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Chromosome damage in mitosis induces BubR1 activation and prometaphase arrest

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Abstract The effect of double-strand DNA breaks (DSBs) on the spindle assembly checkpoint (SAC) has important implications with respect to the relationship between SAC function and chromosome instability of cancer cells. Here, we demonstrate that induction of DSBs in mitosis results in prolonged hyper-phosphorylation of the SAC protein BubR1 and association of BubR1 with kinetochores in mammalian cells. Combining single cell time-lapse microscopy with immunofluorescence, flow cytometry, and Western blot analysis in synchronized cells, we provide evidence that DSBs activate BubR1, leading to prometaphase arrest. Accordingly, elimination of BubR1 expression by siRNA resulted in the abrogation of mitotic delay in response to chromosome damage. These results suggest that BubR1 links DNA damage to kinetochore-associated SAC function.

Keywords: Double-strand DNA break (DSB); Spindle assembly checkpoint (SAC); BubR1; Prometaphase; Kinetochore

1. Introduction

Growing cells experience continuous genotoxic damage. To ensure that progeny are genetically equivalent, cells possess checkpoint controls in response to genotoxic insults that could compromise genetic integrity. In eukaryotic cells, G1 and G2 checkpoints mediated by the ATM and ATR kinases detect DNA damage and ensure that the damage is repaired before entry into mitosis [1,2]; however, rapidly growing cells such as tumor cells, which often have mutations in the G1 and G2 checkpoints, may encounter DNA damage after they have committed to mitosis when the chromosomes are already condensed. If the cell is to maintain genetic integrity in this situation, it must either commit suicide or develop another checkpoint control within mitosis to delay progression of mitosis and manage the damage. Studies in yeast [3] and flies [4,5] indicate that DNA damage induces a mitotic delay through activation of the spindle assembly checkpoint (SAC); however, it is not clear yet whether a similar mechanism exists in mammalian cells.

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Abbreviations: APC/C, anaphase promoting complex/cyclosome; SAC, spindle assembly checkpoint; DSB, double-strand DNA break

Mammalian cells exposed to double-strand DNA breaks (DSBs) during terminal G2 or the early stages of mitosis appear to harbor chromosome fragments that persist until anaphase [6,7], suggesting that DNA repair are not efficient under the constraint of condensed chromosomes. It is thought that mammalian cells tend to correct the damage in the next G1 or experience sudden mitotic cell death [6], also known as mitotic catastrophe [8,9]. In contrast, work from Nitta et al. [10] suggests that the SAC can respond to DNA damage in mammalian cells. They showed that DNA damage activates SAC in p53-dysfunctional cells and leads to mitotic catastrophe. Further, they demonstrated that elimination of SAC components BubR1 or Mad2 abrogates mitotic cell death and result in chromosome mis-segregation. These findings are consistent with the hypothesis that phenotypic aneuploidy in cancer cells arises from impaired DNA repair in conjunction with a dysfunctional mitotic checkpoint [11,12].

The SAC monitors whether microtubule spindles are all attached to kinetochores and whether all kinetochores are under tension from bi-polar spindles. A single unattached chromosome is sufficient to activate the SAC and delay mitosis through inhibition of the APC/C ubiquitin ligase, which degrades cyclin B and securin [13–15]. BubR1, like Mad2, inhibit APC/C by binding to Cdc20, or through inhibition of APC/C ubiquitin ligase activity as components of the mitotic checkpoint complex (MCC) [15,16]. Notably, previous study using laser microsurgery showed that extensive DNA damage in mammalian cells that are committed to divide delays the metaphase–anaphase transition through SAC component Mad2 [17]. It is not known whether BubR1 is involved.

The aim of this study was to determine which stage of mitosis after chromosome condensation is affected by DSB. We were particularly interested to know if BubR1 responds to DSB in mitosis because BubR1 has been shown to respond to DSBs in flies [4], is a critical component of SAC signaling [18–22], and is also implicated in tumorigenesis [23–25].

2. Results

2.1. DNA damage in mitosis induces prometaphase arrest

We first confirmed the presence of DNA damage in mitotic cells by immunostaining with antibody to γ-H2AX, since phosphorylated H2AX (γ-H2AX) is detected at sites of DNA damage [26]. To avoid complications caused by activation of interphase checkpoints, we used cells that were synchronized at prometaphase in all experiments. Normal mouse embryonic

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fibroblasts (MEFs) and HeLa cells were synchronized by nocodazole then challenged with the radiomimetic drug doxorubicin for 6 h and immunostained with antibodies to γ-H2AX. CREST serum or anti-BubR1 antibodies were used to mark the kinetochores. Doxorubicin-treated nocodazole arrested cells contained γ-H2AX-positive foci (Fig. 1). CREST or BubR1 staining at the kinetochores and the presence of condensed chromatin, as revealed by DAPI staining, indicate that the cells were in prometaphase. These data confirm that treatment with doxorubicin induced DSBs in prometaphase in normal MEFs as well as HeLa cells, even when the chromosomes were condensed.

We next investigated the effect of DSB on the checkpoint control for mitotic progression. HeLa cells were synchronized with nocodazole for 13 h, washed, and treated with doxorubicin or mitomycin C to induce DNA damage. At 2 h and 5 h after nocodazole release and drug treatment, cells were fixed and stained with DAPI to score for their stage in mitosis. When asynchronously growing cells were treated with doxorubicin for 13 h, the majority of the cells were arrested in prophase or G2 (Fig. 2A), while nocodazole-treated cells were arrested at prometaphase (Fig. 2B). Asynchronous cells treated with mitomycin C were arrested in prophase (50%) and prometaphase (46%). The effects of DNA damage on synchronized cells were noticeably different. Two hours after nocodazole release, untreated cells progressed to anaphase (6%) and cytokinesis (18%) and 5 h after nocodazole release, 31% of the cells were in cytokinesis. In sharp contrast, synchronized cells treated with 2.4 μM doxorubicin or 10 μM MMC were arrested in prometaphase even at 5 h after release.

DNA topoisomerase II (topo II) activity is required for metaphase–anaphase transition [27,28]. To distinguish between the effects of DSB formation and inhibition of decatenation, we compared two different topo II inhibitors, doxorubicin and ICRF-193. Doxorubicin inhibits topo II activity by stabilizing DNA-topo II complexes thereby blocking re-ligation and causing DSB, but does not affect the catalytic decatenation activity. ICRF-193 inhibits the catalytic activity of topo II by blocking formation of the covalent intermediate between the DNA strands and topo II without causing DNA damage [29].

In contrast to doxorubicin treatment, inhibition of topo II catalytic activity by 100 μM ICRF-193 induced metaphase arrest for 2 h after release. Cells progressed to anaphase by

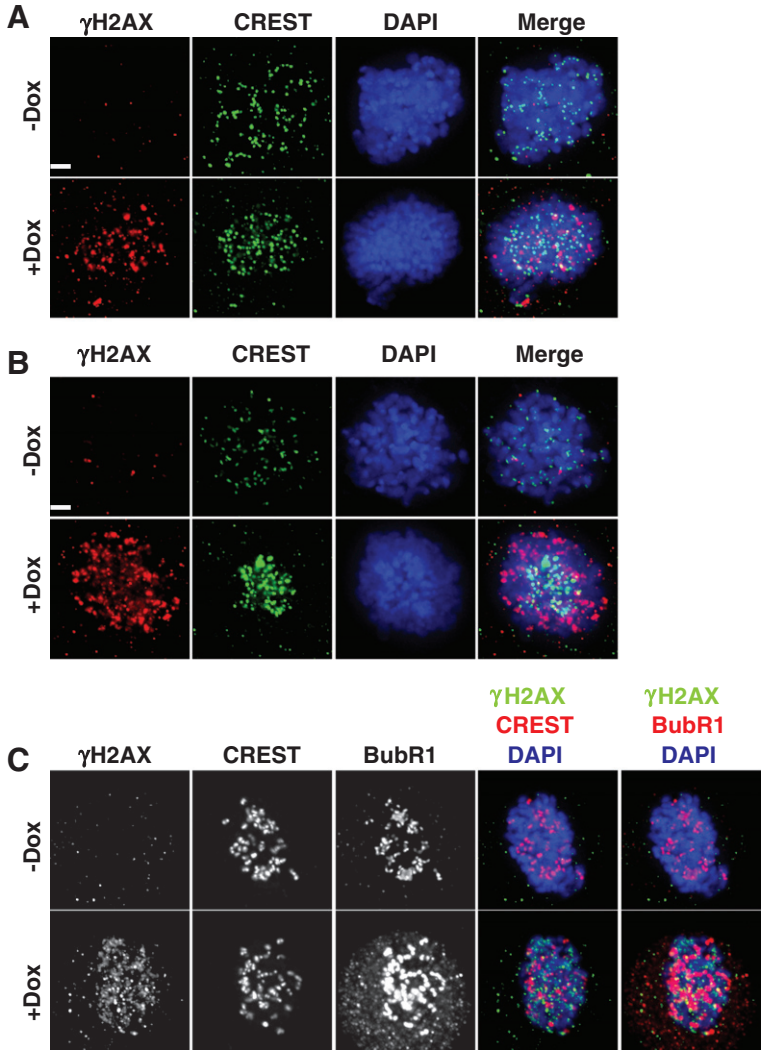

Fig. 1. DSB in prometaphase chromosomes. (A, B) MEFs were synchronized at prometaphase then treated with 2.4 μM doxorubicin for 6 h (+Dox) or untreated (−Dox). Cells were fixed and co-stained with γ-H2AX and CREST (A) or anti-BubR1 antibodies (B). Blue represents DAPI-stained chromosomes. (C) HeLa cells were treated and immunostained as in MEFs. Images were obtained and merged as marked.

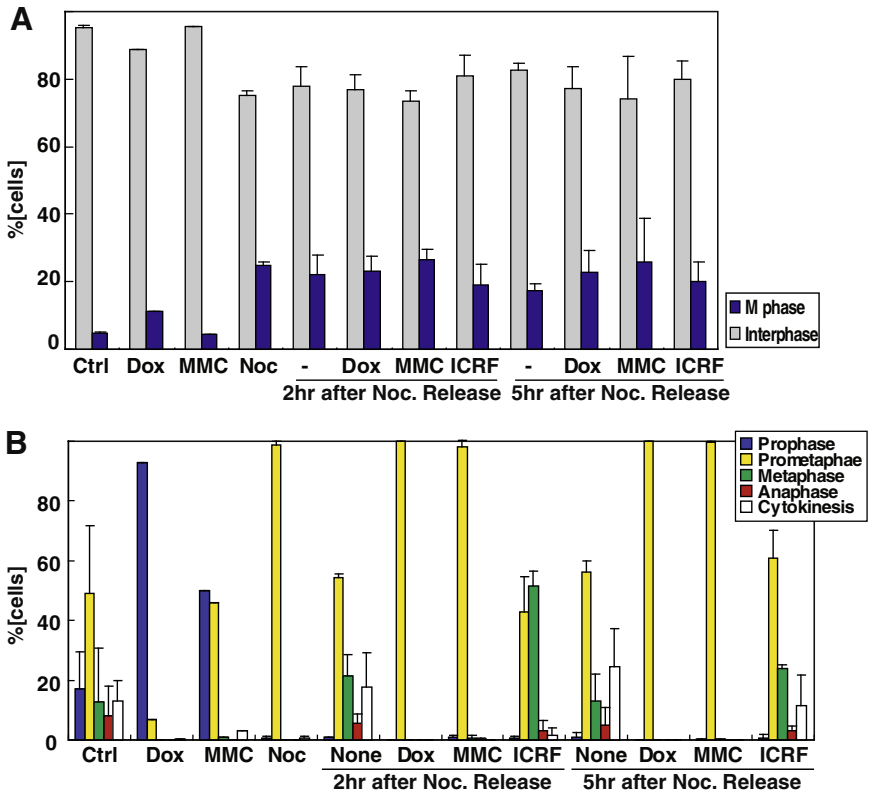

Fig. 2. Mitotic phase distribution following DNA damage. (A) Asynchronously growing HeLa cells were treated with Noc, Dox, MMC, or 100 μM ICRF-193 (ICRF) for 13 h. When assessing the effects of DNA damage in mitosis, nocodazole arrested cells were washed, then challenged with the drugs indicated for 2 h and 5 h, respectively. In each experimental set, 1000 DAPI-stained cells were scored. (B) Mitotic phase from (A) were analyzed further in detail. Approximately 200 cells from (A) were counted and scored. The data represent average scoring of two independent experiments.

5 h (Fig. 2B). This is consistent with reports that inhibition of DNA decatenation interferes with metaphase–anaphase transition [27,30,31] and suggests that DSB and decatenation induces distinct checkpoint controls. Skoufias et al. [31] previously reported that DNA damage does not activate the SAC; the discrepancy with our data may be due to differences in the drug concentration used or in the method of analysis. We used half the concentration of drugs compared to Skoufias and colleagues' work, because we observed massive cell death from high concentration of drug treatment.

We then analyzed mitotic timing following treatment with doxorubicin at the single cell level. *H2B-GFP*-encoding plasmids were transfected into T98G cells before nocodazole arrest and synchronization to facilitate observation of chromosome movement in live cell imaging. Time-lapse microscopy showed that anaphase was delayed for up to 13 h when prometaphase cells encountered DSBs, whereas untreated cells entered anaphase within 45 min of release (Fig. 3A and Supplementary movies 1 and 2). Similar results were obtained in normal MEFs (Fig. 3B and Supplementary movies 3–5), indicating that DSB induction after chromosome condensation induces prometaphase arrest in both normal and cancer cell lines.

2.2. DNA damage-induced prometaphase arrest requires BubR1

We next examined the role of BubR1 in the induction of prometaphase arrest by DNA damage. The phosphorylation status of BubR1 is closely linked to SAC activity and is crucial for mitotic timing. Notably, BubR1 monitors kinetochore-microtubule attachments [20,32] and the signal generated by unattached kinetochores induces hyper-phosphorylation of BubR1 [19], which appears as a characteristic slower migrating band [33].

Synchronized HeLa cells were released into the cell division cycle with or without doxorubicin treatment and assessed for BubR1 status by western blot, and analyses for cell cycle stage were performed at different time points after release (Fig. 4). Cycloheximide treatments were included to block de novo synthesis of the protein. Cells that were not treated with doxorubicin progressed into anaphase 2 h after nocodazole release (Fig 4B), and the level of phospho-BubR1 was markedly reduced at this point (Fig. 4A). In comparison, treatment with doxorubicin resulted in attenuated phosphorylation of BubR1 that persisted for more than 6 h, concomitant with a delay in cell division.

Next, we assessed the intracellular localization of BubR1 following induction of DSBs. Direct inhibition of APC/C by the MCC, which requires BubR1, can occur away from the kinetochores [34], whereas the role of BubR1 in monitoring kinetochore-microtubule attachments [20,32] and checkpoint signaling [18,19,21] requires its localization to kinetochores. In the presence of DSB induced by doxorubicin or MMC, BubR1 was predominantly associated with prometaphase kinetochores. Interestingly, BubR1-positive foci were larger in DNA damaged cells than in normal cycling cells (Fig. 5). The nature of this large BubR1-containing complex warrants future investigation.

Finally, we assessed the role of BubR1 in mitotic progression when cells encountered DSB. HeLa cells transfected with siRNA for BubR1 or control siRNA were synchronized, then challenged with doxorubicin as in Fig. 4. Doxorubicin treatment in control cells showed attenuated phospho-BubR1 and delay in

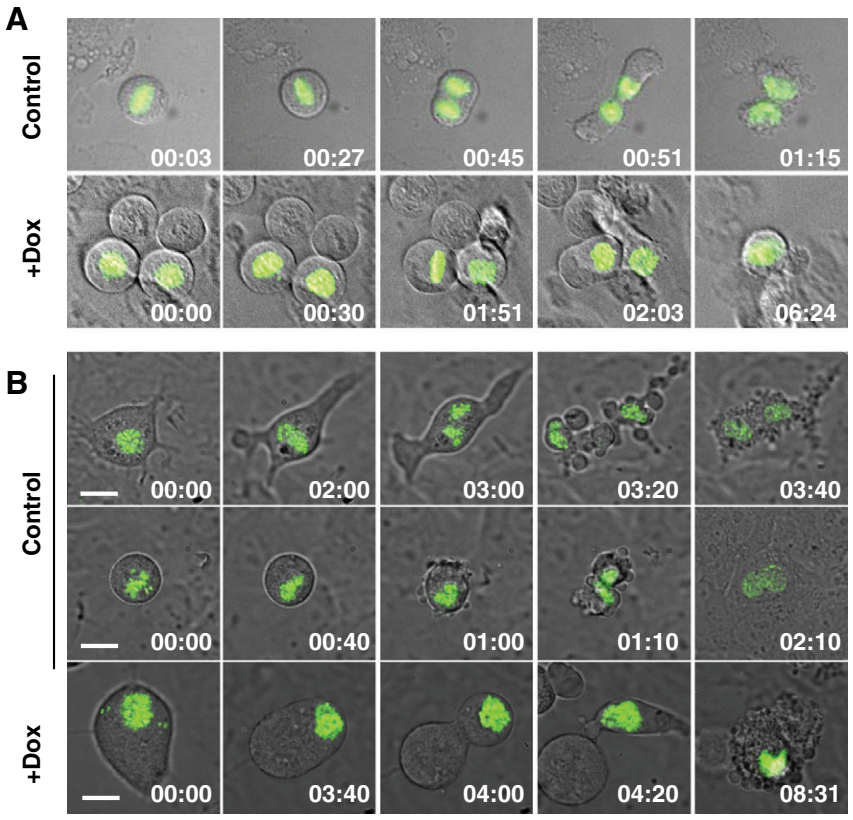

Fig. 3. DNA damage after chromosome condensation delays chromosome segregation. (A) T98G cells were transfected with *H2B-GFP* expression construct and synchronized with nocodazole. Following release from nocodazole arrest, cells were either untreated or treated with doxorubicin and subjected to time-lapse microscopy. Images were captured at indicated time points. (B) Time-lapse microscopy of synchronized normal MEFs transfected with *H2B-GFP*. Captured images are representative of at least five different time-lapse microscopic analyses.

mitotic progression for ~9 h (Fig. 5B & C, siControl). In comparison, doxorubicin treatment in cells repressed for BubR1 expression (Fig. 5B, siBubR1) did not show apparent checkpoint response but entered anaphase in 2 h as in untreated cells (Fig. 5C, siBubR1). Collectively, these results suggest that DSB after chromosome condensation induces kinetochore-associated BubR1 phosphorylation and SAC activation.

3. Discussion

Our results show that formation of DSBs in mitosis induces prolonged hyper-phosphorylation and kinetochore association of BubR1 leading to prometaphase arrest. In contrast, inhibition of topo II catalytic activity resulted in metaphase arrest. This difference implies that DSB formation and inhibition of DNA decatenation generate distinct signals. We speculate that inhibition of decatenation does not induce DSBs but affects the tension between sister chromatids, whereas DSBs might alter chromosome topology and hence the organization of kinetochores. Our findings therefore suggest that the decatenation checkpoint [31,35,36] is distinct from the DSB-induced SAC and indicate that multiple checkpoint mechanisms may control mitotic progression.

Then what is the significance of BubR1 phosphorylation and kinetochore association in the presence of DSBs? BubR1 plays an essential role in kinetochore localization and assembly of other SAC components [19], and also regulates chromosome-spindle attachments [20]. Therefore, it is possible that BubR1 responds to altered chromosome topology caused by DSBs because this affects kinetocore-microtubule attachment and associated events. Alternatively, DSB-induced BubR1 hyperphosphorylation may be required to induce mitotic cell death in the end [10]. Studies in yeast [12] and BRCA2-deficient cells [11] suggested that DNA repair may be coordinated with mitotic checkpoints. Therefore, one possibility is that SAC activation in response to DSB cross-talks with an as-yet-unidentified DNA repair. In this vein, it is interesting to note that a key component in DNA damage checkpoint, Chk1, is required for sustained anaphase delay upon taxol treatment [37].

Our study indicates that BubR1 plays an important role in the DNA damage response. Since accumulated DNA lesions cause cancer, our data suggest that inactivation of BubR1 may be involved in the initiation stage of tumorigenesis: impaired BubR1 phosphorylation or insufficient level of kinetochore-associated BubR1 due to mutation of BubR1-regulating factors may initiate chromosome instability without apparent BubR1 mutation.

4. Materials and methods

4.1. Antibodies and Western blot analysis

Antibodies were purchased as indicated: anti-β-Actin (AC-15) and anti-Flag (M2), Sigma–Aldrich; anti-BubR1, BD Biosciences; CREST, Cortex Biochem; anti-γH2AX, TREVIGEN. Anti-Lamin A/C monoclonal antibody was a gift from Prof. F. McKeon (Harvard Medical School).

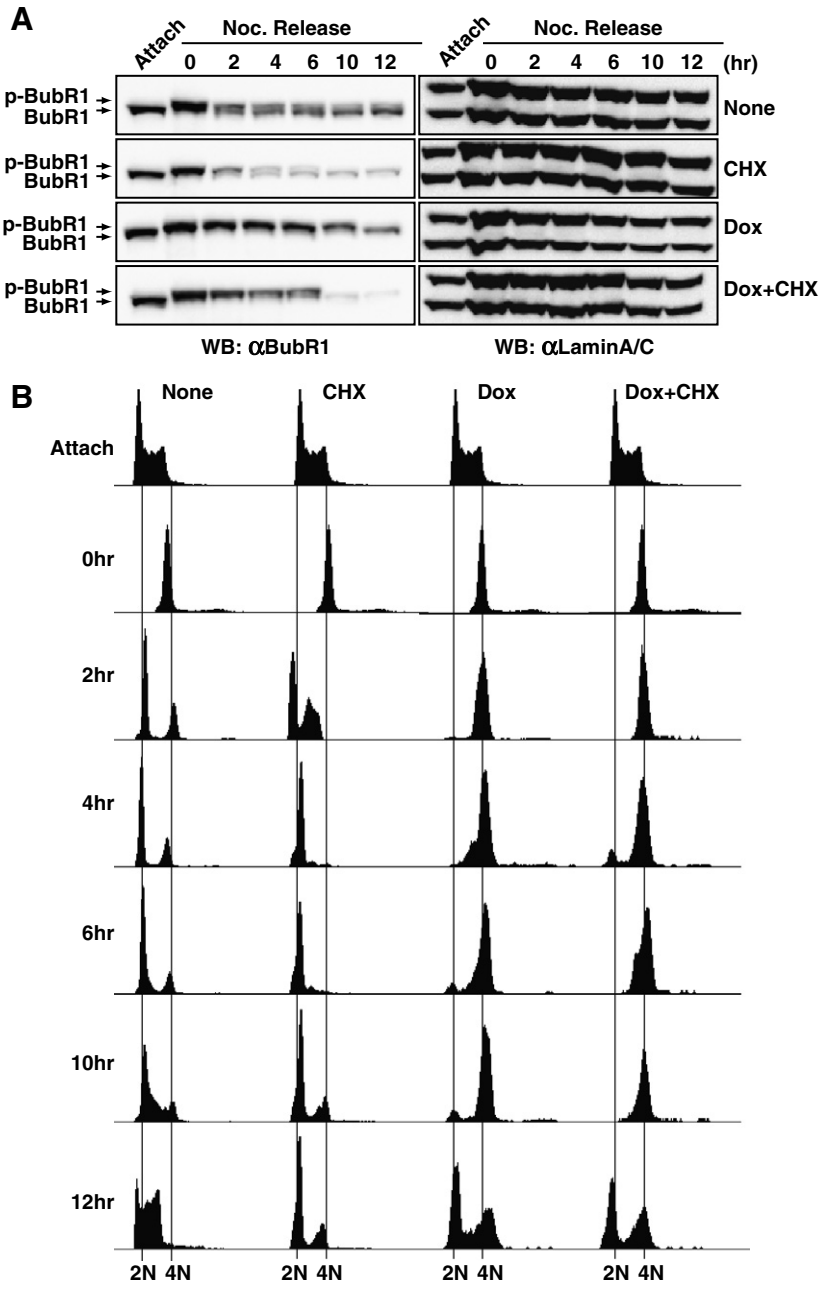

Fig. 4. Chromosome damage results in sustained BubR1 phosphorylation concomitant with delayed progression into anaphase. (A) Western blot analysis of BubR1. Cells were synchronized in prometaphase with nocodazole and subjected to mitotic-shake off, then washed and released into the cell cycle (None). Cells remained attached after mitotic-shake off, thus in interphase, were included for control (Attch). Nocodaozle released cells were treated with Dox with or without 100 μg/mL cycloheximide (CHX) for the time points indicated. The blots were reprobed with anti-Lamin A/C antibody to normalize protein levels. (B) Effects of DNA damage on cell cycle progression at different time points following Dox treatment as in (A). 2N and 4N DNA contents are marked.

4.2. Knock-down expression of BubR1

Synthetic siRNA for BubR1 were designed against 3′-UTR (GUCUCACAGAUUGCUGCCUTT) and siRNA for GFP (GUUCAGCGUGUCCGGCGAGTT) was employed for control. Duplex siRNAs were obtained from Samchully Pharmacy Com. (Daejeon, Korea). 240 nM of duplex siRNAs were transfected into HeLa cells using oligofectamine (Invitrogen).

4.3. Time-lapse video microscopy

Cells were grown in 35-mm Delta-T dishes (Bioptechs Inc., Butler, PA) and transfected with pBOS-GFP-H2B. For synchronization in prometaphase, T98G cells were treated for 13 h with 200 ng/mL nocodazole, or 400 ng/mL nocodazole for MEFs. Synchronized cells were washed three times with PBS and incubated in fresh media with or without doxorubicin. Time-lapse fluorescence and DIC microscopy were performed with a CoolSnap HQ-cooled CCD camera on a DeltaVision Spectris Restoration microscope built around an Olympus IX70 stand, with a 20×/0.75 NA lens (AppliedPrecision). Images were acquired every 3 min for T98G cells and every 10 min for MEFs.

4.4. Immunofluorescence microscopy

For indirect immunofluorescence microscopy, cells were fixed with 4% paraformaldehyde, permeabilized twice in PBS containing 0.5% Triton X-100 for 15 min at room temperature, then incubated in blocking buffer (10% goat serum in 0.1% PBST) for 1 h at room

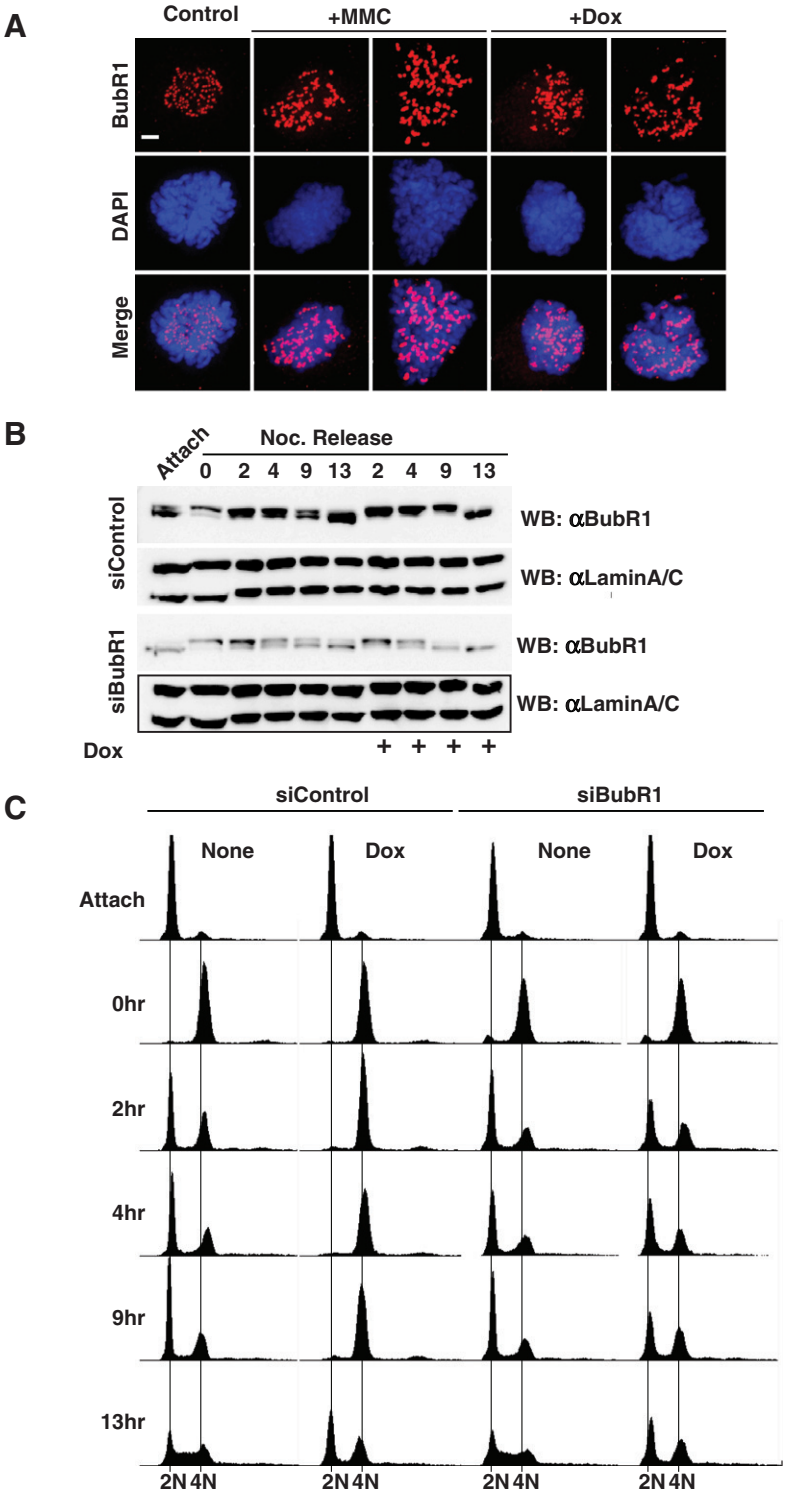

Fig. 5. BubR1 association with prometaphase kinetochores after chromosome damage. HeLa cells were arrested in prometaphase, washed, and treated with Dox (+Dox) or MMC (+MMC) for 2 h prior to staining with anti-BubR1 antibodies and DAPI. (B, C) HeLa cells were transfected with siRNA for BubR1 (siBubR1) or control (siControl), synchronized in prometaphase as in Fig. 4, released, challenged with or without doxorubicin, then analyzed for their cell cycle stages at the indicated time points (C). Concomitantly, BubR1 level was assessed by Western blot. Same blot was reprobed for anti-Lamin A/C (B). Cells remained attached after mitotic-shake off, were included for control (Attch).

temperature. Cells were incubated with primary antibodies diluted in blocking buffer for 1 h, followed by incubation with secondary antibodies in blocking buffer. After a final rinse, the cells were mounted on microscope slides in VECTASHIELD containing DAPI. Images obtained from DeltaVision were acquired as a series of 0.4 μm-thick image sections and merged.

4.5. Flow cytometric analysis of cell cycle

Cells were fixed with cold 70% ethanol, and stained with propidium iodide (PI) for FACS analysis (FACSCalibur; Becton Dickinson, Franklin Lakes, NJ). Data were processed using CellQuest software.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.febslet.2008.04.028.

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