Interplay between the DNA Damage Proteins MDC1 and ATM in the Regulation of the Spindle Assembly Checkpoint

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Background: The spindle assembly checkpoint (SAC) and the DNA damage response (DDR) are two distinct pathways designed to ensure genomic stability. ATM and MDC1 regulate the SAC.

Results: ATM phosphorylation of H2AX at mitotic kinetochores recruits MDC1. ATM and MDC1 modulate kinetochore localization and proper assembly of key SAC factors.

Conclusion: ATM and MDC1 regulate the SAC.

Significance: DDR core proteins maintain genomic stability also by regulating mitosis progression.

To avoid genomic instability, cells have developed surveillance mechanisms such as the spindle assembly checkpoint (SAC) and the DNA damage response. ATM and MDC1 are central players of the cellular response to DNA double-strand breaks. Here, we identify a new role for these proteins in the regulation of mitotic progression and in SAC activation. MDC1 localizes at mitotic kinetochores following SAC activation in an ATM-dependent manner. ATM phosphorylates histone H2AX at mitotic kinetochores, and this phosphorylation is required for MDC1 localization at kinetochores. ATM and MDC1 are needed for kinetochore localization of the inhibitory mitotic checkpoint complex components, Mad2 and Cdc20, and for the maintenance of the mitotic checkpoint complex integrity. This probably relies on the interaction of MDC1 with the MCC. In this work, we have established that ATM and MDC1 maintain genomic stability not only by controlling the DNA damage response, but also by regulating SAC activation, providing an important link between these two essential biological processes.

Proper response to DNA damage is essential for maintaining genome stability. Eukaryotic cells have evolved the DNA damage response (DDR) to sense and repair DNA damage. The DDR includes repair of the damage, checkpoint activation, and, when required, activation of cellular senescence or apoptosis. Among the different types of DNA lesions, DNA double-strand breaks (DSBs) are the most harmful because if not responded to properly, they can result in genomic instability. Following the formation of a DSB, DDR proteins accumulate at the chromatin surrounding the break. This focus formation is an essential step in DDR activation.

ATM, a phosphatidylinositol-3 kinase-like family member, is the key transducer kinase in the response to DSBs. It is activated immediately following DSB induction and recruited to DSBs where it phosphorylates numerous DDR substrates, hence regulating their activity. Moreover, ATM is important for the activation of the G1/S, intra-S phase and G2/M checkpoints, and for DSB repair. One of the first targets of ATM is histone H2AX, which is phosphorylated on serine 139 at the chromatin surrounding the DSBs. The phosphorylated form of histone H2AX (referred to as γ-H2AX) is crucial for the recruitment of DDR proteins to DSBs and thus for an intact DDR.

One of ATM substrates is MDC1, which is a central member of the DDR. MDC1 is an adaptor protein that is composed of several protein-protein interacting modules. Promptly after DSB induction, MDC1 is recruited to DSBs, where it directly binds γ-H2AX, as well as other DDR members. MDC1 recruits numerous proteins to sites of damage. It is essential for DSB repair and for the activation of the G2/M and intra-S phase checkpoints.

The mitotic spindle assembly checkpoint (SAC) is another mechanism employed by cells to avoid genomic instability. It regulates metaphase to anaphase transition by ensuring the fidelity of chromosome segregation. The kinetochores, which are large proteinaceous complexes, assemble on the centromeres and monitor the microtubule attachment state. At prometaphase, kinetochores start to interact with the microtubules and by the end of metaphase, all chromosomes are bi-oriented, with sister kinetochores connected to microtubules from opposite spindle poles. Incorrectly attached or unattached kinetochores during metaphase activate the SAC.

The primary target of the SAC is Cdc20, a co-activator of the anaphase-promoting complex/cyclosome, which is an E3-ubiquitin ligase that targets cell cycle regulators for degradation. During the SAC, the mitotic checkpoint complex (MCC; composed of Mad2, BubR1, and Bub3) binds Cdc20 to form an inhibitory complex, which prevents Cdc20 from activating the anaphase-promoting complex/cyclosome. Once bi-orientation occurs for all chromosomes, the SAC is inactivated. Cdc20 is released from the MCC and can then activate the anaphase-promoting complex/cyclosome to promote the metaphase to anaphase transition.

Increasing evidence support a mitotic role for DDR proteins. The DDR checkpoint kinase Chk1 controls SAC activation by...
regulating the SAC kinases BubR1 and Aurora-B (17). The phosphatidylinositol-3 kinase-like family member DNA-dependent protein kinase catalytic subunit regulates microtubule dynamics and chromosome segregation during mitosis (18). The active form of ATM localizes at centrosomes during mitosis (19, 20). When excessive DNA damage is introduced to mitotic cells, ATM inhibits centrosome-dependent spindle formation, leading to a transient mitotic arrest, probably through SAC activation (21). Additionally, following spindle disruption, ATM relocates from centrosomes to distinct cellular foci, which also contain activated p53 (22). Recently, it was shown that ATM phosphorylates the mitotic protein NuSAP, leading to an ATM-dependent mitotic arrest (23). Moreover, aneuploidy was observed in human and mice cells depleted of ATM as well as in clinical samples from ataxia telangiectasia (A-T; deficient of a functional ATM) patient brains (24–26). Furthermore, ATM is phosphorylated on serine 1403 during mitosis by the Aurora-B kinase. This mitotically activated ATM phosphorylates the SAC protein Bub1 (27). Loss of ATM function results in shortened mitotic timing and a defective SAC (27). Finally, MDC1 is hyperphosphorylated in cells arrested in mitosis (28–30).

In this study, we established a mitotic role for MDC1 and ATM in regulating SAC activation. MDC1-depleted cells complete mitosis and escape mitotic blockage by SAC activation faster than control cells. We further demonstrated that cells lacking ATM undergo mitosis faster than control cells. We found that MDC1 is a kinetochore protein that interacts with the MCC. We also show that histone H2AX is phosphorylated at mitotic kinetochores by ATM. This phosphorylation is needed for the localization of MDC1 at kinetochores. Interestingly, both MDC1 and ATM are required for proper localization of Mad2 and Cdc20 at kinetochores and for the formation of an intact MCC during SAC activation. Our findings reveal a novel regulatory role for MDC1 and ATM in the SAC and propose a new link between the DDR and cell cycle regulation.

**EXPERIMENTAL PROCEDURES**

**Extract Preparation and Treatments**—Protein extracts were prepared according to Ref. 31. Cells were treated with 100 nm nocodazole, 25 ng/ml taxol, 100 ng/ml colcemid, ionizing radiation (RX-650; Faxitron X-ray Corp.) or 10 mm KU-55933 (KuDOS Pharmaceuticals). A-T fibroblasts and stable HeLa cell lines containing shRNA-MDC1 and shRNA-LacZ (32) were transfected into cells using Oligofectamine (Invitrogen). A-T fibroblasts and stable HeLa cell lines containing shRNA-MDC1 and shRNA-LacZ (32) were a kind gift from K. Wassmann (University Paris 6, France). Anti-BubR1 antibodies (SBR1.1 (34)) were a kind gift from S. Taylor (Manchester University, Manchester, UK). Human autoimmune antibodies, which recognize centromeres (CREST) were a kind gift from W. Earnshaw (Edinburgh, UK).

**Plasmids**—HA-MDC1-FHA, HA-MDC1-tBRCT and HA-MDC1 were described (33). siRNA-resistant MDC1 was generated by mutating five nucleotides (resulting in silent mutations) in HA-MDC1, using the QuickChange site-directed mutagenesis kit (Stratagene). The plasmid was transfected into cells using Lipofectamine (Invitrogen). HA-H2AX was generated by PCR of H2AX from pQE9-H2AX (a kind gift from S. P. Jackson, Cambridge University, Cambridge, UK) and cloned into pcDNA3-HA. HA-mH2AX was generated by mutating serine 139 to alanine by a site directed mutagenesis performed on HA-H2AX.

**RNA Interference**—siRNA against MDC1 and GFP sequences (33) were transfected into cells using Oligofectamine (Invitrogen). The sequence of an additional siRNA against MDC1 that was used in this study (siMDC1–2) was GUUGUAACUGAAAUCUGACG.

**Time Lapse Microscopy**—Cells were grown on eight-well µ-slides (Ibidi) and filmed by time-lapse microscopy (37 °C in 5% CO2). Bright field images were acquired every 4 to 5 min using a FV-1000 confocal microscope (Olympus) equipped with an IX81 inverted microscope and a 40×/0.6 objective and analyzed using NIH ImageJ software.

**Immunoprecipitation Assays**—IPs were done with the indicated antibodies and protein A or G-Sepharose beads (Santa Cruz Biotechnology). Extracts (1–2 mg) were added to the reaction. Beads were washed with wash buffer (20 mM Tris, pH 7.5, 0.2 mM EDTA, 0.5 mM DTT, 0.2% Triton X-100, 150 mM NaCl), and bound proteins were subjected to SDS-PAGE and Western blot analysis. Western blots were quantified using the Image Gauge software (version 4.0, FUJIFILM Science Lab).

**Mitotic Spreads and Immunofluorescence Assays**—For mitotic spreads, cells were treated for 2 h with 100 ng/ml colcemid or 25 ng/ml taxol. Mitotic cells were obtained by mitotic shake-off. Chromosomes spreads were done by cytocentrifugation on positively charged Superfrost-plus adhesion slides (Thermo Scientific).

For immunofluorescence assays, cells were grown on poly-L-lysine (Sigma-Aldrich) coverslips. 2 h after 100 nm nocodazole addition the cells were fixed in methanol and acetone, or with formaldehyde. The coverslips were rinsed in PBS and blocked for 1 h in 10% FBS in PBS.

Cells or chromosomes were immunostained with primary and secondary Dylight488- or Dylight594-conjugated antibodies (Jackson Immunoresearch Laboratories). Slides were viewed using the Eclipse TE2000-E inverted fluorescence microscope (Nikon) equipped with a 60×/1.4 oil-immersion objective and a Vosskühler COOL-1300Q camera and analyzed via NIH AR software (version 2.3) or with FV-1000 confocal microscope (Olympus) equipped with an IX81 inverted microscope and a 60×/1.4 oil-immersion objective. After scanning, a maximum intensity projection was generated from the serial sections (Olympus software, version 1.6). The fluorescence levels were quantified using the “Analyze Particles” function of NIH ImageJ software.

**Statistical Analysis**—For all comparisons, a two-sample, unequal variance, two-tailed t test was used. Statistical differ-
ences for all tests were considered significant at the $p < 0.05$ level.

RESULTS

MDC1 Regulates Mitotic Progression—To investigate the mitotic role of MDC1, mitotic progression was analyzed in U2OS cells down-regulated for MDC1 or in control cells (GFP siRNA; Fig. 1A). The duration of mitosis, from chromatin condensation until completion of cell division, was measured using time-lapse microscopy. Mitosis duration was shortened by ~40% in MDC1 down-regulated cells compared with control ($p$ value $= 1.1 \times 10^{-13}$; 29.6 ± 3.15 compared with 49.3 ± 5.38 min, respectively; Fig. 1, B and C). The duration of mitosis in MDC1 down-regulated cells complemented with a siRNA-resistant MDC1 (Fig. 1A) was similar to control ($p$ value $= 6.8 \times 10^{-16}$; 49.6 ± 3.95 min; Fig. 1, B and C), verifying that shorter mitosis is due to MDC1 depletion and not due to a siRNA off-target effect. A shorter mitotic duration in MDC1-depleted cells was also observed when using a different MDC1 siRNA duplex in U2OS cells or in HeLa MDC1 down-regulated cells (Fig. 1, D–G). Taken together, these results suggest that MDC1 regulates mitotic progression.

MDC1 Is Involved in the SAC—Because short mitosis may result from an impaired SAC we studied MDC1 down-regulated cells under SAC activation. Treatment of cells with either nocodazole or taxol results in mitotic arrest and SAC activa-
tion, through a similar, but not identical, molecular cascade (8). Nocodazole interferes with microtubules polymerization, rendering all kinetochores unattached. Taxol stabilizes microtubules, causing lack of tension across attached kinetochores. Mitotic arrest under these treatments is not permanent, and after several hours, cells escape this blockage, flatten, and enter G1 phase as tetraploid cells, a process termed mitotic slippage (35). Control U2OS cells or MDC1 down-regulated cells were treated with taxol or nocodazole and mitotic slippage time was measured by time-lapse microscopy, from chromosome condensation until cells started to flatten. Down-regulation of MDC1 led to a shift in the distribution of mitotic slippage times in comparison with control (Fig. 2). MDC1 down-regulated cells escaped mitotic arrest faster than control (taxol; Fig. 2A; 198 ± 13.6 min compared with 272 ± 12.4 min, respectively, a reduction of 28%; p value = 1.3 × 10−12 and nocodazole; Fig. 2B; 246 ± 1.79 min compared with 326 ± 6.86 min, respectively, a reduction of 25%; p value = 9.55 × 10−19). The effect of the MDC1 siRNA on mitotic slippage times is a result of MDC1 depletion because complementation with siRNA-resistant MDC1 resulted in a similar phenotype to control (265 ± 25.1 min (taxol; p value = 7 × 10−19), and 283 ± 5.41 min (nocodazole; p value = 0.0001; Fig. 2)). Hence, these results indicate that MDC1 regulates SAC maintenance (Fig. 2).

**MDC1 Is Localized at Mitotic Kinetochores**—Most SAC proteins are localized at mitotic kinetochores (7, 8). As our results suggest that MDC1 regulates the SAC, we analyzed MDC1 mitotic localization. First, we tested whether MDC1 is part of mitotic kinetochores by examining the binding of MDC1 to the inner kinetochore protein, CENP-C. CENP-C was co-immunoprecipitated with anti-MDC1 antibodies from cell extracts prepared from nocodazole-treated 293T cells (Fig. 3A). The interaction between MDC1 and CENP-C indicates that MDC1 is part of the mitotic kinetochore complex. To further analyze the kinetochore localization of MDC1, mitotic chromosome spreads were prepared from untreated, mitotic HeLa cells, or from HeLa cells treated with taxol or colcemid (causes depolymerization of microtubules, similar to nocodazole). The mitotic spreads were immunostained with antibodies directed against MDC1 and CENP-A, the centromeric variant of histone H3 (Fig. 3B). We detected three types of occupancy for MDC1 at sister kinetochores. In some sister kinetochores, MDC1 staining overlapped the staining of CENP-A at both kinetochores; in some, the overlap was
detected only at one of the two kinetochores, whereas in others, MDC1 staining did not appear at all (Fig. 3B). In addition, we could detect MDC1 at telomeres (Fig. 3B, white arrows), as reported previously (36). MDC1 was identified in about one-third of mitotic kinetochores (Fig. 3C). MDC1 staining was not detected in spreads prepared from MDC1 down-regulated HeLa cells (Fig. 3D). Taken together, MDC1 is part of mitotic, SAC-activated kinetochores.

ATM Regulates the Kinetochore Localization of MDC1—MDC1 is a known substrate of ATM during the DDR (33, 37); thus, ATM may also regulate MDC1 in mitosis. Kinetochore localization of MDC1 in nocodazole-treated 293T cells incu-
bated with KU-55933, a specific inhibitor of ATM (38), was analyzed. ATM inhibition impaired the ability of anti-MDC1 antibodies to co-immunoprecipitated CENP-C from SAC-activated extracts (Fig. 3E). Similar results were obtained with caffeine, a general inhibitor of the phosphatidylinositol-3 kinase-like family (39). Moreover, the localization of MDC1 at kinetochores was compromised in mitotic spreads prepared from A-T patient fibroblasts compared with mitotic spreads prepared from A-T fibroblasts complemented with a functional ATM (Fig. 3F) (40). Hence, our data indicate that ATM controls the localization of MDC1 at mitotic kinetochores.

**H2AX Is Phosphorylated at Mitotic Kinetochores**—During the DDR, ATM phosphorylates histone H2AX, an event that is required for the recruitment and retention of MDC1 at sites of DNA damage. Because we found that the localization of MDC1 at mitotic kinetochores is dependent on ATM, we further examined whether this is mediated by the formation of γ-H2AX at mitotic kinetochores. First, we tested whether histone H2AX is phosphorylated at these kinetochores. γ-H2AX was present at 37% of all kinetochores of SAC induced cells (Fig. 4A and B). When SAC-activated cells were incubated with KU-55933, γ-H2AX was present at 22% of all kinetochores (Fig. 4B; p value = 2.6 \times 10^{-6}), indicating that the presence of γ-H2AX at mitotic kinetochores is ATM-dependent. Notably, there was a remarkable overlap between MDC1 and γ-H2AX staining on mitotic chromosomes (Fig. 4C), suggesting that they are present at the same kinetochores. These results are reminiscent of the results obtained when we analyzed the local-

![Image](https://example.com/image.png)
The phosphorylation of histone H2AX at mitotic kinetochores is required for the assembly of MDC1 at kinetochores.

**ATM and MDC1 Act Together in Regulating Mitotic Progression**—We next assessed whether ATM co-operates with MDC1 in the regulation of mitosis. A-T cells, which have no ATM protein (40), or A-T cells complemented with ATM were down-regulated for MDC1 and mitotic duration was examined. As expected, A-T cells complemented with ATM and down-regulated for MDC1 (ATM+/MDC1−) completed mitosis faster than control cells (ATM+/MDC1+; p value = 4.6 × 10−7; Fig. 5). Likewise, the A-T cells (ATM−/MDC1+) had a shorter mitosis compared with control ATM+/MDC1+ cells (p value = 2.8 × 10−11; Fig. 5). The shortening of mitosis in both cases was similar. Notably, when cells were deficient of both ATM and MDC1 (ATM−/MDC1−), they exhibited a significantly shorter mitosis compared with cells deficient of only one of the proteins (p value = 0.047; Fig. 5). This indicates that ATM, similar to MDC1, regulates mitosis. Our findings that ATM controls MDC1 kinetochore localization (Fig. 3, E and F), suggest that MDC1 is regulated by ATM also in mitosis. However, because cells lacking both MDC1 and ATM exhibit an even shorter mitosis compared with cells lacking one of the proteins, these proteins should also have distinct roles in the regulation of mitosis.

**MDC1 Interacts with the MCC**—MDC1 is an adaptor that binds many proteins during the DDR and recruits them to sites of DNA damage (4). We examined whether MDC1 also binds the MCC, which is the complex that executes the SAC. The MCC subunit BubR1 co-immunoprecipitated MDC1 from 293T cell extracts (Fig. 6A). The interaction was consistently stronger in mitotic cells compared with unsynchronized cells (Fig. 6A). When cells were treated with ionizing radiation to
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Induce DSBs, the MDC1-BubR1 interaction was abolished (Fig. 6A), probably because ionizing radiation-induced cells activate the G2/M checkpoint, resulting in less mitotic cells in the population (41, 42). MDC1 contains many protein-protein interaction modules (4); thus, we studied which domain in MDC1 binds to the MCC. The tandem BRCA1 C-terminal (tBRCT) domain of MDC1 was sufficient for the interaction with the MCC (Fig. 6B). Our data did not rule out the possibility that other domains in MDC1 can also mediate the interaction with the MCC. Additional studies are required to establish whether the interaction between MDC1 and the MCC is direct. Taken together, these results imply that the MCC binding region in MDC1 is the tBRCT domain and that MDC1 regulates the SAC via its interaction with the MCC.

**MDC1 and ATM Modulate together the Formation of the MCC**—During the DDR, ATM modulates several of the protein-protein interactions involving MDC1, and thus, its activity is essential for the localization of these interacting partners at DSBs (4). We examined whether MDC1 acts as a scaffold protein that mediates the kinetochore localization of MCC components in an ATM-dependent manner. MDC1 or GFP (as a control) were down-regulated in HeLa cells. The cells were then treated with nocodazole alone or with nocodazole and the ATM inhibitor and subjected to immunofluorescence studies. Notably, the localization of Mad2 and Cdc20 at mitotic kinetochores (detected by co-localization with CREST centromeric auto-immune anti-sera) was impaired in cells in which both MDC1 was downregulated and the activity of ATM was inhibited (Fig. 7, A and B). The combination of ATM inhibition and MDC1 depletion was required for this significant reduction (Fig. 7, A and B). Therefore, either the recruitment or retention of Mad2 and Cdc20 at kinetochores is affected. Interestingly, there was no change in the recruitment of BubR1 to kinetochores upon MDC1 down-regulation and ATM inhibition (Fig. 7, A–C).

**FIGURE 7.** MDC1 and ATM together regulate Mad2 and Cdc20 kinetochore localization and MCC formation. A–C, control (siRNA GFP) or MDC1 down-regulated (siRNA MDC1) HeLa cells incubated with nocodazole were either treated with the ATM inhibitor KU-55933 or untreated. A and B, MDC1 and ATM regulate Mad2 and Cdc20 kinetochore localization. The cells were stained by immunofluorescence using antibodies directed against CREST (red) and Mad2 (A) or Cdc20 (B; green). Right panel, quantification of Mad2 (A) and Cdc20 (B) fluorescence levels at kinetochores; each kinetochore was analyzed separately using the “analyze particles” tool of NIH ImageJ software. Asterisks represent a significant reduction in Mad2 (A; p value = 0.0012) or Cdc20 (B; p value = 0.0016) fluorescence intensities at CREST-stained kinetochores. C, cells were stained by immunofluorescence using antibodies directed against CREST (red) and BubR1 (green). Right panel, quantification of BubR1 fluorescence levels at kinetochores. The plots in A–C show the average level of the relevant protein per kinetochore. Error bars represent S.E. from analyses of five to seven cells. The images were compiled using a confocal microscope under similar conditions (same times and intensities of exposure). D, HeLa cells down-regulated for MDC1 and ATM were treated with nocodazole and stained by immunofluorescence using antibodies directed against CENP-E (red) and BubR1 (green). E, MDC1 is required for an intact MCC. HeLa cells down-regulated for MDC1 and ATM (shMDC1 + ATM) and control (shLacZ) cells were treated with nocodazole and subjected to co-immunoprecipitation experiments using antibodies directed against BubR1 or control IgG. Antibodies directed against Mad2 or Cdc20 were used to detect the proteins on Western blots. Right panel, quantification of co-immunoprecipitated Mad2 and Cdc20 levels normalized to the levels of retrieved BubR1. Bars represent the ratio between normalized levels of proteins retrieved from shMDC1 + ATM cells and from shLacZ cells. Error bars represent S.E. from three (Cdc20) or four (Mad2) independent experiments. Asterisks represent the significant reduction in Mad2 (left panel; p value = 0.004) or Cdc20 (right panel; p value = 0.015) binding to BubR1. The arrowhead marks Mad2 position the upper band is the antibody light chain.
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7C), probably due to the fact that BubR1 arrives at kinetochores separately from Mad2 and Cdc20 (8).

Lack of MDC1 and ATM does not result in a general kinetochore disruption because the kinetochore localization of BubR1 and the mitotic kinetochore component CENP-E are intact (Fig. 7, C and D). Taken that Mad2 localization at kinetochores is crucial for proper SAC activation (43), this result suggests that MDC1 and ATM regulate the SAC by ensuring proper recruitment and/or maintenance of Mad2-Cdc20 complex at mitotic kinetochores.

Our findings that MDC1 and ATM are required for Mad2 and Cdc20 kinetochore localization while not affecting BubR1 localization (Fig. 7, A–C) and that MDC1 binds to the MCC (Fig. 6) propose that MDC1 and ATM are also needed for maintaining the integrity of the MCC during SAC activation. BubR1 was immunoprecipitated from extracts prepared from HeLa cells stably expressing a shRNA, which depletes MDC1 and highly reduces ATM levels (36), or a control LacZ shRNA. Interestingly, the interaction of BubR1 with Cdc20 and Mad2 was disrupted when both ATM and MDC1 were depleted (Fig. 7E). Similar results were obtained when A-T cells were down-regulated for MDC1. However, in A-T cells or in A-T cells complemented with ATM and down-regulated for MDC1, MCC integrity was not impaired (data not shown). This occurred without reducing the protein levels of Mad2 and Cdc20 (Fig. 7F). These results indicate that MDC1 and ATM together regulate the SAC. Both proteins are required for the formation of an intact MCC and for proper kinetochore localization of the Mad2-Cdc20 complex during SAC activation.

DISCUSSION

Here, we revealed a novel role for ATM and MDC1, key members of the DDR, in regulation of the SAC. We found that MDC1 and ATM control the SAC by regulating both the proper localization of the MCC members, Mad2 and Cdc20, at kinetochores and the integrity of the MCC.

In search of the mitotic role of MDC1, we found that MDC1 down-regulated cells complete mitosis significantly faster than control (Figs. 1 and 5), implying that MDC1 regulates mitotic progression. Townsend et al. (44) reported that MDC1 down-regulated cells undergo a very long mitotic phase and presumably blocked at mitosis. In our work, there was never such a growth arrest. On the contrary, cells without MDC1 had a shorter mitosis. This result occurred using different siRNAs and cell lines (Figs. 1 and 5). When we complemented MDC1 down-regulated cells with an siRNA-resistant MDC1, mitosis times returned to normal, indicating that the short mitosis is due to MDC1 depletion and not to siRNA off-targets. The discrepancy found between our work and Townsend et al. (44) may be due to off target effects of the siRNAs used by the latter group. In accordance with our results, Bu et al. (45) has showed that MDC1-depleted cells exhibit less histone H3 serine 10 phosphorylation (a marker of mitotic cells), indicating that the fraction of mitotic cells in this population is lower.

Here, we describe a role for MDC1 in the activation of the SAC. When control cells were viewed by live cell imaging, we occasionally detected a movement of the condensed chromosomes on the equatorial plane of the cell, presumably waiting for all kinetochores to be properly attached. In MDC1 down-regulated cells, such a movement was hardly detected, suggesting that the metaphase chromosomes did not wait for proper kinetochore attachment. This observation, although not quantifiable, strengthened our conclusion that MDC1 plays a mitotic role in SAC activation.

MDC1 acts differently than classical SAC proteins; the effect of depleting MDC1 on mitotic slippage is moderate compared with the effect of classical SAC member depletion (Fig. 2 and 46, 47). For example, it was demonstrated that the SAC is not activated in the absence of Mad2 (46, 47). Unlike other SAC proteins, which are found at all mitotic kinetochores, MDC1 is detected at ~35% to 40% of mitotic kinetochores (Figs. 3C and 4C). Because the localization of MDC1 at kinetochores seems to be randomly distributed, with no regard to sister kinetochore, we did not consider individual chromosomes but individual kinetochores in our analyses (Fig. 3, B and C). This partial localization can be attributed to the fact that some kinetochores may have low levels of MDC1, which are below detection levels by the anti-MDC1 antibodies. Alternatively, the binding of MDC1 to kinetochores may be dynamic so that at a given time point not all kinetochores contain MDC1. This may also be due to an indirect SAC activation, which is DNA damage-dependent (see below).

We demonstrate here that ATM modulates the association of MDC1 with kinetochores through the phosphorylation of histone H2AX at kinetochores, followed by the binding of MDC1 to γ-H2AX (Figs. 3, G and F, and 4). Inhibition of ATM activity reduces the phosphorylation of histone H2AX at kinetochores (by ~40%, Fig. 4B). This reduction is not complete probably due to redundancy between ATM, ATR, and DNA-dependent protein kinase catalytic subunit in histone H2AX phosphorylation at kinetochores, similar to what occurs at DSBs (48, 49). We believe that this partial reduction of γ-H2AX at kinetochores leads to a partial reduction (of ~40%) in the localization of MDC1 at kinetochores of A-T cells (Fig. 3F). Supporting this is the experiment in Fig. 4C. When we interfered with the ability of histone H2AX to be phosphorylated by any kinase, there was a reduction of 80% in the MDC1 localization at kinetochores (Fig. 4C).

A similar reduction in mitosis times was detected in A-T fibroblasts (40) and in MDC1 down-regulated cells. When cells are deficient of both proteins, mitosis durations further shorten (Fig. 5), indicating that MDC1 and ATM probably have both related and distinct roles in mitotic regulation. Here, we show that ATM and MDC1 together control the localization of Mad2 and Cdc20 at mitotic kinetochores and the integrity of the MCC (Fig. 7). Although aneuploidy was observed in cells lacking ATM in animal models and clinical samples from A-T patient brains (24–26), only recently, data emerged regarding the role of ATM in mitosis regulation, which is not related to its DDR role. ATM phosphorylates the mitotic spindle-associated protein NuSAP, which induces mitotic arrest and regulates mitotic progression (23). In addition, ATM is phosphorylated and activated by Aurora-B. ATM then phosphorylates Bub1 (27); however, it was not demonstrated how this phosphorylation event contributes to SAC activation. Depletion of Aurora-B or Bub1 results in impairment in the localization of BubR1 and Mad2 at kinetochores (50–52). We could not detect any changes in BubR1 phosphorylation, which is Bub1-dependent (data not shown) in the absence of ATM, and we could only
show defects in Mad2 and Cdc20 kinetochore localization when both ATM and MDC1 were absent (Fig. 7A). Notably, we did not find any changes in BubR1 localization at kinetochores in this study (Fig. 7B). We thus believe that the requirement of Aurora-B and Bub1 for the localization of Mad2 and BubR1 at kinetochores does not involve ATM and MDC1 and is regulated by a different pathway.

The MCC localizes at mitotic kinetochores where it executes the SAC by binding to Cdc20 to prevent anaphase-promoting complex/cyclosome activity (8). We reveal that MDC1 and ATM are required for proper kinetochore localization of Mad2 and Cdc20 but not for the localization of BubR1 (Fig. 7, A–C). This is in line with the finding that the binding of BubR1 to Cdc20 requires an earlier binding of Mad2 to Cdc20 (6, 53). We show that during SAC activation MDC1 interacts with the MCC (Fig. 6), probably via direct binding of MDC1 to Cdc20 (44). We propose a model for the involvement of ATM and MDC1 in SAC activation. ATM phosphorylates histone H2AX at certain mitotic kinetochores and MDC1 binds the MCC sub-complex Mad2-Cdc20 and together with ATM is required for its recruitment to kinetochores.

FIGURE 8. A model describing the involvement of MDC1 and ATM in the formation of the MCC during SAC activation. Shown is the involvement of ATM and MDC1 during the SAC. ATM phosphorylates histone H2AX at certain mitotic kinetochores and γ-H2AX serves as a docking platform for MDC1 at these kinetochores. MDC1 binds the MCC sub-complex Mad2-Cdc20 and together with ATM is required for its recruitment to kinetochores.

The function of MDC1 and ATM in SAC activation does not rely on DNA damage induction because no such damage was generated. Several studies indicate that the SAC can be activated by exogenous DNA damage (56–58) and that ATM can activate the SAC due to excessive DNA damage in mitosis (21, 59). We cannot distinguish whether the role of ATM and MDC1 in SAC activation is due to a DNA damage-independent function of these DDR proteins or that the SAC is activated by the DDR. In the first scenario, a novel role for MDC1 and ATM in the SAC, which is distinct from their DDR role, is shown here. In the second scenario, we assume that MDC1 and ATM act in the SAC as DDR proteins and that the SAC can also be activated by DNA damage. If so, it is possible that DSBs exist at centromeric regions during mitosis. This damage, if it exists, results from endogenous means and probably causes a subtle effect on cells. This may explain the mild effect of MDC1 depletion on mitotic slippage (Fig. 2). Hence, it is likely that ATM and MDC1 are not core SAC protein and the role they play in the SAC is DNA damage-dependent. There is a recent report showing that a DSB induced in yeast centromeric DNA activates the SAC (57). However, as mammalian and yeast centromeres are different, the effect of DSBs on mammalian centromeres is yet to be shown; thus, we cannot exclude either one of the possibilities.

This study establishes a novel connection between the DDR proteins ATM and MDC1 and the SAC. ATM and MDC1 regulate the SAC together through an interaction with the MCC. They are required for both the localization of Mad2 and Cdc20 at kinetochores and the formation of an intact MCC. The integrity of the MCC is crucial for an intact SAC, which when deregulated can cause chromosomal aberrations and genomic instability. Thus, ATM and MDC1 bring about another level of regulation to this already highly regulated cellular mechanism.

Acknowledgments—We thank laboratory members Yifat Oren and Amit Tzur for discussions, Michael Brandeis and Batshaev Kerem for advice and reagents, Michal Schwartz and Efrat Ozeri-Galai for technical help, Naomi Melamed-Book for confocal analyses, and Katja Wasmann, Stephen Taylor, Stephen P. Jackson, William Earnshaw, and Yosef Shiloh for reagents.
REFERENCES

1. Smeenk, G., and van Attikum, H. (2013) The chromatin response to DNA breaks: leaving a mark on genome integrity. Annu. Rev. Biochem. 82, 55–80.
2. Lavín, M. F., and Kozlowski, S. (2007) ATM activation and DNA damage response. Cell Cycle 6, 931–942.
3. Stucki, M., Clapperton, J. A., Mohammad, D., Yaffe, M. B., Smerdon, S. J., and Jackson, S. P. (2005) MDC1 directly binds phosphorylated histone H2AX to regulate cellular responses to DNA double-strand breaks. Cell 123, 1213–1226.
4. Coster, G., and Goldberg, M. (2010) The cellular response to DNA damage: A focus on MDC1 and its interacting proteins. Nucleus 1, 166–178.
5. Jungmichel, S., and Stucki, M. (2010) MDC1: The art of keeping things in focus. Chromosoma 119, 337–349.
6. Lara-Gonzalez, P., Westhorpe, F. G., and Taylor, S. S. (2012) The spindle assembly checkpoint. Curr. Biol. 22, R966–980.
7. May, K. M., and Hardwick, K. G. (2006) The spindle checkpoint. J. Cell Sci. 119, 4139–4142.
8. Musacchio, A., and Salmon, E. D. (2007) The spindle–assembly checkpoint in space and time. Nat. Rev. Mol. Cell Biol. 8, 379–393.
9. Cheeseman, I. M., and Desai, A. (2008) Molecular architecture of the kinetochore–microtubule interface. Nat. Rev. Mol. Cell Biol. 9, 33–46.
10. Kops, G. J., Weaver, B. A., and Cleveland, D. W. (2005) On the road to cancer: aneuploidy and the mitotic checkpoint. Nat. Rev. Cancer 5, 773–785.
11. Vdovina, G., Maia, A. F., and Lens, S. M. (2008) The chromosomal passenger complex and the spindle assembly checkpoint: kinetochore-microtubule error correction and beyond. Cell Div. 3, 10.
12. Vagnarelli, P., Ribeiro, S. A., and Earnshaw, W. C. (2008) Centromeres: old tales and new tools. FEBS Lett. 582, 1950–1959.
13. Braunstein, I., Miniowitz, S., Moshe, Y., and Hershko, A. (2007) Inhibitory factors associated with anaphase-promoting complex/cyclosome in mitotic checkpoint. Proc. Natl. Acad. Sci. U.S.A. 104, 4870–4875.
14. Logan, E., and Boussaba, H. (2008) Kinetochore-microtubule interactions ‘in check’ by Bub1, Bub3 and BubR1: The dual task of attaching and signalling. Cell. Cycle 7, 1763–1768.
15. Manchado, E., Eguren, M., and Malumbres, M. (2010) The anaphase-promoting complex/cyclosome (APC/C): cell-cycle-dependent and -independent functions. Biochem. Soc. Trans. 38, 65–71.
16. Solomon, M. J., and Burton, J. L. (2008) Securin’s M-phase entry. Nat. Cell Biol. 10, 381–383.
17. Zachos, G., Black, E. J., Walker, M., Scott, M. T., Vagnarelli, P., Earnshaw, W. C., and Gillespie, D. A. (2007) Chk1 is required for spindle checkpoint function. Dev. Cell 12, 247–260.
18. Lee, K. J., Lin, Y. F., Chou, H. Y., Yajima, H., Fattah, K. R., Lee, S. C., and Chen, B. P. (2011) Involvement of DNA-dependent protein kinase in normal cell cycle progression through mitosis. J. Biol. Chem. 286, 12796–12802.
19. Trifanelli, A., Oricho, E., Ciccarello, M., Mangiacasale, R., Palena, A., Lavia, P., Suddu, S., and Cundari, E. (2004) p53 localization at centrosomes during mitosis and postmitotic checkpoint are ATM-dependent and require serine 15 phosphorylation. Mol. Biol. Cell 15, 3751–3757.
20. Zhang, S., Hemmerich, P., and Grosse, F. (2007) Centrosomal localization of DNA damage checkpoint proteins. J. Cell. Biol. 101, 451–465.
21. Smith, E., Dejuphon, D., Balestrini, A., Hamep, M., Lenz, C., Takeda, S., Vindigni, A., and Costanzo, V. (2009) An ATM- and ATR-dependent checkpoint inactivates spindle assembly by targeting CEP56. Nat. Cell Biol. 11, 278–285.
22. Oriochio, E., Saladino, C., Iacovelli, S., Suddu, S., and Cundari, E. (2006) ATM is activated by default in mitosis, localizes at centrosomes and monitors mitotic spindle integrity. Cell Cycle 5, 88–92.
23. Xie, P., Li, L., Xing, G., Tian, C., Yin, Y., He, F., and Zhang, L. (2011) ATM-mediated NuSAP phosphorylation induces mitotic arrest. Biochem. Biophys. Res. Commun. 404, 413–418.
24. Iouriev, I. Y., Vorsanova, S. G., Liehr, T., Koloti, A. D., and Yurov, Y. B. (2009) Increased chromosome instability dramatically disrupts neural genome integrity and mediates cerebellar degeneration in the ataxia-telan-}

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G2/M transition in mammalian cells. Biochem. Biophys. Res. Commun. 397, 157–162
46. Meraldi, P., Draviam, V. M., and Sorger, P. K. (2004) Timing and checkpoints in the regulation of mitotic progression. Dev. Cell 7, 45–60
47. Wysong, D. R., Chakravarty, A., Hoar, K., and Ecsey, J. A. (2009) The inhibition of Aurora A abrogates the mitotic delay induced by microtubule perturbing agents. Cell Cycle 8, 876–888
48. Ward, I. M., and Chen, J. (2001) Histone H2AX is phosphorylated in an ATR-dependent manner in response to replicational stress. J. Biol. Chem. 276, 47759–47762
49. Stiff, T., O’Driscoll, M., Rief, N., Iwabuchi, K., Löbrich, M., and Jeggo, P. A. (2004) ATM and DNA-PK function redundantly to phosphorylate H2AX after exposure to ionizing radiation. Cancer Res. 64, 2390–2396
50. Hauf, S., Cole, R. W., LaTerra, S., Zimmer, C., Schnapp, G., Walter, R., Heckel, A., van Meel, J., Rieder, C. L., and Peters, J. M. (2003) The small molecule Hesperadin reveals a role for Aurora B in correcting kinetochore-microtubule attachment and in maintaining the spindle assembly checkpoint. J. Cell Biol. 161, 281–294
51. Johnson, V. L., Scott, M. I., Holt, S. V., Hussein, D., and Taylor, S. S. (2004) Bub1 is required for kinetochore localization of BubR1, Cenp-E, Cenp-F and Mad2, and chromosome congression. J. Cell. Sci. 117, 1577–1589
52. Ditchfield, C., Johnson, V. L., Tighe, A., Ellston, R., Haworth, C., Johnson, T., Mortlock, A., Keen, N., and Taylor, S. S. (2003) Aurora B couples chromosome alignment with anaphase by targeting BubR1, Mad2, and Cenp-E to kinetochores. J. Cell Biol. 161, 267–280
53. Davenport, J., Harris, L. D., and Goorha, R. (2006) Spindle checkpoint function requires Mad2-dependent Cdc20 binding to the Mad3 homology domain of BubR1. Exp. Cell Res. 312, 1831–1842
54. Shah, I. V., Botvinick, E., Bonday, Z., Furnari, F., Berns, M., and Cleveland, D. W. (2004) Dynamics of centromere and kinetochore proteins; implications for checkpoint signaling and silencing. Curr. Biol. 14, 942–952
55. Jazayeri, A., Falck, J., Lukas, C., Bartek, J., Smith, G. C., Lukas, J., and Jackson, S. P. (2006) ATM- and cell cycle-dependent regulation of ATR in response to DNA double-strand breaks. Nat. Cell Biol. 8, 37–45
56. Banerjee, T., Nath, S., and Roychoudhury, S. (2009) DNA damage induced p53 downregulates Cdc20 by direct binding to its promoter causing chromatin remodeling. Nucleic Acids Res. 37, 2688–2698
57. Dotiwala, F., Harrison, J. C., Jain, S., Sugawara, N., and Haber, J. E. (2010) Mad2 prolongs DNA damage checkpoint arrest caused by a double-strand break via a centromere–dependent mechanism. Curr. Biol. 20, 328–332
58. Mikhailov, A., Cole, R. W., and Rieder, C. L. (2002) DNA damage during mitosis in human cells delays the metaphase/anaphase transition via the spindle-assembly checkpoint. Curr. Biol. 12, 1797–1806
59. Lee, H. J., Hwang, H. I., and Jang, Y. J. (2010) Mitotic DNA damage response: Polo-like kinase-1 is dephosphorylated through ATM-Chk1 pathway. Cell Cycle 9, 2389–2398