Food and Chemical Toxicology 133 (2019) 110745

Contents lists available at ScienceDirect

Food and Chemical Toxicology

journal homepage: www.elsevier.com/locate/foodchemtox

Cadmium disrupts the DNA damage response by destabilizing RNF168

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ARTICLE INFO

Keywords:
Cadmium
DNA damage response
Double-stranded break
Ubiquitination
RNF168

ABSTRACT

Cadmium (Cd) is a dispensable element for the human body and is usually considered a carcinogen. Occupational and environmental Cd exposure leads to sustained cellular proliferation in some tissues and tumorigenesis via an unclear mechanism. Here, we evaluated the role of Cd in the DNA damage response (DDR). We found that Cd exposure causes extensive DNA double-strand breaks (DSBs) and prevents accumulation of ubiquitination signals at these sites of DNA damage. Cd treatment compromises 53BP1 and BRCA1 recruitment to DSBs induced by itself or DNA damaging agents and partially inactivates the G2/M checkpoint. Mechanistically, Cd directly binds to the E3 ubiquitin ligase RNF168, induces the ubiquitin–proteasome pathway that mediates RNF168 degradation and suppresses RNF168 ubiquitin-ligase activity *in vitro*. Our study raises the possibility that Cd may target RNF168 to disrupt proper DSB signaling in cultured cells. This pathway may represent a novel mechanism for carcinogenesis induced by Cd.

1. Introduction

DNA double-strand breaks (DSBs) are the most toxic form of DNA damage. DSBs can occur during normal cellular processes, such as the development of the immune system and meiotic recombination, or can be induced by endogenous and exogenous genotoxic agents. A series of proteins and protein complexes are activated in response to DSBs to sense, transduce and amplify DSB's signals, to either induce cell-cycle arrest and apoptosis or fix damaged DNA (Bassing and Alt, 2004; Khanna and Jackson, 2001). These processes are regulated by multiple mechanisms, particularly those involving post-translational modifications (PTMs) on crucial DSB response proteins. PTMs occurring on DSB sites recruit DNA damage response (DDR) factors to sites of damage to form supramolecular complexes, namely DDR foci. These foci can be readily detected by immunostaining and monitored to establish the DDR molecular network and assess DNA damage signal transduction (Goodarzi and Jeggo, 2013; Jackson and Bartek, 2009). Deficiencies in DSB signaling and repair promote mutation accumulation and carcinogenesis and lead to developmental defects and embryonic lethality (Miki et al., 1994; Stewart et al., 1999; Varon et al., 1998).

Phosphorylation is the most common and well-defined PTM in the DSB response pathway. Upon DSB damage, ataxia telangiectasia-mutated (ATM) kinase is recruited to DSB sites and activated by interacting with Nbs1 (Nijmegen breakage syndrome 1). Activated ATM then phosphorylates a series of proteins, including the H2A variant H2AX, to initiate DNA damage signal transduction and DNA repair. Phosphorylated H2AX (γH2AX) is detectable within seconds of DNA damage around DSB sites, and is essential to accumulate downstream repair and signaling factors at DSB sites (D'Amours and Jackson, 2002; Lee and Paull, 2004; Marechal and Zou, 2013; Smith et al., 2010).

MDC1 (mediator of DNA damage checkpoint protein 1) is the major mediator of γH2AX and downstream DDR factors. It is well established that MDC1 directly binds γH2AX through its BRCT (BRCA1 C-terminal) domains and γH2AX–MDC1 interaction is essential for accumulation of MDC1 at break sites and DDR focus formation. In the vicinity of a DNA break site, MDC1 serves as a molecular platform for assembly of many DDR factors. MDC1 FHA (forkhead-associated) domain and SDTD (short-repeat sequences that shared the consensus motif Ser-Asp-Thr-

Abbreviations: Cd, cadmium; DDR, DNA damage response; DSBs, DNA double-strand breaks; PTM, post-translational modification; ATM, ataxia telangiectasia-mutated; MDC1, mediator of DNA damage checkpoint protein 1; BRCA1, breast and ovarian cancer type 1 susceptibility protein; 53BP1, p53-binding protein 1; HR, homologous recombination; NHEJ, nonhomologous end-joining; IR, ionizing radiation; CSR, class switch recombination; H3pS10, phosphorylation of histone H3 at serine 10

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https://doi.org/10.1016/j.fct.2019.110745
Received 9 May 2019; Received in revised form 30 July 2019; Accepted 30 July 2019
Available online 31 July 2019

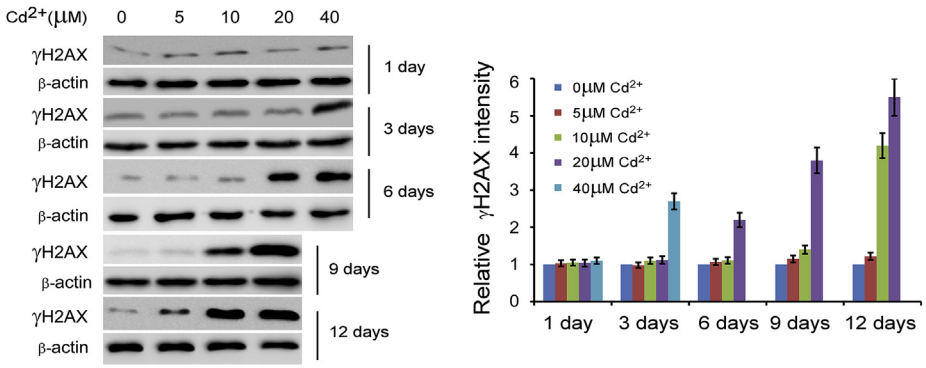

Fig. 1. Cd exposure causes DSB formation. U2OS cells were treated with different concentrations of Cd and lysed at the indicated time points. Left: western blots showing Cd-induced DSB formation marked by γH2AX. Right: quantitative analysis of the relative γH2AX levels after Cd treatment. At each time point, the intensity of γH2AX without Cd treatment was assigned as 1. Error bars represent standard deviation (SD) of three independent experiments.

Asp) repeats interact with phosphorylated ATM and Nbs1, respectively, and allow spreading and amplification of ATM signaling surrounding DSBs, while phosphorylation of the tandem TQXF (short-repeat sequences that shared the consensus motif Thr-Gln-X-Phe) repeats on MDC1 mediates the interaction between MDC1 and RNF8, which is important for the recruitment of RNF8 to DSB sites and the initiation of ubiquitin-dependent signaling at break sites (Goldberg et al., 2003; Huen and Chen, 2010; Lou et al., 2003, 2006; Melander et al., 2008; Mochan et al., 2003; Stewart et al., 2003; Xu and Stern, 2003b).

Ubiquitination occurs by various mechanisms and has functions in the regulation of DDR that differ from those of phosphorylation. Ubiquitination is the covalent attachment of a 76-amino acid ubiquitin polypeptide via its C terminus to lysine residues of a target protein. The reaction requires a cascade of enzymes, including an E1 activating enzyme (E1), an E2-conjugating enzyme, and an E3 ubiquitin ligase (Pickart, 2001; Pickart and Eddins, 2004). Together with E2 ubiquitin-conjugating enzyme UBC13, RNF8 poly-ubiquitylates H1 or lethal (3) malignant brain tumor like 2 (L3MBTL2) (Nowsheen et al., 2018; Thorslund et al., 2015) and provides a docking site for another newly identified E3 ligase, RNF168. RNF168, a RIDDLE (radiosensitivity, immunodeficiency, dysmorphic features, and learning difficulties) syndrome E3 ligase, is recruited to DSB sites by recognizing RNF8-generated poly-ubiquitin chains, where it also associates with Ubc13 to ubiquitinate H2A and H2AX. This process results in the generation of K63-linked ubiquitin chains on H2A/H2AX. These ubiquitin chains are required to accumulate and retain downstream DDR factors, including breast and ovarian cancer type 1 susceptibility protein (BRCA1) and p53-binding protein 1 (53BP1) on DSB sites (Huen and Chen, 2010; Mattiroli et al., 2012; Panier and Durocher, 2013; Zhao et al., 2014).

DSBs are highly deleterious lesions. Misrepaired DSBs cause chromosome breaks and translocations that are associated with genomic instability and related diseases (Kasparek and Humphrey, 2011; Khanna and Jackson, 2001). DSBs are repaired primarily by homologous recombination (HR) and nonhomologous end-joining (NHEJ). The elimination of DSBs via HR, which uses the homologous template to guide accurate repair, generates error-free repair products, whereas NHEJ simply rejoins the two broken ends with removal of damaged nucleotides. 53BP1 and BRCA1 play pivotal roles in DSB repair. 53BP1 may promote NHEJ in various systems, and depletion of 53BP1 significantly reduces the efficiency of NHEJ, as determined using an I-SceI inducible reporter system. 53BP1 is also required for class switch recombination (CSR) and variable (V), diverse (D) and joining (J) recombination, which are mainly mediated by NHEJ-related mechanisms and are involved in the development of immunoglobulin and T cell receptors (TCRs) respectively (Manis et al., 2004; Minter-Dykhouse et al., 2008; Xie et al., 2007). BRCA1 functions primarily in HR-mediated repair, but also has some roles in NHEJ (Bau et al., 2006; Durant and Nickoloff, 2005; Moynahan et al., 1999; Snouwaert et al., 1999). Recent reports have indicated that one essential function of BRCA1 in DSB repair is removing 53BP1 from DSB sites to promote DSB end resection and initiate HR (Bunting et al., 2010; Cao et al., 2009).

Cadmium (Cd) is a well-known carcinogenic metal that is abundant in the environment and poses a serious threat to human health. Many epidemiological studies have shown that occupational and environmental exposure to Cd is associated with prostate, breast and lung cancer development. Cd was classified as a Group I carcinogen by the International Agency for Research on Cancer (IARC, 1993;). It has been described that Cd exposure causes carcinogenesis by multiple mechanisms including aberrant gene expression, inhibition of DNA damage repair, induction of oxidative stress, and inhibition of apoptosis. (Joseph, 2009; Liu et al., 2009; Luevano and Damodaran, 2014; Marettova et al., 2015; Waisberg et al., 2003). However, the molecular and cellular mechanisms underlying these effects are still poorly understood.

Cadmium chloride ($CdCl_2$) is a commonly used cadmium salt in toxicological experiments in most previous publications. However, a recent study indicated that cadmium nitrate $Cd(NO_3)_2$, one of the major products from cigarette smoke, may have stronger cytotoxicity than other Cd-derived compounds (Lee et al., 2018). In this study, we systematically analyzed the effects of Cd on the DDR in mammalian cells using $Cd(NO_3)_2$ as a source of Cd ions. Our findings provide important insights into the roles of Cd in interfering with a proper DDR.

2. Results

2.1. Cd exposure promotes DSB formation in cultured cells

When DSBs occur, γH2AX responds to the initial damage signals and a series of DDR factors involving both DNA damage checkpoints and DSB repair proteins are recruited to the damage sites. Thus, DSBs caused by exogenous agents or cellular metabolism are commonly detected by visualizing the expression or focus formation of γH2AX (Kuo and Yang, 2008; Solovjeva et al., 2017). To study genotoxic effects of Cd in mammalian cells, we treated cells with Cd and detected DSBs by western blotting using γH2AX antibody. Consistent with previous reports (Viau et al., 2008), we found that Cd exposure indeed caused abundant DSB formation in cells (Fig. 1). Intriguingly, the effects of Cd on DSB formation were dependent on exposure time and Cd concentration. Specifically, exposure to 10 μM Cd for 12 days in U2OS cells induced more severe DSB damage than exposure to 40 μM Cd for 3 days. These findings suggest that long-term exposure to low concentrations of Cd may cause damage to a similar extent to that induced by short-term exposure to high concentrations of Cd. This finding indicates that the genotoxicity of Cd can accumulate in cells as Cd exposure time increases. Accordingly, long-term occupational or environmental Cd exposure may cause genotoxic effects similar to those of acute Cd exposure or poisoning.

2.2. Cd impaired recruitment of BRCA1 and 53BP1 to DSB sites

We then asked whether Cd impairs DDR factors recruitment to DSB sites. Etoposide is a topoisomerase II inhibitor, and is widely used to

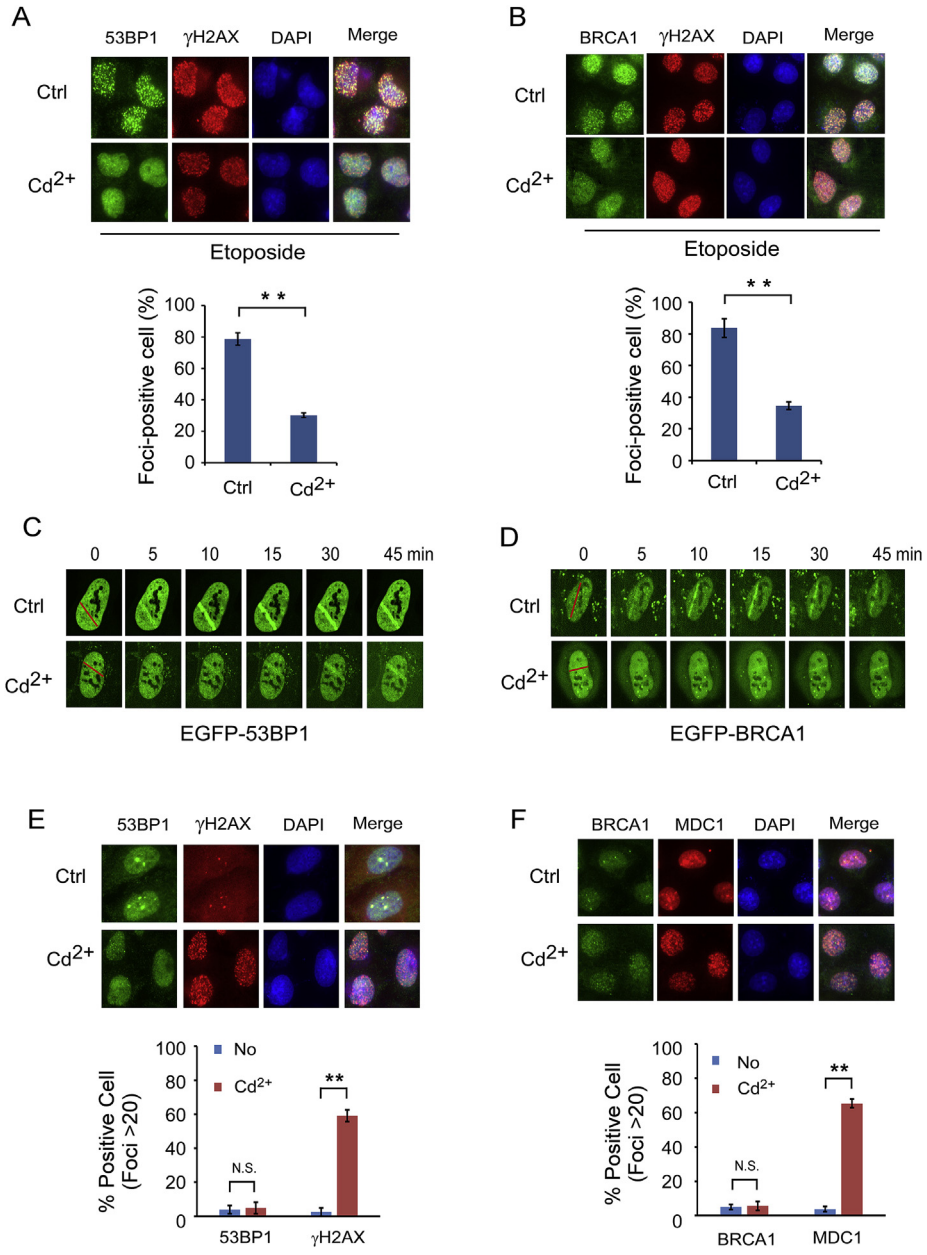

Fig. 2. Cd impedes recruitment of 53BP1 and BRCA1 to DSB sites. (A and B) Cd influenced recruitment of BRCA1 and 53BP1 to DSB sites. U2OS cells were untreated (Ctrl) or pretreated with 80 μM Cd for 4 h and then exposed to 2 μM etoposide for 2 h. Immunofluorescence staining was carried out using the indicated antibodies. Top: representative images. Bottom: percentage of 53BP1 (A) or BRCA1 (B) focus-positive cells among γH2AX focus-positive cells for each sample. The data represent the means ± SD of three independent experiments (> 100 total cells counted for each experiment). The *p*-value is indicated as $^{**}p < 0.01$. (C and D) U2OS cells expressing EGFP-53BP1 or EGFP-BRCA1 were untreated (Ctrl) or pretreated with 80 μM Cd for 4 h. Live-cell imaging was performed following laser-induced micro-irradiation. Representative cells showing the accumulation of EGFP-53BP1 (C) or EGFP-BRCA1 (D) in laser-micro-irradiated disk regions (indicated by red lines in the 0-min cell images). (E and F) Cd treatment impaired 53BP1 and BRCA1 recruitment to DSBs induced by themselves. U2OS cells were exposed to 80 μM Cd for 4 h. Following Cd treatment, the cells were fixed for immunofluorescence staining with the indicated antibodies. Top: representative images. Bottom: percentage of focus-positive cells for each sample. The data represent the means ± SD of three independent experiments (> 100 total cells counted for each experiment). The *p*-value is indicated as $^{**}p < 0.01$ and N.S. = not significant. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

induce DSBs in mammalian cells. Upon exposing cells to etoposide, we could easily detect both BRCA1 and 53BP1 foci, which colocalized with γH2AX at DSB sites. By contrast, pre-exposure to 80 μM Cd followed by etoposide prevented the accumulation of BRCA1 and 53BP1 foci in the Cd-treatment group, despite enhanced γH2AX focus formation (Fig. 2A and B). We then performed live-cell imaging to examine the recruitment of exogenously expressed EGFP-BRCA1 and EGFP-53BP1 to DSBs. As expected, 80 μM Cd for 4 h markedly reduced the accumulation of EGFP-BRCA1 and EGFP-53BP1 to DNA damage stripes generated by micro-irradiation (Fig. 2C and D). Taken together, these results reveal that Cd treatment impairs BRCA1 and 53BP1 recruitment to DSB sites induced by other DNA-damaging agents.

To explore whether Cd-induced DSBs also can trigger a cascade of DDR events similar to those induced by other genotoxic agents, we exposed U2OS cells to Cd and monitored the formation of foci of known DDR factors by immunofluorescence. As shown in Fig. 2E and F, 80 μM Cd treatment for 4 h induced DSBs, as evidenced by marked γH2AX and MDC1 foci. These findings indicate that DDR signal transduction pathways remained intact through the signal mediator MDC1. 53BP1 and BRCA1 recruitment to DSB sites, however, was blocked as no obvious 53BP1 or BRCA1 foci were detected on Cd-induced DSB sites (Fig. 2E and F). These data suggest that Cd exposure indeed causes the formation of DSBs in cultured cells but recruitment of the DDR factors 53BP1 and BRCA1 to these DSBs is blocked. Cd-induced DNA damage signals were suspended between MDC1 and BRCA1/53BP1.

We performed AnnexinV/PI-based flow cytometry analyses to detect acute Cd exposure-induced cell death. Our data indicated that a 4-h treatment with 80 μM Cd did not induce a marked increase in the amount of dead cells (Supplementary Fig. 1). However, more than 60% of the cells are γH2AX-positive in this condition (Fig. 2E). We also carried out a clonogenic survival assay to determine viability of cells with long-time, low-dosage Cd treatment. As shown in Supplementary Fig. 2, treatment of U2OS cells with 10 μM or lower concentrations of Cd for 12 days did not significantly influence cell survival. However, γH2AX was obviously activated in this condition (Fig. 1). This suggested that Cd induced γH2AX activation and DDR should not result from apoptotic cell death.

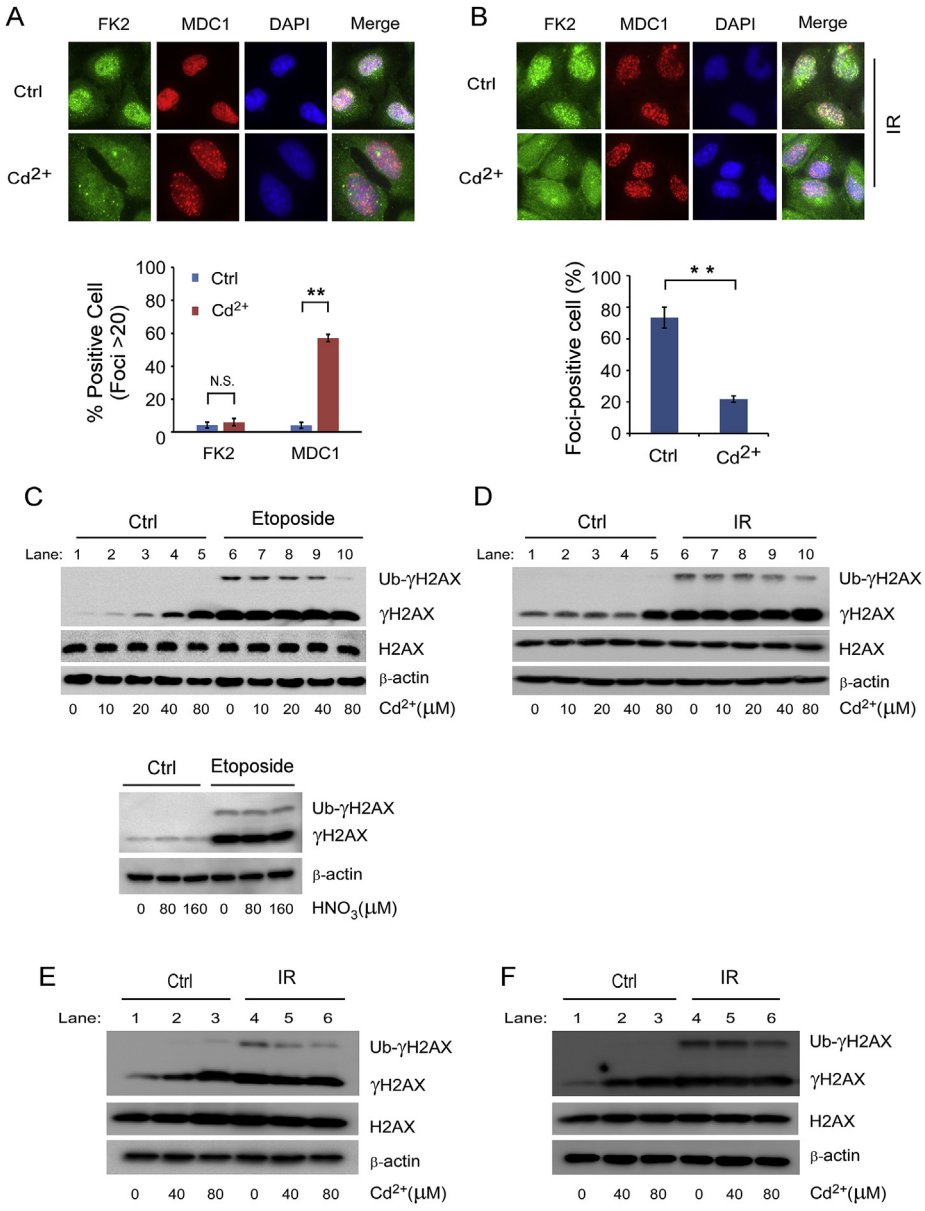

Fig. 3. Cd suppresses DNA damage-induced ubiquitination at DSB sites. (A) Cd-induced DSBs failed to assemble ubiquitin at DSB sites. U2OS cells were exposure to 80 μM Cd for 4 h. Following Cd treatment, cells were fixed for immunofluorescence staining with the indicated antibodies. Top: representative images. Bottom: percentage of focus-positive cells for each sample. The data represent the means ± SD of three independent experiments (> 100 total cells counted for each experiment). The *p*-value is indicated as $^{**}p < 0.01$ and N.S. = not significant. (B) Cd suppresses IR-induced ubiquitination at DSB sites. U2OS cells were untreated (Ctrl) or pretreated with 80 μM Cd for 4 h, irradiated with IR (10 Gy, recovered for 1 h) and fixed. Immunofluorescence staining was carried out using the indicated antibodies. Top: representative images. Bottom: percentage of FK2 focus-positive cells among MDC1 focus-positive cells for each sample. The data represent the means ± SD of three independent experiments (> 100 total cells counted for each experiment). The *p*-value is indicated as $^{**}p < 0.01$. (C and D) Cd pre-treatment suppressed DSB-induced γH2AX mono-ubiquitination. U2OS cells were pretreated with different concentrations of Cd $(NO_3)_2$ (top) or HNO_3 (bottom) for 4 h. After treatment, cells were exposed to 10 μM etoposide for 2 h (C), or irradiated with 10 Gy IR (D) and cell extracts were analyzed by western blotting using the indicated antibodies. (E and F) Effects of Cd on γH2AX formation and mono-ubiquitination are independent of p53. HCT116 WT (E) or p53 KO cells (F) were pretreated with different concentrations of Cd for 4 h, irradiated with IR (10 Gy, recovered for 1 h), lysed, and subjected to western blotting.

2.3. Cd blocked ubiquitination of γH2AX/H2A at DSBs sites

Histone H2AX/H2A is poly-ubiquitinated at DSBs sites. This PTM can be detected by immunofluorescence staining using anti-Ub FK2 antibody that recognizes conjugated ubiquitin (Huen et al., 2007; Mailand et al., 2007; Mattiroli et al., 2012). Because BRCA1 and 53BP1 recruitment at DSB sites depends on poly-ubquitination of γH2AX/H2A, we investigated the effects of Cd on FK2 focus formation. Using MDC1 foci as a DSB marker, we found that Cd-induced DSBs also failed to assemble ubiquitin at DSB sites to form FK2 foci (Fig. 3A). Cd pre-treatment (80 μM for 4 h) dramatically reduced the accumulation of FK2 signals at DSBs induced with 10 Gy ionizing radiation (IR) (Fig. 3B). We also found that γH2AX mono-ubiquitination, which routinely occurs after DSB induction, was lost in Cd-induced γH2AX, as detected by western blotting (Fig. 3C top and D, lane 5). Consistent with reduced FK2 foci, increasing concentrations of Cd pre-treatment suppressed γH2AX mono-ubiquitination after etoposide exposure (Fig. 3C top, lanes 6–10) or IR (Fig. 3D, lanes 6–10). These data imply that Cd exposure may block the initiation of γH2AX/H2A ubiquitination at DSB sites, which could result in defective BRCA1 and 53BP1 recruitment and the interruption of DSB signaling. The tumor suppressor p53 plays a central role in DDR. A previous report indicated that Cd impairs p53 function by inducing conformational changes (Meplan et al., 1999). In wild-type HCT116 cells or p53-negative HCT116 cells, we observed similar effects of Cd on γH2AX formation and mono-ubiquitination with or without IR radiation (Fig. 3E and F). This suggested that the genotoxic effects of Cd described here are independent of p53.

2.4. Cd exposure destabilizes E3 ubiquitin ligase RNF168

Next, we examined the molecular mechanisms through which Cd affects DSB signal transduction and DSB repair mediated by ubiquitination. At DSB sites, histone H2A/H2AX ubiquitination is mainly mediated by the RING-type ubiquitin ligases RNF8/RNF168 and Polycomb repressive complex 1 in mammals (Doil et al., 2009; Stewart et al., 2009). RNF8/RNF168 mediates 53BP1 and BRCA1 recruitment to DSB sites. We thus clarified whether Cd targets RNF8/RNF168 E3 enzymes to regulate the DDR. As expected, Cd exposure reduced RNF168 protein levels in a dose-dependent manner (Fig. 4A) and mildly affected RNF8 protein levels (data not shown) in U2OS cells. The protease inhibitor MG132 fully restored RNF168 protein levels (Fig. 4A). Importantly, there were no noticeable changes in *RNF168* mRNA levels

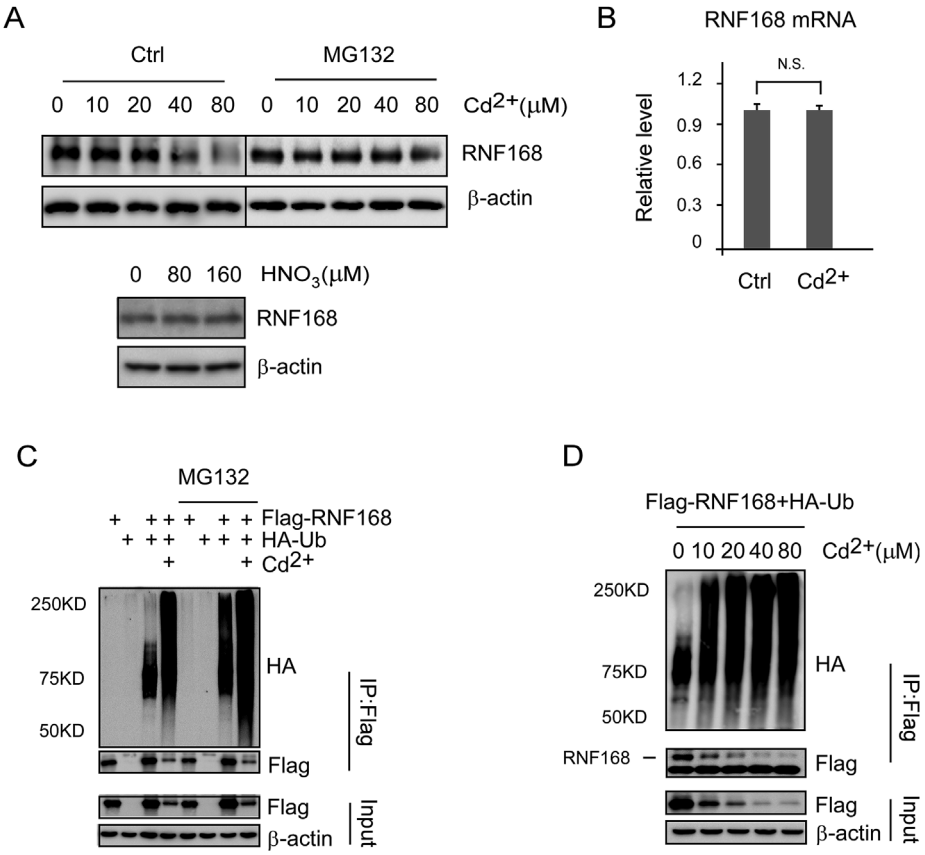

Fig. 4. Cd promotes proteasome-mediated RNF168 degradation. (A) Cd causes RNF168 degradation. U2OS cells were exposed to different concentrations of Cd $(NO_3)_2$ (top) or HNO_3 (bottom) and then treated with DMSO (Ctrl) or the proteasome inhibitor MG132. Cell extracts were analyzed by western blotting using the indicated antibodies. (B) mRNA level of RNF168 is not affected by Cd treatment. U2OS cells were untreated (Ctrl) or pretreated with 80 μM Cd for 4 h and harvested. The relative expression level of *RNF168* mRNA was analyzed by quantitative RT-PCR. The data represent the means ± SD of three independent experiments. The *p*-value is indicated as N.S. (not significant). (C and D) Cd promotes poly-ubiquitination of RNF168.293T cells were co-transfected with Flag-tagged RNF168 and HA-tagged Ub expression constructs and treated with 80 μM Cd and MG132 (C) or different concentrations of Cd for 4 h (D) as indicated. Cell extracts were subjected to immunoprecipitation followed by immunoblotting with indicated antibodies.

following Cd treatment (Fig. 4B). These data suggest that Cd exposure may reduce RNF168 protein levels through an ubiquitin–proteasome-dependent degradation pathway. To verify this result, we performed poly-ubiquitination assays in 239T cells using epitope-tagged RNF168 and ubiquitin. Here, Cd exposure triggered extensive poly-ubiquitination of RNF168 in a concentration-dependent manner (Fig. 4C and D). Poly-ubiquitinated RNF168 will be degraded by the proteasome system, thus resulting in reduced RNF168 levels.

2.5. Cd directly binds to RNF168

To determine whether Cd directly binds RNF168 and disrupts its E3 ubiquitin ligase activity, we developed an *in vitro* ion pull-down assay. The RNF168 N-terminal contains a RING domain (Fig. 5A) that is stabilized by the coordination of two Zn ions and has an essential role in the DDR (Campbell et al., 2012; Zhang et al., 2013). Therefore, we examined whether Cd could bind directly to the RING domain. We incubated purified GST-RNF168 protein (Fig. 5B) with Cd to determine whether Cd could replace Zn ions from the RING domain and could then be “pulled down” by RNF168 protein from the supernatant (Fig. 5C). We found that a significant amount of Cd was pulled from the buffer by wild-type RNF168 protein but not by a RING domain-deletion mutant (Fig. 5D and E). Moreover, Zn ions were released from wild-type RNF168 into the supernatant (Fig. 5F). These results support that Cd and Zn competitively bind to the RNF168 RING domain and that Cd can displace Zn.

We next examined whether Cd also sustains RNF168 E3 ligase activity, in a similar to the effects of Zn. We performed *in vitro* assays to assess the E3 ligase activity of RNF168/Cd. Here, 20 μM Cd completely abolished RNF168 enzyme activity, suggesting that Cd fails to stabilize the RNF168 RING domain (Fig. 5G and H).

2.6. Cd exposure caused defects in the G2/M transition checkpoint

RNF168 is required for the DNA damage-induced G2/M transition checkpoint (Bohgaki et al., 2011). When DNA damage occurs in the G2 phase of the cell cycle, healthy cells are arrested in the G2 phase and will not enter mitosis until DNA repair is completed (Lobrich and Jeggo, 2007; Niida and Nakanishi, 2006). Cells undergoing the G2/M transition can be monitored by immunostaining for the mitotic marker H3pS10 (phosphorylation of histone H3 at serine 10) (Lobrich and Jeggo, 2007; Niida and Nakanishi, 2006). We carried out G2/M checkpoint assays with increasing concentrations of Cd to test the potential effects of Cd on this cellular function (Fig. 6A). Upon Cd treatment, we found that the percentage of cells in G2/M gradually decreased (Fig. 6B, top panel), indicating that Cd exposure partially activated the G2/M checkpoint. Following etoposide treatment, more cells were arrested in the G2 phase and no longer entered mitosis due to the functional G2/M checkpoint in these cells (Fig. 6B, lane 1). However, increasing Cd concentrations reduced the restriction of the etoposide-activated G2/M checkpoint, increasing the proportion of cells entering mitosis in a concentration-dependent manner (Fig. 6B, lanes 2–5, and 6C).

3. Discussion

Efficient DNA damage signal transduction relies on the sequential assembly of numerous DDR factors at DNA break sites. Impaired DSB signaling is always associated with defective DNA damage checkpoints, dysfunctional DSB repair, and genome instability, which eventually leads to tumorigenesis, developmental defects, and embryonic lethality. Here, for the first time, we systematically studied the effects of Cd on DDR, including both DSB signaling and repair. We provided robust evidence suggesting that Cd binds directly to the E3 ubiquitin ligase RNF168 and destroys the RNF168-mediated chromatin ubiquitination

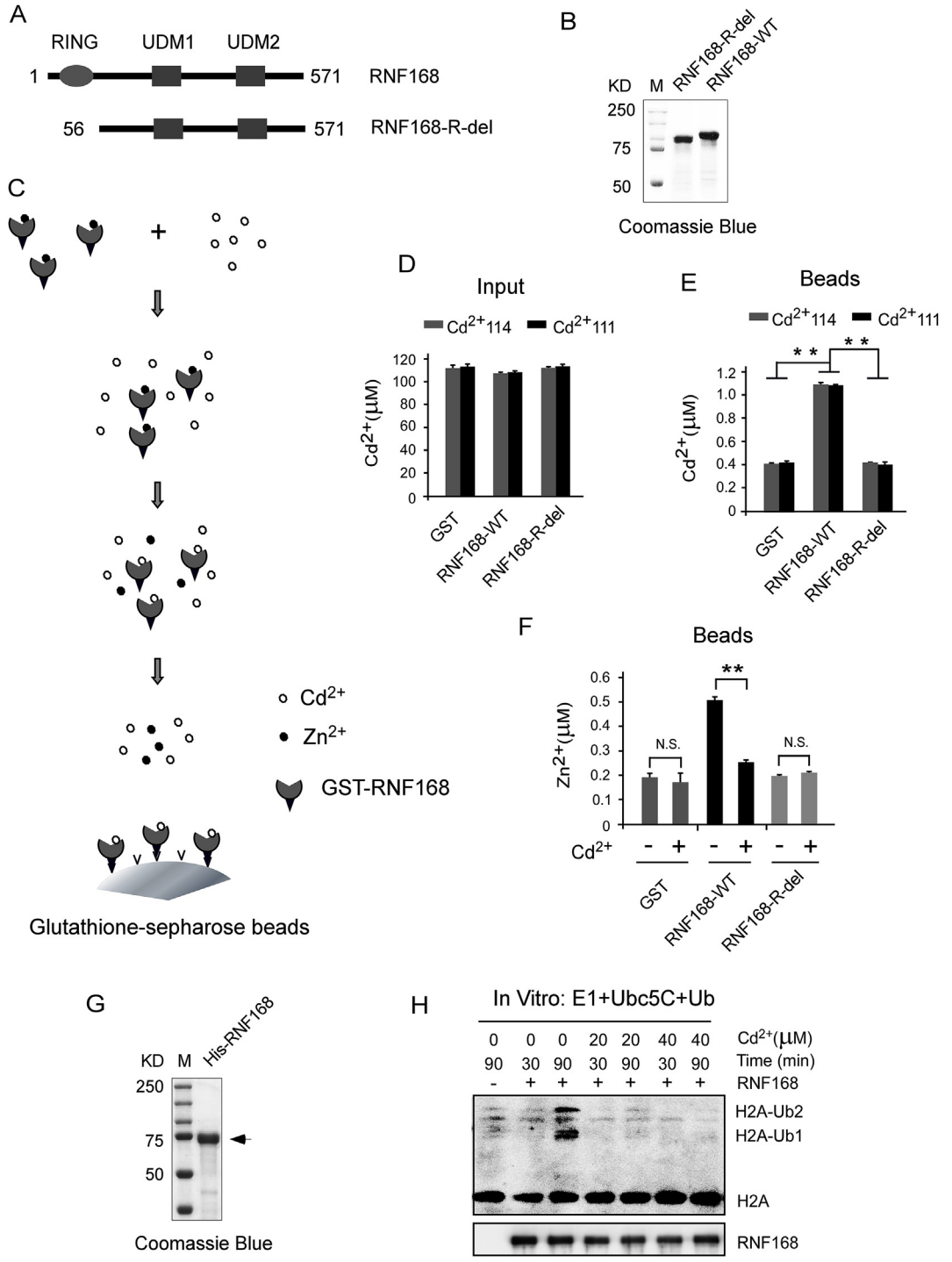

Fig. 5. Cd substitutes for Zn in RNF168 and suppresses its E3 ligase activity. (A) Schematic of RNF168-WT (wild-type) and RNF168-R-del (RING domain deletion) used in the following experiments. (B) The purity of GST-RNF168-WT and GST-RNF168-R-del proteins was analyzed by SDS-PAGE with Coomassie blue staining. (C) Schematic of GST-RNF168 ion pull-down experiments. The same concentration of GST-RNF168-WT or RNF168-R-del proteins was incubated with Cd-containing buffer. If the RING domain of RNF168-immobilized Zn is replaced by Cd, Cd will tightly associate with GST-RNF168 and Zn will be released into the buffer solution. After incubation, all GST-fusion proteins and associated ions are captured by glutathione-sepharose beads and separated with solution by centrifugation. The Cd and Zn contents on "beads" were detected by ICP-MS, which represents how many ions were pulled down by bait from buffer. (D) Initial concentration of Cd in the buffer was determined and shown. (E) Wild-type RNF168 but not RING domain deletion mutant pulled down Cd from buffer. Cd in different precipitates was determined by ICP-MS after being digested with nitric acid. (F) Zn in the RING domain of RNF168 was replaced by Cd. Zn in indicated precipitates was determined and shown. All data in (D) to (F) represent the means ± SD of three independent experiments. The *p*-value is indicated as $^{**}p < 0.01$ and N.S. = not significant. (G) Purified His-RNF168 protein was analyzed by SDS-PAGE with Coomassie blue staining. (H) *In vitro* E3 Ub ligase activity assays were performed with commercially provided H2A protein as substrate. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

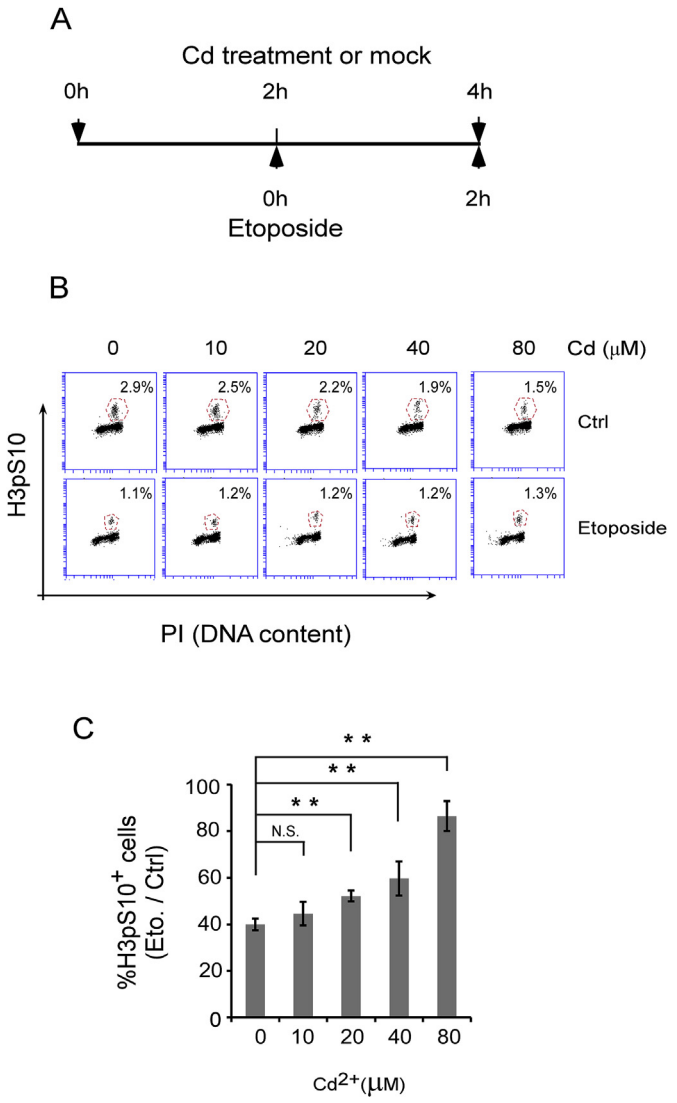

Fig. 6. Cd-attenuated DNA damage induces G2/M checkpoint. (A) Drug exposure scheme. HCT116 cells were treated with increasing concentrations of Cd for 2 h, and then cells were treated with 0.25 μM etoposide for a further 2 h. After drug exposure, G2/M checkpoint assays were performed. (B) Representative flow cytometry analysis. (C) Ratios of mitotic cells with etoposide treatment to those with DMSO treatment (Ctrl) at different concentrations of Cd are shown in the bar graph. Three independent experiments were performed. The data represent the means ± SD The *p*-value is indicated as $^{**}p < 0.01$ and N.S. = not significant.

cascade and related cellular functions. This represents a new indirect genotoxic mechanism of Cd and likely facilitates the development of therapies for acute and chronic Cd poisoning or cancer.

Cd has a long half-life in the human body (~25 years). Normally, Cd concentrations < 20 μM are not considered cytotoxic in many cultured cell lines (Dally and Hartwig, 1997; Meplan et al., 1999; Waalkes et al., 1992). Using our experimental system, we found that long-term exposure to low-concentration Cd (10 μM) also led to an accumulation of DSB damage. This finding suggests that long-term, low-dose occupational or environmental exposure may cause genotoxic effects similar to those of acute, high-dose Cd exposure. In other words, Cd exposure even in the micromolar range may represent a certain degree of Cd contamination.

DSBs can be generated by multiple mechanisms. Etoposide (VP16) is a well-known cancer chemotherapy drug. It binds directly to topoisomerase II (Top II) to block the religation step of the catalytic cycle, thus producing single-strand nicks and DSBs (Chen et al., 1984; Montecucco et al., 2015), while ionizing radiation (IR) damages DNA by imparting energy in the form of ionization clusters that generate clustered damage in the DNA, including DSBs. (Mladenov and Iliakis, 2011; Santivasi and Xia, 2014; Ward, 1988). The detailed mechanism by which Cd induces DSBs in mammalian cells is still not clear. Our data indicated that Cd pre-treatment mildly increased γH2AX levels after exposure to etoposide or IR. The combined effects are not very obvious (Fig. 3C and D). This suggests that the genotoxicity of Cd may partially overlap with both etoposide and IR in terms of DSB formation. Multiple complicated mechanisms might together contribute to the formation of DSBs after Cd exposure. The underlining mechanisms still need further clarification.

The RING-type protein RNF168 is a major E3 ubiquitin ligase that is responsible for conjugation of K63-linked ubiquitin chains onto histone molecules in the vicinity of DSBs. The RING domain, which is stabilized by two Zn ions, is essential for E3 ligase activity and mediating DSB signaling (Campbell et al., 2012; Doil et al., 2009; Stewart et al., 2009; Zhang et al., 2013). Our *in vitro* data suggested that Cd could directly bind to the RING domain of RNF168 and act as a substitute for Zn, suppressing its E3 ligase activity. Although Cd and Zn share similar chemical properties, substitution of Cd for Zn in the RING domain may still cause a certain degree of conformational changes in the protein. RNF168 containing Cd lacks E3 ligase activity and is considered an "abnormal" protein, which is further degraded through the ubiquitin–proteasome system. Our data indicated that many of the effects of Cd on DDR are similar to the reported phenotypes of RNF168-deficient cells, including decreased ubiquitin signals at DSB sites, defective DSB recruitment of 53BP1 and BRCA1, and disrupted G2/M checkpoint function (Bohgaki et al., 2011). These findings suggested that RNF168 may be a main target of Cd in cells in the context of DSB damage signaling and repair. However, we still cannot rule out the possibility that other reported Cd targets including DNA-PKcs, XRCC4, and ligase IV (Li et al., 2015; Viau et al., 2008) may also contribute to its effect on DDR.

RNF168-mediated chromatin ubiquitination is essential for DDR signal transduction. DDR defects lead to genomic instability in cells, aiding in cancer occurrence and development of cancer via mutation accumulation. Mice deficient for DDR factors including MDC1, RNF8, 53BP1 or BRCA1 have increased cancer susceptibility. However, tumor susceptibility was not increased in *RNF168*$^{-/-}$ mice (Bohgaki et al., 2011). A functional ATM–CHK2–p53 pathway successfully prevents tumorigenesis in *RNF168*$^{-/-}$ mice. Inactivation of p53 strongly promoted tumorigenesis when RNF168 was also suppressed. RNF168 and p53 likely collaborate to suppress cancer development in mice (Bohgaki et al., 2011). Intriguingly, our and previously reported data indicated that Cd might inhibit the activities of both RNF168 and p53 in cells, suggesting the tumor-promoting effects of Cd should be stronger than those of other genotoxic drugs that target these two DDR factors individually. Meanwhile, Cd exposure may increase the risk of cancer in patients carrying the p53 mutation.

BRCA1 is a well-known tumor suppressor which maintains genome stability by supporting HR mediated DSB repair (Dagan and Gershoni-Baruch, 1997; Kinzler and Vogelstein, 1997). *BRCA1* is one of the most frequently mutated genes in hereditary breast and ovarian cancer (Venkitaraman, 2002). Loss of heterozygosity (LOH) at the normal BRCA1 allele and retention of the mutated copy is common in the tumor (Li and Greenberg, 2012). A recent study indicated that the RNF168-mediated ubiquitin cascade acts as a backup to BRCA1 at a later stage of HR. The RNF168 pathway is important for preventing genome instability and tumorigenesis in BRCA1 heterozygous mice (Zong et al., 2019). In this respect, Cd contamination probably is more dangerous for BRCA1 mutation carriers than for persons with the wild-type allele.

Like the well-established chemotherapy drugs cisplatin and bleomycin, metal complexes including Cd-containing complexes have been studied as potential anti-cancer drugs (Bjelogrlic et al., 2010; Milacic et al., 2006, 2008). Recently, Zhou et al. (2015) synthesized cadmium-

coordinated thiacalix[4]arene tetrasulfate (TC4ATS-Cd) and indicated this newly designed molecule selectively killed leukemia cells *in vitro* and in a mouse xenograft model. It is worth conducting further experiments to test whether this kind of cadmium-coordinated supramolecules still harbor RNF168-suppressing activity both *in vitro* and *in vivo*. This will help us to evaluate whether we can develop a new Cd and RNF168 inhibition-based targeted therapeutic strategy for treating BRCA1-deficient tumors.

4. Methods

4.1. Cell culture and reagents

Human U2OS and 293T cells were purchased from ATCC and cultured in Dulbecco's modified Eagle's medium with 10% fetal bovine serum (FBS) in the presence of antibiotics. Antibodies against 53BP1 (A300-272A), HA (A190-208A) and H3pS10 (A301-844A) were purchased from Bethyl. Anti-RNF168 (06–1130) and anti-H2AX antibodies (05–636) were purchased from Millipore. Anti-BRCA1 (D9) was purchased from Santa Cruz Biotechnology. Anti-actin (A5441) and anti-FLAG (F1804) antibodies were purchased from Sigma. Anti-FK2 was purchased from Viva-Bioscience. Anti-IgA-allophycocyanin (559353) and fluorescein isothiocyanate (FITC)-conjugated anti-mouse IgM (17–5790) were purchased from BD Company, and anti-MDC1 antibodies were described before (Xu and Stern, 2003a). The standard solution of $Cd(NO_3)_2$ was obtained from the National Institute of Metrology of China.

Human cDNA clones encoding 53BP1, BRCA1, and RNF168 were sub-cloned into pcDNA3.0 with three copies of HA or FLAG epitope at its N-terminus. EGFP-tagged BRCA1 and 53BP1 were generated using the EGFP-C1 expression vector (Clontech). GST-tagged and His-tagged RNF168 and RNF168 mutants were generated using pGEX-6P-1 (GE Healthcare) and pET-28a, respectively.

4.2. Immunobotting, immunoprecipitation and immunostaining

Whole-cell lysis, immunoblotting, immunoprecipitation and immunostaining were performed as previously described (Wang et al., 2012, 2013). Briefly, the cells were lysed with NETN buffer (20 mM Tris, pH 8.0, 1 mM ethylenediaminetetraacetic acid, 150 mM NaCl, and 0.5% NP-40) containing protease inhibitors for 30 min. Cleared cell lysates were then collected for immunoprecipitation or boiled in sodium dodecyl sulfate (SDS) loading buffer and subjected to SDS-polyacrylamide gel electrophoresis (PAGE). For immunostaining, the cells were fixed with ice-cold methanol for 5 min, blocked with 2% bovine serum albumin (BSA) solution in phosphate-buffered saline containing 0.1% Tween-20 for 30 min, and subjected to immunostaining analysis, as previously described (Wang et al., 2012, 2013). Fluorescence analysis was performed using an Olympus IX-81 fluorescence microscope.

4.3. Laser micro-irradiation and live cell imaging

U2OS cells expressing the indicated EGFP-tagged proteins were cultured in sterile glass-bottomed dishes. DSBs were generated in live-cell nuclei by local irradiation with a 365-nm pulsed nitrogen ultraviolet laser (16 Hz pulse, 41% laser output) generated from a micropoint system (Andor). This system was directly coupled to the epifluorescence path of a Nikon A1 confocal imaging system with time-lapse imaging every 30 s for 10 min. Raw images were imported into ImageJ software (NIH, USA) for processing. The fluorescence intensities of the micro-irradiated areas were determined by measuring the mean absolute intensities of the microirradiated areas after subtraction of the mean cellular background intensity. Each data point shown is the average of 10 independent measurements.

4.4. In-vitro ion pull-down and E3 ubiquitin ligase assays

To test the binding between RNF168 and metals, ion pull-down assays were performed using GST-Sepharose-immobilized GST or its fusion protein as “bait”. Equal amounts of GST-fusion proteins were immobilized on GST-Sepharose and examined by Coomassie blue staining. The loaded beads were incubated with Cd ion-containing buffer at 4 °C for 1 h. After extensive washing, the beads were digested with nitric acid and analyzed by inductively coupled plasma mass spectrometry (ICP-MS) to determine the concentrations of Cd and Zn.

In vitro ubiquitin ligase assays were performed as previously described (Mattiroli et al., 2012). Briefly, recombinant His-RNF168 protein was added to 0.4 mM E2 enzyme (Ubc5C recombinant), 0.0125 mM E1 (UBE1; Boston Biochem), and 16 mM ubiquitin (Boston Biochem). Reaction buffer was comprised of 50 mM Tris-HCl (pH 8.0), 1 mM DTT, 3 mM ATP, 5 mM $MgCl_2$, and 1 μM $ZnCl_2$.

4.5. Quantitative real-time reverse transcription polymerase chain reaction (qRT-PCR)

Total RNA from cells was prepared using the TRIzol reagent (Invitrogen) and then reverse-transcribed with a SuperScript III Cells Direct cDNA Synthesis Kit (Invitrogen), according to the manufacturer's instructions. qPCR was performed using SYBR Green PCR Master Mix (Bio-Rad, Hercules, CA, USA) according to the manufacturer's protocol. The primer sequences used in this study were as follows: *RNF168* forward, 5′-GGATCTGCATGGAAATCCTCG-3′; *RNF168* reverse, 5′-ACTGG AAGCACGGTTTACACA-3′; *GAPDH* forward, 5′-CCCATGTTCGTCATGG GTGT-3′; and *GAPDH* reverse, 5′-TGGTCATGAGTCCTTCCACG-3′.

4.6. G2/M checkpoint assays

G2/M transition checkpoint assays were performed as previously described (Yu and Chen, 2004). Briefly, HCT116 cells were pretreated with Cd and exposed to etoposide to induce DSBs for 2 h. After drug exposure, cells were fixed with ethanol and stained with rabbit anti-phosphohistone H3 antibody (H3pS10), followed by incubation with FITC-conjugated anti-rabbit secondary antibodies. The stained cells were treated with RNase, incubated with propidium iodide, and analyzed by flow cytometry.

Funding

This work was supported by the National Basic Research Program of China (grant number 2015CB910602), the National Natural Science Foundation of China (grant number 31370841) and the Beijing Natural Science Foundation (grant number 5182003) awarded to H.W.; the National Natural Science Foundation of China (grant numbers 31530016 and 31761133012), the National Basic Research Program of China (grant number 2015CB910601) and the Shenzhen Science and Technology Innovation Commission JCYJ20170412113009742 to X.X.

Competing interests

The authors declare that they have no competing interests.

Declaration of interests

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgements

We would like to thank Yu Zhang (National Institute of Biological Sciences, Beijing), Jun Tang (China Agricultural University) and

Xiaohua Wu (Scripps Research Institute, CA) for providing reagents. We would also like to thank members of the Wang laboratory for their constructive discussions and assistance with the conception and interpretation of this study.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.fct.2019.110745.

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