RAD50 phosphorylation promotes ATR downstream signaling and DNA restart following replication stress

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The MRE11/RAD50/NBN (MRN) complex plays a key role in detecting DNA double-strand breaks, recruiting and activating ataxia-telangiectasia mutated and in processing the breaks. Members of this complex also act as adaptor molecules for downstream signaling to the cell cycle and other cellular processes. Somewhat more controversial are the results to support a role for MRN in the ataxia-telangiectasia and Rad3-related (ATR) activation and signaling. We provide evidence that RAD50 is required for ATR activation in mammalian cells in response to DNA replication stress. It is in turn phosphorylated at a specific site (S635) by ATR, which is required for ATR signaling through Chk1 and other downstream substrates. We find that RAD50 phosphorylation is essential for DNA replication restart by promoting loading of cohesin at these sites. We also demonstrate that replication stress-induced RAD50 phosphorylation is functionally significant for cell survival and cell cycle checkpoint activation. These results highlight the importance of the adaptor role for a member of the MRN complex in all aspects of the response to DNA replication stress.

INTRODUCTION

Response to DNA damage is a complex process involving the participation of multiple proteins that function in recognition of the damage, signaling to the cell cycle and other cellular processes and ultimately repair of the damage (1). The most lethal form of damage, the DNA double-strand break (DSB), affects all organisms and is a threat to the genome, causing chromosomal abnormalities and increasing the risk of cancer and other pathologies (2). The MRE11/RAD50/NBN (MRN) complex is a key player in different aspects of the response to DNA damage (3). It functions in the recognition of DNA DSB, in the processing of these breaks and in signaling to cellular processes to enhance cell survival (4–6).

Deletion of these genes in mice causes embryonic lethality with hypomorphic mutations in MRE11 giving rise to ataxia-telangiectasia-like disorder (ATLD) (7). In Nijmegen breakage syndrome (NBS), the defective gene is NBN (8) and deficiency in RAD50 has been described for a single patient, Nijmegen breakage syndrome-like disorder (9). The cellular phenotype of all these disorders is similar in that they exhibit radiosensitivity, cell cycle abnormalities and a defective response to DNA damage (9–11). However, the clinical phenotype of these disorders points to two groups (12). ATLD shares many of the clinical features of ataxia-telangiectasia (A-T), which includes neurodegeneration, ataxia, progressive cerebellar atrophy, abnormal eye movements and dysarthria (11). In contrast, NBS and NBS-like disorders do not exhibit spinocerebellar ataxia but rather microcephaly and mental retardation (9,13). On the other hand, there is a report of compound heterozygous mutations in the Mre11 gene in two unrelated patients with Nijmegen breakage-like severe microcephaly (14), mental retardation, chromosomal instability and radiosensitivity. Typical of NBS, neither patient showed immunodeficiency or cancer predisposition. Since cells from these patients had efficiently activated ataxia-telangiectasia mutated (ATM) (mutated in A-T patients)
Unlike the more classical ATLD where ATM is not activated, it is possible that increased apoptosis caused by ATM activity accounts for the microcephaly. All of these proteins together with ATM respond to DNA DSB but differ in response during development and in the adult nervous system (12). One of the earliest events in the response to DNA DSB is the localization of MRN to the break (15–17).

In Thermotoga maritima, MR adopts an open form with a central MRE11 nuclease dimer and two peripheral RAD50 molecules where it forms an ATP-controlled transient molecular clamp at the DNA DSB (18). The complex then recruits ATM to the DNA DSB where it is activated by autophosphorylation to phosphorylate a number of key substrates at the break site including H2AX, MDC1 and 53BP1 (19,20). Members of the MRN complex are also phosphorylated by ATM whereafter they play adaptor roles in mediating the phosphorylation of a multitude of substrates involved in cell cycle control, DNA repair and other cellular processes (21–23). ATR-dependent phosphorylation of NBN is required for correction of the S phase defect in NBS cells (24) and there is some evidence for a requirement for NBN phosphorylation for cell survival post irradiation (25–27). On the other hand, phosphorylation of MRE11 leads to inactivation of the MRN complex and its dissociation from chromatin (22). The third member of the complex, RAD50, is phosphorylated at a single site (S635) by ATM in response to DNA DSB (23). Phosphorylation of RAD50 plays a key regulatory role as an adaptor for specific ATM-dependent downstream signaling through SMC1 for DNA repair and cell cycle checkpoint control in the maintenance of genome integrity. In contrast to the data for ATM-dependent signaling, there is some disagreement on the role of MRN in ATR checkpoint signaling. Depletion of MRE11 or NBN has been shown to reduce ataxia-telangiectasia and RAD3-related (ATR) checkpoint activation in one study (28), yet in other studies that was not the case (29–31). Furthermore, while ATR-dependent G2/M arrest is NBN-dependent, no such dependence was observed for MRE11 (32). More recently, Duursma et al. (33) using Xenopus extracts showed that the MRN complex binds specifically to the ATR-activating structure and is required for ATR-dependent checkpoint activation through recruitment of the ATR activator TopBP1. Our previous data show that not only is the MRN complex important for signaling through the other members of the MRN complex. Deficiency in UV-induced signaling is also evident in Seckel-ATR-deficient cells supporting a role for ATR in this process (Fig. 1A). We also observed reduced levels of MRN proteins in Seckel cells but we are unsure of the significance of this, since it has not been reported elsewhere. A normal or close-to-normal response to UV was obtained for A-T cells (Fig. 1A). Similar results were obtained when RAD50-deficient cells were exposed to the DNA replication inhibitor hydroxyurea (HU) (Fig. 1B). Not surprisingly, when RAD50 was downregulated using siRNA, HU-induced signaling was also abrogated (Fig. 1C). This was also the case when ATR was downregulated. We also examined UV-induced signaling in NBN- and MRE11-deficient cells observing reduced levels of phosphorylation of Chk1 and SMC1 in NBS cells and a more marked effect with ATLD2 extracts (Supplementary Material, Fig. S1). We previously showed that MRE11 staining was predominantly cytoplasmic in RAD50-deficient cells and that no MRE11 foci were detected after exposure to IR (9). We demonstrate here that RAD50 is recruited to sites of DNA replication stress by its co-localization with RPA (Fig. 2A). Under these conditions, 19–23% of cells showed foci, which is in keeping with the percentage of cells in the S phase as determined by flow cytometry. As expected, no RAD50 foci were detected in F239 cells but RPA still localized to sites of damage. In Seckel cells, RAD50 was still capable of recruitment to these sites of damage, revealing that there is no dependence on ATR for this recruitment as reported previously for other members of the MRN complex. On the other hand, there are conflicting data on the requirement for the MRN complex for ATR activation (30–33). However, exposure of RAD50-deficient cells to HU failed to recruit ATR to sites of replication stress (Fig. 2B). Further evidence for a role for the MRN complex in recruiting ATR to sites of damage was demonstrated by the failure of ATRIP to localize with RPA in RAD50-deficient cells after HU treatment (Fig. 2C). In keeping with previous data, neither ATR nor ATRIP co-localized with RPA in Seckel cells. Failure of ATR recruitment to sites of damage in RAD50-deficient cells is not due to reduced levels of ATR in these cells (Fig. 1). Further evidence for supporting a role for RAD50 in ATR activation is provided by the failure to observe HU-induced phosphorylation of Chk1 in RAD50-deficient cells (Fig. 1A and B). Recent data using Xenopus extracts reveal that Chk1 phosphorylation is dependent on the recruitment of TopBP1 to single-strand DNA/double-strand DNA junctions (33). Accordingly, we examined TopBP1 binding to chromatin in RAD50-deficient cells. While TopBP1 levels in whole cell extracts were comparable in control and RAD50-deficient cells, the amount bound to chromatin is reduced in the RAD50-deficient cells (Fig. 2D). It is also of note that while ATR levels are comparable in control and

RESULTS

RAD50 is required for ATR-dependent signaling

We have previously shown that RAD50-deficient cells are characterized by chromosomal instability, radiosensitivity and impaired activation of ATM and downstream signaling in response to radiation-induced DNA DSB (9). We also demonstrated that at least some of these pathways are mediated through ATM-dependent phosphorylation of RAD50 (23). In order to investigate a possible role for RAD50 phosphorylation in DNA replication stress, we initially determined the response of F239-RAD50-deficient cells to agents that block DNA replication. Exposure of F239 cells to 20 J/m² of ultraviolet (UV) radiation failed to induce phosphorylation of a number of downstream substrates including p53, SMC1, Chk1 and NBN (Fig. 1A). As observed previously, loss of RAD50 leads to destabilization of the other members of the MRN complex. Deficiency in UV-induced signaling is also evident in Seckel-ATR-deficient cells supporting a role for ATR in this process (Fig. 1A). We also observed reduced levels of MRN proteins in Seckel cells but we are unsure of the significance of this, since it has not been reported elsewhere. A normal or close-to-normal response to UV was obtained for A-T cells (Fig. 1A). Similar results were obtained when RAD50-deficient cells were exposed to the DNA replication inhibitor hydroxyurea (HU) (Fig. 1B). Not surprisingly, when RAD50 was downregulated using siRNA, HU-induced signaling was also abrogated (Fig. 1C). This was also the case when ATR was downregulated. We also examined UV-induced signaling in NBN- and MRE11-deficient cells observing reduced levels of phosphorylation of Chk1 and SMC1 in NBS cells and a more marked effect with ATLD2 extracts (Supplementary Material, Fig. S1). We previously showed that MRE11 staining was predominantly cytoplasmic in RAD50-deficient cells and that no MRE11 foci were detected after exposure to IR (9). We demonstrate here that RAD50 is recruited to sites of DNA replication stress by its co-localization with RPA (Fig. 2A). Under these conditions, 19–23% of cells showed foci, which is in keeping with the percentage of cells in the S phase as determined by flow cytometry. As expected, no RAD50 foci were detected in F239 cells but RPA still localized to sites of damage. In Seckel cells, RAD50 was still capable of recruitment to these sites of damage, revealing that there is no dependence on ATR for this recruitment as reported previously for other members of the MRN complex. On the other hand, there are conflicting data on the requirement for the MRN complex for ATR activation (30–33). However, exposure of RAD50-deficient cells to HU failed to recruit ATR to sites of replication stress (Fig. 2B). Further evidence for a role for the MRN complex in recruiting ATR to sites of damage was demonstrated by the failure of ATRIP to localize with RPA in RAD50-deficient cells after HU treatment (Fig. 2C). In keeping with previous data, neither ATR nor ATRIP co-localized with RPA in Seckel cells. Failure of ATR recruitment to sites of damage in RAD50-deficient cells is not due to reduced levels of ATR in these cells (Fig. 1). Further evidence for supporting a role for RAD50 in ATR activation is provided by the failure to observe HU-induced phosphorylation of Chk1 in RAD50-deficient cells (Fig. 1A and B). Recent data using Xenopus extracts reveal that Chk1 phosphorylation is dependent on the recruitment of TopBP1 to single-strand DNA/double-strand DNA junctions (33). Accordingly, we examined TopBP1 binding to chromatin in RAD50-deficient cells. While TopBP1 levels in whole cell extracts were comparable in control and RAD50-deficient cells, the amount bound to chromatin is reduced in the RAD50-deficient cells (Fig. 2D). It is also of note that while ATR levels are comparable in control and

RAD50-deficient cells, reduced levels of ATR are also associated with chromatin in the RAD50-deficient cells either in the presence or in the absence of HU treatment (Fig. 2D). The MRN-dependent recruitment of TopBP1 for ATR activation observed above is supported by evidence for a physical interaction between NBN and TopBP1 in vivo (34). Thus, we determined whether RAD50 interacted with TopBP1 using co-immunoprecipitations. The results in Figure 2E reveal that both proteins co-immunoprecipitated and this does not occur in RAD50-deficient cells. Together, these data show that RAD50 is recruited to the sites of DNA replication stress and is required for ATR signaling through substrates such as Chk1.

**Figure 1.** SMC1 and Chk1 phosphorylation events are defective in RAD50-deficient cells following replication stress. Control NFF, A-T, Seckel-ATR-deficient and F239-RAD50-deficient cells were either left untreated (−) or treated (+) with either 20 J/m² UV or 2 mM HU and harvested 1 or 2 h after treatments respectively. Whole-cell extracts were prepared and resolved on SDS–PAGE. (A and B) ATR and RAD50-deficient cells are defective in UV and HU-induced phosphorylation of SMC1 S957, NBN pS343 and Chk1 pS317. (C) Knockdown of either ATR or RAD50 impaired SMC1 and Chk1 phosphorylation events post-HU treatment. Control NFF cells were transfected with control siRNA, ATR siRNA or RAD50 siRNA and also compared with untransfected RAD50-deficient cells. Whole-cell extracts were immunoblotted with the indicated antibodies.

**RAD50 phosphorylation occurs in response to replication stress**

We previously showed that RAD50 is phosphorylated at a specific site (S635) in response to DNA DSB by ATM kinase where it plays a key regulatory role as an adaptor for specific ATM-dependent downstream signaling, for DNA repair and
cell cycle control to maintain the integrity of the genome (23). To
determine whether DNA replication stress had a similar effect,
we looked for UV and HU-induced phosphorylation of
RAD50 with an antibody recognizing this site. The results in
Figure 3A show that HU and UV are as effective as IR in
causing specific phosphorylation at S635 on RAD50. Phosphor-
ylation was not observed in RAD50-deficient cells. Evidence
that this was ATR-mediated was provided by an ATR in vitro
kinase assay using a RAD50 substrate (GST588–666) containing
the S635 site (Supplementary Material, Fig. S2A). No activity
was observed in ATR immunoprecipitates from ATR or
RAD50-deficient cells. Using a phospho-specific antibody
against this site, RAD50 pS635, we observed increasing
phosphorylation with time after UV treatment (Supplementary
Material, Fig. S2B) and in a dose-dependent manner in response
to both UV and HU (Supplementary Material, Fig. S2C and D).
Under these conditions, we also observed that Chk1 phosphory-
ation increased with increasing HU in control cells (Supplemen-
tary Material, Fig. S2E). We also demonstrated that knockdown
of ATR attenuated the phosphorylation of RAD50 (Fig. 3B).
As expected, when RAD50 was downregulated there was no
detectable phosphorylation. These data suggest that ATR inter-
acts with RAD50. Immunoprecipitation of RAD50 led to
co-immunoprecipitation of ATR, which occurred both in the
presence and absence of DNA damage (Fig. 3C). Reverse immu-
noprecipitation with an antibody against ATR confirmed this

![Figure 2. Recruitment of ATR-ATRIP to the sites of stalled DNA replication forks is defective in the absence of RAD50. Cells were treated with 2 mM HU and allowed to recover for 2 h. (A) Stalled replication forks induce co-localization of RAD50 and RPA34. Cells were immunostained with anti-RAD50 (red) and anti-RPA34 (green) antibodies. This co-localization was determined in control NFF, A-T, and Seckel-ATR-deficient cells. (B) RPA is recruited normally to sites of the replication stress but ATR is undetectable in F239-RAD50-deficient cells. Cells were immunostained with ATR (red) and RPA34 (green) antibodies. Replication arrest triggers the co-localization of ATR and RPA34 foci only in the control NFF and in A-T cells. (C) Co-localization of ATRIP and RPA. Cells were immunostained with ATRIP (red) and RPA34 (green) antibodies. This co-localization was only observed in the control NFF and A-T cells. (D) Chromatin association of ATR and TopBP1 proteins was reduced in RAD50-deficient cells. Control and F239-RAD50-deficient cells were either left untreated (−) or treated (+) with 2 mM HU and harvested 2 h later. Cell extracts and chromatin fractions were resolved on SDS–PAGE and analyzed with the indicated antibodies. (E) Physical interaction between TopBP1 and RAD50. Control NFF and RAD50-deficient cells were either left untreated or subjected to 2 mM HU and harvested 2 h later. Whole cell lysates were subjected to immunoprecipitation with RAD50 antibody and immunoblotted as indicated.](image)
interaction (Fig. 3D). These data demonstrate that specific phosphorylation of RAD50 on S635 occurs in response to replication stress and is ATR-dependent.

**RAD50 phosphorylation is required for ATR signaling**

To determine the functional significance of ATR-dependent phosphorylation of RAD50, we employed full-length wild-type (Wt) and phosphosite mutant (S635G) RAD50 cDNA constructs (23). Transient transfection of these into RAD50-deficient cells gave rise to comparable levels of Wt and S635G phosphosite mutant RAD50 protein as well as correcting the MRE11 protein deficiency previously observed in these cells (Fig. 4A, right hand panel). Introduction of RAD50 Wt into RAD50-deficient cells restored HU-induced phosphorylation of both SMC1 and Chk1. However, the phosphosite mutant (S635G) failed to restore phosphorylation of these proteins (Fig. 4A, right-hand panel). These data suggest that not only is it necessary for RAD50 to be recruited to sites of stalled replication forks to initiate ATR-dependent signaling but it should also be phosphorylated to initiate downstream signaling. To distinguish between recruitment and the downstream effect, we determined localization of RAD50 and RAD50 pS635 at sites of DNA damage. Transfection of RAD50-deficient cells with RAD50 Wt led to the appearance of RAD50 pS635 at damaged sites co-localizing with RPA (Fig. 4B). As expected, no signal was detected in cells transfected with RAD50 S635G even though RAD50 protein was expressed at the same level as in RAD50 Wt transfected cells (Supplementary Material, Fig. S3) and RAD50 protein is present at these sites of DNA damage as observed by co-localization of RAD50 with RPA (Fig. 4C). We also demonstrated that ATR is recruited to sites of DNA damage and co-localizes with Rad50 in cells transfected with Rad50 S635G (Fig. 4D), consistent with the model that ATR is recruited to sites of replication stress in a Rad50-dependent manner and then phosphorylates Rad50 for downstream signaling. We confirmed that these were sites of stalled collapsed replication forks by demonstrating that both RAD50 and RAD50 pS635 foci co-localized with sites of DNA DSB by γH2AX staining (Supplementary Material, Fig. S4A and B). These results demonstrate that RAD50 plays a key role in recruiting the ATR–ATRIP complex to sites of stalled replication forks where it is activated. In addition, they demonstrate that once recruited to these structures, RAD50 is phosphorylated in an ATR-dependent manner to mediate ATR signaling.
Figure 2. Continued
RAD50 phosphorylation is required for restart of DNA replication forks

When DNA replication forks encounter DNA damage, they stall and because of their fragility may collapse and give rise to DNA DSB (35). Restart requires a variety of different factors and recombination mediated mechanisms (36,37). We determined whether RAD50 and/or the phosphorylated form of RAD50 might play a role in DNA replication fork restart after HU treatment. We employed a pulse labeling protocol to label ongoing replication with the 5-chloro-2-deoxyuridine analog (CldU) prior to HU treatment, followed by pulsing with IdU to label restart of DNA replication after damage. The results in Figure 5A reveal significant recovery of DNA replication in both control and A-T cells in response to HU as evidenced by extensive overlap of CldU and IdU labeling. However, RAD50-deficient cells failed to restart replication under these conditions. It is also evident that Seckel-ATR-deficient cells are also defective in restart.

To investigate whether RAD50 phosphorylation might play a role in replication restart, we transfected RAD50-deficient cells with RAD50 Wt and RAD50 S635G phosphosite mutant and used the same labeling protocol. RAD50-deficient cells transfected with RAD50 Wt restarted DNA replication at comparable levels to control (Fig. 5C). However, the RAD50 phosphosite mutant failed to show restart, similar to that in vector only transfected cells. This difference between RAD50 mutant and Wt is particularly evident when these experiments are quantitated (Fig. 5D).

Recent data on budding yeast show that when DNA synthesis is impeded, the structural maintenance of chromosomes complex, cohesin, accumulates at replication sites and is critical to the recovery of stalled forks (38). Furthermore, there was a significant decrease in enrichment of the cohesin protein SCC1 at ARS306, an early origin of replication in HU-arrested RAD50△ cells. Since origins of replication are not as well defined in mammalian cells, we looked for association of SCC1, RAD50 and other proteins at sites of replication restart by immunoprecipitation of IdU-labeled sites of replication, followed by detection of bound proteins by immunoblotting. As observed in yeast, SCC1 accumulated at sites of replication in HU-treated control cells but there was no evidence for this association in RAD50-deficient cells consistent with their failure to restart replication (Fig. 6A, left-hand panel). Failure to observe signal in untreated cells is very likely due to rapid incorporation of CldU into higher molecular size fragments, whereas replication fork restart is likely to persist as low-molecular-weight material for some time with detection of CldU signal. Input data analysis is demonstrated in Figure 6A, right hand panel. While the levels SMC1, SCC1, TopBP1, ATR and RPA are normal in F239 cells, the input data provide evidence for reduction in all of these. This suggests that these proteins are not present on chromatin in RAD50-deficient cells after cross-linking. We observed a double band for RAD50 in these
experiments which might be explained by the cross-linking step. Furthermore, when RAD50-deficient cells were transfected with RAD50 Wt cDNA, RAD50, SMC1 and SCC1 accumulated at sites of DNA replication restart (Fig. 6B, left-hand panel). As expected from the failure to induce DNA replication restart, after transfection of these cells with RAD50 phosphosite mutant, there was no evidence for association of cohesin. Interestingly, the presence of the mutant form of RAD50 was able to correct the chromatin localization of the cohesin complex proteins as described by cross-linking (Fig. 6B, right panel). Under these conditions, the ATR-dependent phosphorylated forms of RAD50 and SMC1 also associated with newly replicating DNA. Furthermore, the use of RAD50 antibody led to co-immunoprecipitation of the phosphorylated forms of both proteins (Fig. 6C). Input analysis is demonstrated in Figure 6B, right hand panel. Again as in Figure 6A, SMC1 and SSC1 are reduced in the vector only RAD50-deficient cells but both Wt and mutant RAD50 restore these proteins to normal levels.

### Functional importance of RAD50 S635 phosphorylation

Exposure of cells to UV or HU leads to a decrease in cell survival, which can be explained at least in part by DNA damage resulting from DNA replication forks collapse. Since RAD50-deficient cells and those transfected with the RAD50 phosphosite mutant (S635G) failed to restart replication after HU treatment, we predicted that they would be more sensitive to these agents. Exposure of RAD50-deficient cells (vector) to UV revealed significantly increased sensitivity over the dose range 5–20 J/m² (Fig. 6D). Reintroduction of Wt RAD50 into these cells restored survival to values similar to control. However, the phosphosite RAD50 mutant had only a minimal effect on restoring survival. Delay of entry into mitosis in response to DNA replication stress is mediated by ATR (39). Therefore, we examined the effect of RAD50 phosphorylation on the G2/M checkpoint using status of histone H3 phosphorylation (40). There was a marked drop in entry of control and A-T cells into mitosis after exposure to UV (Fig. 6E, left-hand panel). However, RAD50-deficient and ATR-deficient cells passed into mitosis in a relatively unimpeded fashion in response to damage. A similar picture was obtained when cells were exposed to HU (Fig. 6E, right-hand panel; Supplementary Material, Fig. S5). When the phosphorylation status of RAD50 on mitotic entry was determined after transfection, reintroduction of RAD50 Wt restricted entry in a manner comparable to control (Fig. 6F, left-hand panel; Supplementary Material, Fig. S6). On the other hand, transfection with phosphosite mutant RAD50 showed no inhibition of entry to mitosis and was similar to vector only cells (Fig. 6F, right-hand panel). Together, these data demonstrate that ATR-dependent phosphorylation of RAD50 mediates cell survival and checkpoint control after replication stress.

### DISCUSSION

We reveal here that while the MRN complex is required for ATR activation, ATR-dependent phosphorylation of RAD50 is essential for all aspects of downstream signaling and for DNA
replication restart. These data further help to resolve the conflict in support of a role for the complex in ATR signaling. We show that both UV- and HU-induced ATR signaling are completely ablated in RAD50-deficient cells. Consistent with this, we find that ATR signaling is also not detectable in MRE11-deficient cells (ATLD2). This is not surprising since deficiency in either RAD50 or MRE11 results in very much reduced levels of both proteins (7,9,23). ATR-dependent signaling was also reduced in NBN-deficient cells but to a lesser extent. Previous data using a single NBS cell line revealed defective ATR signaling but they failed to observe this in ATLD (32). Depletion of MRE11 or NBN reduced checkpoint activation in some cases but not in others (28–31). Recent data using Xenopus extracts reveal specific association of the MRN complex with an ATR-activating structure and evidence that the complex plays a direct role in ATR checkpoint activation (33). They also showed that knockdown of NBN in human cells caused a defect in ATR activation in response to low-dose HU.

In response to DNA damage, activation of ATR and ATM leads to the phosphorylation of hundreds of downstream substrates that control a multitude of cellular pathways (41). The MRN complex too is part of this cellular network of substrates, but it occupies a special place as an adaptor and phosphorylation of members of the complex is required to fulfill that role (21,23). All three members of the complex are phosphorylated in response to DNA damage and specific functional sites have been identified for both NBN and RAD50 (23,24). In the case of NBN, phosphorylation is observed at S282 and S343 both of which are required for correction of the S phase checkpoint in NBS cells (21,24). Mutation at these sites prevents phosphorylation and signaling through downstream substrates linked to DNA repair, cell cycle checkpoint activation and cell survival (25–27). Phosphorylation at a single site on RAD50 (S635) is required for correction of DNA repair, cell cycle checkpoint activation and cell survival in RAD50-deficient cells in response to DNA DSB (23). However, it is evident that phosphorylation of different members of the complex has different outcomes. Di Virgilio et al. (22) using Xenopus extracts identified several sites of phosphorylation on MRE11, but these events lead to inactivation of the complex and its dissociation.
from chromatin suggesting that it was a signal, perhaps to control extent of processing of DNA ends. Phosphorylation of Rad50 is significant since a phosphosite mutant failed to correct ATR-dependent signaling through Chk1 and SMC1 phosphorylation in RAD50-deficient cells. This is further borne out by the failure of the phosphosite mutant to correct the cell cycle checkpoint and cell survival defects in RAD50-deficient cells. Exposure of RAD50-deficient cells to HU was accompanied by failure to restart DNA replication. DNA replication restart was restored by Wt Rad50 but the phosphosite mutant failed to restore synthesis, pointing to the importance of RAD50 phosphorylation in this process. Recent data in yeast show that when DNA synthesis is impeded, cohesin (SCC1) accumulates at replication sites and is critical to the recovery of stalled forks (38). Furthermore, in RAD50Δ mutants there was a significant decrease in SCC1 accumulation at early origins of replication. Our results in mammalian cells are consistent with this since we showed that under conditions of DNA replication stress RAD50-deficient cells fail to accumulate SCC1 or restart replication. Both SCC1 accumulation and replication restart were restored with transfection of Wt RAD50 but neither of these occurred when the RAD50 phosphosite mutant (S635G) was introduced into RAD50-deficient cells. Tittel-Elmer et al. (38) demonstrated the importance of structural features of RAD50 including the hook, the coiled-coil regions and the tethering capability of the MRX complex for cohesin accumulation to forks during replication stress (38). Our results reveal another critical feature of RAD50, its ATR-dependent phosphorylation at a specific site, for cohesin loading and replication fork restart. It is of interest that this phosphorylation occurs in the region of the Zn^2+ hook and may function to stabilize RAD50 conformation and enhance tethering between sister chromatids. At this stage, it is unclear as to how RAD50 promotes the accumulation of cohesin at stalled or damaged replication sites. Tittel-Elmer et al. (38) propose that MRX stabilizes transiently bound cohesin at replication sites following fork arrest and that RAD50 hook-mediated bridging is essential for cohesin localization by maintaining sister chromatid conformation (38). Our data enlarge upon that model and extend it to show that RAD50 and/or its specific phosphorylation function in the
recognition, signaling and restart of blocked replication forks. It is also consistent with another model which describes a direct role for MRN in ATR activation through recruitment of TopBP1 (33). In RAD50-deficient cells, recruitment of both TopBP1 and ATR to chromatin is deficient. We envisage that in this case, as observed by others, RAD50 as part of the MRN complex plays an essential role in recruiting the ATR-activating complex to sites of blocked or collapsed replication forks. Once this occurs, ATR is activated to phosphorylate RAD50 at a specific site (S635) and as expected, this is not observed in RAD50-deficient cells. This phosphorylation of RAD50 is essential for ATR signaling, since a phosphosite mutant introduced into RAD50-deficient cells failed to restore ATR-dependent phosphorylation of Chk1 or SMC1. Once the signaling events are complete, the fork is restored and the ATR-activating complex is removed from this region. However, the MRN complex remains, presumably still tethering the sister chromatids behind the fork. At this stage, the presence of the complex is required for cohesin loading and DNA replication restart. As observed in yeast, RAD50 plays an important role in restart and we have shown here that specific phosphorylation of RAD50 increases that process presumably by assisting in the stabilization of the cohesin complex.

MATERIALS AND METHODS

Cell cultures and RAD50 transfections

Human hTERT-transformed fibroblast cell lines of NFF (control), F02–98 (Seckel-ATR deficient), NBS1-ILB1 and ATLD2 were used (42). Fibroblast cell lines of A-T (AT4B1) and F239-RAD50-deficient cells were described previously (9,23). All cells were cultured in DMEM medium supplemented with 10% fetal calf serum (FCS) and 1% penicillin and streptomycin. The cloning of full-length RAD50-FLAG into pcDNA3.1 mammalian expression vector (Invitrogen) and the generation of RAD50 mutant S635G were described previously (23). F239-RAD50-deficient cells were transiently transfected with empty vector, full-length Wt or RAD50 mutant S635G constructs using NEON electroporation (Invitrogen) at 1400 V,
20 mm width and two pulses. All experimental cells were grown at 37°C with 5% CO2. These cells were exposed to either 2 mM HU or 20 J/m² UV radiation and were incubated for 2 and 1 h, respectively, before harvesting unless otherwise indicated.

Lysate preparation, immunoprecipitation and immunoblotting

Whole cell extracts were prepared by resuspending cells in ice-cold lysis buffer (50 mM Tris–HCl, pH 7.4, 150 mM NaCl, 2 mM ethylene glycol tetraacetic acid (EGTA), 2 mM ethylenediaminetetraacetic acid (EDTA), 25 mM sodium fluoride, 25 mM β-glycerophosphate, 0.1 mM sodium orthovanadate, 0.1 mM PMSF, 0.2% Triton X-100, 0.3% NP40 and a cocktail of protease inhibitors [Roche-Diagnostics]) and incubated on ice for 30 min. Supernatants were collected following centrifugation at 14 000 g for 15 min. For immunoprecipitations, protein extracts were precleared with protein A beads for 1 h at 4°C. The supernatants were then incubated with the required antibody for 4 h at 4°C in the presence of 100 ng/ml ethidium bromide. The immunocomplexes were collected with protein A and G beads and then washed twice with lysis buffer. Cell lysates and immunoprecipitates were resolved on SDS-PAGE and transferred to nitrocellulose membranes using 1xTowbin’s buffer (25 mM Tris–Base, 192 mM glycine, 20% methanol and 0.02% SDS) at 100 V for 1 h at 4°C. Membranes were immunoblotted with the required antibodies and analyzed using ECL (PerkinElmer Life Science, MA, USA). RAD50 anti-S635 phospho-specific antibody was generated as described previously (23). Anti-rabbit NBN was from Novus Biologicals, CO, USA, anti-mouse β-actin was from Sigma-Aldrich, MO, USA; anti-rabbit p53, anti-mouse p53 pS15, anti-mouse Chk1 and anti-rabbit polyclonal Chk1 pS317 antibodies were from Cell Signaling Technology, MA, USA. Anti-mouse monoclonal antibodies to RAD50 and NBN pS343 were from Upstate, NY, USA. Anti-mouse monoclonal (ATM-2C1, MRE11 and RPA34) and anti-rabbit polyclonal (SCC1, SMC1 and SMC1 pS957) antibodies were from GeneTex, Inc., CA, USA. Anti-goat polyclonal antibody to ATR (ATR-N19) was from Santa Cruz Biotechnology, USA. Anti-mouse monoclonal TopBP1 antibody was from BD Bio-sciences, CA, USA. Anti-mouse and anti-rabbit secondary antibodies were from Merck Millipore, MA, USA, and Rockland, USA, respectively.

ATR kinase assay

The GST-RAD50 constructs spanning the full-length of RAD50 have been described previously in ATM kinase assays in response to DNA DSBs (23). A short GST-RAD50, containing S635 (aa588 – 666), was also prepared for substrate verification.
ATR kinase assays were carried out as described previously (43). Control, F239-RAD50-deficient and Seckel-ATR-deficient fibroblasts were harvested at 1 h after treatment with 20 J/m² UV radiation. Cells were lysed in ice-cold lysis buffer (25 mM hydroxyethyl piperazineethanesulfonic acid (HEPES), pH 7.4, 150 mM NaCl, 1.5 mM MgCl₂, 1 mM EGTA, 0.5% Triton X-100, 0.5 mM sodium orthovanadate and 1 mM sodium fluoride and a cocktail of protease inhibitors [Roche-Diagnostics]). ATR immunoprecipitates were prepared with anti-rabbit polyclonal ATR antibody and protein G-Sepharose for 4 h at 4°C. Immunocomplexes were washed twice with lysis buffer, twice with lysis buffer containing 1 M NaCl and twice with kinase buffer (10 mM HEPES, pH 7.4, 50 mM NaCl, 10 mM MgCl₂ and 1 mM EGTA). The kinase reaction was prepared by resuspending washed beads in a final volume of 30 μl kinase buffer (10 μM ATP, 10 μCi γ³²P-ATP) containing 1 μg of soluble GST-RAD50 fusion protein for 30 min at 30°C. Kinase reactions were terminated by the addition of 15 μl of loading dye resolved on SDS–PAGE and phosphorylated proteins were visualized by autoradiography.

siRNA transfections

siRNAs directed against RAD50 sequence [pBD872 (pEBVsiRAD50-274)], ATR sequence [pBD962 (pEBVsiATR-854)] or control siRNA [pBD650 (pEBV-siControl)] were obtained from the Biard Lab in France (44). Cells were seeded in six-well plates at 1.5 × 10⁴ cells/well and transfection with siRNAs was performed using lipofectamine 2000 (Invitrogen), according to the manufacturer’s suggested protocol. The transfected cells were incubated for 24 h before treatments with either UV or HU as indicated.

Immunofluorescence microscopy

For measurement of nuclear foci formation, Cells were grown on coverslips in six-well plates for 48 h before treatment with 2 mM HU for 2 h. After treatment, cells were washed in phosphate buffered saline (PBS), fixed with PBS containing 4% paraformaldehyde and permeabilized with PBS containing 0.5% Triton X-100 for 10 min. Cells were then blocked in PBS containing 5% bovine serum fraction V albumin (BSA) for 1 h at room temperature. As indicated, cells were incubated with primary antibodies of goat polyclonal ATR 1:200 (ATR-N19, Santa Cruz Biotechnology, USA), mouse monoclonals RPA34 1:800 and ATR 1:2000 (GeneTex, Inc., USA), rabbit polyclonal ATRIP 1:200 (Upstate), rabbit polyclonal RAD50 1:200 (Sigma), rabbit polyclonal RAD50 pS635 1:1000 (23) or mouse monoclonal γH2AX 1:1000 (Merk Millipore, MA, USA) in BSA blocking solution for 1 h at room temperature. After washing with PBS, cells were incubated with Alexa-Fluor-594 and Alexa-Fluor-488 (Invitrogen) at 1:1000 dilution for 1 h at room temperature. Nuclei were counterstained with 4,6-diamidine-2’-phenylindole (DAPI) for 10 min at room temperature. After three final washing steps, the coverslips were mounted in Vectashield mounting medium (Vector Laboratories, Burlingame, CA, USA). Images were analyzed using Axioskop2 Zeiss fluorescence microscopy equipped with a digital camera (AxioCam MRn) and the AxioVision LE4 software.

Sub-cellular fractionation

The cellular protein fractionations were performed as described previously (45). The cells were either mock-treated or treated

Figure 5. Continued
with 2 mM HU and allowed to recover for 2 h. Cells were then washed with cold PBS and resuspended in buffer A [10 mM HEPES pH 7.9, 10 mM KCl, 1.5 mM MgCl₂, 0.34 M sucrose, 10% glycerol, 1 mM sodium fluoride, 1 mM sodium orthovanadate and cocktail of protease inhibitors (Roche-Diagnostics)]. Triton X-100 was added to a final concentration of 0.1% and the cells were incubated on ice for 5 min. Cytoplasmic fractions were collected by centrifugation at 1300g for 4 min.

Figure 6. RAD50 associates with the accumulation of cohesin complex at sites of replication in response to HU, which is an event dependent on phosphorylation of RAD50. (A) RAD50 phosphorylation at S635 associates with early phosphorylated SMC1 and SCC1 at sites of replication in control NFF but not in F239-RAD50-deficient cells. Cells were either mock treated (−) or treated (+) with 2 mM HU for 2 h, released from HU stress and then pulsed with ClidU. Cells were cross-linked and chromatin fractions were immunoprecipitated with anti-BrdU antibody. These immunocomplexes (left-hand panel) and input (right hand panel) were immunoblotted with indicated antibodies. (B) Frequencies of cohesin complex accumulation at the replication sites were restored in RAD50-deficient cells complemented with RAD50 Wt. RAD50-deficient cells were transfected with Wt RAD50, RAD50 mutant S635G forms or vector only. Cells were treated and processed as in A. BrdU immunocomplexes (left-hand panel) and input (right-hand panel) were immunoblotted with the indicated antibodies. (C) Interaction between RAD50 and cohesin. Control NFF and F239-RAD50-deficient cells were either untreated or treated with 2 mM HU and harvested 2 h later. Whole-cell lysates were subjected to immunoprecipitation with RAD50 antibody. RAD50 immunocomplexes (left-hand panel) and input (right-hand panel) were immunoblotted with the indicated antibodies. (D) RAD50 phosphorylation mediates cell survival in response to replication stress. Expression of RAD50 mutant failed to complement UV sensitivity in RAD50-deficient cells. RAD50-deficient cells were transfected with Wt RAD50, RAD50 mutant S635G forms or vector only and exposed to 0, 10, or 20 J/m² UV radiation. Colony survival was expressed as a percentage of irradiated/un-irradiated cells. Error bars represent (SEM) standard error of the mean, n = 3. (E) RAD50-deficient cells are defective for arrest at G2/M checkpoint either post-UV (left-hand panel) or HU (right-hand panel) treatments. Cells were either treated with 20 J/m² UV or 2 mM HU or allowed to recover for 1 or 2 h, respectively. Cells were stained with Histone H3-pS10 conjugated antibody and stained with PI for DNA content. Cellular fluorescence was measured to analyze for Histone H3 phosphorylation and DNA content. Control NFF and A-T cells exhibited a marked decrease in the percentage of G2 cells entering mitosis after replication fork stalling, whereas Seckel-ATR and F239-RAD50-deficient cells failed to show G2/M arrest. The error bars represent the standard deviation for three independent experiments. (F) Left-hand panel; RAD50-deficient cells complemented with RAD50 Wt restored G2/M checkpoint post-HU treatment. F239-RAD50-deficient cells were transiently transfected with RAD50 Wt, RAD50 mutant S635G or vector only for 48 h. For the last 2 h, RAD50-complemented cells and control NFF cells were either mock treated or treated with 2 mM HU. Cells were stained with Histone H3-pS10-conjugated antibody and PI. Cellular fluorescence was measured to analyze Histone H3 phosphorylation and for DNA content (PI staining). RAD50-deficient cells complemented with RAD50 Wt and Control NFF cells exhibited a marked decrease in the percentage of G2 cells entering mitosis, while RAD50-deficient cells transfected with either RAD50 phospho mutant S635G or vector only failed to show G2/M arrest. Right-hand panel; immunoblot analysis of RAD50 expression in RAD50-deficient cells transfected with RAD50 Wt, RAD50 mutant S635G or empty vector containing FLAG epitope.
and then clarified by centrifugation at 16 000g for 15 min. The pellets of nuclei, examined microscopically, were washed once with buffer A at high speed and then lysed for 10 min on ice in buffer B (3 mM EDTA, 0.2 mM EGTA, 1 mM DTT and protease inhibitors). Soluble nuclear fractions were separated from chromatin by centrifugation at 1700g for 4 min. The chromatin pellets were washed with buffer B at high speed. Isolated chromatin was resuspended in SDS sample buffer and
then sheared by sonication to obtain chromatin bound fractions (CBFs).

**BrdU immunoprecipitation of proteins present at stalled replication sites**

Immunoprecipitation of proteins localized to chromatin following short treatment with HU has been carried out as described previously (46). Briefly, cells were treated with 2 mM HU for 2 h, HU was removed and cells were labeled with 100 μM CldU for 40 min. Cells were cross-linked in 1% paraformaldehyde for 15 min in DMEM medium without FCS. The cytoplasmic protein fraction was removed by incubation in hypotonic buffer [10 mM HEPES, pH 7, 50 mM NaCl, 0.3 mM sucrose, 0.5% Triton X-100 and protease inhibitor cocktail (Roche-Diagnostics)] for 10 min on ice and centrifugation at 1500 g for 5 min. Nuclear soluble fraction was removed by incubation with nuclear lysis buffer (10 mM HEPES pH 7, 200 mM NaCl, 1 mM EDTA, 0.5% NP-40 and protease inhibitor cocktail) for 10 min on ice and then centrifuged at 13 000g for 2 min. Pellets were resuspended in lysis buffer (10 mM HEPES pH 7, 500 mM NaCl, 1 mM EDTA, 1% NP-40 and protease inhibitor cocktail), sonicated, centrifuged for 30 s at 13 000g and supernatants containing chromatin fractions were collected. Total chromatin protein of 200 μg of each sample was used for immunoprecipitation with 2 μg of anti-CldU antibody (rat-anti-BrdU; OBT0030F Abd Serotec). The immunocomplexes were collected with protein A and G beads and were washed twice with nuclear lysis buffer and twice with washing buffer (10 mM HEPES, 0.1 mM EDTA and protease inhibitor cocktail), incubated in 2 × sample loading buffer (100 mM Tris–HCl, pH 6.8, 100 mM DTT, 4% SDS, 0.2% bromophenol blue and 20% glycerol) for 30 min at 90 °C and then resolved on 6% SDS–PAGE for immunoblotting analysis.

**DNA replication recovery assays**

The DNA replication restart assays were carried out as described previously (47,48). Cells were grown on coverslips and marked by pulse labeling for 30 min with 50 μM of CldU and then arrested for 6 h by treating with 2 mM HU. The medium was removed and cells were then incubated with 50 μM of IdU in fresh complete medium for 60 min. All the following steps were carried out at room temperature. Cells were fixed at a concentration of 10⁶ cells/ml in 2 ml of ice-cold 70% ethanol for 24 h at −20 °C. After blocking in PBS containing 1% BSA, cells were incubated with Alexa Fluor-488 conjugated Phospho-H3-pS10 antibody (Cell Signaling Technology, MA, USA) at 1:30 dilution for 3 h at room temperature. Cells were resuspended in 25 μg/ml propidium iodide (PI) and RNase A 0.1 mg/ml in PBS for 30 min at room temperature. Cellular fluorescence was measured using a Becton Dickinson (San Jose, CA, USA) FACSCalibur flow cytometry and analyzed using BD Cell Quest Pro Version 5.1.1.

**Cell survival**

Clonogenic cell survival assay was carried out as described previously (9). Control and A-T fibroblasts and RAD50-deficient cells transfected with Wt RAD50, RAD50 mutant S635G or vector only were resuspended at 2 × 10⁵ cells/ml in culture medium. Cells were exposed to 0, 10 or 20 J/m² UV radiation. Colony survival was expressed as a percentage of irradiated/un-irradiated cells. Error bars represent SE using data from three independent experiments.

**SUPPLEMENTARY MATERIAL**

Supplementary Material is available at HMG online.

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