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Interaction between human MCM7 and Rad17 proteins is required for replication checkpoint signaling

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Human Rad17 (hRad17) is centrally involved in the activation of cell-cycle checkpoints by genotoxic agents or replication stress. Here we identify hMCM7, a core component of the DNA replication apparatus, as a novel hRad17-interacting protein. In HeLa cells, depletion of either hRad17 or hMCM7 with small-interfering RNA suppressed ultraviolet (UV) light- or aphidicolin-induced hChk1 phosphorylation, and abolished UV-induced S-phase checkpoint activation. Similar results were obtained after transfection of these cells with a fusion protein containing the hMCM7-binding region of hRad17. The hMCM7-depleted cells were also defective for the formation of ATR-containing nuclear foci after UV irradiation, suggesting that hMCM7 is required for stable recruitment of ATR to damaged DNA. These results demonstrate that hMCM7 plays a direct role in the transmission of DNA damage signals from active replication forks to the S-phase checkpoint machinery in human cells.

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Introduction

Genomic integrity in eukaryotic cells is continuously challenged by DNA-damaging agents generated as by-products of normal cellular metabolism or derived from environmental sources. To maintain the stability of their genomes, cells have evolved a battery of DNA damage response pathways, termed cell-cycle checkpoints, which are differentially activated in response to distinct types of genotoxic stress (e.g., DNA double-strand breaks (DSBs), ultraviolet (UV)-induced photoproducts, or abnormal replication forks). Defects in checkpoint signaling lead to genetic instability, heightened sensitivity to DNA-damaging agents, and increased susceptibility to cancer development in mammals (Elledge, 1996; Storchova and Pellman, 2004).

The process of DNA replication is particularly sensitive to genotoxic stress, and requires continuous surveillance in order to ensure that duplication of the genome is executed with high fidelity during S phase. Loss or malfunction of proteins that monitor DNA replication fidelity, including ATR, hRad17, and hChk1, leads to genomic instability and cell death, even in the absence of extrinsic genotoxic agents (Brown and Baltimore, 2000; Wang *et al*, 2003). Recently, the mechanisms whereby S-phase cells detect and respond to damaged or aberrantly structured DNA have come under intensive scrutiny. Studies in yeast and *Xenopus* demonstrated that interfering with replication fork progression activates the S-phase checkpoint, and that mutations in the replication machinery led to defective S-phase checkpoint signaling (Lupardus *et al*, 2002; Stokes *et al*, 2002; Osborn and Elledge, 2003; Tercero *et al*, 2003). Although the underlying mechanisms are not well defined, mammalian cells also mount strong checkpoint responses to lesions that impede replication fork progression, such as UV-induced cyclobutane–pyrimidine dimers (Heffernan *et al*, 2002). Compelling evidence implicates two phosphatidylinositol 3-kinase-related kinases, ATR and ATM, as proximal transducers in S-phase checkpoint signaling pathways in mammalian cells (Abraham, 2001; Shiloh, 2003; Shechter *et al*, 2004). ATM is activated in response to DNA DSBs, which trigger specific alterations in histone structure (Bakkenist and Kastan, 2003), or the loading of the MRE11–Rad50–NBS1 complex at the site of strand breakage (Carson *et al*, 2003; Uziel *et al*, 2003; Lee and Paull, 2004). In contrast, ATR, together with its putative regulatory subunit, ATRIP (Cortez *et al*, 2002), is attracted to single-stranded DNA (ssDNA) coated with replication protein A (RPA) (Zou and Elledge, 2003), which is a common intermediate produced during the processing of damaged DNA. Accumulating evidence suggests that checkpoint signaling through ATR is intimately linked to the process of DNA replication (Hekmat-Nejad *et al*, 2000; Michael *et al*, 2000; Casper *et al*, 2002; Lupardus *et al*, 2002; Tercero *et al*, 2003; Marheineke and Hyrien, 2004; Shechter *et al*, 2004; Ward *et al*, 2004a, b).

During G_1 phase of the cell cycle, replication origins in DNA are licensed by the assembly of a pre-replication complex (pre-RC) comprising the origin recognition complex (ORC), Cdc6, and MCM (*m*ini*c*hromosome *m*aintenance) complex (Waga and Stillman, 1998; Tye, 1999). Upon entry of the cell into S phase, pre-RCs are converted into active replication forks by phosphorylation events carried out by the cyclin E–cdk2 and Dbf4–Cdc7 kinases. During fork progression through duplex DNA, unwinding and denaturation of the DNA is required to create a template for the replicative polymerases. The hexameric MCM complex, consisting of the hMCM2–7 proteins, is believed to function as a replicative helicase that mediates unwinding of DNA at origins of replication, as well as DNA in the path of active replication forks (You *et al*, 1999, 2002; Labib *et al*, 2000; Lei and Tye, 2001).

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Previous studies demonstrated physical and functional interactions between ATR and the key checkpoint protein hRad17 during the cellular response to damaged DNA or replication stress (Bao *et al*, 2001; Stokes *et al*, 2002; Zou *et al*, 2002; Wang *et al*, 2003). DNA damage incurred during G_1, S, or G_2 phase of the cell cycle is sensed by hRad17, which, acting in a complex with the replication factor C (RFC) 2–5 subunits, loads three PCNA-related proteins, hRad9, hHus1, and hRad1 (termed the '9-1-1 complex'), near the damaged site (Burtelow *et al*, 2000; Bermudez *et al*, 2003). Although the ATRIP-ATR and 9-1-1 complexes are recruited independently to DNA damage sites (Kondo *et al*, 2001; Melo *et al*, 2001; Zou *et al*, 2002), the phosphorylation of many ATR substrates, including hChk1 and hRad17 itself, is highly dependent on the 9-1-1 complex (Zou *et al*, 2002; Jiang *et al*, 2003).

To further understand the checkpoint signaling functions of hRad17, we performed a yeast two-hybrid screen aimed toward the identification of novel hRad17-interacting proteins. In this study, we show that the MCM protein hMCM7 is a functionally important binding partner for hRad17 in human cells. The present findings indicate that, in addition to its contributions to normal DNA replication, hMCM7 is centrally involved in the activation of the ATR-dependent S-phase checkpoint by agents that induce DNA replication stress.

Results

Using full-length hRad17 as the bait, we performed a yeast two-hybrid screen with a human fetal brain cDNA library as the prey. Of the 33 positive clones that emerged from this screen, one encoded the carboxyl-terminal region of hMCM7, a component of the preinitiation complex assembled at origins of DNA replication (Waga and Stillman, 1998). To verify the yeast two-hybrid results, we cotransfected HeLa cells with HA-tagged hMCM7 and FLAG-tagged hRad17, and observed a significant amount of FLAG-hRad17 in the α-HA immunoprecipitates from these cells (Figure 1A, upper panel). The converse co-immunoprecipitation experiment revealed a modest but readily detectable amount of α-HA immunoreactivity in FLAG-Rad17 immunoprecipitates from the doubly transfected cells.

In subsequent experiments, we demonstrated that α-hRad17 antibodies co-precipitated endogenous hMCM7 from nontransfected HeLa cells (Figure 1B, left panel). Interestingly, exposure of cells to aphidicolin (Aph) or UV light increased the amount of hMCM-7 that co-immunoprecipitated with the endogenous hRad17. This experiment was repeated with three additional human cell lines (U2OS, A549, and human embryonic kidney 293T), and yielded similar results (not shown). Based on the prediction that ATR and its partner protein, ATRIP, reside in close proximity to the replication fork, we tested whether α-ATRIP immunoprecipitates also contained hMCM7 and/or hRad17. Both proteins were indeed present in the α-ATRIP immunoprecipitates, and, once again, the associations were increased by Aph or UV light exposure (Figure 1B, lower panel). Collectively, these results suggest that hMCM7 is closely associated with both ATRIP-ATR and hRad17 in both the absence and presence of agents that induce replication stress.

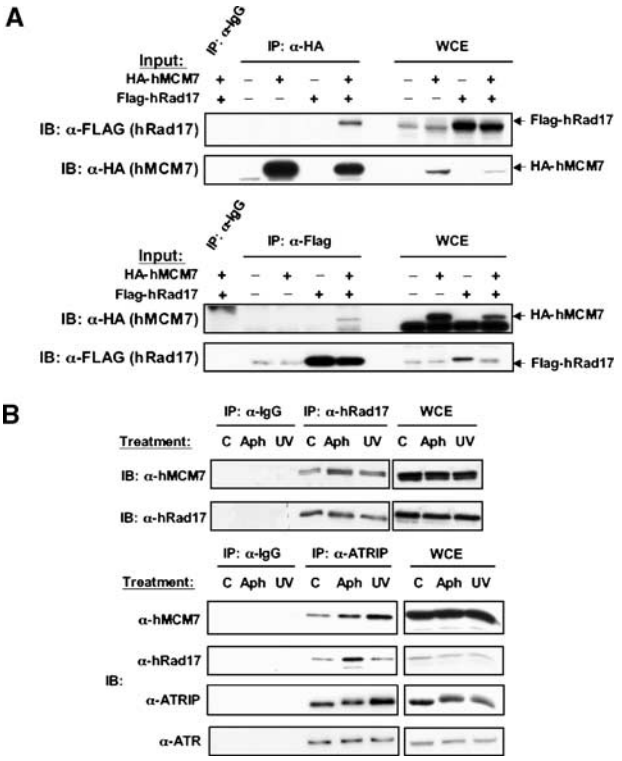

Figure 1 Co-immunoprecipitation of hRad17 with hMCM7. (**A**) Co-immunoprecipitation of FLAG-tagged hRad17 and HA-tagged hMCM7. HeLa cells were cotransfected with the indicated plasmids, and cellular extracts (0.5 mg protein) were immunoprecipitated (IP) with α-FLAG or α-HA antibodies. (**B**) Genotoxic stress-induced co-immunoprecipitation of hRad17, hMCM7, and ATRIP. HeLa cells were exposed for 4 h to 200 J/m^2 UV-B light or 1 μM Aph. Cellular extracts (1 mg protein) were immunoprecipitated and immunoblotted with the indicated antibodies.

Treatment of cells with inhibitors of DNA replication or UV light activates the ATR-dependent S-phase checkpoint (Abraham, 2001; Shiloh, 2003). To determine whether the interaction between hRad17 and hMCM7 was involved in genotoxic stress-induced ATR activation, we silenced hRad17 or hMCM7 gene expression in HeLa cells by transfection with specific siRNAs. These reagents reduced the expression of their respective target proteins by at least 80%, relative to the control cells that received luciferase (Luc)-specific siRNA. Both hRad17- and hMCM7-depleted cells displayed significant defects in hChk1 phosphorylation at Ser-345 after UV light exposure (Figure 2A). Furthermore, reduced expression of hMCM7 impaired UV-induced phosphorylation of the Ser-635 site in hRad17. These modifications of hChk1 and hRad17 are mediated by ATR in UV-damaged cells (Liu *et al*, 2000; Bao *et al*, 2001; Bartek and Lukas, 2003). Loss of hMCM7 did not lead to global defects in checkpoint signaling, as the phosphorylation of hChk2 provoked by ionizing radiation (IR) was not perturbed in hMCM7 siRNA-treated cells, whereas this response was clearly blunted in hRad17-depleted cells. The latter results are consistent with the model that ATM, rather than ATR, serves as the primary upstream activator of hChk2 in response to IR-induced DSBs (Bartek and Lukas, 2003; Shiloh, 2003).

During DNA replication, hMCM7 interacts with DNA as one member of the hexameric hMCM2–7 complex (Tye, 1999;

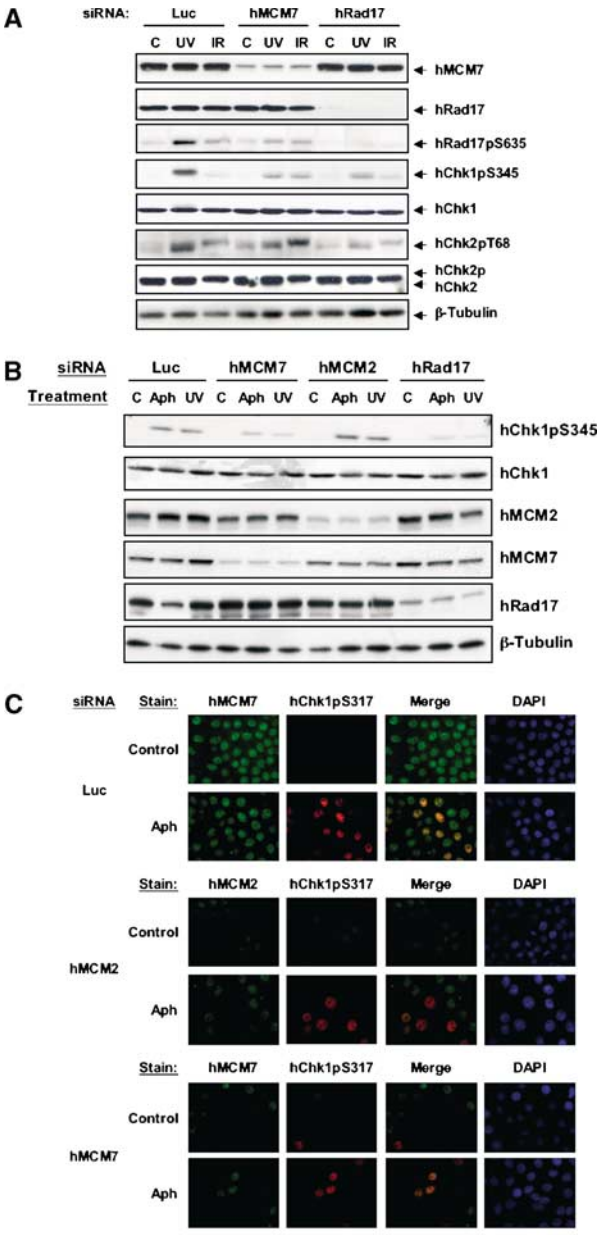

Figure 2 Depletion of hMCM7 and hRad17 inhibits genotoxic stress-induced hChk1 phosphorylation. (**A**) HeLa cells were transfected with hMCM7 or hRad17 siRNA, and then treated with 200 J/m^2 of UV or 20 Gy IR at 48 h post-transfection. Cellular extracts were harvested at 1 h after UV or IR treatment, and cell extracts (50 μg protein) were immunoblotted with the indicated antibodies. β-Tubulin served as a sample-loading control. (**B**) hMCM7, hMCM2, or hRad17 siRNA-transfected HeLa cells were treated for 4 h with 200 J/m^2 UV light or 1 μM Aph, and cellular extracts were separated by SDS–PAGE and immunoblotted with the indicated antibodies. (**C**) Cells were transfected with hMCM7 or hMCM2 siRNA, and were treated with 1 μM Aph. Cells were fixed after 4 h and stained with the indicated antibodies. Cell nuclei were stained with DAPI.

Labib and Diffley, 2001). To determine whether the S-phase checkpoint functions of hMCM7 was uniformly dependent on the MCM2–7 proteins, we silenced hMCM2 gene expression in HeLa cells by transfection with specific siRNA. Exposure to either siRNA reduced expression of the cognate MCM protein by at least 80%, relative to the Luc siRNA-treated control cells. We noted that depletion of hMCM2 caused a moderate reduction of hMCM7 expression and *vice versa*, suggesting that loss of one MCM protein might negatively impact the stability of other members of the hMCM2–7 complex (Figure 2B). Nonetheless, reduced expression of hMCM2 had no effect on genotoxic stress-induced hChk1 phosphorylation, whereas this response was profoundly suppressed in the hMCM7 siRNA-treated cells (Figure 2B and C). Signal relay from ATR to hChk1 was also not perturbed in hMCM4 siRNA-treated cells (not shown). Thus, these observations indicate that the efficiency of ATR signaling to hChk1 is particularly sensitive to reduced expression of the hMCM7 subunit.

We previously reported that UV light-induced DNA damage triggers the appearance of ATR-containing nuclear foci, which represent surrogate markers for ATR activation at sites of DNA damage (Tibbetts *et al*, 2000). In the present study, we determined whether the formation of these ATR nuclear foci was impaired in hMCM7- or hRad17-depleted cells. As shown previously (Tibbetts *et al*, 2000), the majority of nuclei in nonirradiated cells exhibited a diffuse nuclear staining pattern for ATR, which shifted to a punctate staining pattern at 6 h post-UV irradiation (Figure 3A). The formation of ATR nuclear foci was not impaired in hRad17-depleted cells (Figure 3A); indeed, these cells displayed a clear in-

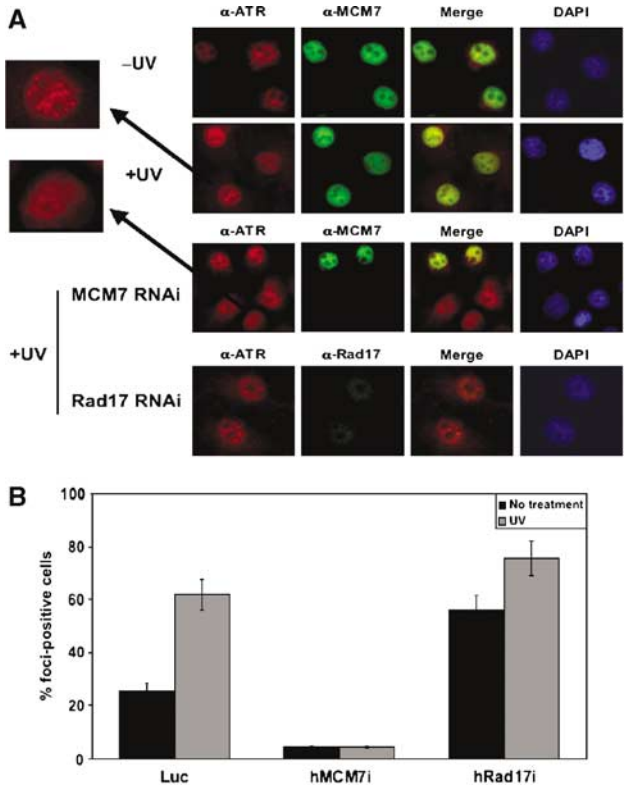

Figure 3 Loss of hMCM7 interferes with the formation ATR nuclear foci in UV-damaged cells. (**A**) Immunofluorescence microscopy. HeLa cells were either left untreated or irradiated with 200 J/m^2 UV light. Cells were fixed after 6 h, and immunostained with α-ATR or α-hMCM7 antibodies. In the bottom two rows, the cells were transfected with the indicated siRNAs, and were irradiated with UV light after 48 h. DAPI staining was used to mark cell nuclei. (**B**) Quantitation of ATR nuclear foci in the cell populations shown in panel A. Triplicate samples (minimum, 100 cells per sample) were counted manually by microscopy. Error bars indicate standard error of the mean from the three sample populations.

crease in the number of nuclear foci-positive cells in the absence of UV exposure (Figure 3B). The latter results suggest that knockdown of hRad17 expression triggers the accumulation of spontaneous, unrepaired DNA damage in cycling HeLa cells. The consequences of hMCM7 protein depletion were strikingly different, as the appearance of ATR nuclear foci was virtually abolished in both nonirradiated and UV-irradiated cells (Figure 3A and B). Thus, hMCM7 expression appears to be critical for the recruitment of ATR into nuclear foci following cellular exposure to certain types of genotoxic stress.

Under normal conditions, recognition of DNA damage during S phase slows replication fork progression and inhibits the firing of late origins of replication (Lopes *et al*, 2001; Tercero and Diffley, 2001). We predicted that impairment of the UV-induced DNA damage response might yield a UV-resistant DNA synthesis (UVDS) phenotype, which is analogous to the radio-resistant DNA synthesis (RDS) defect associated with loss of ATM and other proteins involved in the checkpoint response to DNA DSBs incurred during S phase (Painter and Young, 1980). We found that hMCM7 siRNA-treated A549 cells displayed elevated levels of DNA synthesis at 30–120 min after UV light exposure (Figure 4A and B), consistent with the induction of UVDS. In contrast, these cells did not exhibit the RDS defect after IR exposure, indicating that hMCM7 expression was specifically required for S-phase checkpoint activation in response to UV-induced DNA damage. As expected, given the more global role of hRad17 in DNA damage signaling, transfection of the cells with hRad17 siRNA evoked both UVDS and RDS (Figure 4B and C).

Based on the above-noted defects in ATR function in hMCM7-depleted cells, we predicted that these cells might fail to delay S-phase progression in response to UV-induced DNA damage. However, testing of this prediction was complicated by the possibility that reduced hMCM7 might lead to an intrinsic defect in S-phase progression. Consequently, we examined S- to M-phase progression in hMCM7 siRNA-treated HeLa cells with a nocodazole capture assay. Flow cytometric analyses of propidium iodide (PI)-stained cells revealed that both hRad17- and hMCM7-depleted cells accumulated with 4*N* DNA content in the presence of nocodazole, indicating that these cell populations had successfully replicated their DNA (Supplementary Figure S1A). To monitor more accurately cell-cycle progression in hMCM7 siRNA-treated cells, we pulse-labeled these cells with BrdU and monitored the cell-cycle status of the labeled cells by PI staining over the following 24 h. Relative to the Luc siRNA-treated control cells, BrdU-positive cells depleted of hMCM2, hMCM4 (not shown), or hMCM7 displayed little or no defect in S-phase progression (Supplementary Figure S1B and C). In subsequent experiments, we transfected HeLa cells with siRNAs targeted against green fluorescent protein (GFP), hMCM7, or hRad17, and treated the cells with 200 J/m^2 UV and examined their cell-cycle distributions at 24 h postirradiation. In GFP siRNA-transfected cells, UV irradiation provoked an increase in the percentage of S-phase cells after 24 h, consistent with a checkpoint-mediated delay of cell-cycle progression through S phase (Figure 5A). In contrast, hMCM7 siRNA-treated cells arrested predominantly in G_2/M phase after UV light exposure. These results indicate that impaired S-phase checkpoint activation in the hMCM7-depleted cells favors the progression of cells bearing damaged DNA into G_2 phase, where they are captured by the G_2 DNA damage checkpoint.

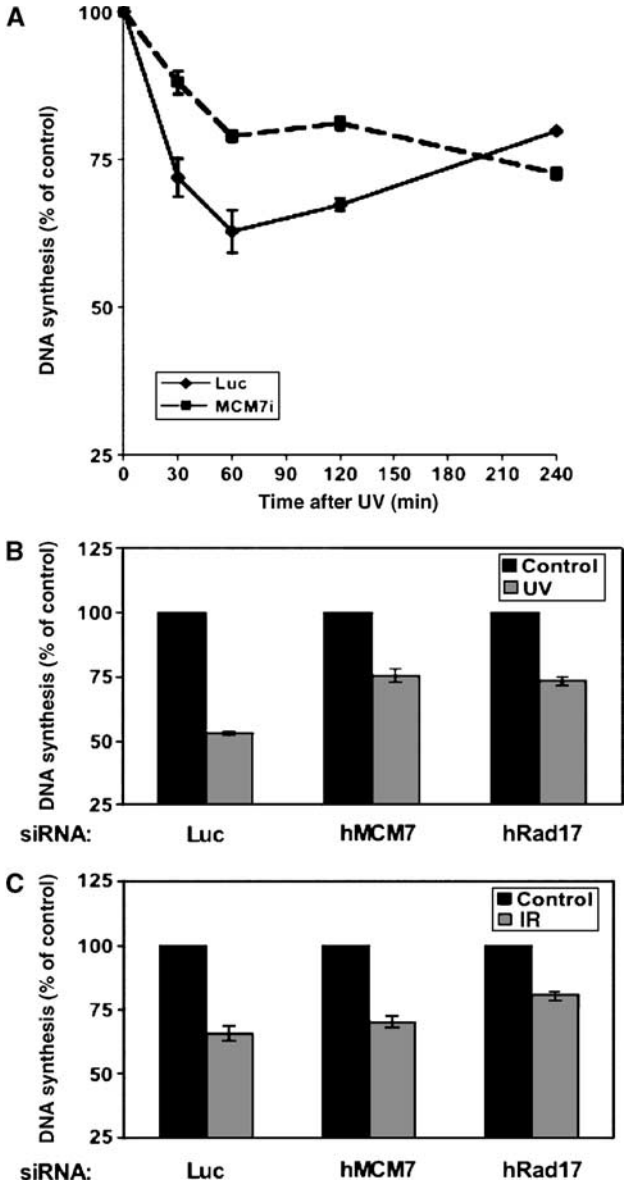

Figure 4 hMCM7 depletion confers a UVDS phenotype. (**A**) Time course of UVDS. A549 cells were treated with the indicated siRNAs, and, after 48 h, were irradiated with 200 J/m^2 UV light. DNA synthesis was measured at the indicated times after UV light exposure as described in Materials and methods. Error bars indicate standard deviation from quadruplicate samples. (**B**) Effect of hMCM7 versus hRad17 siRNA treatment on UVDS. A549 cells were treated with the indicated siRNAs, and were irradiated with 200 J/m^2 UV light at 48 h post-transfection. DNA synthesis was determined at 1 h after radiation exposure. (**C**) Effect of hMCM7 versus or hRad17 depletion on RDS. A549 cells were treated with the indicated siRNAs, and were irradiated with 20 Gy IR at 48 h post-transfection. DNA synthesis was determined at 1 h after radiation exposure.

Cell-cycle checkpoint malfunctions are frequently associated with heightened sensitivity to killing by DNA-damaging agents. Treatment of hMCM7- or hRad17-depleted HeLa cells with UV light revealed that both of these cell populations were sensitized to the antiproliferative and/or cell-killing

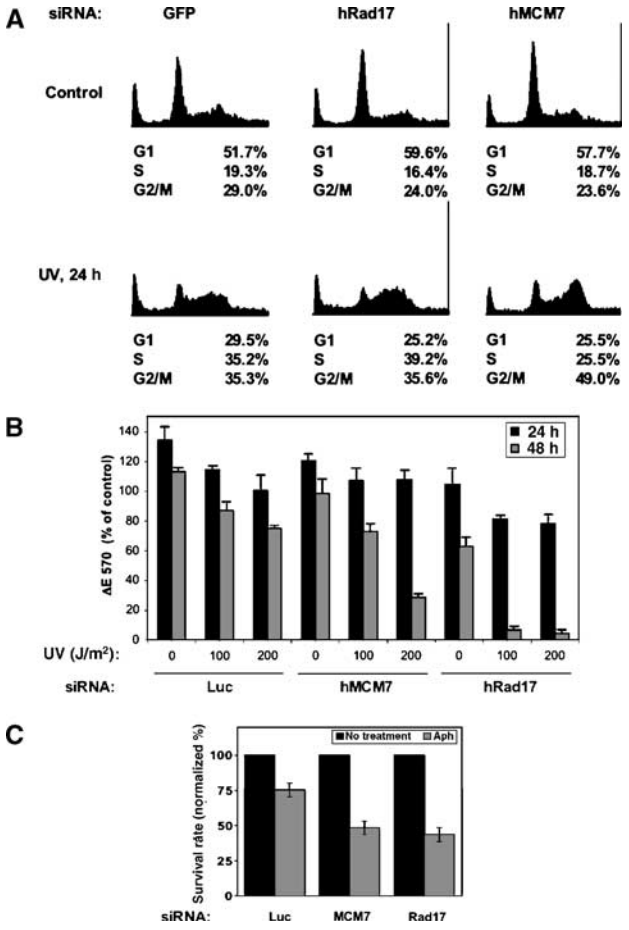

Figure 5 Effects of hMCM7 or hRad17 depletion on UV-induced S-phase checkpoint activation and cell survival. (**A**) Impaired S-phase checkpoint in hMCM7-depleted cells. HeLa cells were transfected with the indicated siRNAs, and then exposed to 200 J/m^2 UV light at 48 h after transfection. Cell-cycle distributions were examined at 24 h postirradiation. (**B**) Cell survival after UV irradiation. HeLa cells were transfected with indicated siRNA and, after 48 h, were exposed to the indicated doses of UV light. Cell survival was determined after 24 or 48 h in triplicate samples with an MTT dye conversion assay. Absorbance values were normalized to the zero time control for each cell population, which was arbitrarily set at 100%. Error bars indicate standard deviations. (**C**) Effect of hMCM7 or hRad17 depletion on cellular recovery from an Aph block. HeLa cells were transfected with the indicated siRNAs, and were replated after 24 h for survival assays. After 24 h in culture, the cells were treated for 16 h with 1 μM Aph, and then released into drug-free medium. Cell survival was determined in quadruplicate samples as described in Materials and methods. Values were normalized to the untreated control for each cell population, and error bars indicate standard deviations.

activities of UV light, relative to that observed in the Luc siRNA-treated control cells (Figure 5B). Finally, we examined the clonogenic survival of hMCM7- or hRad17-depleted cells after transient DNA replication stress imposed by Aph. Once again, recovery of the cells after release from Aph-induced replication arrest was impaired in the hMCM7 and hRad17 siRNA-treated cell populations (Figure 5C).

Mapping studies of the hRad17–hMCM7 interaction with the yeast two-hybrid system revealed that a 43-amino-acid peptide fragment (designated MCM7-5) from hMCM7 was sufficient to bind detectably to full-length hRad17 (Supplementary Figure S2). Conversely, the minimal hRad17-derived peptide capable of binding to an amino-terminally truncated hMCM7 bait protein (fragment designated R27 in the hMCM7 deletion series) encompassed 249 amino acids from the carboxyl terminus of hRad17 (indicated as Rad17-5 in Supplementary Figure S2). We constructed GFP fusion proteins containing the MCM7-5 or Rad17-5 fragments with the idea in mind that these fusion proteins, when ectopically expressed in human cells, might interfere with the physical and functional interactions between endogenous hMCM7 and hRad17.

In preliminary studies, we found that transient expression of a GFP-tagged fusion protein containing the MCM7-5 fragment completely blocked S-phase entry and progression in otherwise unperturbed HeLa cells (results not shown). In contrast, HeLa cells transfected with the GFP-Rad17-5 construct continued to cycle normally, a finding that prompted us to focus on this fusion protein as a potential inhibitor of signal relay between hMCM7 and hRad17 in UV light- or Aph-stressed cells. Co-immunoprecipitation experiments revealed that endogenous hMCM7 associated with the ectopically expressed GFP-Rad17-5 fusion protein in transfected HeLa cells (Figure 6A). In contrast, α-hMCM7 immunoprecipitates from GFP-transfected cells contained no detectable GFP, in spite of the fact that intact GFP was expressed at much higher levels than the GFP-Rad17-5 fusion protein. Furthermore, GFP-Rad17-5 expression reduced the level of endogenous hRad17 present in α-hMCM7 immunoprecipitates (Figure 6A), consistent with the idea that the Rad17-5 fragment competitively interferes with the hMCM7–hRad17 interaction in intact cells. In subsequent studies, we found that expression of GFP-Rad17-5 strongly suppressed the phosphorylation of hChk1 in UV- and Aph-treated cells (Figure 6B). Furthermore, like their hMCM7-depleted counterparts, the GFP-Rad17-5-expressing HeLa cells displayed the UVDS phenotype (Figure 6C).

To rule out the possibility that the GFP-Rad17-5 construct was simply acting as a dominant suppressor of hRad17 function, we determined whether the GFP-Rad17-5-transfected cells retained the ability to phosphorylate hChk2 after IR exposure. This ATM-dependent response also hinges on the function of hRad17 (see Figure 2A). Indeed, the GFP-Rad17-5-transfected cells displayed a strong increase in basal hChk2 phosphorylation, which was modestly increased by IR or UV exposure, while hChk1 activation was virtually abolished (Figure 6D). The basal increase in hChk2 phosphorylation in GFP-Rad17-5-expressing cells is consistent with the observation that these cells suffer spontaneous DNA damage (see Figure 7A), possibly due to disruption of the replication checkpoint. Regardless, we conclude from these results that the GFP-Rad17-5 construct is a selective, rather than global, inhibitor of hRad17 functions in radiation-damaged cells.

ATR-deficient cells rapidly accumulate damaged DNA in the absence of extrinsic genotoxic stress, likely due to a breakdown in the fidelity of DNA replication (Brown and Baltimore, 2000; de Klein *et al*, 2000). Given the evidence that GFP-Rad17-5 expression disrupts signal transmission through ATR to hChk1, we predicted that cells transfected with this construct might also display elevated levels of spontaneous DNA damage. Consequently, we stained the cells with a phosphospecific antibody that recognizes an ATM/ATR-dependent phosphorylation site (Ser-139) in histone H2AX. The phosphorylated form of H2AX (γH2AX) accumulates rapidly

at sites of DNA damage in cells treated with IR, UV, or DNA replication inhibitors (Rogakou *et al*, 1998, 1999; Ward and Chen, 2001). Expression of GFP-Rad17-5 in HeLa cells provoked a greater than seven-fold increase in γH2AX-positive nuclei, compared with the GFP-transfected control cells (Figure 7A and B). We repeated these experiments with hMCM7 siRNA-treated cells, and observed that loss of hMCM7 also caused a clear increase in γ-H2AX-positive nuclei (Figure 7C). In contrast, no increase in γH2AX staining was seen in the hRad17-depleted cells. The latter results suggest that loss of hRad17 does not lead to spontaneous DNA damage, although we have not ruled out the possibility that hRad17 expression is required for maximal phosphorylation of histone H2AX in UV-damaged cells.

Discussion

The hexameric MCM complex plays central roles in DNA replication initiation and elongation (Labib *et al*, 2000). In this study, we have defined an additional role for the hMCM7 subunit of the MCM complex in the activation of the S-phase DNA damage checkpoint by UV light and Aph. Our results argue that hMCM7 participates in functionally important interactions with at least two key elements of the S-phase checkpoint machinery—hRad17 and ATRIP-ATR. Manipulations that interfere with hMCM7 function strongly inhibit the formation of ATR nuclear foci, as well as ATR-dependent hChk1 activation in UV- or Aph-stressed cells. Furthermore, disruption of hMCM7 function triggered a DNA damage response in the absence of extrinsic genotoxic stress, consistent with previous evidence that the ATR–Chk1 pathway plays a continuous role in genome maintenance during normal DNA replication (Casper *et al*, 2002; Cha and Kleckner, 2002; Marheineke and Hyrien, 2004). Taken together, these results strengthen the notion that components of the DNA replication fork function as both DNA damage sensors and effectors of checkpoint signaling in S-phase cells (Tercero *et al*, 2003).

Previous studies identified at least three binding partners for hRad17: Ku-interacting protein (Chang *et al*, 1999), hRad1 (Rauen *et al*, 2000), and protein phosphatase 5 (Ali *et al*, 2004). With the exception of the hRad1 interaction, the functional significance of these associations with hRad17 remains unclear. Our results indicate that binding of hRad17 to hMCM7 is critical for S-phase checkpoint signaling in response to replicative stress. Studies in fission yeast have shown that Rad17 is constitutively bound to chromatin, and that the level of chromatin-bound Rad17 is increased during replication stress (Kai *et al*, 2001). More recent results indicate that the hRad17–RFC2–5 complex binds most avidly to DNA duplexes containing single-stranded gaps coated with RPA (Zou *et al*, 2003). These structures are abundant in the vicinity of active replication forks (Waga and Stillman, 1998). In light of the current findings, we propose that RPA recruits

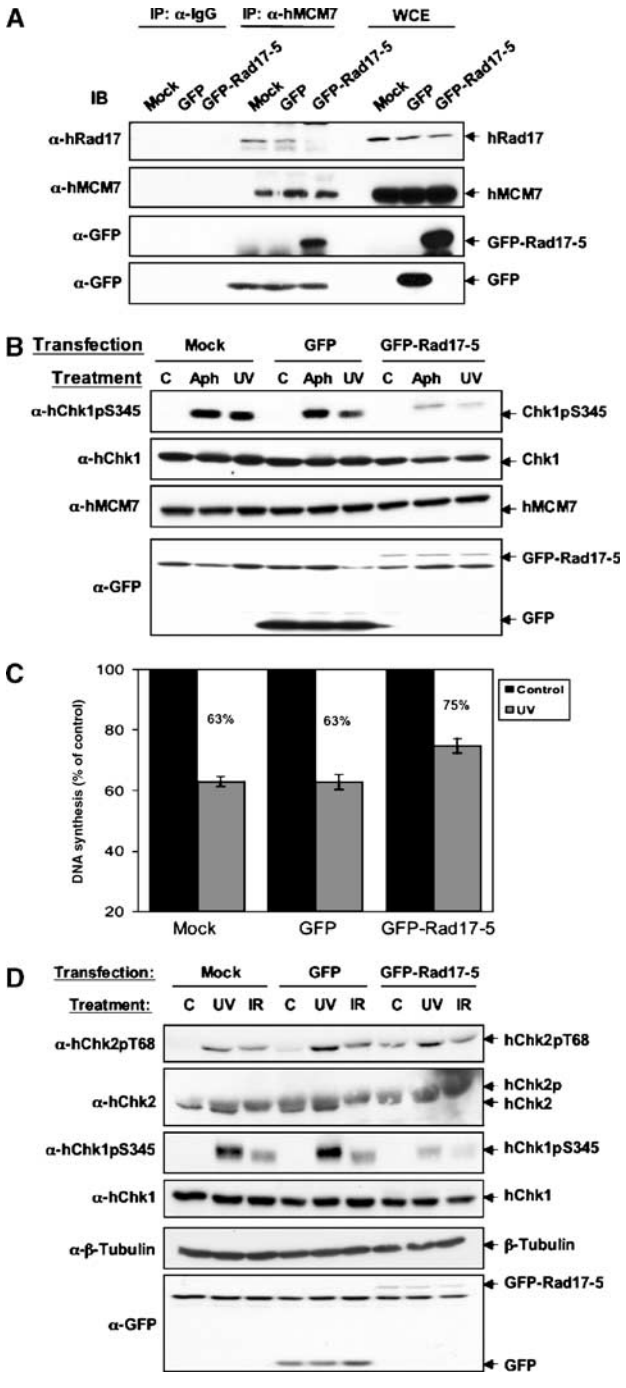

Figure 6 Disruption of S-phase checkpoint signaling by ectopic expression of the hMCM7-binding region of hRad17. (**A**) Binding of ectopically expressed GFP-Rad17-5 to endogenous hMCM7. HeLa cells were transfected with GFP or GFP-Rad17-5 fusion construct (encoding amino acids 421–670 of hRad17). At 24 h post-transfection, the cells were harvested, lysed, and detergent-soluble protein (1 mg) was immunoprecipitated with α-hMCM7 antibody. (**B**) hChk1 phosphorylation. HeLa cells were transfected with GFP or GFP-Rad17-5, and then treated with 200 J/m^2 UV light or 1 μM Aph at 16 h after transfection. Cellular extracts (50 μg protein) were prepared after 2 h, and proteins were immunoblotted with the indicated antibodies. (**C**) Ectopically expressed GFP-Rad17-5 confers a UVDS phenotype. HeLa cells were transiently transfected with either GFP- or GFP-Rad17-5-encoding plasmids, and, after 24 h, were irradiated with 200 J/m^2 UV light. DNA synthesis was determined at 1 h after radiation exposure as described in Materials and methods. (**D**) hChk2 phosphorylation. HeLa cells were transfected with GFP or GFP-Rad17-5, and then treated with 200 J/m^2 UV light or 20 Gy of IR at 16 h after transfection. Cellular extracts (50 μg protein) were prepared after 2 h, and proteins were immunoblotted with the indicated antibodies.

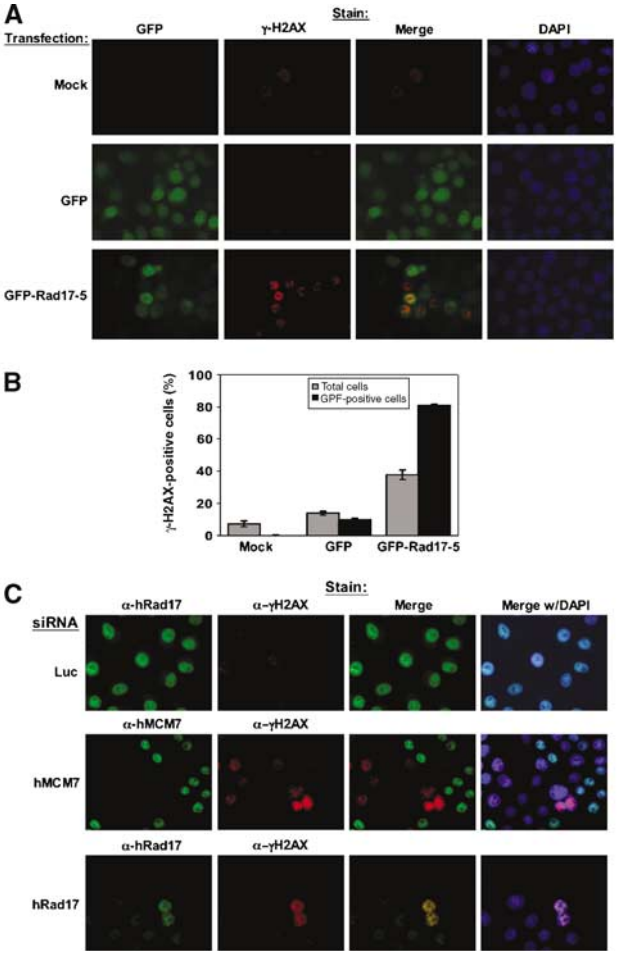

Figure 7 Histone H2AX phosphorylation. (**A**) γH2AX staining. HeLa cells were transiently transfected with either GFP- or GFP-Rad17-5-encoding plasmids, then were fixed and permeabilized after 16 h in culture. The cells were stained with γH2AX-specific antibody (red), and cell nuclei were counterstained with DAPI (blue). (**B**) Quantitation of γH2AX-positive cell nuclei. A minimum of 100 cells from each of the cell populations depicted in panel A were scored for nuclear γH2AX staining. (**C**) γH2AX staining in hMCM7- versus hRad17-depleted HeLa cells. The indicated siRNA-transfected cells were costained with α-γH2AX antibody (red) and either hRad17 or hMCM7 antibody (green) after 60 h in culture. Cell nuclei were counterstained with DAPI (blue).

the hRad17–RFC2–5 complex to sites of DNA replication, where hRad17 encounters and binds to hMCM7 in the vicinity of the replication fork. We further speculate that replication fork stalling induced by agents such as UV or Aph triggers a local increase in the level of RPA-bound ssDNA, an event that provokes the recruitment of sufficient hRad17–RFC2–5, together with ATRIP-ATR, to elicit a full-blown S-phase damage checkpoint response. This model is consistent with the notion that DNA polymerase stalling induced by Aph causes DNA helicase activity to become uncoupled from DNA polymerases in the *Xenopus* egg extracts, an event that would lead to abnormally long stretches of ssDNA (Walter and Newport, 2000).

Although the present study has focused exclusively on hMCM7, this protein is generally believed to function in the context of the hMCM2–7 complex during DNA replication. Remarkably, however, depletion of either hMCM4 or hMCM2 failed to disrupt the activation of hChk1 by UV or Aph, indicating that the checkpoint signaling function of hMCM7 may not be obligatorily linked to its role in DNA replication. A recent report provides strong support for the idea that MCM proteins are functionally intertwined with the S-phase checkpoint machinery (Cortez *et al*, 2004). This study demonstrated that two MCM proteins, hMCM2 and hMCM3, are phosphorylated by ATR and ATM, respectively, in cells exposed to genotoxic stress. These findings complement an earlier study, which showed that hMCM4 also undergoes rapid phosphorylation in cells exposed to UV light or DNA replication inhibitors (Ishimi *et al*, 2003). Although the functional significance of these phosphorylation events is unknown, it seems clear that, as is the case for ATR and hRad17, the interplay between ATR and the MCM proteins will prove to be bidirectional. Cortez and Elledge also found that hMCM7 was a direct binding partner for the ATR-associated protein ATRIP, and showed that partial depletion of hMCM7 interfered with UV-induced hChk1 activation in U2OS cells. Taken together with our findings, these results provide compelling evidence to support the hypothesis that hMCM7 plays a specialized role in the relay of DNA damage signals from the replication fork to proximal elements of the S-phase checkpoint pathway.

Costanzo *et al* (2003) have defined a distinct mechanism of S-phase checkpoint initiation in *Xenopus* egg extracts (Costanzo *et al*, 2003). These investigators demonstrated that the Dbf4–Cdc7 kinase was a critical target of the ATR-dependent S-phase checkpoint induced by the topoisomerase II inhibitor etoposide. Inhibition of Dbf4–Cdc7 by this checkpoint mechanism interferes with the phosphorylation of the MCM complex by this protein kinase, thereby suppressing conversion of pre-RCs to active replication forks. The inhibition of replication origin firing via this checkpoint mechanism complements the pathway outlined in the present study, which relies on hMCM7 function to slow the progress of and/or stabilize pre-existing replication forks that encounter fork-stalling lesions. The common element that leads to the recruitment of ATR in both of these S-phase checkpoint pathways is the formation of RPA-coated ssDNA (Costanzo *et al*, 2003; Zou and Elledge, 2003; Zou *et al*, 2003).

Our results indicate that hRad17 and hMCM7 interact in a pathway that includes ATR and mediates S-phase checkpoint activation in response to genotoxic stress. However, it is clear that binding to hMCM7 is not the only mechanism by which hRad17 receives and transmits DNA damage signals in human cells. For example, we observed that UV- or IR-induced hChk2 phosphorylation was not impaired in hMCM7-deficient cells, whereas this event was strongly suppressed in cells depleted of hRad17. This outcome is consistent with previous evidence that hChk2 phosphorylation and activation are more tightly coupled to ATM than to ATR (Matsuoka *et al*, 2000; Bartek and Lukas, 2003). The checkpoint functions of hRad17 are not confined to S phase (Elledge, 1996; Caspari and Carr, 1999), suggesting that, at other points in the cell cycle, DNA damage signals are relayed to hRad17 through RPA and hRad17-interacting proteins other than hMCM7.

A striking outcome of our studies was that decreased hMCM7 expression abolished the formation of ATR-containing nuclear foci in UV-treated cells. In contrast, depletion of hRad17 actually promoted the formation of such foci in the absence of genotoxic agents. These observations reinforce the idea that the ATRIP-ATR and 9-1-1 complexes are recruited

independently to damaged chromatin (Zou *et al*, 2002). While RPA may be required for efficient recruitment of ATRIP-ATR to ssDNA (Zou and Elledge, 2003), the present findings, together with those of Cortez *et al* (2004), suggest that hMCM7 is centrally involved in the assembly and/or stabilization of ATRIP-ATR-containing foci in S-phase cells. In line with this model, we found that α-ATRIP immunoprecipitates from cycling cells contain both hMCM7 and hRad17, and that the amounts of co-precipitating hMCM7 and hRad17 are increased after cellular exposure to UV or Aph (Figure 1B).

In summary, the present findings highlight the hMCM7 protein as a critical node for checkpoint signal transmission from stalled replication forks. A major unresolved issue is whether the checkpoint signaling functions of hMCM7 are strictly tied to its role in DNA replication as part of the MCM complex, or whether this facet of hMCM7 function is independent of its contributions to normal DNA replication. Regardless, the present studies add significant strength to the idea that the replication fork functions as both a sensor of DNA damage and an effector of checkpoint signaling in cells that incur DNA damage during S phase (Cimprich, 2003; Katou *et al*, 2003; Tercero *et al*, 2003; Oehlmann *et al*, 2004).

Materials and methods

Cell culture and antisera

U2OS osteosarcoma and HeLa cells were maintained in Dulbecco's modified Eagle's medium (DMEM) medium supplemented with 10% fetal bovine serum (FBS). A549 lung carcinoma cells were grown in high-glucose DMEM/F12 supplemented with 10% FBS. Polyclonal antibodies against ATRIP were prepared by immunizing rabbits with a glutathione *S*-transferase fusion protein containing amino acids 1–181 from human ATRIP. Polyclonal antibodies against hMCM2 were kindly provided by Dr Wei Jiang (The Burnham Institute). Commercial antibodies were obtained from the following sources (in parentheses): α-hMCM4 (Abcam); α-hMCM7 (Sigma); α-hRad17 and α-hChk1 (Santa Cruz); α-phospho-hChk1 (pSer-317 and pSer-345) and α-Chk2 (Cell Signaling); α-ATR (Affinity Bioreagents); α-γH2AX (Cell Signaling); monoclonal α-FLAG M2 (Sigma); Alexa488-conjugated monoclonal α-BrdU (Molecular Probes); α-HA, clone 12CA5 (BabCo), α-phospho-Chk2 (pT-68) (R&D systems). Aph was prepared as a stock solution in dimethylsulfoxide and stored at −80°C until use.

Plasmids and constructs

Plasmid expression vectors for HA-tagged mouse MCM7 and FLAG-tagged human hRad17 were constructed in pcDNA3.1 (Invitrogen). For yeast two-hybrid screening, fragments of hRad17 and hMCM7 were subcloned into pBridge and pACT2 vectors (Clontech), respectively. The interacting fragments of hRad17 and hMCM7 were subcloned into pEGFP-C2 (Clontech). U2OS cells were transfected with Fugene 6 (Roche) in accordance with the manufacturer's suggested protocol. HeLa cells were transfected with plasmid DNAs with Lipofectamine 2000 (Invitrogen).

siRNA transfections

The siRNA duplexes targeted against hMCM7, hMCM2, hMCM4, hRad17, GFP, and Luc were purchased from Dharmacon Research Inc. (Lafayette, CO). The hRad17 siRNA target sequence was designed as previously reported (Zou *et al*, 2002) and the target sequences for the hMCM2, hMCM4, and hMCM7 siRNAs are TCATCGGAATCCTTCACCA, TGTTACAGGCATCTATCGA, and CTCGGGAAGAAGCAGTTCA, respectively. Cells were transfected with siRNA duplexes using Oligofectamine (Invitrogen) according to the manufacturer's suggested protocol.

Cell-cycle analysis

For fluorescence-activated cell sorting (FACS) analysis, cells were ethanol-fixed, washed in phosphate-buffered saline (PBS), and were stained for 30 min at 37°C with 10 μg/ml RNase A and 20 μg/ml PI. For PI staining of GFP-expressing cells, the cells were first fixed for 10 min in 0.5% paraformaldehyde on ice, washed twice in PBS, and stained as described above. Cells were analyzed with a flow cytometer (FACSort, BD Sciences) and CellQuest Pro software.

Yeast two-hybrid screen

The full-length human hRad17 cDNA was subcloned into a yeast multicopy plasmid carrying the GAL4-binding domain (pBridge, Clontech, La Jolla, CA). The construct was then transformed into yeast strain A109. The transformants were selected, and then secondarily transformed with a human fetal brain cDNA library constructed in pACT2 vector (Clontech). Transformants were screened for growth on plates lacking adenine, tryptophan, histidine, and leucine. After 3–6 days at 30°C, approximately 10^6 colonies were obtained, and positive clones were further examined for α-galactosidase expression. Plasmids harboring interacting cDNAs were rescued from positive yeast colonies, transformed into *Escherichia coli* KC8 strain, and positive transformants selected for growth on leucine-deficient M9 medium. The interacting cDNAs were characterized by nucleotide sequencing.

Co-immunoprecipitation

Cells were harvested by trypsinization and centrifugation, and then were resuspended in lysis buffer (150 mM sodium chloride, 5 mM β-glycerophosphate, 5 mM EDTA, pH 7.4, containing 1 mM DTT and 1% NP-40, supplemented with 10 μg/ml PMSF, 10 μg/ml leupeptin, 10 μg/ml aprotinin, and 1 μg/ml pepstatin. After 15 min on ice, the lysate was centrifuged for 5 min at 9000 *g*, and cleared extracts were immunoprecipitated with 2 μg of primary antibody. Depending on the type of primary antibody, either mouse monoclonal immunoglobulin G (IgG) or polyclonal rabbit α-mouse IgG was used as a negative control. Immune complexes were precipitated for 2 h at 4°C with either protein A–Sepharose for polyclonal antibodies or anti-mouse IgG–Sepharose for monoclonal antibodies. Immunoprecipitates were washed three times with lysis buffer, resuspended in 40 μl 2 × SDS–PAGE sample buffer, and samples were heated for 5 min at 100°C. Soluble proteins were resolved by SDS–PAGE, transferred to PVDF membrane, and immunoblotted with the indicated antibodies.

UV-resistant DNA synthesis assay

A549 cells were labeled for 24 h with 20 nCi/ml methyl-^{14}C-thymidine (Amersham) at the time of siRNA transfection. The medium was removed and replaced with fresh medium. The ^{14}C-labeled cells were then treated with the indicated doses of UV-B light. After 1 h, the cells were pulsed for 40 min with 2 mCi/ml methyl-^{3}H-thymidine (Amersham). The doubly labeled cells were washed with PBS, fixed in 80% ethanol, and stored at −20°C for at least 3 h. The cells were then washed two times with 80% ethanol. NaOH (0.5 ml, 0.25 M) was added to each cell pellet, and incorporation of ^{14}C and ^{3}H into DNA was measured by dual-isotope liquid scintillation counting. The ratio of ^{3}H-labeled DNA to ^{14}C-labeled DNA was determined for each sample, and normalized to the ratio obtained in the nonirradiated control samples.

Immunofluorescence microscopy

HeLa cells were grown on glass coverslips coated with poly-L-lysine (Sigma). At the indicated time points, the cells were washed twice with PBS, and were fixed for 10 min in PBS containing 3.75% formaldehyde and 0.2 M sucrose. Samples were rehydrated in PBS, and incubated for 30 min in blocking solution (PBS containing 10% FBS and 0.4% Triton X-100). Samples were subsequently overlaid for 1 h with primary antibodies at room temperature. After three washes in PBS and 0.1% Triton X-100, the samples were overlaid for 1 h with Texas red-conjugated goat anti-rabbit IgG or FITC-conjugated goat anti-mouse IgG diluted 1:500 in PBS and 0.1% Triton X-100. Samples were then washed and incubated for 5 min in 1 μg/ml 4′,6′-diamidino-2-phenylindole hydrochloride (DAPI) to stain nuclear DNA. After extensive washing, the specimens were mounted with coverslips and an aqueous anti-fade mounting reagent (Vectashield, Vector Laboratories).

Cell proliferation and clonogenic assays

Effects of UV on cell proliferation and viability were determined with an 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) assay (Mosmann, 1983). Briefly, HeLa cells were seeded into 12-well dishes, transfected with indicated siRNA, and

then UV irradiated at 48 h after transfection. Samples were prepared by removal of the culture medium and addition of 250 µl of fresh medium containing 0.5 mg/ml MTT. After 2 h at 37°C, the cells were lysed with 250 µl 2-butanol/2-propanol/1 N HCl (16/8/1, v/v/v). The soluble material was transferred to flat-bottomed 96-well plates and the absorbance at 570 nm was determined with an automated late reader.

For clonogenic assay, HeLa cells were transfected with siRNAs, and, after 24 h, the cells were replated in 60-mm dishes and treated as described in the figure legend. Colonies were stained with crystal violet, and dye-bound protein was solubilized at 37°C with 0.5% SDS in 50% ethanol. Samples were analyzed by absorbance spectroscopy at 590 nm.

Supplementary data

Supplementary data are available at *The EMBO Journal* Online.

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