoblotting with antibodies against indicated molecules. (**B**) Cells were exposed to IR, stained with phosphorylated ATM and then counterstained with DAPI. (**C**) Control or ZNF668-deficient U2OS cells were exposed to IR (10 Gy), and cell lysates were prepared at indicated time points after treatment. Cell lysates were analyzed using western blotting and immunoblotting with antibodies against indicated molecules.

however, this interaction is not observed after ZNF668 knockdown (**Fig. 3D**). Next, we assess how ZNF668 affects the Tip60-H2AX interaction. Immunoblotting showed that ZNF668 knockdown does not affect Tip60 expression (**Fig. S2A**), while chromatin fractionation revealed that ZNF668 knockdown did not reduce the level of chromatin-associated Tip60 after IR (**Fig. S2B**). However, immunoprecipitation analysis revealed that Tip60 interacts with ZNF668 (**Fig. 3E**). ZNF668 deletion analysis shows that amino acid regions 84–190 and 428–534 are required for interaction with Tip60 (**Fig. 3F**). It is interesting to note that the 84–190 region is also required for ZNF668 interaction with MDM2 and p53.[14] Together, these results suggest that ZNF668 interaction with Tip60 facilitates Tip60-mediated H2AX lys5 acetylation after DNA damage.

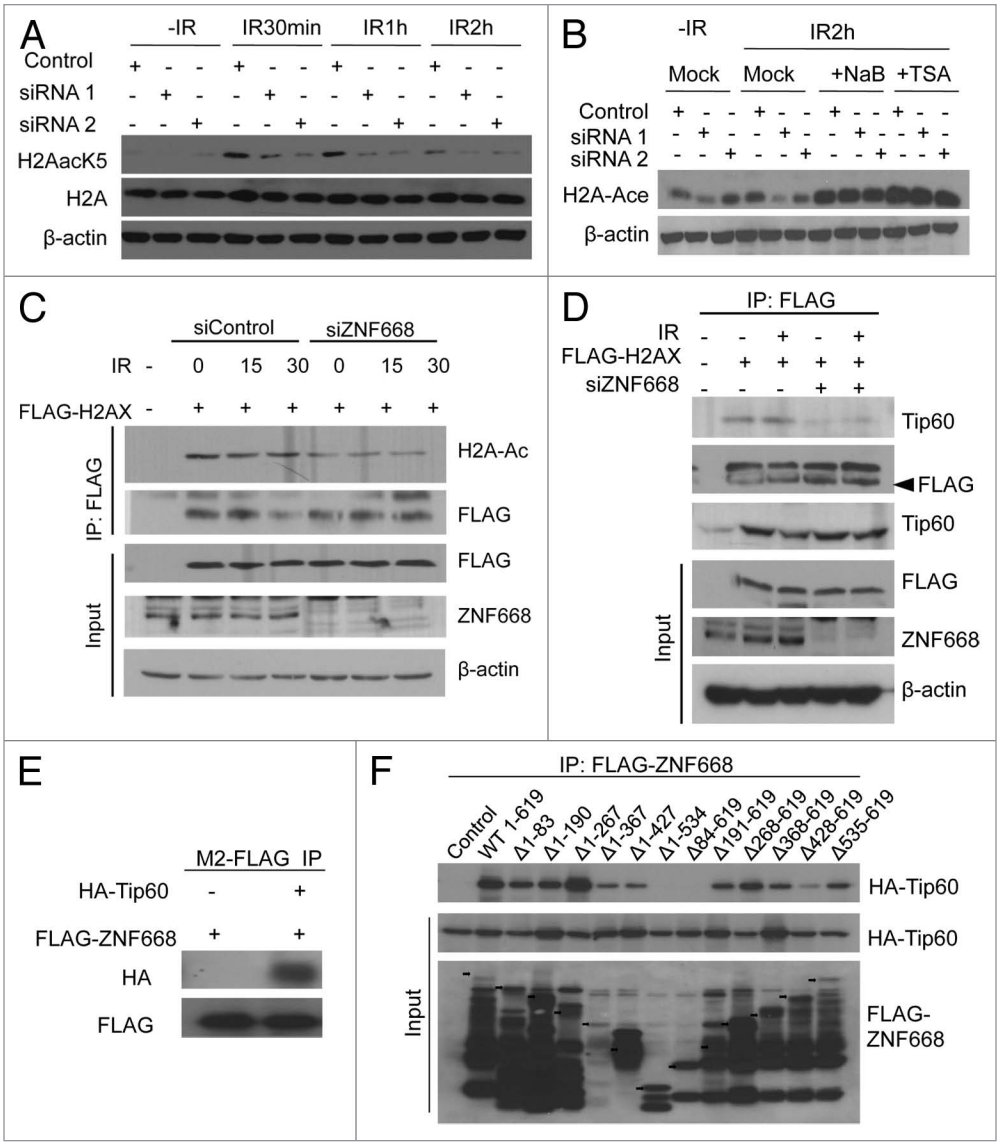

Figure 3. ZNF668 interaction with Tip60 promotes H2AX acetylation after DNA damage. (**A**) Control or ZNF668-deficient U2OS cells were exposed to IR (10 Gy) and cell lysates were prepared at indicated time points after treatment. Cell lysates were analyzed using western blotting and immunoblotting with antibodies against indicated molecules. (**B**) Control or ZNF668-deficient U2OS cells were exposed to IR and treated with or without NaB and TSA. Cell lysates were analyzed by western blotting. (**C**) ZNF668-deficient and control U2OS cells transfected with a Flag-H2AX expression vector were exposed to IR (10 Gy) and allowed to recover for the indicated times. Immunoprecipitation was performed using M2-Flag beads, and proteins were analyzed by immunoblotting with antibodies against H2A acetylated on K5. (**D**) U2OS cells were transfected as indicated. Immunoprecipitation was performed using M2-Flag beads, and proteins were analyzed. (**E and F**) U2OS cells were transfected as indicated. Cells lysates (3 mg) were immunoprecipitated and analyzed.

ZNF668 promotes chromatin relaxation. H2AX lys5 acetylation is known to facilitate HR-mediated DSB repair by relaxing the chromatin around the damaged DNA to allow DDR protein recruitment.[21] We have shown that reduced ZNF668 expression compromises H2AX lys5 acetylation after DNA damage (**Fig. 3A and B**), suggesting that ZNF668 plays a role in chromatin relaxation. Therefore, to further show that the ZNF668-Tip60 interaction promotes HR, we investigated whether ZNF668 regulates chromatin relaxation. Since chromatin relaxation promotes DDR foci formation,[22] we examined U2OS cells for DDR foci in the context of ZNF668 expression. Rad51 is one of the key proteins in HR and is required for the strand invasion step in HR.[9] Immunofluorescence data show that ZNF668-deficient cells exhibited a significantly lower amount of Rad51 foci compared with ZNF668-containing cells after IR treatment (**Fig. 4A**). However, treatment with NaB significantly reversed this effect (**Fig. 4B**). NaB was also shown to rescue defective HR in ZNF668 knockdown cells, as shown by the DR-GFP signal in the HR reporter assay (**Fig. 4C**). The combined data suggest that ZNF668 promotes HR by coordinating Tip60-mediated H2AX lys5 acetylation to allow chromatin relaxation and DDR protein recruitment to damaged DNA.

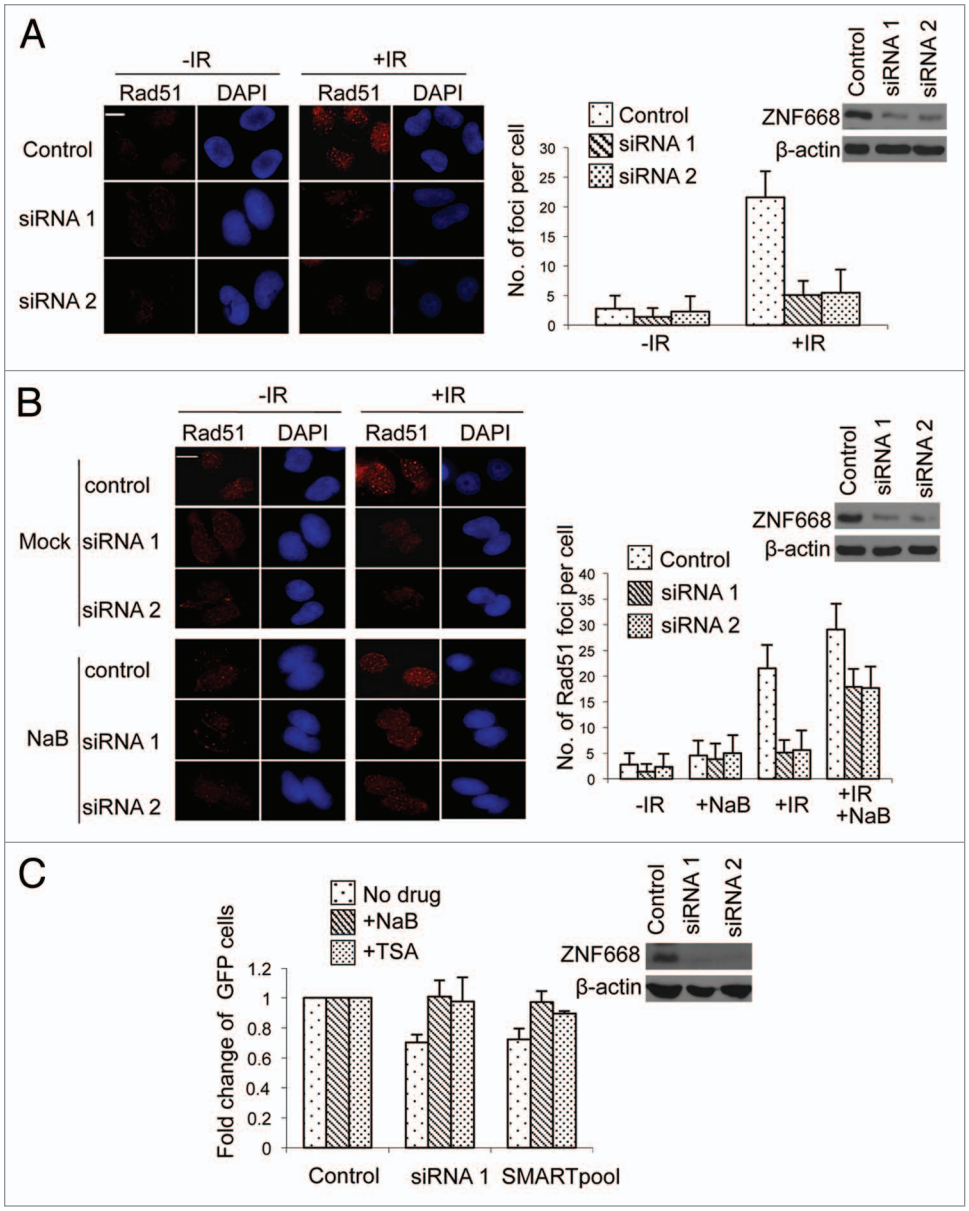

Figure 4. Loss of ZNF668 impairs chromatin relaxation. (**A**) Cells were exposed to IR, stained with Rad51 and then counterstained with DAPI. Left: representative immunostaining images are shown. Scale bar, 10 μM. Right: Data are given as the mean ± SD from three independent experiments; Student's t-test. Western blots demonstrating the effectiveness of ZNF668 knockdown are shown to the right of the graph. (**B**) Cells were exposed to IR (10 Gy) in the presence or absence of chromatin relaxation reagents (NaB, 5 mM or TSA, 200 ng/ml). Cells were prepared and subjected to immunostaining 3 h after exposure to IR radiation. Left: representative immunostaining images are shown. Scale bar, 10 μM. Right: Data are given as the mean ± SD from three independent experiments; Student's t-test. (**C**) Cells were subjected to HR analysis in the presence or absence of chromatin relaxation reagents. Each value represents the mean ± SD from three independent experiments; Student's t-test. Western blots demonstrating the effectiveness of ZNF668 knockdown are shown to the right of the graphs.

ZNF668 promotes RPA activation at UV-induced DNA damage sites. To determine the impact of ZNF668 on UV damage response, the ability of the ZNF668 knockdown cells to recover from the UV damage was investigated. Cell survival assay revealed that ZNF668-deficient cells were more sensitive to UV-induced DNA damage than control cells (**Fig. 5A**). Single-strand DNA (ssDNA) lesions induced by UV lead to replication fork stalling and activation of ATR signaling and Chk1 phosphorylation.[23] Western blotting revealed that treatment with UV induced similar levels of Chk1 phosphorylation in ZNF668-containing and ZNF668-deficient cells (**Fig. S3**), suggesting that ZNF668 doesn't affect the ATR pathway following UV-induced DNA damage. In the presence of UV-induced DNA damage, replication protein A (RPA) binds to ssDNA and is hyperphosphorylated.[24] Although ATR/Chk1 signaling was not impaired in ZNF668-deficient cells, we found that RPA phosphorylation was significantly lower compared with control cells (**Fig. 5B**). This is consistent with previous findings that, besides ATR, other protein kinases may be responsible

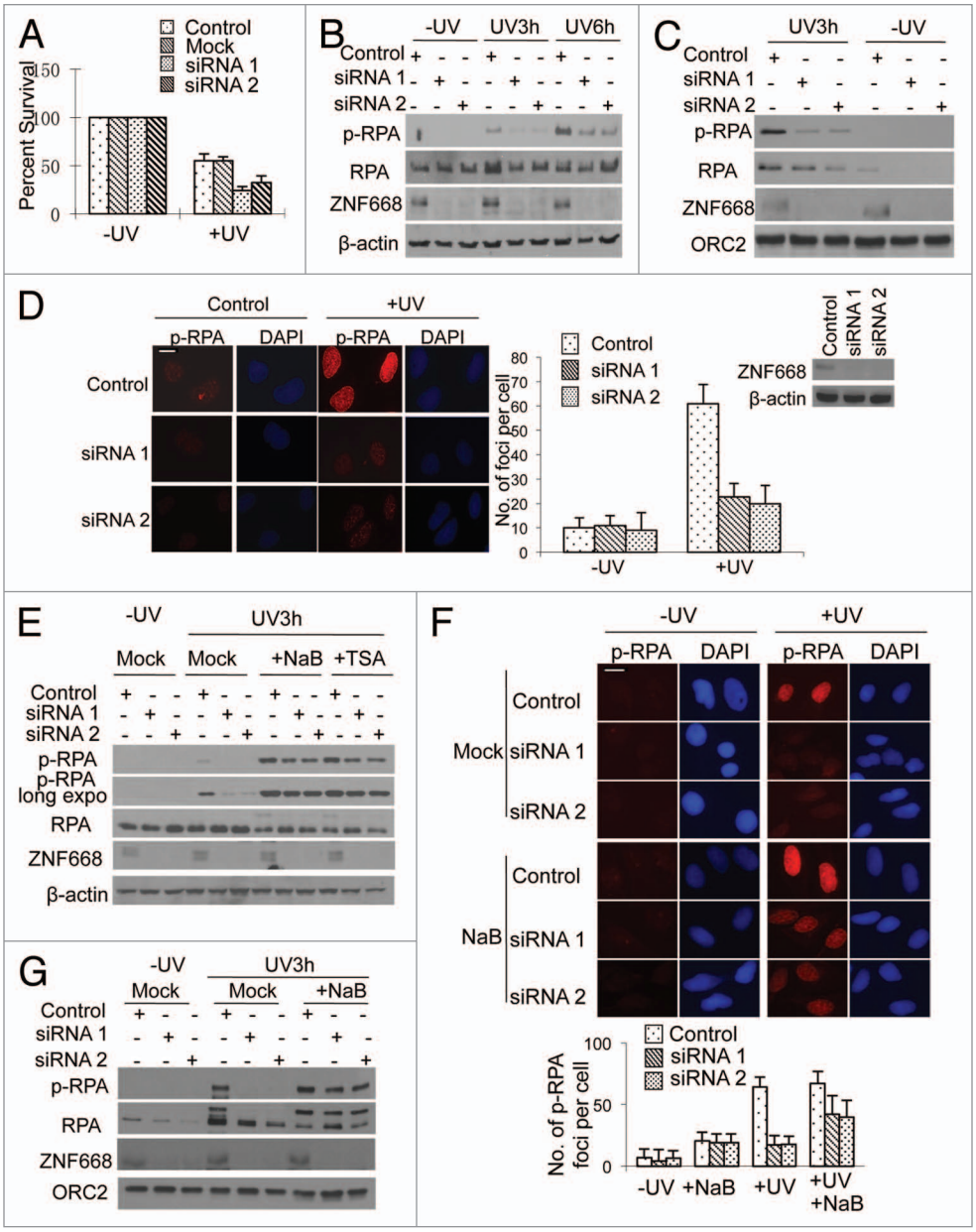

Figure 5. ZNF668 knockdown sensitizes cells to UV and impairs RPA phosphorylation. (**A**) Control or ZNF668-deficient U2OS cells were seeded and exposed to UV. Viable cell colonies in three plates were counted. The graphs represent the mean ± SD of three independent experiments. U2OS cells were treated with UV radiation (50 J/m^2). (**B**) Cell lysates were harvested at indicated time points after UV radiation and subjected to immunoblotting with antibodies against indicated molecules. (**C**) Chromatin fractionation of U2OS cells that were exposed to UV radiation (50 J/m^2) were prepared and analyzed. (**D**) U2OS cells were exposed to UV and stained with phosphorylated RPA34. Left: representative immunostaining images are shown. Scale bar, 10 μM. Right: Data are given as the mean ± SD from three independent experiments; Student's t-test. Western blots demonstrating the effectiveness of ZNF668 knockdown are shown to the right of the graphs. (**E**) Cells were exposed to 50 J/m^2 of UV in the presence or absence of chromatin relaxation reagents (NaB, 5 mM or TSA, 200 ng/ml). Cell lysates were prepared and subjected to western blotting analysis 3 h after exposure to UV radiation. (**F**) Cells were prepared and subjected to immunostaining 3 h after exposure to UV radiation. Upper: representative immunostaining images are shown. Scale bar, 10 μM. Lower: quantitative analysis of phosphorylated RPA34 foci induced in ZNF668-deficient cells from multiple experiments. At least 50 cells were scored in each sample. (**G**) Chromatin-enriched fractions were subjected to western blot analysis 3 h after UV radiation.

for RPA phosphorylation.[25-27] To determine why RPA phosphorylation is lowered in ZNF668-knockdown cells, we checked the efficiency of RPA recruitment to UV-damaged DNA. Chromatin fractionation revealed that ZNF668-deficient cells had lower levels of chromatin-associated RPA and phosphorylated RPA than control cells (**Fig. 5C**). In addition, immunofluorescence staining revealed that ZNF668-deficient cells had reduced levels of phosphorylated (**Fig. 5D**) and total (**Fig. S4**) RPA foci. In light of the above findings that ZNF668 promotes chromatin relaxation, we checked whether NaB and TSA could rescue this phenomenon. Amazingly, chromatin relaxation rescued the phosphorylation (**Fig. 5E**), foci formation (**Fig. 5F**) and chromatin binding

(**Fig. 5G**) of RPA in ZNF668-deficient cells in response to UV. Together, these data indicate that loss of ZNF668 impairs RPA access to chromatin, compromising RPA activation at the site of UV-induced DNA damage.

Discussion

ZNF668 is implicated to have a role in inhibiting cancer initiation and progression in part because of its regulation of p53 tumor suppressor function.[14] Whether additional pathways are controlled by ZNF668 for tumor suppression is unknown. In the present study, we investigated the role of ZNF668 in response to two common DNA damage agents, IR and UV radiation. DNA damage response is a highly conserved but complicated process with multiple steps, including the initial recognition of DNA lesions, access of repair proteins to the damaged DNA and restoration of chromatin to its original state.[1,2,8] In the case of IR-induced DNA DSBs signaling, ATM kinase activity is immediately activated to phosphorylate a number of downstream effectors, including histone H2AX and Chk2.[16,17] Because of its rapid response, phosphorylation of H2AX (γH2AX) is now used in many clinical studies as a quantitative DNA DSBs biomarker.[28] Similarly, ATR protein senses UV damage and participates in signal transduction via phosphorylation of many of the same effectors, including Chk1.[23] Our studies reveal that rapid phosphorylation of H2AX and activation of ATM/ATR signaling following IR and UV are not compromised by ZNF668 loss, despite impaired cell survival after DNA damage (**Figs. 1, 2 and 5**). These results indicate that ZNF668 functions downstream of H2AX and Chk1/Chk2 phosphorylation in the DNA damage signaling and repair pathway. Furthermore, although we observed that ZNF668 was not required for the initial recognition and signaling of DNA lesions followed both IR and UV treatment, it was required for the recruitment of DNA repair proteins to the lesions, indicating that ZNF668 is an important protein in the later stages of DNA damage response.

Alterations in chromatin structure are emerging as key control points during DNA repair. Reorganization of the chromatin structure is necessary for efficient recruitment of DNA damage repair proteins to the damage sites.[29-31] The dynamic chromatin structure change is a result of both chromatin remodeling process and post-translational modification of histones.[31] Post-translational modifications of histones, including acetylation and ubiquitination, are important for the proper disassembly of chromatin after DNA damage to facilitate the timely repair events. Following the induction of DSBs, the H2B ubiquitin ligase RNF40 cooperates with the suppressor of Ty homolog-16 (SUPT16H) to induce dynamic change of chromatin structure, which facilitates proper checkpoint activation and timely DNA repair.[32] Recent studies have suggested a role of chromatin acetylation by histone acetyltransferase (HAT) complexes in DNA-damage detection and DNA repair.[33-35] The histone acetylation mediated by Tip60 acetyltransferase is required for the subsequent recruitment of repair proteins to the DSBs sites. However, the precise underlying mechanisms mediating the function of HAT complex remain to be established. In the present study, we found through IR treatment that ZNF668 participates in HR by remodeling chromatin through the regulation of posttranslational modifications of histones. On the basis of our findings, we propose a model of ZNF668 in DSB repair, in which the induction of a DSB results in ZNF668-mediated Tip60-HAT recruitment to DNA damage sites and H2AX acetylation (**Fig. S5**). Acetylated histones facilitate access and binding of DNA-repair machinery to chromatin DNA.

Our findings suggest that ZNF668-mediated Tip60 recruitment to DNA damage sites are important for efficient DNA repair and cell survival. These results are consistent with recent reports that HAT complexes help regulate transcription and DNA damage response and thus may a play role in cancer development.[36,37] For example, Tip60 haploinsufficiency is associated with breast and colon cancer development. Disruption of the p400:Tip60 ratio in colorectal cancer cells contributes to loss of oncogene-induced DNA damage responses.[38] In addition, mutation or inhibition of HAT complexes in yeast and mammalian cells has resulted in hypersensitivity to DNA-damage-inducing agents and compromised DNA repair.[39,40] Therefore, the factors that are involved in regulation of HAT complexes and chromatin structure contribute to cancer development. Our finding that ZNF668 is involved in the HAT regulation process during DNA repair further suggests that ZNF668 plays an important role in cancer development.

Our data indicate an important role of ZNF668 in RPA phosphorylation and DNA repair followed by UV damage. RPA is the ssDNA-binding protein and is essential for recruitment and activation of ATR.[25] RPA itself is also a downstream target of ATR. Although we didn't observe impaired ATR activation in ZNF668-deficient cells, interestingly, we detected a decrease of RPA phosphorylation at S4 and S8 sites, indicating that phosphorylation of S4 and S8 is independent of ATR. These data are consistent with previous findings that phosphorylation of S4 and S8 is primarily catalyzed by the DSB-responsive protein DNA-PK.[26] Phosphorylation of RPA at S4 and S8 sites has been implicated in mitotic delay and facilitates DNA DSB repair in response to UV damage.[27] In ZNF668-deficient cells, the impaired RPA phosphorylation and its recruitment to DNA damage sites further support the role of ZNF668 in regulation of RPA activity and DNA damage response.

Further investigation is needed to fully understand ZNF668's involvement in the regulation of chromatin structure in both IR- and UV-induced DSBs repair. Other researchers have proposed that the acetylation of H2AX by Tip60 facilitates the release of H2AX from sites of DNA damage and enables repair proteins to access DSB sites.[18,36] The findings of the present study indicate that ZNF668 is required for Tip60-H2AX binding, which is necessary for H2AX acetylation after DNA damage. However, because Tip60 has broad substrate specificities, ZNF668 may also participate in the acetylation of other core histones, such as H2A and H4. Furthermore, the details regarding the interplay between ZNF668 and other types of histone posttranslational modifications, such as phosphorylation, ubiquitination and sumoylation, warrant additional investigation. In addition to post-translational modification of histones, ATPase-dependent

chromatin remodeling is required for nucleosome destabilization at DSBs. A recent study by Xu et al. demonstrated that histone acetylation in the absence of p400 ATPase activity did not lead to nucleosome destabilization, suggesting a co-operation between histone modification and chromatin remodeling during DSBs repair.[41] However, whether ZNF668 plays a role in altering p400 activity and chromatin remodeling is unclear. Therefore, future studies should aim to clarify the effects of ZNF668 on histone modifications and chromatin remodeling in DNA repair.

Materials and Methods

Cell culture and transfection. U2OS human osteosarcoma cells were purchased from the American Type Culture Collection. These cells were maintained in McCoy's 5A modified medium (Cellgro, 10-050-CV) supplemented with 10% FBS with glutamine, penicillin and streptomycin. Cells were incubated at 37°C in a humidified incubator with 5% CO_2 and transfected with FuGENE 6 (Promega, E2691) or Oligofectamine (Invitrogen, 58303).

Plasmids and small interfering RNAs. FLAG-tagged ZNF668 was constructed as described previously.[14] The FLAG-tagged ZNF668 deletion mutants ZNF668-Δ 1~Δ 12 were generated as described previously.[14] FLAG-H2AX plasmid was a gift from Dr Claudia Lukas (Institute of Cancer Biology and Centre for Genotoxic Research). ZNF668 expression was knocked down in U2OS cells using siRNA. The ON-TARGETplus ZNF668 siRNA duplex and ON-TARGETplus SMARTpool ZNF668 siRNA mixture were purchased from Dharmacon Research, Inc. ON-TARGETplus nontargeting siRNA was used as a control in siRNA reactions. RNA duplexes or SMARTpool (final concentration, 100 nM) were transfected into the U2OS cells using Oligofectmine according to the manufacturer's protocol. Cells transfected with ZNF668 siRNA were incubated for 2 or 3 d. Western blotting was used to assess protein levels.[14]

Antibodies and reagents. An anti-ZNF668 antibody was generated as described previously.[14] Anti-FLAG M2-agarose affinity gel was purchased from Sigma (A2220). Replication protein A (RPA) antibody was purchased from NeoMarkers (MS-691-B0). An anti-phosphorylated RPA32 (S4/S8) antibody was purchased from Bethyl (A300-245A). Rad51 was purchased from Calbiochem (PC130). ATM, phosphorylated H2AX were purchased from Cell Signaling Technology (2873 and 2595, respectively). An anti-histone H2A (acetyl K5) antibody was from Abcam (ab1764). Anti-Tip60 and phosphorylated ATM antibodies were purchased from Millipore (07-038 and 05-740, respectively). Fluorochrome-conjugated secondary antibodies were purchased from Jackson ImmunoResearch Laboratories. Cisplatin was purchased from Sigma (P4394). Sodium butyrate (NaB) and trichostatin A (TSA) were obtained from Sigma (B5887 and T8552, respectively) and used at 5 mM or 200 ng/mL, respectively.

Cell survival assay. U2OS cells were transfected with ZNF668 siRNAs. Seventy-two hours after the transfection, the cells were plated at low density and exposed to UV radiation or ionizing radiation (IR). Cells were incubated for 2–3 wk to allow colonies to form. The resulting colonies were detected by staining with crystal violet. Colonies containing 50 or more cells were counted.

Comet assay. DSB repair in U2OS cells was analyzed by neutral comet assay using the Trevigen's Comet Assay Kit (4250-050-K) according to the manufacturer's instructions. Cells were exposed to 10 Gy γ-irradiation (IR) and subjected to comet analysis at indicated time points.

HR analysis. HR in ZNF668-knockdown cells was analyzed to determine the role of ZNF668 in DSB repair. U2OS-DR-GFP cells were transfected with ZNF668 siRNA with or without FLAG-ZNF668. Forty-eight hours after transfection, the cells were transfected with pEGFP-C1 as for transfection efficiency control or pCBASce plasmid for detection of HR efficiency. Forty-eight hours later, GFP-positive cells were detected via flow cytometric analysis using a FACScalibur apparatus with the Cellquest software program (Becton Dickinson). Cells were incubated for 16 h in sodium butyrate (5 mM) or trichostatin (200 ng/mL) to induce chromatin relaxation before flow cytometric analysis.

Affinity purification of ZNF668 and the H2AX protein complex. U2OS cells were transiently transfected with an empty FLAG plasmid, FLAG-ZNF668 plasmid or FLAG-H2AX plasmid. Forty-eight hours later, whole-cell extracts were prepared with RIPA buffer (50 mM TRIS-HCl, pH 7.4, 1% NP-40, 10% sodium deoxycholate, 150 mM NaCl, 1 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, 1 mM sodium orthovanadate, 1 mM NaF) and immunoprecipitated with anti-FLAG M2 affinity gel overnight. Bead-bound immunocomplexes were eluted with 3× FLAG peptide (Sigma, F4799). Immunocomplexes were separated using 7 or 12% SDS-PAGE.

Immunofluorescence microscopy. For detection of DNA damage-induced foci of p-RPA34, RPA34 and Rad51, immunofluorescent staining was performed essentially as described previously.[42] Cells were treated with cytoskeleton and stripping buffer, fixed with 4% paraformaldehyde and then permeabilized with 0.5% NP-40 and 1% Triton X-100. The primary antibodies used in this staining were mouse anti-RPA (1:1,000), rabbit anti-pRPA (1:1,000) and rabbit anti-Rad51 (1:500). The cells were incubated with the primary antibodies for 2 h at room temperature and incubated with secondary antibodies for 1 h at room temperature. Slides were mounted in medium containing DAPI (Vector Laboratories, H-1000) and analyzed under a fluorescence microscope. The number of foci per cell in at least 50 cells per sample was scored. Chromatin relaxation was induced by incubating cells with NaB (5 mM) after exposure to UV or IR and then stained for DNA damage foci.

Immunoblotting and chromatin fractionation analysis. Cells were washed in PBS and lysed in urea buffer or modified RIPA buffer. For chromatin fractionation, cells were lysed in (10 mM HEPES pH 7.9, 1.5 mM $MgCl_2$, 10 mM KCl, 0.34 M sucrose, 10% glycerol, 1 mM dithiothreitol and 0.1% Triton X-100), and nuclear extracts were lysed in a buffer (3 mM EDTA, 0.2 mM EGTA and 1 mM dithiothreitol). After clarification, cell pellets were resuspended in an SDS sample buffer.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Acknowledgments

This work was supported by the Department of Defense Breast Cancer Research Program Predoctoral Traineeship Award BC083020 to R.H.

Supplemental Materials

Supplemental materials may be found here: www.landesbioscience.com/journals/cc/article/25064

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