The MCM-associated protein MCM-BP is important for human nuclear morphology

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Summary

Mini-chromosome maintenance complex-binding protein (MCM-BP) was discovered as a protein that is strongly associated with human MCM proteins, known to be crucial for DNA replication in providing DNA helicase activity. The *Xenopus* MCM-BP homologue appears to play a role in unloading MCM complexes from chromatin after DNA synthesis; however, the importance of MCM-BP and its functional contribution to human cells has been unclear. Here we show that depletion of MCM-BP by sustained expression of short hairpin RNA (shRNA) results in highly abnormal nuclear morphology and centrosome amplification. The abnormal nuclear morphology was not seen with depletion of other MCM proteins and was rescued with shRNA-resistant MCM-BP. MCM-BP depletion was also found to result in transient activation of the G2 checkpoint, slowed progression through G2 and increased replication protein A foci, indicative of replication stress. In addition, MCM-BP depletion led to increased cellular levels of MCM proteins throughout the cell cycle including soluble MCM pools. The results suggest that MCM-BP makes multiple contributions to human cells that are not limited to unloading of the MCM complex.

Key words: MCM complex, DNA replication, Helicase, G2 checkpoint

Introduction

The integrity of the human genome depends on its faithful replication, a process that is highly regulated in order to ensure that each DNA segment is duplicated. This involves the orchestrated assembly of pre-replicative complexes at the origins of replication, which include the minichromosome maintenance (MCM) complex. The MCM complex comprises MCM proteins 2–7, which are conserved in all eukaryotes and interact with each other to form a hexameric ring. The MCM complex is important for origin licensing, origin melting and subsequently for replication fork progression (reviewed by Forsburg, 2004; Maiorano et al., 2006). Considerable evidence indicates that the MCM complex provides the DNA helicase activity necessary to unwind double stranded DNA during replication and that this activity is greatly stimulated by interactions with Cdc45 and GINS (Ives et al., 2010; Moyer et al., 2006).

MCM2–MCM7 are members of the AAA+ ATPase family of proteins and share a region of homology encompassing the ATPase motifs referred to as the MCM box (Forsburg, 2004; Koonin, 1993). They form stable complexes that are loaded onto DNA at the end of mitosis and remain DNA-associated throughout most of the cell cycle, dissociating in mitosis just after the dissociation of MCM2–MCM7. TAP-tagging or immunoprecipitation of MCM core subunits from human cells recovered MCM-BP in addition to MCM proteins MCM2–MCM7. TAP-tagging or immunoprecipitation of MCM-BP recovered MCM3–MCM7 but not MCM2, whereas TAP-tagging of MCM2 recovered MCM3–MCM7 but not MCM-BP. The results indicate that alternative MCM complexes exist in human cells that contain MCM-BP but lack MCM2. Similarly to other MCM proteins, MCM-BP is largely nuclear with a proportion that is chromatin-associated throughout most of the cell cycle, dissociating in mitosis just after the dissociation of MCM2–MCM7 (Sakwe et al., 2007). Although MCM-BP lacks extensive homology to other MCMs, it contains a 15 amino acid region of homology with MCM4, MCM6 and MCM7 that overlaps with the Walker B motif and also has some homology to the C-terminal region of MCMs (particularly MCM7) from diverse organisms and archaeal MCM (Sakwe et al., 2007).

MCM-BP is conserved in most eukaryotes, with the notable exception of *Saccharomyces cerevisiae* and *Caenorhabditis elegans*. *Studies in Arabidopsis thaliana* identified the MCM-BP homologue (ETG1) as an E2F target and showed that loss of ETG1 leads to reduced DNA replication, activation of a G2 cell cycle checkpoint and reduced sister chromosome cohesion (Takahashi et al., 2008; Takahashi et al., 2010). Like human MCM-BP, ETG1 was shown to bind MCM proteins (Takahashi...
Fig. 1. Effects of MCM-BP depletion on human cells. (A) Immunofluorescence microscopy of HeLa cells 4 days after transfection with two shRNA-containing plasmids targeting MCM-BP (shMCM-BP1 and shMCM-BP2) or empty plasmid (control). DNA was stained with DAPI (blue), and α-tubulin was detected with anti-α-tubulin antibody (red). (B) Levels of the indicated MCM proteins were determined by immunoblotting total HeLa cell extracts transfected with shMCM-BP1 or shMCM-BP2. Actin is shown as a loading control. (C) The percentage of multi-lobed nuclei observed in A. Average values with standard errors are shown from three separate experiments in which at least 200 cells were counted (P<0.0005 for both shMCM-BP samples). (D) Immunofluorescence microscopy of U2OS cells 4 days after transfection with plasmids expressing shMCM-BP1. Top and bottom panels are images of different cells from the same experiment. DNA was stained with DAPI (blue), and α-tubulin was detected with anti-α-tubulin antibody (green). (E) The percentage of multi-lobed nuclei observed in D and in the same experiment performed with shMCM-BP2 and empty plasmid (control). Average values with standard error are shown from three separate experiments in which at least 200 cells were counted (P<0.001 for shMCM-BP1; P<0.05 for shMCM-BP2). (F) Apoptotic cells were determined by immunofluorescence imaging of DAPI-stained nuclei. Intense DAPI staining indicates fragmented DNA in apoptotic cells (arrowheads). (G) Quantification of the percentage of HeLa cells scored as apoptotic in D. Average values with standard error are shown from three separate experiments in which at least 100 cells were counted.
et al., 2008). The MCM-BP homologue of *Xenopus laevis* has also been recently studied (Nishiyama et al., 2011). Like human MCM-BP, immunoprecipitation of *Xenopus* MCM-BP from egg extracts recovered MCM3–7 and a direct interaction with MCM7 was subsequently identified. In an in vitro replication system involving the addition of *Xenopus* sperm nuclei to interphase egg extracts, MCM-BP was observed to enter the nuclei in mid-S phase and this correlated with the dissociation of MCM proteins from chromatin. Furthermore, depletion of MCM-BP from the egg extracts was found to reduce the dissociation of MCMs from the chromatin at the end of S phase, suggesting that MCM-BP has a role in unloading the MCM complex from chromatin after DNA synthesis is complete. siRNA-mediated downregulation of MCM-BP in human cells was also reported to result in a subtle increase in chromatin-associated MCM proteins at the end of S phase, suggesting that MCM-BP also affects MCM unloading in human cells (Nishiyama et al., 2011). In this study we further investigated the importance of MCM-BP in human cells using sustained shRNA-mediated knockdown and identified additional effects of MCM-BP modulation on nuclear morphology, G2 checkpoint responses and MCM protein levels.

### Results

#### MCM-BP has a major effect on nuclear structure

We have previously used short interfering RNA (siRNA) to downregulate MCM-BP in human cells and saw no gross cellular abnormalities (Sakwe et al., 2007). However, because 29 bp short hairpin RNA (shRNA) expression from a plasmid can lead to better depletion of chromatin-associated proteins and more prolonged silencing in general, we explored the use of two different 29 bp shRNAs to downregulate MCM-BP. Transfection of HeLa cells with either of these plasmids (shMCM-BP1 or shMCM-BP2), followed by selection for the plasmids led to multi-lobed and donut-shaped nuclei that became apparent after 2 days of selection (4 days post-transfection), which were not seen in cells transfected with the empty plasmid (Fig. 1A). Western blots confirmed that MCM-BP was downregulated with both shRNA plasmids but to a greater degree by shMCM-BP1, without downregulation of other MCM proteins (Fig. 1B). In keeping with these results, shMCM-BP1 resulted in approximately 46% of cells with abnormal nuclei as opposed to 22% after shMCM-BP2 treatment (Fig. 1C). The same shRNA experiments were performed in two additional cell lines, U2OS (Fig. 1D,E) and CNE2Z (data not shown) with very similar results. In addition, an increased percentage of apoptotic cells was observed after shMCM-BP1 and shMCM-BP2 treatment (on average 20% and 13%, respectively), as detected by the intense DAPI staining of cells with fragmented DNA (Fig. 1F,G).

Because MCM-BP can form complexes with other MCM proteins, we examined whether depletion of other MCMs resulted in a similar phenotype as MCM-BP depletion. To this end, shRNAs against MCM4 and MCM2 were used to silence these proteins and, consistent with previous reports (Ge et al., 2007; Ibarra et al., 2008), also resulted in reduced levels of other MCM proteins (Fig. 2A). However, these treatments did not result in the multi-lobed or donut-shaped nuclei typical of MCM-BP depletion, and resulted in only a small increase in bi-lobed nuclei over that seen with the negative control plasmid (Fig. 2B,C). Note that although it is possible the shMCM2- and shMCM4-treated cells are not functional nulls because of residual levels of MCM2 and MCM4 in the cells, these proteins were depleted beyond levels detectable in the western blot and the depletion was more extensive than the MCM-BP depletion, which resulted in a striking phenotype. Therefore, the phenotype of the MCM-BP knockdown is not typical of other MCM protein depletion phenotypes.

To further verify that the effect of *MCM-BP* shRNA treatment was due to loss of MCM-BP and not an off-target effect of the shRNAs, we performed a rescue experiment with FLAG-tagged shRNA-resistant MCM-BP (FLAG–MCM-BPr). For these experiments, HeLa cells were co-transfected with the shMCM-BP1 plasmid and a second plasmid expressing FLAG–MCM-BPr with silent mutations making it resistant to shMCM-BP1, and nuclear morphology was examined 4 days post-transfection. Although only a small proportion of the cells expressed the FLAG–MCM-BPr, virtually all of the cells expressing this shRNA-resistant MCM-BP (FLAG-positive) had normal nuclear morphology in contrast to neighbouring FLAG-negative cells, of which approximately 50% were abnormal (Fig. 3A,B). An additional control was also performed in which the shMCM-BP1 plasmid was co-transfected with a plasmid expressing FLAG–β-gal in place of FLAG–MCM-BPr. As expected, the FLAG–β-gal-expressing cells failed to rescue the nuclear morphology defect caused by shMCM-BP1, resulting in approximately 60% of the FLAG-positive cells having multi-lobed nuclei (shBP1+LacZ in Fig. 3A,B). This is in marked contrast to the 5% of FLAG-expressing cells with multi-lobed nuclei, when cells were

![Fig. 2. Multi-lobed nuclear phenotype is not typical of MCM depletion.](image)

(A,B) HeLa cells were transfected with plasmids expressing shRNA against MCM2 (shMCM2) or MCM4 (shMCM4) or empty plasmid. Cell extracts were analyzed 4 days later by western blotting using the indicated antibodies (A; actin served as the loading control), or cells were fixed, stained with DAPI and imaged by fluorescence microscopy (B). (C) The percentage of abnormally shaped nuclei observed in B, shown in comparison with the shMCM-BP1 (shBP1) treatment from Fig. 1. Average values with standard error are shown from three separate experiments in which at least 200 cells were counted.
co-transfected with shMCM-BP1- and FLAG–MCM-BPr-expressing plasmids. Furthermore, overexpression of wild-type FLAG-tagged MCM-BP in HeLa cells (without MCM-BP depletion) resulted in an approximate twofold increase in nuclei with multiple lobes compared with the cells treated with the empty FLAG expression plasmid and, 2 days later, cells were stained with DAPI (blue) and anti-FLAG antibody (green). Examples of FLAG–MCM-BP-positive cells with abnormal morphology are shown in both top and bottom panels. (D) The percentage of FLAG-positive cells with multi-lobed nuclei from C. Average values with standard error are shown from three separate experiments in which at least 200 cells were counted (P<0.01).

MCM-BP-depleted cells proliferate with additional centrosomes

To better understand the defect associated with the abnormal nuclei resulting from MCM-BP depletion, we first performed a FACS analysis of the DNA content of cells treated with shMCM-BP1 and shMCM-BP2 (Fig. 4A). These profiles were very similar to those of control cells, indicating that the abnormal nuclei were not arrested at a particular phase of the cell cycle. However, a small increase in the G2–M peak was observed with both shMCM-BP constructs and a shoulder on the G1 peak of the shMCM-BP1 profile was consistent with increased apoptotic cells as observed above. To determine whether cells depleted of MCM-BP were able to undergo DNA replication, shMCM-BP1-treated cells were pulse labelled with bromodeoxyuridine (BrdU), a thymidine analogue, for 30 minutes and then imaged by immunofluorescence microscopy using an anti-BrdU antibody. Cells with normal and multi-lobed nuclei were both observed to incorporate BrdU with similar frequency, indicating that the abnormal nuclei were...
Fig. 4. Modest effect of MCM-BP depletion on the cell cycle. (A) DNA content of HeLa cells transfected with shMCM-BP1 or shMCM-BP2 as in Fig 1A was determined using flow cytometry. (B) HeLa cells treated with shMCM-BP1 as above were pulsed with BrdU for 30 minutes (left panel) followed by a 9-hour chase without BrdU (right panel). BrdU was detected with anti-BrdU antibody following denaturation of the DNA (green), and total DNA was detected by DAPI staining (blue). Right bottom panel shows cells that have incorporated BrdU and progressed to mitosis (arrowheads).

Fig. 5. MCM-BP depletion results in centrosomal amplification. (A) Mitotic HeLa cells transfected with plasmids expressing shMCM-BP1 or shMCM-BP2 or empty plasmid (control). Images were taken 4 days post-transfection (two examples of each silencing plasmid are shown). DNA was stained with DAPI (blue) and γ-tubulin was visualized with anti-γ-tubulin antibody (red). (B) The percentage of metaphase HeLa cells from A that appeared abnormal for any reason. Average values with standard error are shown from three separate experiments in which at least 100 cells were counted (P<0.0005). (C) The percentage of mitotic HeLa cells from A with more than two centrosomes (as determined by γ-tubulin staining). (D) Images of HeLa cells after transfection with a plasmid expressing shMCM-BP1 or empty plasmid (control) as in A. DNA was stained with DAPI (blue), and γ-tubulin (red) and pericentrin (green) were visualized using antibodies specific for these proteins.
able to undergo DNA synthesis (Fig. 4B, left panels). When BrdU-pulsed cells were released into BrdU-free medium for 9 hours, mitotic cells positive for BrdU incorporation became evident, indicating that MCM-BP-depleted cells are able to move from S phase to mitosis (Fig. 4B, right panels). Together the results indicate that MCM-BP-depleted cells retain the ability to cycle despite their abnormal nuclear morphology.

Fig. 6. See next page for legend.
In addition to abnormal interphase cells, examination of DAPI-stained shMCM-BP1- or shMCM-BP2-treated cells also showed a high frequency of abnormal mitotic cells. In particular, approximately 70% of metaphase cells had misaligned and branched DNA after either shMCM-BP treatment (Fig. 5A,B). Staining with γ-tubulin revealed that approximately 50% and 20% of the shMCM-BP1- and shMCM-BP1-treated mitotic cells, respectively, had more than two spindle poles, compared to only 3% of cell treated with the negative control plasmid (Fig. 5A,C). An example of an abnormal mitosis with two centrosomes is also shown (Fig. 5A, bottom panel). Therefore multiple spindle poles are likely to account for at least a proportion of the abnormal DNA alignments. Why this proportion is larger with shMCM-BP1 than shMCM-BP2 is unclear but could be due to the less effective MCM-BP downregulation by shMCM-BP2. Co-staining with pericentrin and γ-tubulin confirmed that the multiple γ-tubulin foci were centrosomes (Fig. 5D). Therefore MCM-BP depletion leads to centrosome amplification. Interestingly, the percentage of mitotic cells with additional centrosomes (50% for shMCM-BP1 and 20% for shMCM-BP2) corresponds well to the percentages of cells with multi-lobed nuclei after these treatments (see Fig. 1C), suggesting that the multilobed nuclear morphology results from passage through mitosis with many centrosomes.

**MCM-BP depletion leads to transient G2 checkpoint activation**

Centrosome amplification can result as a consequence of G2 checkpoint activation (Dodson et al., 2004), prompting us to look for evidence of a G2 checkpoint. Western blotting performed on total cell lysates from log-phase cells showed that, compared with the control transfection, shMCM-BP1 treatment resulted in an increase in the level of phosphorylated Cdc2 (at Tyr15), a phosphorylation that prevents progression into mitosis by affecting Cdc2–cyclin B activity (Fig. 6A,B, compare lanes 1 and 2 in each). Wee1, the kinase responsible for this phosphorylation, was concurrently elevated, whereas levels of Cdc25C, the protein that dephosphorylates Tyr15, were decreased (Fig. 6A, lanes 1 and 2). This pattern is consistent with G2 checkpoint activation. Because ATM and ATR kinases are integral to G2 checkpoint signalling, we assessed whether their activities were required for the Cdc2 inactivation resulting from MCM-BP depletion. To this end, we inhibited ATM and/or ATR function using caffeine and analyzed Cdc2 Y15 phosphorylation with and without MCM-BP depletion. Caffeine treatment decreased Cdc2 phosphorylation in response to both shMCM-BP1 and shMCM-BP2 treatments, without affecting the levels of Cdc2, verifying that this is a checkpoint response (Fig. 6B). In keeping with this conclusion, MCM-BP downregulation by both shMCM-BP1 and shMCM-BP2 triggered phosphorylation of Chk1 at Ser317, without affecting Chk1 levels (Fig. 6C).

Because shMCM-BP1 and shMCM-BP2 treatments did not lead to cell cycle arrest, we also analyzed the persistence of checkpoint signalling using a time course experiment (Fig. 6A). To this end, samples were taken every 12 hours, starting at 3 days post-transfection (one day after puromycin selection to kill untransfected cells) and analyzed by western blotting. Changes in the levels of checkpoint markers relative to the transfection control samples, showed induction of checkpoint signalling at 3 days and to a lesser degree at 3.5 days but, at subsequent times, the levels of the markers were not consistent with G2 checkpoint activation, even though MCM-BP remained silenced throughout the time course (Fig. 6A, compare + and – lanes at each time point). Rather, the decreased levels of Wee1 seen at 4 and 4.5 days are consistent with checkpoint recovery (Bartek and Lukas, 2007). The results suggest that MCM-BP depletion transiently activates G2 checkpoint signalling.

Activation of the G2 checkpoint was further examined by determining the effect of MCM-BP depletion on cell cycle progression. To this end, we synchronized MCM-BP-depleted cells to the G1–S boundary using a double thymidine block and examined the cell cycle profile of released cells by measuring DNA content using FACS analysis (Fig. 6D). Control and shMCM-BP1-treated cells showed similar progression through S phase (2- to 8-hour samples) but the G2–M peak persisted for longer after MCM-BP silencing (10- and 12-hour samples). In a similar block and release experiment, western blots for histone H3 phosphorylated at Ser10 (pH3), a marker of mitosis, showed a delay in the accumulation of phosphorylated H3 with shMCM-BP1 treatment (Fig. 6E). This suggests that the increase in the G2–M peak in 10- and 12-hour MCM-BP-depleted samples was due to an increased G2 population. Cell cycle progression was also examined by a BrdU pulse–chase experiment in MCM-BP-depleted cells in which the progression of the labelled S-phase cells was followed through the cell cycle (supplementary material Fig. S1). As in the synchronization experiment, slower progression through G2–M was evident in the MCM-BP-depleted samples compared with mock-treated cells. Together the results indicate that MCM-BP depletion leads to slower progression through the G2 phase of the cell cycle.

**MCM-BP depletion results in increased replication stress**

G2 checkpoint activation as well as impaired cell cycle progression can result from improper DNA replication leading to single-stranded DNA regions. These single-stranded regions...
are typically bound by the single-stranded DNA binding protein, replication protein A (RPA), and large stretches of single stranded DNA are apparent as RPA foci by microscopy. We examined RPA foci in cells 5 days post-transfection with shMCM-BP1 or shMCM-BP2 and observed an approximate threefold increase in cells with three or more RPA foci compared with control cells (Fig. 6F,G). An increase in chromatin-associated RPA after shMCM-BP treatment was also detected by western blotting of the chromatin-enriched fraction (Fig. 6H,I). This suggests that MCM-BP depletion caused a problem during DNA synthesis resulting in single-stranded DNA regions, which could be responsible for the impaired cell cycle progression observed following MCM-BP silencing. In addition to RPA foci, we also examined the effect of MCMBP downregulation on γ-H2AX, a marker of double-stranded DNA breaks. Although some increase in the levels of chromatin-associated γ-H2AX was observed (Fig. 6H,I), we did not see an increase in γ-H2AX foci (data not shown). These results suggest that depletion of MCM-BP results in extended regions of single-stranded DNA, resulting in replication stress.

**MCM-BP affects MCM protein levels**

Studies in *Xenopus* egg extracts and in HeLa cells have shown that MCM-BP depletion results in increased levels of MCM proteins on chromatin at mid to late S phase (Nishiyama et al., 2011). However, in the course of our studies we noticed that the effect of prolonged MCM-BP depletion was not limited to chromatin-associated MCMs but appeared to have a more global effect on MCM protein levels. In fact, increased MCM protein levels after treatment with shMCM-BP1 or shMCM-BP2 were apparent in whole cell extracts of log-phase cells (Fig. 7A). However, these treatments did not affect the level of another replication protein, proliferating-cell nuclear antigen (PCNA; Fig. 7A), nor did they increase the level of MCM transcripts (data not shown), suggesting that MCM-BP specifically affects MCM proteins at the post-transcriptional level. Fractionation of

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**Fig. 7.** MCM-BP depletion results in elevated levels of MCM proteins. (A) Total HeLa cell lysates were analyzed by western blotting for the indicated MCM protein or for PCNA (loading control) 5 days post-transfection with shMCM-BP1 (shBP1), shMCM-BP2 (shBP2) or empty plasmid. (B) Cells were treated as in A then fractionated into soluble and chromatin protein fractions before western blotting with the indicated antibodies. Equal cell equivalents were analyzed for soluble and chromatin fractions. (C,D) HeLa cells were transfected with plasmids expressing shMCM-BP1 or empty plasmid, and 5 days later synchronized at the G1–S boundary using a double thymidine block, and released for the indicated number of hours. Levels of the indicated MCM proteins were measured by western blotting equivalent amounts of whole cell extracts (C) and soluble (D) fractions. Actin was used as a loading control.
the log-phase cells into soluble and chromatin fractions showed that the soluble fraction of MCM proteins was most affected by MCM-BP depletion (Fig. 7B). Because G1 is the most prevalent phase in log-phase cells, we also examined MCM levels through S, G2 and M phases using HeLa cells synchronized at the G1–S boundary (similar to those described in Fig. 6D) and released for various times. Examination of both whole cell extracts (Fig. 7C) and soluble proteins (Fig. 7D) showed that MCM-BP depletion resulted in increased levels of MCM4, 5 and 7 through S (2– to 6-hour time points) and G2–M (8– to 10-hour points) phases. Together, the results indicate that MCM-BP can affect total cellular levels of the MCM proteins throughout the cell cycle, which might be a factor affecting their chromatin association.

Discussion

We have investigated MCM-BP function through its prolonged depletion in human cells. We have shown that lack of MCM-BP leads to centrosome amplification and nuclei with altered morphology. It is probable that the unusual multi-lobed and donut-shaped configurations of the DNA are a result of multiple mitotic spindles emanating from the amplified centrosomes, leading to abnormal mitotic events. Similar abnormal nuclear morphology, including donut-shaped nuclei, have been reported following silencing of the centromeric protein CENPA and following inhibition of the Aurora B mitotic kinase with hesperadin (Goshima et al., 2003; Hauf et al., 2003). In each of these cases the abnormal nuclear morphology has been attributed to chromosome misalignment and missegregation. Some DNA replication proteins, including Orc1, Orc2 and MCM5 have been shown to be partly associated with centrosomes and to have additional roles in controlling centrosome copy number (Ferguson et al., 2010; Hemerly et al., 2009; Prasanth et al., 2011). However, we have not detected any noticeable association of MCM-BP with centrosomes by immunofluorescence microscopy. In addition, although a proteomic analysis of human centrosomes identified MCM4, 6 and 7 in the centrosome fraction, MCM-BP was not detected (Andersen et al., 2003). Therefore the centrosome amplification observed after MCM-BP depletion might be an indirect effect of cell cycle perturbation.

Both the detection of G2 checkpoint markers as well as the increased frequency of RPA foci following MCM-BP depletion point to problems with DNA replication and suggest a role for MCM-BP in this process. Studies on Arabidopsis thaliana lacking the MCM-BP homologue, ETG1, came to a similar conclusion, as these cells exhibited G2 checkpoint signalling and decreased proliferation (Takahashi et al., 2008). In the case of human MCM-BP depletion, markers of the G2 checkpoint were only detected transiently, indicating recovery from the checkpoint. However, increased levels of RPA foci and slowed progression through G2 persisted. Although only a small percentage of cells scored positive for RPA foci after MCM-BP depletion, this was significantly higher than with control shRNA treatment and in line with the cellular percentages reported for MCM3 depletion in HeLa cells (Ibarra et al., 2008). In Arabidopsis, ETG1 disruption was also found to impair sister chromatid cohesion, possibly as an indirect effect of improper DNA synthesis (Takahashi et al., 2010). The same study also found that MCM-BP silencing with siRNA interfered with sister chromatid cohesion in HEK-293T cells. However, we have not observed any noticeable effect of MCM-BP depletion with our shRNAs on chromosome cohesion, possibly because of the presence of residual MCM-BP on the chromosomes. Therefore, the effects of MCM-BP depletion that we have observed are unlikely to be due to impaired sister chromatid cohesion.

Studies on the Xenopus laevis MCM-BP homologue in egg extracts have shown that MCM-BP only enters added sperm nuclei late in S phase, a time corresponding to the dissociation of MCM complexes from chromatin (Nishiyama et al., 2011). In addition, depletion of MCM-BP from these extracts resulted in increased levels of MCM proteins on chromatin, and addition of exogenous MCM-BP increased the dissociation of MCM proteins from chromatin late in S phase and dissociated MCM2–7 hexamers. Taken together, these results suggest that Xenopus MCM-BP can unload MCM complexes from chromatin at the end of S phase. In human cells, increased association of MCMs with chromatin at mid to late S phase was also observed after MCM-BP depletion, consistent with a role of MCM-BP in MCM unloading (Nishiyama et al., 2011). However, other explanations are also possible, such as increased loading of MCM complexes on chromatin after MCM-BP depletion, perhaps driven by increased levels of MCM proteins, as reported here. In addition, our finding that MCM-BP depletion inhibits Cdc2 might be a factor in the degree to which MCMs associate with chromatin, as Cdc2 inactivation has been previously reported to increase the level of MCMs on chromatin in G2 (Fujita et al., 1998). Regardless, our current data show that the increased association of MCM proteins with chromatin in late S phase is not simply due to a shift of the MCM proteins from the soluble to chromatin-associated forms but rather that MCM-BP has a more global effect on MCM protein levels in human cells. Our finding that MCM-BP depletion increased the soluble and total cellular levels of MCM proteins at all stages of the cell cycle does not appear to be a transcriptional effect because quantitative RT-PCR performed on the mRNA encoding several MCM proteins did not detect increased levels of MCM transcripts following MCM-BP depletion (data not shown). Therefore we favour a scenario where MCM-BP affects the stability of MCM proteins. It is presently unclear whether the increase in MCM protein levels itself could result in some of the phenotypes associated with MCM-BP depletion, as the role of the soluble pool of MCM proteins is currently unknown. However, it is interesting to note that a recent study in mice has shown that severely small decreases in MCM protein levels can affect MCM localization and result in replication stress (Chuang et al., 2010). It remains to be determined whether increases in MCM levels also have detrimental effects.

Differences in the observed contributions of MCM-BP in Xenopus and human systems could be due to differences in the localization of MCM-BP in Xenopus and humans. The effect of MCM-BP in Xenopus would only be expected to be manifested at the end of S phase because this is when it enters the nucleus. By contrast, the nuclear localization of MCM-BP in human cells throughout the cell cycle means that it would be available for MCM interactions throughout S phase and in other stages of the cell cycle. It is also probable that the checkpoint activation and replication stress detected following MCM-BP depletion in human cells, but not in Xenopus extracts, reflects additional roles for MCM-BP in human cells arising from localization differences. In this regard the results in human cells more closely resemble those in plant cells where MCM-BP is also constitutively nuclear (Takahashi et al., 2008).

We observed that MCM-BP overexpression leads to nuclear abnormalities similar to those seen with MCM-BP depletion...
in human cells, indicating that excess MCM-BP could be as detrimental as a lack of MCM-BP. This requirement for a precise level of MCM-BP might reflect the need for formation of particular MCM-BP-containing complexes. TAP-tagging and co-immunoprecipitation of MCM-BP from human cells and Xenopus extracts revealed interactions with MCM-3–7 (Nishiyama et al., 2011; Sakwe et al., 2007). Similarly TAP-tagging of ETG1 in Arabidopsis cells recovered MCM-2–7 (Takahashi et al., 2008). MCM-BP was also shown to associate with the MCM4, 6 and 7 core sub-complex when these proteins were coexpressed in insect cells (Sakwe et al., 2007). In summary, our results suggest that MCM-BP makes multiple contributions to DNA replication in human cells. As a result, a lack of MCM-BP manifests as cells with increased apoptosis and abnormal nuclei exhibiting signs of replication stress and slowed G2 progression. The importance of MCM-BP is further supported by its high degree of conservation in eukaryotes and by the finding that the gene encoding the MCM-BP/ETG-1 homologue in Schizosaccharomyces pombe (SPAC1687.04) is essential (http://www.sanger.ac.uk/Projects/S_pombe/). In addition, human genome-wide studies have identified MCM-BP as significantly upregulated in brain cancer, breast cancer and seminoma (http://www.oncomine.org) as well as in stem cells (Van Hoof et al., 2006), further supporting the importance of this protein in cell proliferation.

Materials and Methods

shRNA and plasmids

shRNA in pRS plasmids, which include a puromycin resistance cassette, were obtained from the Origene HuSH-29 collection (Cambridge Bioscience Ltd, Cambridge, UK). The following shRNA sequences were used to target specific genes: MCM-BP1, 5′-CAGGACACGTCGTAAGAGGACGGTGCT-3′; MCM-BP2, 5′-AAGGACTCCACAGGCCATTTCGCTGTTCCGTCATT-3′; MCM2, 5′-GGCCTACAAGCTCCAGGACAATGCGGATCGAGAG-3′; MCM4, 5′-GAGCTGTTGGCCTATTCGAGTCATACCAAG-3′. The pRS empty plasmid was used as a negative control for shRNA experiments. For experiments involving MCM-BP overexpression, MCM-BP was expressed from plasmid pMZSF-MCM-BP described previously (Sakwe et al., 2007). To generate the shRNA and plasmids

Transfections

Transfections were performed using Lipofectamine 2000 (Invitrogen) according to the manufacturer’s protocol. For shRNA experiments, 0.5–1.0 × 106 HeLa cells were transfected with 10 μg pRS plasmid expressing the shRNA of interest (or empty pRS as a negative control) for 48 hours and then puromycin was added to a final concentration of 2.5 μg/ml. Cells were grown under selection for another 2–3 days, as noted (referred to as 4- and 5-day post-transfection). For experiments involving caffeine treatment, 2 mM caffeine was added 2 hours prior to harvesting the cells. For MCM-BP overexpression, 0.5–1.0 × 106 HeLa cells were transfected with 10 μg pMZF6-MCM-BP for 48 hours, then immediately processed for immunofluorescence microscopy. For MCM-BP rescue experiments, 0.5–1.0 × 106 HeLa cells were transfected with 5 μg each of shMCM-BP1 (or empty pRS plasmid) and MCM-BP1 (or pMZSF-lacZ) for 48 hours, then selected for 48 hours in puromycin (2.5 μg/ml) prior to processing for immunofluorescence microscopy.

Antibodies

Antibodies used in this study were: MCM2 (9839; Santa Cruz), MCM3 (9850; Santa Cruz), MCM4 (22779; Santa Cruz), MCM5 (165993; Santa Cruz), MCM6 (9843; Santa Cruz), MCM7 (22782; Santa Cruz), histone H3 Ser10-P (8656-R; Santa Cruz), histone H2B (Upstate), Cdc25C (56209; Santa Cruz), Weel (5285; Santa Cruz), γ-tubulin (17787; Santa Cruz), RP32 (16855; Abcam), Cdc2 Tyr15-P (9111S; Cell Signaling), Cdc2 (954; Santa Cruz), Chk1 (A300-162A; Bethyl Laboratories), Chk1 Ser317-P (A300-163A; Bethyl Laboratories), γ-H2AX (A38548; Abcam), FLAG M2 (Sigma), BrdU–FITC (11 202 693; Roche), actin (Calbiochem), pericentrin (4448; Abcam). The antibody against MCM-BP has been described previously (Sakwe et al., 2007). All secondary antibodies were obtained from Santa Cruz Biotechnology.

Cell synchronization

HeLa cells were transfected with the pRS plasmid expressing MCM-BP1 or pRS alone for 48 hours then selected in puromycin for 24 hours before synchronization at the G1–S boundary by a double thymidine block. Briefly, thymidine (Sigma) was added to a final concentration of 2 mM for 19 hours, followed by two washes in PBS and release into complete DMEM for 10 hours. Thymidine was then added again to 2 mM for 17 hours and cells were either harvested directly or released to complete medium for the indicated times.

Cell fractionation

Transfected cells were fractionated into soluble and chromatin-bound fractions as described previously (Rizzi et al., 2003; Sakwe et al., 2007). Briefly, cells were harvested by trypsinisation, washed twice with ice-cold PBS and resuspended in hypotonic lysis buffer (10 mM Hepes pH 7.9, 10 mM KCl, 1.5 mM MgCl2, 0.34 M sucrose, 10% glycerol, 0.1% Triton X-100). After 15 minutes on ice, the lysate was subjected to centrifugation at 1300 g for 5 minutes. The supernatant, comprising the soluble protein fraction, was transferred to a new tube and the pellet washed once with the same buffer. After centrifugation, the pellet was resuspended in RIPA buffer (50 mM Tris-HCl, pH 7.4, 1% NP-40, 150 mM NaCl, 1 mM EDTA, 0.5% sodium deoxycholate, 0.1% SDS) and incubated on ice for 30 minutes to extract the chromatin-bound proteins. Extracted proteins were clarified by centrifugation at 16000 g for 10 minutes before analysis. For the experiments shown in Fig. 7B, equal cell equivalents of soluble and chromatin fractions (1/25th of each sample) were analyzed by western blotting. Total cell extracts were prepared in 9 M urea in 50 mM Tris pH 6.8 and sonicated for 15 seconds at 50% amplitude. Equal protein amounts (30 μg) were then analysed by western blotting.

Immunoprecipitation

For immunoprecipitation of Chk1, HeLa cells were transfected as described above then lysed in RIPA buffer. Chk1 antibody (1 μg) was incubated with 50 μl Immunocruz F beads (45043; Santa Cruz) for 2 hours at 4°C with rotation. The beads were washed three times in PBS and incubated with 500 μg HeLa lysate overnight at 4°C. The following day, the PBS washes were repeated, and immunoprecipitated protein was eluted in 15 μl SDS protein loading buffer. Eluted proteins were analyzed by western blotting.

Immunofluorescence microscopy

Cells to be imaged were fixed in cold methanol for 10 minutes at −20°C. Cells were then incubated with the indicated primary antibody for 1 hour at room temperature, washed three times with PBS and incubated with the appropriate secondary antibodies for 1 hour at room temperature. Nuclear DNA was stained with DAPI containing ProLong Gold antifade (Invitrogen). Images were collected using a Leica DMIRB2 inverted epifluorescence microscope with 40× or 60× oil-immersion objectives. Images were captured with a digital cooled charge-coupled device camera and OpenLab, version 4.0 software (Improvision Inc.), using the same exposure time between samples. For BrdU incorporation analysis by microscopy, cells were pulsed with 40 mM BrdU 5 days post-transfection with the pRS plasmids and either fixed directly or released into BrdU-free complete medium before fixing and permeabilising in cold methanol. The DNA was then denatured using 2 M HCl for 30 minutes at room temperature, and BrdU was detected using FITC-conjugated anti-BrdU antibody (Roche).

Flow cytometry

For DNA content analysis, cells were fixed in 70% ethanol overnight at −20°C, washed in PBS with 0.5% BSA, treated with 100 μg/ml RNase A for 1 hour at 37°C and stained with 50 μg/ml propidium iodide. All samples were analyzed using a FACS Calibur flow cytometer (BD Biosciences) and data were collected using CellQuest software. Cell cycle analysis was performed using FlowJo software (Treestar Inc., Ashland, OR). For BrdU pulse experiments, cells at days 5 post-transfection with pRS plasmids were pulsed with 40 mM BrdU for 30 minutes. The labelled cells were washed twice with PBS and then restained and fixed or released into BrdU-free complete medium for the indicated times before harvesting and fixation in 70% ethanol overnight at −20°C. DNA was denatured using 2 M HCl at room temperature for 30 minutes. BrdU was detected using FITC-conjugated anti-BrdU antibody (Roche), and DNA was stained with 50 μg/ml propidium iodide.

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**References**


