DNA damage response (DDR) is vital for genomic stability, and its deficiency is linked to tumorigenesis. Extensive studies in interphase (G1-S-G2) mammalian cells have revealed the mechanisms of DDR in great detail; however, how mitotic cells respond to DNA damage remains less defined. We report here that a full DDR is suppressed in mitotic mammalian cells until telophase/cytokinesis. Although early DDR markers such as the phosphorylations of ataxia telangiectasia mutated (ATM) and histone H2A.x (H2AX) can be readily detected, the ionizing radiation-induced foci (IRIF) formation of late DDR markers such as breast cancer type 1 susceptibility protein (BRCA1) and p53-binding protein 1 (53BP1) are absent until the telophase/cytokinesis stage. We further showed that the IR-induced ubiquitination cascade around DNA damage sites did not occur in mitotic cells, which explains, at least in part, why BRCA1 and 53BP1 cannot be recruited to the damaged sites. These observations indicate that DDR is suppressed in mitotic cells after the step of γH2AX formation. Not surprisingly, we found that the absence of a full DDR in mitotic cells was associated with the high cyclin-dependent kinase 1 (CDK1) activities. More 53BP1 IRIF could be detected when the irradiated mitotic cells were treated with a CDK1 inhibitor. Further, the activation of CDK5 in interphase cells impedes the formation of 53BP1 IRIF. Together, these results suggest that the DDR is suppressed by the high CDK1 activity in mitotic mammalian cells.

To maintain genomic integrity, damaged DNA in a cell has to be repaired in a timely and efficient manner. The DNA damage response (DDR) is a complex signal transduction network that detects different types of DNA damage and activates appropriate DNA repair machineries. It was evolutionarily conserved from yeasts to humans. Genetic studies in yeasts have identified a large number of genes that take part in DDR, and a majority of them have homologues in mammals. Studies in human cells and mouse models have showed definitively that deficiencies in various DDR components are linked to human diseases and heightened cancer risks (for review, see Refs. 1, 2).

A well studied example of DDR is how mammalian cells deal with double strand breaks (DSBs). DSBs are quickly detected by the Mre11-Rad50-NBS1 (MRN) complex and the subsequent retention of MRN complex at DSB sites is crucial for the activation and chromatin tethering of ataxia telangiectasia mutated (ATM) kinase. ATM is a master kinase during DDR. It phosphorylates a large number of substrates including the histone H2A variant H2AX at serine 139 and NBS1. Both phosphorylated H2AX (γH2AX) and NBS1 can bind and recruit the mediator protein MDC1 for phosphorylation by ATM (for review, see Refs. 3, 4). The phosphorylated MDC1 then recruits the E3 ubiquitin ligase RNF8 to initiate a localized ubiquitination cascade that eventually leads to the recruitment of BRCA1 and 53BP1 to the damaged sites for further signaling and repair (5–8).

To prevent damaged DNA from being replicated or transmitted to daughter cells, cell cycle progression is usually halted by DNA damage checkpoints. Depending on the phase of the cell cycle when the damage is encountered, the DNA damage checkpoint utilizes different mechanisms to arrest cell cycle progression for DNA repair. In general, the cyclin-dependent kinases (CDKs) are inhibited in response to DNA damage as they are the driver for cell cycle progression. On the other hand, certain CDK activities seem necessary for proper DNA damage response. A number of DDR components can be phosphorylated by CDKs, and these modifications regulate checkpoint signaling and repair pathway choices (for review, see Ref. 9). Studies in budding yeasts suggest that CDK1 activity promotes DNA damage checkpoint activation, DNA DSB end resection, and homologous recombination-dependent repair (10, 11). The treatment of human cells with CDK inhibitor roscovitine results in deficient DDR signaling and DNA repair (12, 13). More specifically, the loss of CDK1-mediated phosphorylation of BRCA1 was shown to compromise its recruitment to the damage sites and the S phase DNA damage checkpoint signaling (14), whereas the CDK2-dependent phosphorylation of ATR-interacting protein (ATRIP) was shown to be required for an efficient G2-M DNA damage checkpoint (15). Interestingly, it was shown recently in murine cells with triple knock-out of interphase CDKs (CDK2, 4, 6) that the overall CDK activity rather than some specific CDK is required for the activation of DDR (16).
**DDR Is Suppressed in Mitotic Mammalian Cells**

Despite a vast body of knowledge about DDR in interphase (G$_1$–S–G$_2$) cells, little is known about how mitotic cells deal with DNA damage. We previously showed that when nocodazole (NOC)-arrested (mitotic) HeLa cells were irradiated and released from the arrest, these cells proceeded through mitotic division until late telophase when they were blocked from finishing cytokinesis and became binucleated (17). This observation leads to the suggestion that there exists a mitotic exit DNA damage checkpoint in mammalian cells which prevents the segregation of broken chromosome arms as demonstrated in yeasts (18–22). It is intriguing that although H2AX is robustly phosphorylated in prometaphase after ionizing radiation (IR), the later steps of canonical DDR including the recruitment of BRCA1 and CHK1 (checkpoint kinase 1); activation only take place in late mitosis at telophase stage (17). Why the cells do not mount a full DDR in early mitosis is unclear. In this report, we examined the DDR beyond the H2AX phosphorylation step in mitotic cells. We provide evidence suggesting that the high mitotic CDK1 activity may interfere with DNA damage response in early mitosis.

**EXPERIMENTAL PROCEDURES**

**Cell Culture and Treatment**—HeLa and U2OS cells were grown in high glucose DMEM (Mediatech) supplemented with 10% FBS and 1× penicillin-streptomycin (Invitrogen). The cells were cultured in a humidified incubator (Forma II; Thermo) at 37 °C in 5% CO$_2$ environment. The IR was introduced by a $^{137}$Cs source (Gammacell 1000 irradiator; Best Theratronics).

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To arrest cells in mitosis, 100 ng/ml NOC (Sigma) was added into the medium for 16 h. Mitotic cells were collected through shake-off. To inhibit mitotic CDK1 or PLK1 activity, 3 μM alsterpaullone (Sigma) or 200 nM BI2536 (ChemieTek) was added to the medium containing NOC.

**Plasmids and Transfection**—pcDNA3-CDK5-GFP (#1346) and pCMV-myc-p35 (#1347) were obtained from Addgene. HeLa cells were transfected with different plasmids using Lipofectamine 2000 (Invitrogen) following the manufacturer's instruction. These two plasmids were transfected at the ratio of 1:3 to ensure that GFP$^+$ cells represented the cells that were transfected with both plasmids. 48 h after the transfection, the cells were given 10 Gray IR and then fixed 1 h later with 4% paraformaldehyde in PBS for immunofluorescence staining.

**Immunofluorescence Microscopy**—After various treatments, the cells (grown on coverslips) were fixed in 4% paraformaldehyde in PBS for 15 min at room temperature, permeabilized with 0.5% Triton X-100 in PBS for 10 min, and blocked in 2% BSA in PBS for 1 h at room temperature. The cells were then incubated with primary antibodies at 4 °C overnight. After being washed three times in PBS, the cells were incubated with Cy3-conjugated goat anti-rabbit/mouse secondary antibodies (Jackson ImmunoResearch) or FITC-conjugated goat anti-rabbit/mouse secondary antibodies (Jackson ImmunoResearch) for 1 h at room temperature. The cells were washed again twice in PBS and counterstained with 1 μg/ml DAPI (Sigma) for 10 min at room temperature. After a final wash in PBS, the coverslips were mounted onto slides with Gel Mount medium (Sigma) and were examined on an inverted microscope Axiosvert 200M (Carl Zeiss) using a 40× objective. Images were processed using AxioVision 4.6 software (Carl Zeiss).

Primary antibodies and dilutions used for immunofluorescence were: mouse anti-γH2AX (Millipore, 05-636) 1:200, rabbit anti-53BP1 (Novus, NB100-304) 1:500, mouse anti-α-tubulin (from the Developmental Studies Hybridoma Bank at University of Iowa, AA4.3) 1:50, mouse anti-multibiquitin (FK2) (Assay Designs, SPA-205) 1:100, rabbit anti-hBRITI 1:200.

**Immunoblotting and Immunoprecipitation**—For protein extraction, the cells were lysed in EBC lysis buffer (50 mM Tris-Cl, pH 8.0, 120 mM NaCl, 0.5% Nonidet P-40 (v/v), 100 mM NaF, 0.2 mM Na$_3$VO$_4$) supplemented freshly with 1 mM PMSF, mixture protease inhibitor (Roche Applied Science), and phosphatase inhibitors (5 mM β-glycerophosphate and 5 mM sodium pyrophosphate). After centrifugation (16,000 × g, 15 min., 4 °C), the supernatants were collected as soluble fractions. The pellets were further subjected to the extraction of chromatin-associated proteins or histones. For chromatin-associated proteins, the above mentioned pellets were resuspended (10$^7$ cells/ml) in EBC buffer supplemented with an additional 0.3 mM NaCl. The suspension was cleared with a sonicator (Misonix) for 2 min (10 s on/5 s off) on ice, incubated on ice for another 10 min, and spun again (16,000 × g, 15 min., 4 °C) to obtain the supernatant as chromatin associated-protein fraction. For histone extraction, the pellets were washed once in EBC and again resuspended in 0.2 mM HCl (10$^7$ cells/ml). The suspension was incubated overnight at 4 °C and spun again (16,000 × g , 15 min., 4 °C). The supernatant was collected as the histone fraction. The histone fraction was neutralized with 1 mM NaOH before further analyses. Protein concentrations in all fractions were measured using the Bradford method. Equal amounts of proteins weremixed with 2× SDS-PAGE loading buffer and boiled for 5 min before being resolved with 8–10% SDS-polyacrylamide gel for immunoblotting analysis. We used the enhanced chemiluminescence (Western Lighting ECL, PerkinElmer Life Sciences) for detection of the blotted proteins.

For the immunoprecipitation of FLAG-BRIT1, mouse anti-FLAG antibodies were added into the chromatin-associated protein extract to a final concentration of about 5 μg/ml. After overnight incubation on a rocking platform at 4 °C, protein G beads (GE Healthcare) were added and further incubated for 1 h at 4 °C. The immune complexes immobilized on the beads were washed three times in cold EBC buffer, boiled in 1× SDS-PAGE loading buffer for 5 min, resolved in a SDS-polyacrylamide gel, and analyzed by immunoblotting.

For calf intestine phosphatase treatment, the immunoprecipitated FLAG-BRIT1 complexes on protein G beads were suspended in 40 μl of 1× NEB buffer 3 (New England Biolabs) plus 2 μl of calf intestine phosphatase (New England Biolabs), incubated at 37 °C for 1 h. The dephosphorylation reaction was stopped with the addition of 40 μl of 2× SDS-PAGE loading buffer and boiling.

Primary antibodies and dilutions used in immunoblotting were: mouse anti-ATM-pS1981 (200-301-400; Rockland) 1:500; mouse anti-ATM (ab78; Abcam) 1:1000; mouse anti-α-tubulin (same as above) 1:500; rabbit anti-γH2AX (#2577; Cell Signaling) 1:500; rabbit anti-H3 (H0164; Sigma) 1:5000; mouse anti-GFP (G096; ABM) 1:1000; mouse anti-myc (sc-40; Santa Cruz) 1:500.
DNA Damage Response in Mitosis

**RESULTS**

**Late DDR Marker 53BP1 Cannot Be Detected on Damaged DNA during Mitosis Until Telophase**—We previously observed that in mitotic HeLa cells, although the early DDR marker γH2AX was strongly induced after IR, the IR-induced foci (IRIF) of the late DDR marker BRCA1 could not be detected (17). To confirm this result, we tested whether another late DDR component, 53BP1, could form IRIF in HeLa cells arrested by NOC treatment. As expected, 53BP1 IRIF was also absent in mitotic cells despite the presence of γH2AX on the condensed chromosomes (Fig. 1A). A similar observation has been reported recently (23, 24).

To determine when 53BP1 is recruited to damaged DNA during mitosis, irradiated HeLa cells were released into fresh medium from NOC-mediated prometaphase arrest. The cells were harvested at different time points and analyzed. We found that 53BP1 foci began to appear only at late telophase or early cytokinesis stage (Fig. 1C). In contrast, γH2AX staining persisted throughout mitosis in damaged cells (Fig. 1, A and B). The kinetics of 53BP1 IRIF formation in mitotic is identical to that of BRCA1 IRIF reported in our previous study (17).

The absence of BRCA1 and 53BP1 IRIF in prometaphase was not due to insufficient time for a full response because neither BRCA1 nor 53BP1 IRIF could be detected even when the irradiated cells were kept in NOC-containing medium for up to 6 h (data not shown, and also see Ref. 17). To exclude the possibility that the NOC treatment-elicited strong activation of the spindle assembly checkpoint somehow interrupts DDR signaling, we obtained mitotic HeLa cells through two other methods. In the first method, the NOC-arrested cells were released into MG132-containing medium for 2 h to allow the cells progress into and be arrested (by MG132) at metaphase. The cells were then irradiated and washed to remove MG132. One hour after MG132 was washed off, the cells were processed for 53BP1 staining. In the second method, HeLa cells were enriched in G2 by CDK1 inhibitor alsterpaullone treatment (16 h) and then released. When the majority of the cells had entered mitosis (in ~7 h, judged by the typical roundup mitotic morphology), they were irradiated and cultured for another 2 h before being processed for the staining of DNA damage marker 53BP1. In both cases, 53BP1 IRIF were undetectable until the telophase/cytokinesis stage (supplemental Fig. S1). Thus, the lack of a full DDR in irradiated NOC-arrested prometaphase cells is unlikely a result of the activation of the spindle assembly checkpoint.

It was reported previously that heavy phosphorylation of histones (such as H3S10) in mitotic cells interfered with the detection of other chromatin modifications, and a pretreatment with λ-phosphatase largely removed the interference (25). To determine whether this interference also prevented us from detecting DDR markers, we fixed and pretreated the NOC-arrested and irradiated cells with λ-phosphatase before immunostaining. The pretreatment effectively eliminated the robust γH2AX staining in mitotic cells, but it did not lead to the staining of 53BP1 IRIF (supplemental Fig. S2). The 53BP1 IRIF staining in the neighboring interphase cells was not affected by the λ-phosphatase treatment.

**IR-induced Ubiquitination Cascade Is Compromised in Mitotic Cells**—To determine at which step the DDR signaling is attenuated in mitotic cells, we first examined the status of master DNA damage checkpoint kinase ATM. As expected, the phosphorylation at serine 1981 after IR was similar in mitotic cells as in unsynchronized interphase cells (Fig. 2A,B). Next, we analyzed H2AX. Consistent with the immunostaining, the formation of γH2AX after IR in mitotic cells was unaffected. In fact, it was even more robust than in interphase cells. However, the IR-induced formation of monoubiquitinated γH2AX was compromised in mitotic cells (Fig. 2B). Because the ubiquitination of H2AX is an indicator for later DDR ubiquitination-dependent signaling, we suspected that the typical polyubiquitination signaling around damage sites might be compromised as well. Indeed, the IR-induced polyubiquitin foci detected with FK2 antibodies was absent in mitotic cells (Fig. 2C). Taken together, these results suggest that DDR in mitotic cells cannot elicit efficient ubiquitination signaling cascade around DNA damage sites, compromising the subsequent recruitment of...
BRCA1 and 53BP1. This conclusion is further supported by the recent finding that the ubiquitin E3 ligases RNF8 and RNF168, which are required for efficient ubiquitination cascade around DNA damage sites and for the formation of BRCA1 and 53BP1 IRIF (5–8, 26, 27), were not recruited to DNA damage sites in mitotic cells (23).

**Inhibition of CDK1 Activity Promotes Formation of 53BP1 IRIF in Mitotic Cells**—One of the most prominent features of early mitosis is the robust CDK1 activity. We wondered whether this high CDK activity could block DDR signaling downstream of H2AX phosphorylation. This certainly makes sense as MDC1 (23, 28), RNF8 (23), and 53BP1 (23, 24) are phosphorylated during mitosis and the phosphorylation may suppress their functions or interactions with other DDR components for signaling. To investigate the role of CDK1 in blocking DDR signaling, we took advantage of a potent CDK1 selective inhibitor alsterpaullone (29). We collected mitotic HeLa cells through shake-off and kept the cells in the NOC-containing medium. The cells were either released into fresh medium without NOC or kept in NOC medium for certain time points before the analysis. As expected, acute CDK1 inhibition led to the decondensation of chromatin and the loss of chromosomal Aurora B staining (Fig. 3A). The inhibition of CDK1 also led to the formation of 53BP1 IRIF, whereas dimethyl sulfoxide treatment had no such effects (Fig. 3). This result suggests that high CDK1 activity suppresses late DDR signaling. Full DDR signaling becomes possible only in late mitotic cells when cyclin B1 is degraded and CDK1 activity drops dramatically.

**Ectopic Expression of CDK5-p35 Compromises DDR Signaling in Interphase Cells**—To test the idea further that high CDK1 activity inhibits DDR signaling, we examined whether ectopic activation of CDK1 in interphase cells can also block DDR signaling. However, such activation will force cells entering mitosis as demonstrated by the overexpression of nondegradable cyclin B1 (30, 31). Further, ectopic activation of other cell cycle-related CDKs may also cause unwanted cell cycle effects that may complicate the analysis. We therefore turned our attention to CDK5, a noncanonical CDK. CDK5 does not play a role in mammalian cell cycle progression although it shares 60% sequence identity to CDK1. Moreover, CDK5 is not activated by cyclins but by its specific partner p35 or p39. Despite the lack of cell cycle function, CDK5 can phosphorylate many CDK1 substrates (for review, see Refs. 32, 33). We therefore decided to express CDK5-p35 ectopically and determine whether DDR signaling can be suppressed. A similar strategy was recently used to demonstrate the role of CDK1 in autophagy (34). We transfected HeLa cells with plasmids encoding CDK5-GFP and myc-p35 and then examined the formation of 53BP1 IRIF. As shown in Fig. 4A, 53BP1 IRIFs were largely abrogated in CDK5- and p35-co-expressing cells. In contrast, 53BP1 IRIFs were formed normally in cells transfected with CDK5-GFP or myc-p35 alone (Fig. 4, A and B). More significantly, the IR-induced γH2AX foci formation was not affected by CDK5-p35 expression (Fig. 4A).
4C). These results suggest that the ectopic activation of CDK5 in interphase cells can also inhibit DDR signaling downstream of the γH2AX step, reminiscent of the mitotic DDR.

High Cdk1 or Cdk5 Activity Also Inhibits Formation of BRIT1 IRIF—In the effort to identify the step of DDR signaling that is suppressed during early mitosis, we found that another early DDR component, BRIT1/MCPH1 (35, 36), could not form IRIF until telophase/cytokinesis stage, similar to BRCA1 and 53BP1 (Fig. 5A). Furthermore, the formation of BRIT1 IRIF was also abrogated in cells expressing CDK5-p35 (Fig. 5B). BRIT1 is known to play an important early role in DDR, and it functions at the step just downstream of γH2AX formation (37, 38). These results suggest that the DDR function of BRIT1 is suppressed during early mitosis as well, which could contribute to the lack of a full DDR signaling in mitotic cells. Interestingly, BRIT1 is also hyperphosphorylated during mitosis, as the slow migrating band of mitotic FLAG-BRIT1 can be reversed to normal status after phosphatase treatment (Fig. 5C). Furthermore, ectopic CDK5 activity can phosphorylate BRIT1 just as mitotic CDK1 can (Fig. 5D). These results further support the notion that high CDK1 activity in early mitosis is responsible for the lack of a full DDR.

**DISCUSSION**

Results from several studies including ours together demonstrate that the DDR in mitotic mammalian cells are distinct from that in interphase cells. First, the early mitotic cells cannot...
mount a full DNA damage response. The DDR stops at the phosphorylation of H2AX step. Second, the early mitotic cells do not stop the cell cycle immediately after DNA damage as in budding yeasts; instead, they continue mitotic progression to the telophase/cytokinesis stage where the DNA damage checkpoint is fully activated and blocks cytokinesis, generating binucleated/tetraploid cells (17, 39, 40). Whether the cytokinesis block depends on the severity of damage. If the DNA damage is not severe (for example, 2-Gray IR), the majority of the damaged cells can finish mitosis successfully and repair their DNA in the next G1 phase (17, 23). If the DNA is severely damaged (for example, 10-Gray IR), most of the cells will fail to complete cytokinesis and become tetraploid/binucleated, leading to a p53-dependent cell cycle withdrawal (17, 39).

An intriguing question is why the DDR signaling is blocked during early mitosis. One possibility is that this is due to the high CDK1 activity in early mitosis. As soon as anaphase begins, the activity of CDK1 drops due to anaphase promoting complex-mediated ubiquitination and degradation of cyclin B1 (41). By telophase, CDK1 activity is reduced to minimal, and a full DDR can be mounted. Thus, DDR in mitosis negatively correlates with CDK1 activity, suggesting that CDK1 may interfere with DDR. Indeed, acute inhibition of endogenous CDK1 activity in mitotic cells restored DDR signaling, and the introduction of CDK1-like activity through CDK5-p35 expression in interphase cells inhibited DDR signaling (this work).

An alternative explanation for the lack of a full DDR in early mitosis is that the highly condensed nature of chromatin prevents the assembly of DDR signaling complexes. However, there are several lines of evidence that indicate otherwise. First, it is well established now that DNA damage can induce robust γH2AX formation during mitosis, indicating that the activated ATM kinase can access H2AX in the condensed chromosomes. Second, a recent study also showed that the MRN complex and MDC1 could be efficiently recruited to DNA damage sites on condensed mitotic chromosomes (23). Third, the ectopic expression of CDK5-p35 complex in interphase cells did not induce apparent chromatin condensation as judged by DAPI staining (Fig. 4B), but 53BP1 IRIF were greatly compromised in these cells. Finally, during meiosis, chromosomes are also condensed, and yet they are broken at certain frequencies purposely to initiate homologous recombination. These endogenous DNA DSBs do elicit a full DDR signaling for efficient homologous recombination (42).

Given these considerations, we favor the explanation that the lack of a full DDR in early mitotic cells is due to the high CDK1 activity. It has been shown that several DDR components, including MDC1 (23, 28), RNF8 (23), RPA2 (39, 40), 53BP1 (23, 24), BRCA2 (43), and BRIT1 (Fig. 5C) are hyperphosphorylated during mitosis, and these proteins all contain a number of consensus CDK1 sites. Such phosphorylation may very well block the functions of these DDR signaling molecules. For instance, sequence analysis of BRIT1 protein indicates that its C-terminal BRCT domain contains two consensus CDK1 sites, and this domain is known to be required for BRIT1-H2AX interaction at DNA damage sites to promote late DDR signaling (38, 44). It will be of importance in the future to demonstrate that the mitotic phosphorylation of BRIT1, RNF8, 53BP1, or other components negatively regulates their functions in DDR.

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