Wee1 controls genomic stability during replication by regulating the Mus81-Eme1 endonuclease

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Correct replication of the genome and protection of its integrity are essential for cell survival. In a high-throughput screen studying H2AX phosphorylation, we identified Wee1 as a regulator of genomic stability. Wee1 down-regulation not only induced H2AX phosphorylation but also triggered a general deoxyribonucleic acid (DNA) damage response (DDR) and caused a block in DNA replication, resulting in accumulation of cells in S phase. Wee1-deficient cells showed a decrease in replication fork speed, demonstrating the involvement of Wee1 in DNA replication. Inhibiting Wee1 in cells treated with short treatment of hydroxyurea enhanced the DDR, which suggests that Wee1 specifically protects the stability of stalled replication forks. Notably, the DDR induced by depletion of Wee1 critically depends on the Mus81-Eme1 endonuclease, and we found that codepletion of Mus81 and Wee1 abrogated the S phase delay. Importantly, Wee1 and Mus81 interact in vivo, suggesting direct regulation. Altogether, these results demonstrate a novel role of Wee1 in controlling Mus81 and DNA replication in human cells.

Introduction

To defend the integrity of the genome, cells have developed an extensive network of pathways that acts in concert to detect and signal DNA damage for subsequent processing and repair. The DNA damage–dependent delay in cell cycle progression critically depends on the phosphatidylinositol 3-kinaselike kinases (PIKKs) ataxia telangiectasia mutated (ATM) and ataxia telangiectasia and Rad3 related (ATR). Whereas ATM signaling is predominantly activated by DNA double strand breaks (DSBs), which are triggered by ionizing radiation, ATR responds to a wider variety of DNA lesions, including UV-induced base damage, replication stress, and DSBs (Shiloh, 2006; Cimprich and Cortez, 2008).

Key components of the ATM–ATR signaling pathway are the Chk2 and Chk1 kinases, which transmit the upstream signal to the downstream cell cycle proteins (Matsuoka et al., 1998; Liu et al., 2000). Triggering ATM–ATR signaling depends on several checkpoint mediator proteins such as 53BP1, MDC1, and TopBP1, which have been proposed to assist in promoting interactions between PIKKs and their substrates and/or aid the retention of critical factors in close proximity to DNA lesions (Bartek and Lukas, 2007). In the ATR–Chk1 pathway, the Rad9–Rad1–Hus1 complex and Claspin are additionally required for Chk1 activation (Kumagai and Dunphy, 2000; Weiss et al., 2002). To delay cell cycle progression, Chk1 and Chk2 are able to inhibit several isoforms of Cdc25, which are phosphatases that remove the inhibitory phosphorylation of cyclin–Cdk complexes (Peng et al., 1997; Sanchez et al., 1997; Mailand et al., 2000). In contrast, Chk1 is also able to activate the tyrosine kinase Wee1, which phosphorylates and thereby inhibits the cyclin B–Cdk1 complex at the G2/M transition of the cell cycle (Lee et al., 2001). During the recovery from the DNA damage checkpoint, Claspin and Wee1 are degraded in an SCF-βTrCP1/2–dependent manner, and cell cycle progression is resumed (Freire et al., 2006).

A common target of PIKKs is histone H2AX, which is phosphorylated on serine 139 (γ-H2AX; Rogakou et al., 1999).

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Abbreviations used in this paper: ATM, ataxia telangiectasia mutated; ATR, ataxia telangiectasia and Rad3 related; DDR, DNA damage response; DSB, double strand break; HU, hydroxyurea; MCM, minichromosome maintenance; PIKK, phosphatidylinositol 3-kinaselike kinase.

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Upon the induction of DSBs, H2AX phosphorylation can be observed by its accumulation into nuclear foci that are thought to represent sites of DNA lesions. γ-H2AX focus formation was proposed to facilitate the recruitment of mediator proteins MDC1, RNF8, RNF168, 53BP1, BRCA1, and the Mre11–Rad50–Nbs1 complex to sites of DNA damage, which is required both for signaling to DNA repair and promoting cell cycle delay (Jackson and Bartek, 2009; van Attikum and Gasser, 2009). γ-H2AX foci do not only quickly appear after the induction of DSBs, but phosphorylation of H2AX also occurs upon replication stress and treatment with other DNA-damaging agents that do not directly induce DSBs, such as UV light (Burma et al., 2001; Ward and Chen, 2001; Stiff et al., 2004; Hanasoge and Ljungman, 2007). In the latter case, γ-H2AX staining is dependent on ATR and is mainly detected in S phase cells in which DSBs are believed to be the result of stalled replication forks (Ward and Chen, 2001; Hanasoge and Ljungman, 2007).

DNA replication is a tightly regulated process initiated by the association of origin recognition complex proteins to the DNA replication origins at telophase and early G1 followed by the loading of CDT1 and CDC6 proteins (Sclafani and Holzen, 2007). Then, the six minichromosome maintenance (MCM) proteins (MCM2–7), which are thought to be the core of the replicative DNA helicase, are loaded onto the origins constituting the prereplication complexes. Activation of the S phase–promoting Cdks and Dbf4-dependent kinase then triggers the recruitment of replication factors such as Cdc45, replication protein A, GINS complex, and DNA polymerases to form functional bidirectional replication forks (Sclafani and Holzen, 2007). Cells have developed several mechanisms to monitor and solve perturbations during replication, for example when forks are blocked. In addition to their role in triggering a DNA damage response (DDR) upon DNA lesions during S phase, several proteins in the ATR–Chk1 pathway are involved in maintaining replication fork integrity when forks are temporally stalled (i.e., as a result of depletion of the deoxyribonucleoside triphosphate pool; Petermann and Caldecott, 2006). Also, proteins involved in homologous recombination are important to repair DNA lesions that result in fork stalling (Heyer et al., 2010). The heterodimeric Mus81-Eme1 structure-specific endonuclease plays a critical role in the initial step of the repair by homologous recombination by cleaving the DNA at the stalled fork and temporally producing DSBs (Hanada et al., 2007). Other enzymes that have been implicated in resolving recombination intermediates are Blm, Gen1, and Exo1 (Segurado and Diffley, 2008; Wechsler et al., 2011).

Here, we set out to identify novel proteins controlling genomic stability by performing an siRNA screen of the human kinome. Wee1 down-regulation was found to induce high levels of γ-H2AX and a general activation of the DDR. Interestingly, Wee1-depleted cells accumulate in S phase and show a reduced replication speed. Notably, both the S phase progression delay and the DDR in the absence of Wee1 are dependent on Mus81-Eme1, and Wee1 and Mus81 are interaction partners, demonstrating a direct and previously unknown link between Wee1 and Mus81.

Results

Wee1 down-regulation triggers a DDR

To search for new genes controlling genome stability, we performed a human kinome siRNA screen using γ-H2AX staining as a read out. Each kinase was down-regulated using a pool of four different siRNA oligonucleotides. The screen showed that down-regulation of Wee1 and Chk1 produces increased levels of γ-H2AX (Fig. S1 A). Wee1 is a well-known regulator of the cell cycle, but its role in maintaining genomic stability is much less well described. Therefore, we decided to focus our further studies on this kinase. First, the result obtained in the primary screen was validated by carrying out down-regulation of Wee1 with each individual siRNA oligonucleotide of the siRNA pool used in the screen. Indeed, knocking down Wee1 with three out of the four siRNAs resulted in increased γ-H2AX staining, confirming that this phenotype is not a result of an off-target effect (Fig. S1 B). γ-H2AX antibody stained the nucleus homogenously, similar to the staining observed in cells with DNA damage during S phase (Fig. S1 C), and occurred as early as 24 h after the siRNA transfection, a time at which Wee1 is efficiently down-regulated (Fig. S1 D). This indicates that genomic integrity, as measured by γ-H2AX staining, is dependent on Wee1.

Because the formation of γ-H2AX is dependent on ATR and ATM, we studied the effect of Wee1 down-regulation on other DDR proteins of both pathways to determine which one was activated. Immunofluorescence analyses demonstrated that Wee1 depletion resulted in characteristic DNA damage–induced foci of 53BP1 and MDC1, two mediators of the ATM pathway, and of replication protein A, TopBP1, and Rad9, which function in the ATR pathway (Fig. 1 A). These results indicate that a general DDR activation occurs in cells depleted of Wee1 and suggest that both double-stranded and single-stranded lesions are formed upon Wee1 depletion. A general activation of the DDR is also reflected by the phosphorylation of Smc1 and Nbs1 in Wee1-depleted cells (Fig. 1 B). Interestingly, we observed a drop in protein levels of Chk1 upon down-regulation of Wee1, accompanied by a decrease in Claspin levels (Fig. 1 B). Furthermore, overexpression of a catalytic inactive version of Wee1 but not the wild-type form or treating cells with a Wee1 inhibitor induced strong phosphorylation of H2AX, demonstrating that the kinase activity of the protein is required to prevent the activation of a DDR (Fig. 1 C).

DDR induction by Wee1 deficiency occurs in S phase

In addition to a pronounced DDR, Wee1 depletion also resulted in a striking accumulation of cells in the S phase of the cell cycle (Fig. 2 A). Therefore, we wondered whether the DDR that is induced by depletion of Wee1 was occurring in this phase. By immunofluorescence analysis of cyclin A, which was not detected in G1 cells, moderately expressed in S phase, and highly expressed during G2, γ-H2AX staining induced by Wee1 depletion was mainly observed in S and G2 phases of the cell cycle (Fig. S1 E). Subsequent flow cytometry analysis showed that in the absence of Wee1, the majority of γ-H2AX–positive cells are in S phase (Fig. 2 B), confirming that depletion of Wee1 predominantly

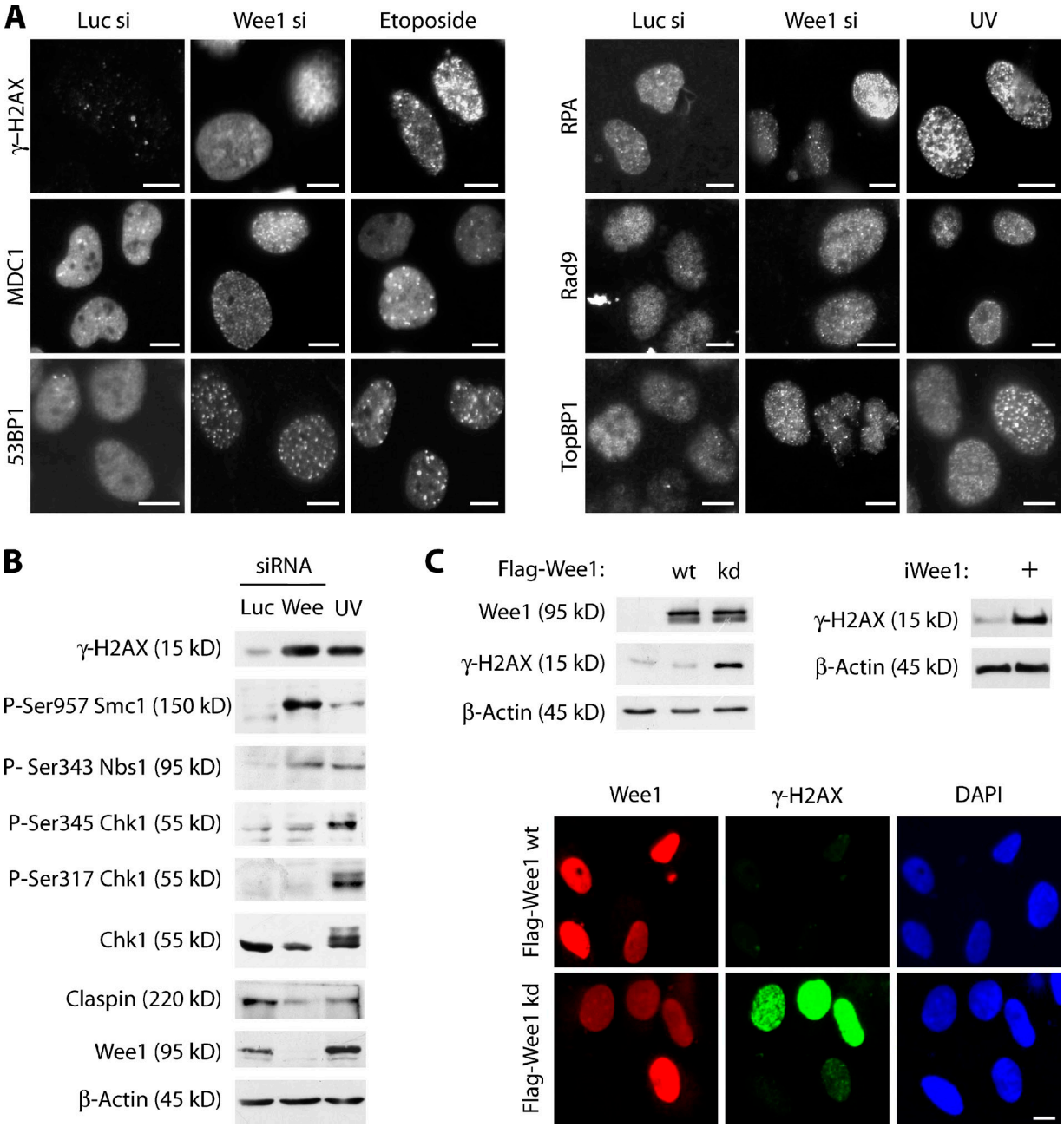

Figure 1. **Wee1 inhibition triggers a general DDR response.** (A) Immunofluorescence analysis of U2OS cells transfected with siRNA oligonucleotides targeting Luciferase (Luc si) or Wee1 (Wee1 si) for 48 h or cells treated with etoposide (20 µM for 1 h) or UV light (40 J/m^2 for 1 h) as positive controls using the indicated antibodies. For every sample, a field with a similar amount of cells is shown. RPA, replication protein A. (B) Immunoblot analysis using cells treated as described in A. (C) U2OS cells were mock transfected or transfected with a wild-type (wt) or kinase-dead (kd; K328A) version of Flag-Wee1 (top left and bottom) or treated with a Wee1 inhibitor for 4 h (top right). Thereafter, phosphorylation of H2AX was analyzed by immunoblot and immunofluorescence analysis. Bars, 10 µm.

triggers a DDR during S phase. Interestingly, Wee1-depleted cells that displayed intense γ-H2AX staining were not actively incorporating BrdU upon incubation for short time periods (Fig. S1 F). When incubating longer, BrdU-positive cells were observed both in Wee1–down-regulated and control cells, indicating a slowdown but not a total inhibition of DNA synthesis (unpublished data).

To further demonstrate that the DDR activation in Wee1-deficient cells occurs during S phase, cells were synchronized at different stages of the cell cycle. As shown in Fig. 2 C, γ-H2AX levels were high in mitotic and early G1 cells of both control and Wee1-depleted cultures, an effect also observed in other studies (Ichijima et al., 2005; Quignon et al., 2007). However, down-regulation of Wee1 in cells progressing through mitosis and G1 did not additionally elevate γ-H2AX levels (Fig. 2 C). In contrast, upon progression through S phase, an increase in H2AX phosphorylation was specifically observed upon Wee1 down-regulation (Fig. 2 D). Importantly, these higher γ-H2AX levels coincided with a delay in S phase progression as compared with control down-regulated cells (Fig. 2 D, right).

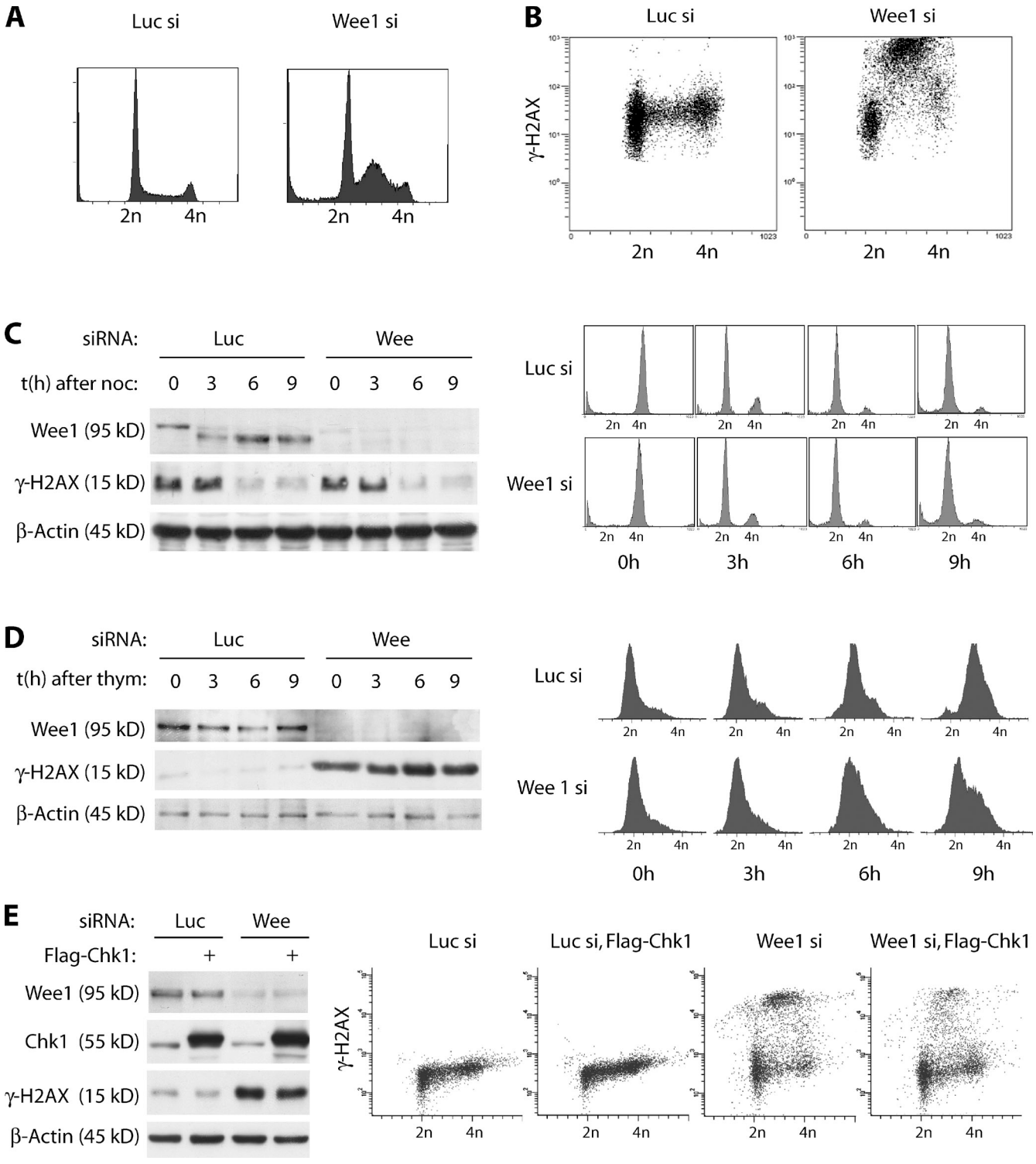

Figure 2. **Wee1 down-regulation affects cell cycle progression during S phase.** (A) U2OS transfected with Luciferase siRNA (Luc si) or Wee1 siRNA (Wee1 si) and cell cycle profiles were determined by flow cytometry analysis after staining with propidium iodide. (B) U2OS cells were transfected with Luciferase or Wee1 siRNA oligonucleotides. 48 h later, cells were fixed and analyzed by flow cytometry for γ-H2AX and DNA content by propidium iodide staining. (C and D) U2OS cells were transfected with the indicated siRNAs at the same time as they were synchronized with nocodazole (noc; C) or thymidine (thym; D) and were subsequently released from the arrest. At the indicated times after the release, cells were collected for flow cytometry analysis for propidium iodide staining or for analysis by Western blotting with the indicated antibodies. (E) Flow cytometry analysis for γ-H2AX/propidium iodide (right) or immunoblotting with the indicated antibodies (left) of U2OS cells transfected with Luc or Wee1 siRNA oligonucleotides and a control or Flag-Chk1 plasmid for 48 h.

DDR in the absence of Wee1 is dependent on Cdk2 but not Chk1 or Cdk1

Next, we investigated the basis of the DDR activation. As mentioned earlier in this section, we observed a decrease in Chk1 protein levels upon Wee1 down-regulation (Fig. 1 B). Lack of Chk1 was reported to raise γ-H2AX levels in S phase cells (Syljuåsen et al., 2005). Therefore, we determined whether H2AX phosphorylation after Wee1 depletion was induced by lowering Chk1 levels. Overexpression of wild-type Chk1 could not prevent the appearance of γ-H2AX in Wee1-depleted cells nor in cells in which Wee1 is catalytically inhibited (Figs. 2 E and S2 A). Moreover, Wee1 knockdown resulted in γ-H2AX induction at early time points after transfection, at which Chk1 levels are not yet affected. In contrast, phosphorylation of H2AX only occurs at late time points after Chk1 down-regulation (Fig. S2 B). Together, these data demonstrate that the DDR activation in Wee1-depleted cells cannot be explained by the drop in Chk1 levels.

As Wee1 is an important inhibitor of cyclin B–Cdk1 complexes during G2 phase by phosphorylating Cdk1 on threonine 14 and tyrosine 15, depletion of Wee1 was expected to result in overactivation of Cdk1, which might lead to activation of the DDR. Thus, we determined whether the lack of Cdk1 inhibition explains the DDR activation observed by studying the formation of γ-H2AX by Western blotting in Wee1- and/or Cdk1-depleted cells that were synchronized in G1–S phase. In addition, cells were also transfected with siRNA against Cdk2, a key regulator in the initiation of DNA replication and continuation of DNA synthesis when associated with cyclins E or A, respectively. Down-regulation of Cdk1 did not inhibit the generation of γ-H2AX in Wee1-depleted cells, indicating that overactivation of Cdk1 cannot explain the DDR observed in Wee1-depleted cells during S phase. However, depleting Cdk2 in Wee1–down-regulated cells did abolish the DDR (Fig. S2 C). To address whether Wee1 influences the phosphorylation status of Cdk2 or Cdk1 during S phase, Cdk1 and Cdk2 inhibitory phosphorylation on tyrosine 15 was studied in synchronized S phase cells that were depleted for Wee1. Phosphorylation of Cdk1 and Cdk2 was inhibited in Wee1–down-regulated cells (Fig. S2 D), suggesting that Wee1 is capable of controlling Cdk1/2 by phosphorylating tyrosine 15 during S phase.

Wee1 controls replication fork movement

The aforementioned results suggest that Wee1 is important for DNA replication. To determine whether Wee1 plays a role during DNA synthesis, we performed DNA combing experiments in cells successively pulse labeled with two thymidine analogues, which permits fork speed analysis (Anglana et al., 2003). The effect of Wee1 down-regulation was studied 24 and 48 h after siRNA treatment. Interestingly, depletion of Wee1 protein significantly decreased the mean replication fork speed from 2.16 to 1.25 kb/min early after Wee1 down-regulation, and this effect persisted at later time points (Fig. 3). To test whether Wee1 also controls replication fork movement in other cell types, similar experiments were performed with lymphoblasts and fibroblasts. As shown in Fig. S3, down-regulation of Wee1 in these cells produced a drop in replication fork speed similar to that observed in U2OS cells.

To determine whether the decrease in fork speed observed in Wee1-depleted cells is directly responsible for the DDR activation, we treated U2OS cells with various concentrations of hydroxyurea (HU). Although treating cells with 5 μM HU led to a similar deceleration of replication fork speed as compared with Wee1 depletion, no significant elevation of γ-H2AX was observed (Fig. 4 A). This indicates that the DDR activation induced by depletion of Wee1 is not only a consequence of the decreased replication fork speed. Instead, we reasoned that Wee1 could protect the stability of stalled replication forks. If this is the case, inhibiting Wee1 in the presence of short exposure to HU should significantly enhance the DDR. Indeed, whereas treatment of the cells with HU or Wee1 inhibitor alone did not result in a notable increase in H2AX phosphorylation, combined treatment resulted in high levels of γ-H2AX by Western blotting and an increased percentage of γ-H2AX–positive cells by immunofluorescence (Fig. 4 B).

Wee1 and Chk1 have complementing but distinct roles during DNA replication

As mentioned earlier in this section, a delay in DNA replication was reported in the absence of Chk1 (Maya-Mendoza et al., 2007; Petermann et al., 2010). To study whether Wee1 and Chk1 have separate functions in preventing genomic instability caused by replication problems, the effect of combined knockdown of Wee1 and Chk1 on γ-H2AX induction and replication fork speed was determined. Although simultaneous down-regulation of Wee1 and Chk1 did not increase γ-H2AX levels as compared with single knockdowns, the vast majority of cells without Wee1 and Chk1 undergo apoptosis, as judged by the sub-G1 population by flow cytometry (Fig. 5 A). In addition, whereas Chk1 down-regulation slows down replication fork speed to a similar extent as Wee1 knockdown, combined depletion of Wee1 and Chk1 resulted in a significant decrease of replication fork speed (Fig. 5 B). Together, these results strongly suggest that Wee1 and Chk1 have separate but complementing roles in securing correct DNA replication.

DDR in the absence of Wee1 depends on the Mus81-Eme1 endonuclease

To study the cause of the decrease in replication fork speed, we monitored replication fork stability by examining levels of replication proteins and their ability to associate with chromatin. Among the components of the MCM2–7 complex studied, a reduced binding of the MCM4 subunit to the chromatin was observed in Wee1-depleted cells as compared with control cultures, as demonstrated by immunofluorescence after a brief permeabilization before fixation (Fig. S4 A) and chromatin fractionation (Fig. S4 B). Wee1 down-regulation did not affect the chromatin binding of MCM2, MCM5, or Cdc45, an initiation factor that interacts with the MCM complex (Fig. S4 B). To resolve whether the absence of MCM4 on chromatin could trigger a DDR, we down-regulated the MCM components MCM2 and MCM4 during S phase. Indeed, depletion of MCM4, but not MCM2, resulted in increased H2AX phosphorylation as compared with control cells (Fig. S4 C). Inhibition of replication upon MCM knockdown was also previously reported (Ibarra et al., 2008),

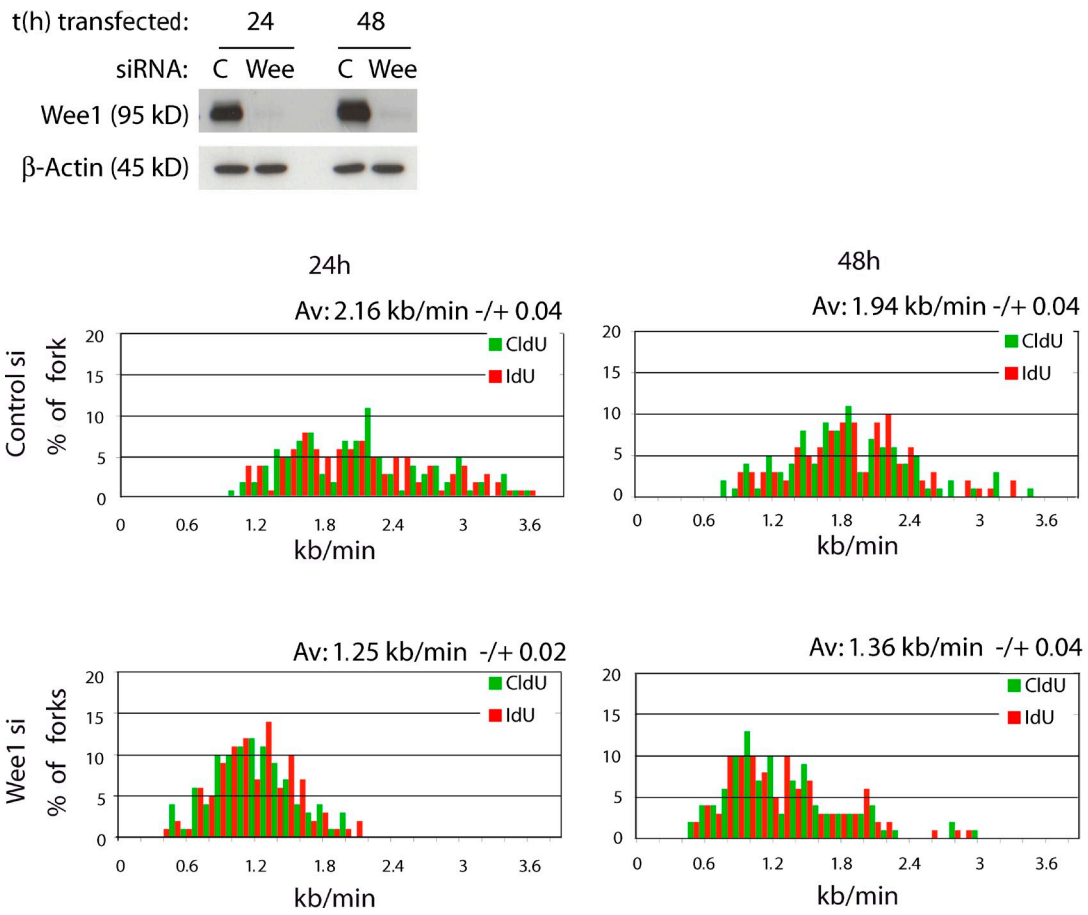

Figure 3. **Fork movement slowdown by Wee1 depletion.** U2OS cells were transfected with control (C) or Wee1 (Wee) siRNA oligonucleotides for the indicated times and thereafter pulsed for IdU and CldU. Fork speed was determined by measuring the length of IdU and CldU tracks on combed DNA molecules. Bar graphs show the percentages of molecules with certain replication speeds from one representative experiment. Above each graph, the average (Av) fork speed with the SEM of three independent experiments is indicated. Western blots show efficiency of Wee1 down-regulation.

and, therefore, we reasoned that Wee1 down-regulation could cause replication fork problems by depleting MCM4 from the chromatin, which subsequently triggers a DDR. However, simply decreasing replication fork speed is insufficient to enhance H2AX phosphorylation (Fig. 4 A), and, therefore, the activation of the DDR is unlikely to be directly caused by the defect in replication. Instead, we hypothesized that the DDR might be triggered by processing of the replication fork by a nuclease. Therefore, we tested whether the endonuclease Mus81-Eme1, which is known to process stalled replication forks (Hanada et al., 2007), could suppress the increase in H2AX phosphorylation induced by depletion of Wee1. Indeed, Mus81 depletion decreased the γ-H2AX signal in Wee1–down-regulated cells, indicating that its activity is critical for the DDR (Fig. 6 A). Fig. S4 (D and E) demonstrates that down-regulation of Exo1, Gen1, or Blm could not revert γ-H2AX induction triggered by Wee1 depletion, indicating that these enzymes are not involved in this response. In contrast, γ-H2AX levels are decreased upon the simultaneous down-regulation of Wee1 and Eme1 (Fig. S4 D), thereby confirming the role of the structure-specific endonuclease Mus81-Eme1 in this process.

Next, we asked whether the H2AX phosphorylation produced by the lack of MCM4 could be suppressed by simultaneous down-regulation of Mus81. Surprisingly, Mus81 down-regulation was not able to revert the DDR induced by MCM4 depletion (Fig. S5 A), suggesting that Mus81 might act upstream of MCM4 in generating the DDR. To further investigate this unexpected result, we depleted Mus81 in cells in which Wee1 was down-regulated and examined MCM4 binding to chromatin in synchronized S phase cells. Consequently, Mus81 down-regulation in Wee1-depleted cells was able to stabilize MCM4 at the chromatin (Fig. S5 B).

To demonstrate that the rescue of the DDR caused by Wee1 depletion by Mus81 is not a result of an indirect effect of preventing cells from entering S phase in the absence of Mus81, we monitored the progression of Mus81–down-regulated cells through S phase. In the absence of Mus81, cells were able to enter and progress through S phase as control cells, as is the case for cells depleted of Cdk2, the other protein we found to rescue the lack of Wee1 effect (Fig. S5 C).

Codepletion of Wee1 and Mus81 restores normal progression during S phase

Based on the aforementioned results, we speculated that Mus81 might have a central and direct role in the S phase delay and DDR observed in Wee1-depleted cells. Therefore, the progression of synchronized S phase cells was studied in conditions of Wee1 and/or Mus81 down-regulation. As observed in Figs. 2 D

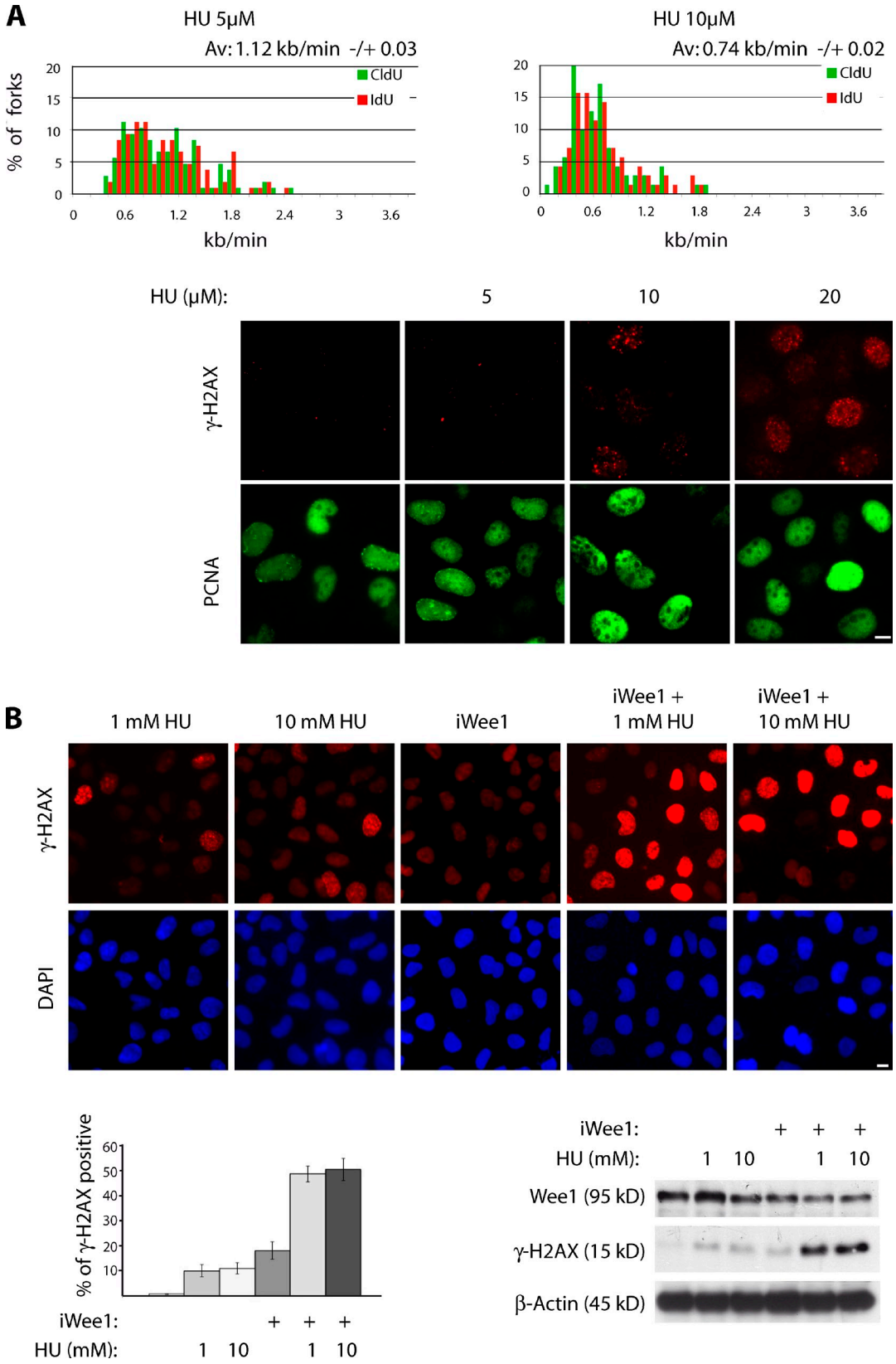

Figure 4. **Wee1 protects the stability of stalled replication forks.** (A) U2OS cells were treated with different concentrations of HU for 24 h. Thereafter, cells were pulsed for IdU and CldU and analyzed as described in Fig. 3 (top) or stained using the indicated antibodies for immunofluorescence (bottom). Av, average; PCNA, proliferating cell nuclear antigen. (B) U2OS cells were treated with Wee1 inhibitor. After 1 h, HU was added at the indicated concentrations, and cells were harvested and analyzed by immunofluorescence for γ-H2AX or immunoblotting using the indicated antibodies. The graph represents the quantification of the immunofluorescence. Represented is the percentage of γ-H2AX–positive cells. A minimum of 1,000 cells was counted for each sample. Error bars represent the SEM of two independent experiments. Bars, 10 μm.

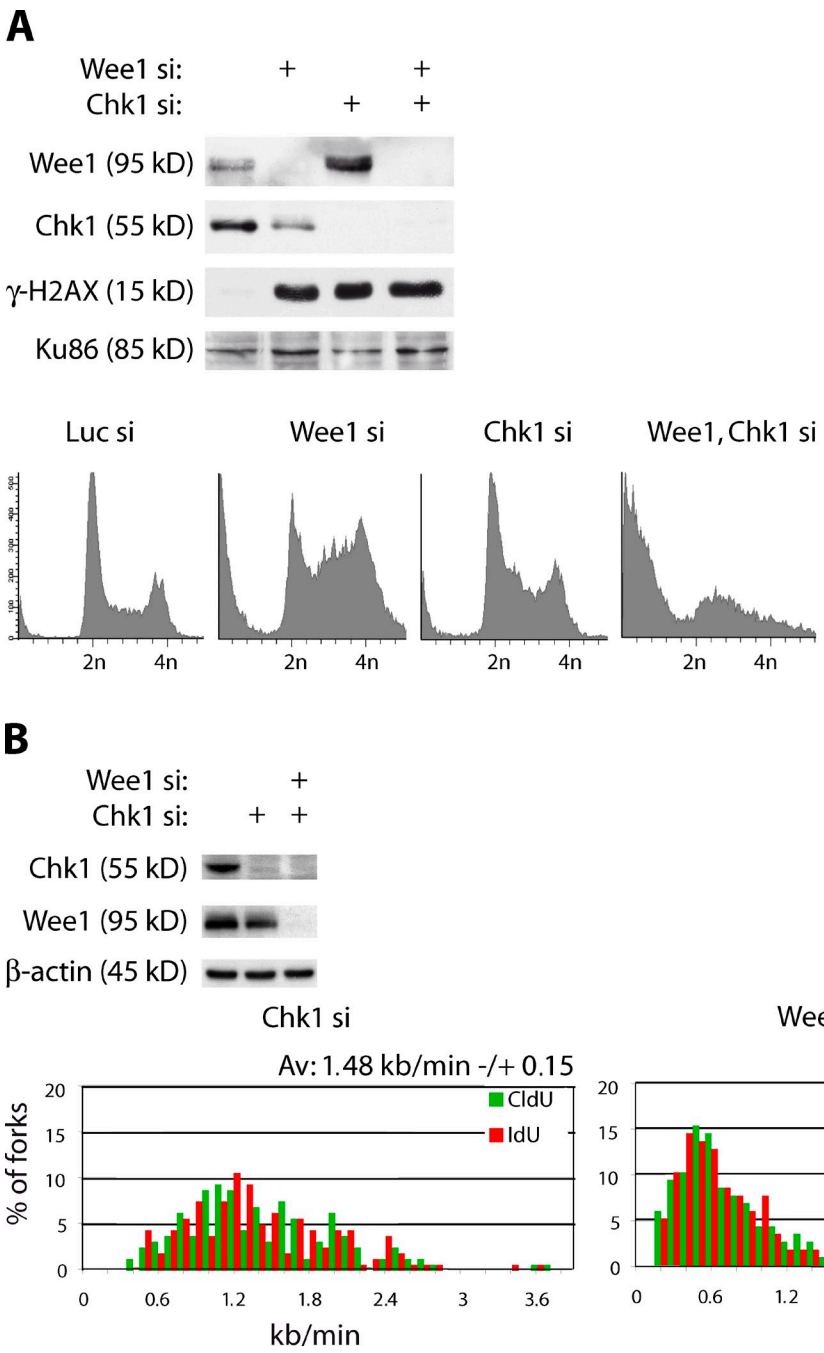

Figure 5. **Wee1 and Chk1 protect from replication problems in a different manner.** (A) Flow cytometry analysis after propidium iodide staining (bottom) and immunoblot analysis (top) of U2OS cells transfected with Luc, Wee1, or Chk1 siRNA oligonucleotides or a combination for 48 h. (B) U2OS cells were transfected with Chk1 with or without Wee1 siRNA oligonucleotides for 48 h and thereafter pulsed for IdU and CldU. (bottom) Fork speed was determined by measuring the length of IdU and CldU tracks on combed DNA molecules. Bar graphs show the percentages of molecules with certain replication speeds from one representative experiment. Above each graph, the mean fork speed with the SEM of three independent experiments is indicated. (top) Levels of the indicated proteins by Western blotting.

and 6 B, synchronized Wee1-depleted cells showed a pronounced inhibition of S phase progression. Interestingly, simultaneous down-regulation of Wee1 and Mus81 resulted in normal progression through S phase (Fig. 6 B), which, together with our previous results, suggests a functional relationship between these two proteins. The strong accumulation of cells in S phase upon Wee1 down-regulation in asynchronous cells was additionally alleviated when Mus81 was depleted simultaneously (Fig. S5 D).

Finally, to define the mechanism of how Mus81 is involved in the DDR induced in the absence of Wee1, we determined whether Mus81 chromatin levels are affected by Wee1 down-regulation. As shown in Fig. S5 E, no major changes in Mus81 association to the chromatin were observed in both asynchronous cells and cells synchronized in S phase, arguing against the exclusion of Mus81 from the chromatin by Wee1. To demonstrate a possible direct link between Wee1 and Mus81,

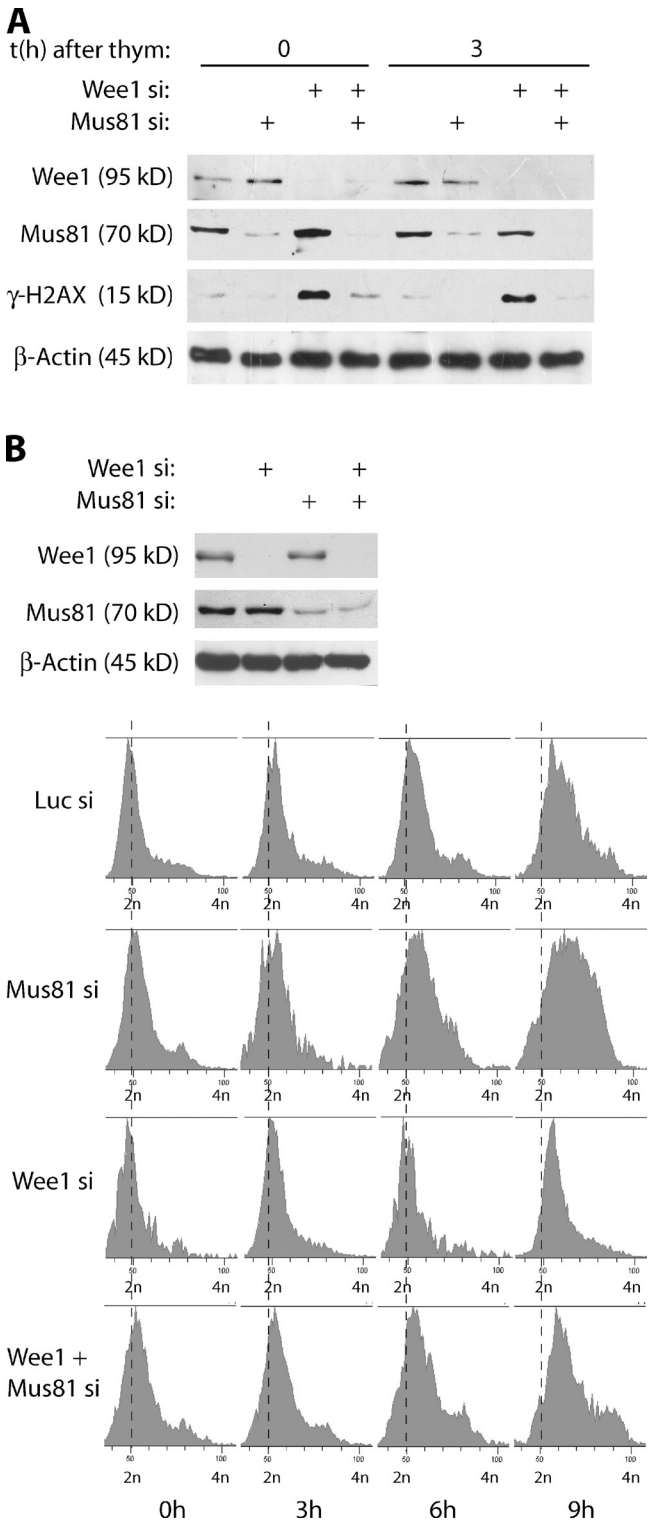

Figure 6. **Mus81 knockdown rescues the DNA damage induction and S phase delay in the absence of Wee1.** (A) U2OS cells were transfected with Luciferase or Mus81 siRNA (Mus81 si) for 48 h in the presence of thymidine (thym). During the last 24 h, they were transfected with Luciferase or Wee1 siRNA (Wee1 si). After that, cells were released or not released from the thymidine block for 3 h before being lysed and analyzed with the indicated antibodies. (B) U2OS cells were transfected with Luciferase siRNA (Luc si) and Mus81 siRNA for 64 h and/or Wee1 siRNA oligonucleotides during the last 24 h, all in the presence of thymidine. Cells were then released from the block and studied at the indicated times for DNA content by flow cytometry. Western blot analysis with the indicated antibodies of whole-cell extracts at time 0 is also shown. Dotted lines indicate the position of the G1 peak at t = 0.

immunoprecipitation experiments were performed. Upon overexpression of both Wee1 and Mus81, the proteins coimmunoprecipitated (Fig. 7 A). An interaction between endogenous Wee1 and Mus81 was also detected (Fig. 7 B). Together, these results indicate an unexpected new function of Wee1 and Mus81 in S phase and suggest that changes in Wee1 function could affect genomic integrity.

Discussion

Here, we report a novel function of Wee1 in maintaining genomic stability by controlling the Mus81-Eme1 endonuclease during DNA replication. By performing a high-throughput siRNA screen to identify kinases involved in genome maintenance using γ-H2AX, a universal DDR marker, as a read out, we identified Wee1 with the highest score. Lack of Wee1 activates a general DDR, predominantly in S phase cells. Wee1 down-regulation activates a Mus81-dependent DDR that slows down the replication fork speed.

In addition to Wee1, we also identified Chk1, an effector kinase in the ATR pathway, in the screen. Chk1 inhibition was reported to cause an increase in γ-H2AX levels in other studies (Syljuåsen et al., 2005; Beck et al., 2010). Importantly, our results show that Wee1 depletion produces a decrease in Chk1 levels, but this is unlikely to account for the increase in γ-H2AX after Wee1 down-regulation, as reintroduction of Chk1 in Wee1-depleted cells did not prevent the DDR activation (Figs. 2 E and S2 A). Moreover, the decrease of Chk1 levels upon Wee1 down-regulation was not observed at early times after siRNA treatment, suggesting that is a side effect of the Wee1 down-regulation and/or the activated DDR (Fig. S2 B). As Chk1 was previously shown to activate Wee1 during the G2/M checkpoint (Lee et al., 2001) and the lack of Chk1 has a similar effect on the replication fork speed as Wee1 down-regulation, we down-regulated both Chk1 and Wee1 to test whether they could be functioning together or in parallel pathways. Cell cycle profiles and measurements of DNA replication speed suggest that during replication, both proteins play a role in different pathways, as combined down-regulation results in more severe phenotypes than single knockdowns (Fig. 5).

Our results demonstrate an accumulation of cells with activated DDR in S phase and replication fork slowdown in the absence of Wee1. In addition, lack of Wee1 amplifies H2AX phosphorylation when replication is challenged (Fig. 4 B). Together, these results suggest that Wee1 protects the stability of stalled replication forks. Because the triggered DDR in replicating cells critically depends on Wee1 kinase activity (Fig. 1 C), catalytic inhibitors of Wee1 might have potent antitumor potential. Indeed, recent publications show that a Wee1 inhibitor enhanced the cytotoxic effects of several DNA-damaging agents specifically in p53-deficient cell lines (Hirai et al., 2009, 2010). Our data suggest that this anticarcinogenic effect could be achieved by the use of the inhibitor on its own and in all replicating cells.

With the exception of Chk1/Claspin levels, Wee1 depletion causes a global activation of the DDR, which indicates the presence of different types of DNA lesions, including DSBs (foci formation of 53BP1 and MDC1 and phosphorylation of Nbs1 and Smc1) and single-stranded lesions/stalled replication forks (foci

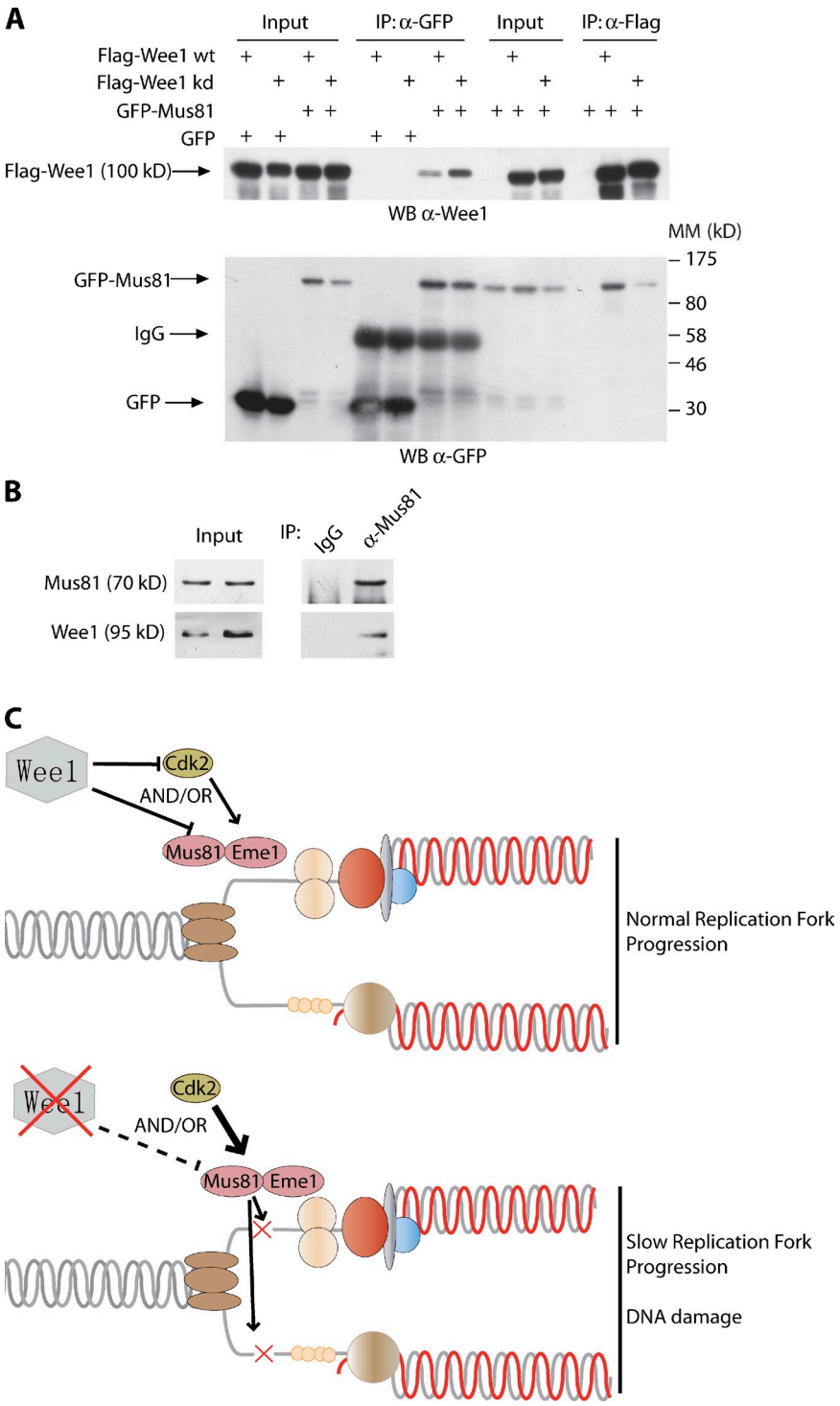

Figure 7. **Interaction between Wee1 and Mus81.** (A) HEK 293T cells were cotransfected with the indicated combinations of plasmids including expression vectors for the wild-type (wt) or kinase-dead (kd; K328A) version of Flag-Wee1, GFP, and GFP-Mus81, and immunoprecipitations (IP) using anti-GFP or anti-FLAG antibodies were performed. Inputs and immunoprecipitations were analyzed with anti-Wee1 and -GFP antibodies. MM, molecular mass; WB, Western blot. (B) Immunoprecipitations of endogenous Mus81 from U2OS cells analyzed by Western blotting using the indicated antibodies. (C) A model for Wee1 regulating DNA replication by controlling Mus81. See Discussion for details.

formation of Rad9 and TopBP1). The magnitude of this DDR in the absence of Wee1 could be explained by the implication of Mus81 in the response upon Wee1 depletion. It is very likely that the endonuclease produces, in addition to DSBs, several other types of lesions by cleaving replication forks, as it shows high affinity for branched structures (Mazina and Mazin, 2008; Taylor and McGowan, 2008). This is in agreement with the ~50× drop in cell viability upon Wee1 down-regulation compared with control cells but no additional sensitivity to other DNA-damaging agents such as UV light and ionizing radiation (unpublished data).

During the course of this project, two other papers appeared describing the identification of Wee1 in an RNAi screen similar to ours (Beck et al., 2010; Murrow et al., 2010). Although the phenotypes observed in these studies are consistent with our work (i.e., Wee1 is required to maintain genomic stability), these studies provide limited insight into the mechanism by which Wee1 controls genomic integrity. Here, we demonstrate for the first time that Wee1 is controlling DNA replication and does so through controlling the Mus81 endonuclease. Although this effect seems independent of Cdk1, Cdk2 inhibition does

rescue the DDR phenotype upon Wee1 depletion (Fig. S2 C). Interestingly, although Wee1 is a known Cdk1 regulator in G2/M, our data show that Wee1 is capable of controlling Cdk1/2 by phosphorylation at the beginning of S phase (Fig. S2 D).

Our results indicate that Wee1 critically regulates the Mus81-Eme1 endonuclease, as depletion of Mus81 or Eme1 in Wee1–down-regulated cells inhibits the DDR and most notably recovers a normal progression through S phase, demonstrating that Mus81 plays a central role early during DNA replication (Figs. 6 and S4 D). This effect seems to be specific for Mus81-Eme1 but not for other endonucleases with similar functions such as Exo1 and Gen1 or Blm helicase, which has been described to be functionally and physically linked to the Mus81–Eme1 complex (Fig. S4, D and E). Human Mus81-Eme1 has been shown to cleave branched structures that resemble replication forks, nicked Holliday junctions, and 3′ flap extensions in vitro (Mazina and Mazin, 2008; Taylor and McGowan, 2008), implying that Mus81 might process similar intermediates arising during normal DNA replication in vivo. Controlling Mus81 activity during S phase might therefore be essential to prevent unscheduled DNA lesions.

How exactly Wee1 regulates Mus81 function is a remaining and interesting issue. One possibility is that Wee1 controls directly or indirectly the association of Mus81 to the DNA, as it occurs in yeast with Cds1 (Kai et al., 2005), but Wee1 knockdown does not affect the association of Mus81 to the chromatin (Fig. S5 E). Therefore, it is possible that Wee1 might negatively regulate the endonuclease activity by phosphorylation during DNA replication. The role of Cdk2 in this process might be activation of Mus81 by phosphorylation. Hyperactivation of Cdk2 in the absence of Wee1 might lead to an inappropriate increase in Mus81 activity, resulting in triggering a DDR (Fig. 7 C). Although more experiments need to be performed to address the molecular mechanism underlying Mus81 activation during S phase, our data showing an in vivo interaction between Wee1 and the Mus81-Eme1 endonuclease implicate Wee1 in the direct inhibition of Mus81-Eme1 activity during S phase. Upon depletion of Wee1, this negative regulation is lost, likely resulting in deregulated dissection of replication forks by the endonuclease, thereby triggering a DDR and an S phase delay.

Materials and methods

Cell lines and plasmids

U2OS and 293T cells, human lymphoblasts, and nontransformed HT1080 fibroblasts were grown using standard procedures. The Flag-Chk1 plasmid was a gift from J. Bartek (Institute of Cancer Biology and Centre for Genotoxic Stress Research, Copenhagen, Denmark), and Flag-Gen1 was a gift from S. West (London Research Institute, Cancer Research UK, Clare Hall Laboratories, London, UK).

The coding sequence for human Wee1 was cloned in frame in pCMV Tag2B (Agilent Technologies) to generate a Flag-tagged version of Wee1. Flag-Wee1 kinase dead was obtained by site-directed mutagenesis of Flag-Wee1 in which lysine 328 was changed to alanine using the QuikChange Site-Directed Mutagenesis kit (Agilent Technologies) with the primers 5′-GATGGATGCATTTATGCCATTCGACGATCAAAAAAGCCATTGGCG-3′ and 5′-CGCCAATGGCTTTTTTGATCGTCGAATGGCATAAATGCATCCATC-3′.

Antibodies and reagents

Antibodies obtained from commercial sources were as follows: cyclin A (H-432), Chk1 (G-4), Wee1 (B-11), Cdk1 (17), and Cdk2 (M2; Santa Cruz Biotechnology, Inc.); rabbit γ-H2AX, pSer957-Smc1, and pSer343-Nbs1 (GenScript); pSer345-Chk1 and pTyr15-Cdk1 (Cell Signaling Technology); pTyr15-Cdk2 and Mus81 (Abcam); pSer317-Chk1 (Bethyl Laboratories, Inc.); β-actin (Sigma-Aldrich); and mouse γ-H2AX and proliferating cell nuclear antigen (Millipore).

MCM2, MCM3, MCM4, MCM5, and Cdc45 antibodies were a gift from J. Méndez (Centro Nacional de Investigaciones Oncológicas, Madrid, Spain). Claspin, 53BP1, TopBP1, Rad9, and BLM antibodies were generated in-house and were previously described (Lee et al., 2001; Toueille et al., 2004; Semple et al., 2007; Rendtlew Danielsen et al., 2009). Selective Wee1 inhibitor II was purchased from EMD and used at 10 µM. For synchronization, cells were incubated with 2.5 mM thymidine for the time indicated in each experiment.

Transfection

The following siRNA oligonucleotides were used: Luciferase 5′-UCGAAGUAUUCCGCGUACG-3′ (Thermo Fisher Scientific), Wee1 #1 5′-AAUAGAACAUCUCGACUUA-3′ (Thermo Fisher Scientific), Wee1 #2 5′-AAUAUGAAGUCCCGGUAUA-3′ (Thermo Fisher Scientific), Wee1 #3 5′-GAUCAUAUGCUUAUACAGA-3′ (Thermo Fisher Scientific), Wee1 #4 5′-CGACAGACUCCUCAAGUGA-3′ (Thermo Fisher Scientific), MCM2 5′-UUCCAUGCCAUCUCCAAUGAGGCUCC-3′ (Invitrogen), MCM4 (SMARTpool; Thermo Fisher Scientific), Mus81 5′-CAGGAGCCAUCAAGAAUAA-3′ (Invitrogen), Cdk1 5′-GAUGUAGCUUUCUGACAAAAA-3′ (Thermo Fisher Scientific), Cdk2 5′-UGCGAUAACAAGCUCCGUCC-3′ (Thermo Fisher Scientific), Chk1 #1 5′-GCGUGCCGUAGACUGUCCA-3′ (Thermo Fisher Scientific), Chk1 #2 5′-GCAACAGUAUUUCGGUAUA-3′ (Thermo Fisher Scientific), Gen1 5′-GUAAAGACCUGCAAUGUUA-3′ (Thermo Fisher Scientific), Eme1 5′-GGAUAAAGAACGCCAGAAU-3′ (Thermo Fisher Scientific), Exo1 5′-CAAGCCUAUUCUCGUAUUU-3′ (Invitrogen), and BLM 5′-UGCAAAUCACAUCGCUGCU-3′ (Invitrogen).

In most experiments, Wee1 was down-regulated using oligonucleotide #2. Cells were transfected using Lipofectamine RNAiMAX (Invitrogen) according to the manufacturer's instructions. Metafectene Pro (Biontex) was used for siRNA and plasmid cotransfection experiments following the manufacturer's guidelines.

Immunofluorescence

Cells grown on coverslips were washed in PBS, fixed with 2% PFA, permeabilized with 0.1% Triton X-100, and blocked using 1% FBS in PBS for 1 h. The incubations of primary antibodies were performed by dilution in 1% FBS for 1 h at RT. After a brief wash in 1% FBS, samples were incubated with Alexa Fluor–conjugated secondary antibodies (Invitrogen). For preextraction, cells were washed in cytoskeletal buffer (10 mM Pipes, pH 7.0, 100 mM NaCl, 300 mM sucrose, and 3 mM $MgCl_2$) and incubated in cytoskeletal buffer supplemented with 0.5% Triton X-100 for 5 min on ice. Subsequently, cells were fixed using 2% PFA, washed in PBS, and extracted with cold methanol for 5 min. Then, samples were blocked and incubated with antibodies as described above.

BrdU incorporation was detected using the BrdU Labeling and Detection kit II (Roche) following the manufacturer's recommendations. The mounting medium used was Vectashield with DAPI (Vector Laboratories). Mounted slides were examined using a fluorescent microscope (Cell Observer) with 20× NA 0.8 or 40× NA 1.3 magnification lenses and equipped with an AxioCam MR3 camera and AxioVision software (Carl Zeiss). Postacquisition image adjustments were performed using Photoshop (CS3; Adobe).

Chromatin fractionation

Biochemical fractionation of cells was performed essentially as previously described (Méndez and Stillman, 2000; Smits et al., 2006). Cells were washed in PBS and resuspended in solution A (10 mM Hepes, pH 7.9, 10 mM KCl, 1.5 mM $MgCl_2$, 0.34 M sucrose, 10% glycerol, 1 mM DTT, and protease and phosphatase inhibitors). Triton X-100 was added to a final concentration of 0.1%, cells were incubated on ice for 5 min, and the cytoplasmic (S1) and nuclear fractions were harvested by centrifugation at 1,300 *g* for 4 min. Isolated nuclei were then washed in solution A, lysed in solution B (3 mM EDTA, 0.2 mM EGTA, 1 mM DTT, and protease and phosphatase inhibitors), and incubated on ice for 10 min. The soluble nuclear (S2) and chromatin fractions were harvested by centrifugation at 1,700 *g* for 4 min. Isolated chromatin (P2) was then washed in solution B, spun down at 10,000 *g*, and resuspended in Laemmli sample buffer. Fractions S1 and S2 were pooled to one soluble fraction. Protein concentrations were determined using the bicinchoninic acid protein assay, and equal amounts of protein were loaded.

Flow cytometry

For cell cycle analysis, cells were collected by trypsinization and fixed in 70% ethanol at 4°C for a minimum of 2 h. After fixation, cells were washed with PBS, and the DNA was stained with propidium iodide.

γ-H2AX staining was performed as previously described (Huang and Darzynkiewicz, 2006) with slight modifications. In brief, cells were fixed in 70% ethanol overnight at 4°C. After washing with BSA-T-PBS (1% BSA/0.2% Triton X-100 in PBS), cells were incubated with mouse γ-H2AX antibody for 1 h at RT. After washing with BSA-T-PBS, cells were incubated with the FITC-conjugated secondary antibody and thereafter with propidium iodide. The samples were analyzed by flow cytometry using an Epics XL-MCL (Beckman Coulter) or LSR II (BD) flow cytometer.

Automated analysis of genomic instability

Cells were grown in 96-well plates in 100 ml of culture medium and transfected using HiPerFect transfection reagent (QIAGEN) according to the manufacturer's guidelines. 48 h after transfection, cells were fixed with 3% formaldehyde, washed in PBS, and postfixed with cold methanol. To check the genomic instability, cells were stained with γ-H2AX antibody and DAPI. Image acquisition was performed using a Cellomics ArrayScan VTI (Thermo Fisher Scientific) with a 10× 0.50 NA objective, and five images were acquired per well, which contained ∼1,000–2,000 cells in total. Image analysis was performed using a high content screening reader (Cellomics ArrayScan). Cells were identified on the basis of DAPI staining and were scored for mean intensity per cell of γ-H2AX. All images and automated image quantifications were visually checked.

DNA combing and image acquisition

Combing was performed as previously described (Anglana et al., 2003). Cells were pulse labeled for 20 min with 100 mM IdU and for 20 min with 100 mM CldU. Genomic DNA was prepared from DNA embedded in low-melting agarose blocks, and DNA fibers were combed on silanized coverslips. Neosynthesized DNA was immunodetected with mouse anti-BrdU FITC (BD), rat anti-BrdU (AbD Serotec), Alexa Fluor 488–conjugated goat anti–mouse (Invitrogen), and Alexa Fluor 555–conjugated goat anti–rat (Invitrogen). DNA fibers were detected with mouse anti–single-stranded DNA (Millipore), Cy5.5-conjugated goat anti–mouse, and Cy5.5-conjugated donkey anti–goat (Abcam). Slides were analyzed with an epifluorescence microscope (Axio Imager.Z2; Carl Zeiss) equipped with a 63× objective lens (NA 0.7–1.4) connected to a charge-coupled device camera (CoolSNAP HQ2; Roper Scientific), and MetaMorph software (Roper Scientific) was used for image acquisition.

Online supplemental material

Fig. S1 shows the kinome screen and its verifications. Fig. S2 demonstrates that lower levels of Chk1 cannot account for the DDR in the absence of Wee1, but Cdk2 can. Fig. S3 shows replication fork speed upon Wee1 depletion in lymphoblasts and HT1080 cells. Fig. S4 demonstrates MCM4 protein by immunofluorescence and Western blot analysis after Wee1 knockdown as well as the effect of depletion of Eme1, Exo1, Gen1, and Blm proteins on the DDR in the absence of Wee1. Fig. S5 demonstrates the effect of simultaneous knockdown of Mus81 and MCM4 or Wee1, the cell cycle effects of down-regulation of Mus81, Cdk2, Wee1, or Mus81/Wee1, and Mus81 chromatin levels upon Wee1 down-regulation. Online supplemental material is available at http://www.jcb.org/cgi/content/full/jcb.201101047/DC1.

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