RIF1 controls replication initiation and homologous recombination repair in a radiation dose-dependent manner

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Key words
DNA repair/ Homologous recombination/ Radiation biology/ RIF1
Summary statement
Homologous recombination repair (HRR) is suppressed in a radiation dose-dependent manner. RIF1 has a role in this dose-dependent HRR suppression by controlling replication initiation.

Abstract
RIF1 controls both the DNA replication timing and DNA double-strand break (DSB) repair pathways to maintain genome integrity. However, it remains unclear how RIF1 links these two processes when exposed to ionizing radiation (IR). Here, we show that homologous-recombination repair (HRR) inhibition by RIF1 occurs in a dose-dependent manner and is controlled via DNA replication. RIF1 inhibits both DNA end resection and RAD51 accumulation after exposure to high doses of IR. Contrastingly, HRR inhibition by RIF1 is antagonized by BRCA1 at a low-dose IR exposure. At high IR doses, RIF1 suppresses replication initiation by dephosphorylating MCM helicase. Notably, the dephosphorylation of MCM helicase inhibits both DNA end resection and HRR even without RIF1. Thus, our data show the importance of active DNA replication for HRR and suggest a common suppression mechanism for DNA replication and HRR at high IR doses, both of which are controlled by RIF1.

Introduction
The exposure of cells to ionizing radiation (IR) induces DNA double-strand breaks (DSBs), which are severely deleterious lesions that may induce reproductive cell death and lead to a predisposition to tumors (Löbrich and Jeggo, 2007). DSBs are largely rejoined through two major repair pathways, non-homologous end-joining (NHEJ) or homologous recombination repair (HRR). HRR is initiated with 5’DNA-end resection at DSB sites and uses undamaged homologous regions, generally from the sister chromatid, as template for the action of RAD51. The current view of the choice between the DSB repair pathways depends on the cell cycle, because HRR was suggested to be active only in the S and G2 phases, in which the sister chromatids are available, whereas NHEJ could be activated throughout the cell cycle. This cell cycle dependence of HRR was evidenced by the fact that CDK promotes HRR by phosphorylating HRR proteins (Esashi et al., 2005; Ira et al., 2004).
Previous studies have shown that the choice between NHEJ or HRR as the repair pathway is regulated by the 53BP1 -BRCA1 circuit (Chapman et al., 2013; Di Virgilio et al., 2013; Escribano-Díaz et al., 2013; Zimmermann et al., 2013). RIF1 (Rap1 Interacting Factor 1), the effector of 53BP1, accumulates at DSB sites via the ATM-dependent phosphorylation of 53BP1, and inhibits HRR by protecting DSB ends from DNA end resection. As a result, RIF1 plays a role in directing the cell towards the use of NHEJ, which is the dominant pathway in G1 phase. On the other hand, BRCA1 inhibits RIF1 accumulation during S and G2 phases, resulting in HRR activation. These studies provided the molecular mechanism for the cell-cycle-dependent regulation of HRR.

There are intimate links between HRR and replication, in which HRR is critical for the successful completion of replication. In bacteria, recombination-mediated D-loop formation can initiate DNA replication in the absence of origins (Kogoma, 1997). This mechanism seems to be conserved in eukaryotes as break-induced replication (BIR) for collapsed DNA replication forks. BIR is well-characterized in budding yeast and consists of a two-strand break at the 5' end of a linear molecule, followed by the resolution of the recombination intermediates to yield a fully replicated chromosome. In mammalian cells, RAD52 is recruited to sites of replication stress and required for fork restart by BIR (Sotiriou et al., 2016). Although the mechanism of BIR activation by the 53BP1-BRCA1 circuit (Isono et al., 2017) and the resulting RIF1-PP1 complex remains unknown, the suppression of replication initiation by RIF1 is a suppressor of replication initiation by the 53BP1-BRCA1 circuit (Isono et al., 2017). This suppression prevents the formation of a replication origin, which is required for the initiation of DNA replication.

In addition to acting in the repair pathway choice, RIF1 plays a major role in the replication timing regulation during S phase, which appears to be a conserved function throughout eukaryotes (Hayano et al., 2012; Ira and Nussenzweig, 2014). Replication initiation begins at replication origins, where the MCM (minichromosome maintenance) helicase complex, composed of MCM2-7, is formed through the sequential assembly of Orc, Cdc6, Cdt1, and MCM, during the G1 phase. Once the S phase is initiated, MCM helicase is converted into an active form in the Cdc45-MCMs-GINS complex (CMG complex), for which MCM2 and MCM4 are phosphorylated by DDK composed of CDC7 and DBF4 (Chuang et al., 2009; Kang et al., 2012). Simultaneously, RIF1 forms a complex with protein phosphatase 1 (PP1), which is poor substrate specificity and requires association with a targeting subunit for the correct substrate recognition (Alver et al., 2017; Hiraga et al., 2014; Hiraga et al., 2017; Yamazaki et al., 2012). The RIF1-PP1 module acts as a suppressor of replication initiation by dephosphorylating MCM2 and MCM4, which maintains MCM helicase in a inactive state until replication forks advance through the genome. This suppression prevents the concurrent suppression of replication initiation by 53BP1-BRCA1, which is required for the suppression of replication initiation by the RIF1-PP1 module. As a result, RIF1 inhibits replication initiation during S phase, which appears to be a conserved function throughout eukaryotes.
studies showed a pivotal role of HRR in restarting replication at one-ended DSBs, the relationship between HRR and replication after IR, which induces two-ended DSBs, is largely unknown.

In order to understand the regulatory mechanisms of HRR following IR, we quantified the HRR activity after IR and demonstrated an IR dose-dependent HRR suppression mediated by RIF1. RIF1 inhibited the accumulation of RAD51 at DSB sites after 3 Gy of IR but not after 0.5 Gy. DR-GFP, an HR reporter assay, showed that RIF1 inhibited HRR only after high IR doses. In these cases, RIF1 accumulated at DSB sites and led to a temporary suppression of replication via promoting dephosphorylation of MCM helicase. Interestingly, dephosphorylation of MCM helicase by a DDK inhibitor or expression of phospho-dead MCM2 mutant suppressed RAD51 accumulation and HRR at DSB sites. Our results suggest that efficient HRR following IR requires active replication, while RIF1 suppresses replication and HRR in an IR dose-dependent manner.

Results

HRR is inhibited by IR in a dose-dependent manner, whereas NHEJ remains unchanged

To functionally assess the extent of HRR and NHEJ activities in the presence of DSBs, HeLa cells harboring a single copy of either DR-GFP (an HRR reporter gene) or pEJ (an NHEJ reporter gene) were transiently transfected with an I-SceI expression vector and exposed to IR (Fig. 1A). I-SceI induced a single two-ended DSB in each specific reporter gene and the number of GFP-positive cells was used as a measure of functional HRR or NHEJ repair (Mansour et al., 2008; Pierce et al., 2001). The DR-GFP assay showed a reduction in HRR, whose frequencies were reduced to 30% and 10% of that of the non-irradiated control after an IR exposure of 3 and 8 Gy, respectively (Fig. 1B). IR did not affect transfection efficiency or gene expression (Fig. S1A), but significantly inhibited HRR in a dose-dependent manner. In contrast, the amount of NHEJ repair was almost constant, even after 8 Gy of IR (Fig. 1C). Similar results were obtained with human U2OS cells (Fig. S1B and S1C). This decrease in functional HRR was confirmed using a chromatin immunoprecipitation (ChIP) assay at an I-SceI DSB site (Niida et al., 2010). The accumulation of RAD51, a recombinase involved in sister-chromatids exchanges, was strongly suppressed after IR exposure (Fig. 1D). Contrastingly, the accumulation of Ku70, an NHEJ protein, remained relatively constant. Thus, the functional HRR and ChIP assays for a two-ended DSB in a specific locus showed a decrease in HRR activity with increasing IR doses.
Next, RAD51 focus formation and the activation of DNA-PKcs, a kinase for NHEJ, were measured in the absence of reporter genes. Phosphorylation of DNA-PKcs increased along with the IR doses (Fig. S1D). In contrast, the number of RAD51 foci per cell was constant regardless of the IR dose (either 0.5 or 3 Gy), even though 3 Gy of IR produced more DSBs than 0.5 Gy (Fig. 1E). Interestingly, the signal intensity of individual RAD51 foci 1 hour after IR exposure was significantly weaker in cells irradiated with 3 Gy of IR than in those irradiated with 0.5 Gy of IR (Fig. 1F and S1E). This is consistent with a previous finding obtained using quantitative image-based cytometry, which showed a reduction in RAD51 focus intensity after increasing IR doses (Ochs et al., 2016). Our results showed that radiation treatment, which generates additional DSBs, inhibits RAD51-mediated HRR in an IR dose-dependent manner both at an I-SceI-induced DSB and at IR-induced DSBs.

**IR inhibition of HRR is mediated by RIF1**

In order to gain insight into the relationship between HRR and NHEJ upon IR treatment, we monitored HRR while inhibiting NHEJ. HeLa-DR-GFP cells were treated with a DNA-PKcs inhibitor after transfection with an I-SceI expression vector and analyzed using a flow cytometer. Unexpectedly, the DNA-PKcs inhibition did not rescue the reduction in HRR after high doses of IR (Fig. S2A). Since RIF1 is supposed to suppress HRR in a different pathway from that of DNA-PKcs, we next measured HRR activity when RIF1 was depleted by siRNA (Fig. S2B). RIF1 depletion alleviated the IR inhibition of HRR, although this repair pathway was not completely restored after exposure to any radiation dose (Fig. 2A). This partial restoration of functional HRR might be explained by either a hyper-end resection after RIF1 depletion (Fig. 2E, lane 4) (Ochs et al., 2016), or a positive role of RIF1 in HRR, which was observed in non-irradiated cells (Fig. 2A, 0 Gy) (Buonomo et al., 2009). To confirm RIF1 function on HRR suppression, RAD51 focus formation was observed in RIF1-depleted cells. The decrease in RAD51 intensity after an IR exposure of 3 Gy was rescued when RIF1 was depleted (Fig. 2B and 2C). RIF1 depletion significantly promoted DNA-end resection in cells treated with 3 Gy of IR, which was detected by the focus formation of RPA2, a single-strand DNA binding protein, and by its phosphorylation (Fig. 2D, S2C, and 2E). These results were not caused by an alteration in the cell cycle after RIF1 depletion (Fig. S2D). These findings were consistent with previous studies (Chapman et al., 2013; Di Virgilio et al., 2013; Escribano-Díaz et al., 2013; Zimmermann et al., 2013). However, the enhanced focus formation of RPA2 and RAD51 in RIF1-depleted cells was prominent after exposure to 3 Gy but not 0.5 Gy of IR, suggesting that RIF1 inhibits HRR only at high IR doses.
BRCA1 antagonizes RIF1 within low IR doses during S/G2 phase

Next, to examine the mechanisms of IR dose-dependent HRR inhibition by RIF1, we analyzed the dose-dependency of 53BP1 phosphorylation, which is a prerequisite for RIF1 accumulation (Chapman et al., 2013; Di Virgilio et al., 2013; Escribano-Díaz et al., 2013; Zimmermann et al., 2013). HeLa cells were synchronized in G1 phase by thymidine block and the arrested cells were released into S phase (Fig. S3A). G1 or S phase cells were exposed to 0.5 or 3 Gy of IR. In G1 phase cells, phospho-53BP1 and RIF1 formed foci regardless of IR doses (Fig. 3A, 3B, S3B and S3C). In contrast, these foci were negligible after 0.5 Gy, but were evident after 3 Gy of IR in S phase cells. Importantly, the number of 53BP1 foci formed in both G1 and S phases were similar, suggesting that 53BP1 phosphorylation after 0.5 Gy of IR was suppressed in S phase cells. Because ATM-mediated Chk2 activation was observed in both G1 and S phase cells exposed to 0.5 Gy of IR, DNA damage checkpoint is not responsible for the suppression of 53BP1 phosphorylation. These results suggest that, in S phase, 53BP1 phosphorylation is controlled by an unknown mechanism.

BRCA1 was reported to play a suppressive role in RIF1 focus formation by promoting dephosphorylation of phospho-53BP1 (Isono et al., 2017). BRCA1 expression was increased in cells that were synchronized in the S phase, in which phospho-53BP1 was suppressed, although the cell cycle synchronization may have artificially affected the BRCA1 expression level (Fig. S3E) (Escribano-Díaz et al., 2013). Therefore, we used siRNA or a BRCA1 overexpression model to test the effect of BRCA1 expression level on phospho-53BP1 and RIF1 accumulation. BRCA1 depletion enhanced the number of phospho-53BP1 and RIF1 foci after 0.5 Gy but not after 3 Gy of IR, suggesting that the endogenous BRCA1 in S phase cells was sufficient to play an antagonistic role after 0.5 Gy but insufficient after 3 Gy of IR (Fig. 3D and 3E). This was confirmed by the observation that BRCA1 overexpression reduced phospho-53BP1 and RIF1 foci, leading to an increased focus formation of RAD51 after 3 Gy of IR (Fig. 3D, 3E, 3F and S3F). These results demonstrated that the HRR activity was intact after 0.5 Gy of IR due to the prominent antagonistic effect of BRCA1 in S phase, while this antagonistic effect was possibly cancelled by an enhanced activity of checkpoint kinases such as ATM after 3 Gy of IR.

IR suppresses DNA replication through the accumulation of RIF1

A foresighted work demonstrated that RIF1 depletion in HeLa cells led to a defect in the intra-S phase checkpoint, as evidenced by radioresistant DNA synthesis (RDS) (Silverman et al., 2004). On the contrary, RIF1 has been characterized as an HRR suppressor (Chapman et al., 2013; Di Virgilio et al., 2013; Escribano-Díaz et al., 2013; Zimmermann et al., 2013). Thus, it
remains elusive whether RIF1 functions in intra-S phase checkpoint and in HRR suppression simultaneously or independently. As we showed an IR dose-dependency of RIF1-mediated HRR suppression (Fig. 2), we investigated relationship between RDS and IR doses. To analyze the intra-S phase checkpoint, HCT116 cells were synchronized in G1 phase by a lovastatin treatment. Once released, the cells in early S phase were irradiated with 0.5 or 3 Gy of IR and S phase progression was monitored. IR exposure of 3 Gy delayed the S phase progression in control cells, whereas 0.5 Gy did not have any impact on the progression (Fig. S4A). In contrast, RIF1-depleted cells progressed without any delay even after 3 Gy of IR (Fig. S4A). Furthermore, DNA synthesis was directly measured by 5-ethyl-2′-deoxyuridine (EdU) incorporation after IR exposure. DNA synthesis in control cells was reduced after 3 Gy but not after 0.5 Gy of IR, and this reduction was alleviated by RIF1 depletion, displaying the IR dose-dependent RDS phenotype (Fig. 4A and S4B).

The DNA synthesis rate originates from two mechanisms: replication initiation (activation of replication origins) and chain elongation (progression of the replication forks). Since elongation appears to be more radioresistant than replication initiation, as previously reported (Lavin and Schroeder, 1988), we next investigated replication initiation. To test this, we distinguished the replication forks by pulse-labeling cells with EdU and PCNA immunostaining (Ge and Blow, 2010). PCNA foci were categorized into two groups: (i) PCNA foci co-localized with EdU foci (origins at which replication had begun before IR exposure), and (ii) PCNA foci localized independently from EdU foci (origins at which replication was initiated after IR exposure, termed replication initiation) (Fig. 4B). The replication initiation (group ii) increased during S phase under non-irradiation conditions and, alternatively, the number of origins already activated (group i) gradually decreased as DNA synthesis at activated replicons was completed (Fig. 4C and 4D). When cells were irradiated with 3 Gy of IR, the number of replication initiations was significantly reduced 1 hour after IR exposure; however, this number was restored after 3 hours (Fig. 4E). Interestingly, RIF1 depletion alleviated the suppression of replication initiation upon 3 Gy of IR, restoring it to the levels observed in non-irradiated cells (Fig. 4F), indicating that RIF1 suppresses replication initiation after IR. Since Doksani et al. reported that DSB triggers firing of a dormant-origin near the DSB site, the effects of RIF1-deficiency on this type of firing was tested (Doksani et al., 2009). To detect the origin firing at DSB sites, we observed EdU incorporation at 53BP1 foci, which indicate DSB sites. After 3 Gy of IR, DNA synthesis rarely occurred at DSB sites in control cells, but increased more than 10-fold in RIF1-depleted cells (Fig. 4G and 4H). These results strongly suggest that
RIF1 has a role in inhibiting replication initiation near the DSB sites in cells exposed to high doses of IR. Consistent with previous work, RIF1 formed nuclear foci in S phase cells upon 3 Gy of IR (Silverman et al., 2004) and this foci formation was inhibited by an ATM inhibitor or by NBS1 depletion (Fig. S4C, S4D, and S4E).

**MCM helicase is a possible molecular link between replication and HRR**

Since RIF1 appeared to mediate inhibition of both replication initiation and HRR upon high-dose IR exposure, the effect of replication blockage in HRR was tested. Transient treatment of cells with a DNA polymerase inhibitor, aphidicolin, abolished RAD51 focus formation in S and G2 phase cells, suggesting an important role of replication in HRR (Fig. S5A and S5B). Recent studies reported that RIF1 binds to PP1 phosphatase and inactivates MCM helicase via the dephosphorylation of MCM2 and MCM4, blocking replication initiation (Alver et al., 2017; Hiraga et al., 2017). Indeed, the phosphorylation of MCM2 was markedly enhanced by RIF1 depletion, which was accompanied with a restoration of the replication block (Fig. 4F and S5C). Immunoprecipitation of RIF1 showed that the exposure to 3 Gy of IR promoted physical interaction between RIF1 and MCM2, implying that RIF1 might dephosphorylate MCM2 in this condition (Fig. S5D and S5E). This interaction is consistent with the obtained results, in which HRR inhibition was marginal after 0.5 Gy, but significant after 3 Gy of IR (Fig. 2A). The importance of MCM phosphorylation in HRR was demonstrated by experiments, in which the Cdc7 inhibitor, XL413 abolished DNA-end resection and subsequent HRR (Hughes et al., 2012) (Fig. 5A, 5B, and 5C). Treatment with XL413 suppressed RAD51 focus formation enhanced by RIF1 depletion to the level of the intact RIF1 control, implying that the phosphorylation of MCMs is a target for RIF1-dependent HRR suppression (Fig. 5D). These results prompted us to test HRR in the phospho-dead mutant of MCM2. Endogenous MCM2 protein was depleted by using auxin-inducible degron (AID) system (Natsume et al., 2017), and wild-type MCM2 or a phospho-dead (S40A, S53A, S108A: 3SA) MCM2 mutant was added back (Fig. 5E). RAD51 focus formation was abolished in MCM2-depleted cells, which is consistent with the observation in HeLa cells treated with siMCM2 (Fig. S5F). RAD51 focus formation was restored by wild-type MCM2 but not by the phospho-dead MCM2 mutant, indicating that the phosphorylation of MCM2 is critical for HRR (Fig. 5F and S5G).

Since the activity of MCMs is tightly regulated and is only active on chromatin during S phase (Chuang et al., 2009; Kang et al., 2012), we finally asked whether RIF1-dependent HRR suppression occurs only in S phase. To test this idea, HeLa cells were synchronized in G2 phase using a CDK inhibitor, RO-3306 (Vassilev et al., 2006). Cell cycle analysis with EdU and PI
staining showed that almost all cells arrested in G2 phase at 24 hours after RO-3306 addition (Fig. S6A). RAD51 focus formation after 3 Gy was not rescued in G2 phase cells by RIF1 depletion, which was in contrast to that of asynchronous cells containing S phase cells (Fig. S6B and S6C). Altogether, the present results suggest that RIF1 has a role in the suppression of HRR only in S phase, in which RIF1 dephosphorylates MCMs and inhibits replication initiation after a high dose of IR.

Discussion

This study demonstrated an IR dose-dependent HRR suppression, in which RIF1 accumulated at DSBs and protected DNA ends from resection, by suppressing replication initiation (Fig. 6). Although the protective role of RIF1 is the same as that in G1 phase (Chapman et al., 2013; Di Virgilio et al., 2013; Escribano-Díaz et al., 2013; Zimmermann et al., 2013), we found that the inhibition of HRR significantly depended on IR doses. Since the antagonistic effect of BRCA1 functions within low dose ranges, RIF1-mediated HRR inhibition seemed to have no or little effect after exposure to a low IR dose such as 0.5 Gy (Fig. 2). RIF1 has been considered as a key protein in the repair choice between HRR and NHEJ, and it was expected to promote NHEJ through HRR inhibition (Chapman et al., 2013; Di Virgilio et al., 2013; Escribano-Díaz et al., 2013; Zimmermann et al., 2013). However, NHEJ activity remained constant regardless of the IR dose, when measured with the pEJ reporter gene and ChIP assays (Fig. 1C and 1D). It appeared that RIF1 exclusively inhibits HRR during the S phase, rather than regulating the choice between repair pathways for rejoining DSBs.

Recently, Ochs and colleagues showed that the number of and the total intensity of RAD51 foci steeply increased at low IR doses, plateaued at intermediate doses, and then gradually declined after high doses such as 10 Gy in U2OS cells. They demonstrated that RAD51-dependent gene conversion, an error-free HRR, switched to RAD52-dependent single strand annealing, a mutagenic HRR, due to a lack of 53BP1 after extremely high IR doses (Ochs et al., 2016). While they demonstrated a dose-dependent suppression of error-free HRR, it is still unclear whether the intermediate doses of IR normally used in radiation therapy can alter the usage of repair pathways. We showed that error-free HRR was suppressed even after intermediate doses of IR such as 3 Gy and that this was controlled by RIF1.

Cells from patients with ataxia-telangiectasia (an ATM deficiency disease) and Nijmegen breakage syndrome (an NBS1 deficiency disease) display abnormal intra S phase checkpoints and RDS (Tauchi et al., 2002). Since RIF1 accumulation is compromised by treatment with an
ATM inhibitor or by NBS1 depletion (Fig. S4C, S4D, and S4E), the RIF1-mediated temporary replication block might be a mechanical basis for these phenotypes. Indeed, a similar defect in the intra-S checkpoint is reported by others using a RIF1-depleted cell, while it remains elusive how RIF1 regulates both the intra-S checkpoint and HRR in the presence of DSBs (Silverman et al., 2004). Our results suggest that RIF1 regulates the phosphorylation of MCM helicase to control both the intra-S checkpoint and HRR inhibition.

The phosphorylation of MCM helicase converts it into the active form, CMG complex, which in turn physically interacts with Polα primase at replication origins to initiate the replication (Gan et al., 2018). A recent paper showed that the 53BP1-RIF1 pathway recruits Polα primase to DSB sites through interaction with the CST complex, which contains proteins that are similar to RPA, resulting in an HRR regulation in G2 phase by Polα-dependent fill-in. Failure in this fill-in causes hyper-resection of DSB ends (Mirman et al., 2018), and indeed RIF1 depletion showed hyper-resection in asynchronous cultured cells (Fig. 2D and 2E). However, our results, in which RIF1-mediated HRR suppression occurred in S phase (Fig. S6), demonstrated that this phenomenon was strongly associated with DNA replication and activation of MCM helicase. Simple explanation for the association of MCM and HRR is that the helicase activity of MCM promotes HRR, as RAD54 helicase is essential for HRR (Essers et al., 1997), although we can not exclude other possibilities. More analyses of RIF1 and MCM helicase in each cell phase are needed to verify the extensive role of RIF1 in HRR regulation.

IR-induced reproductive cell death has been reported to depict a cell survival curve with two phases: an initial upwardly concave curve (the radioresistant region) at low IR doses, and a subsequent linear monotonically declining slope (the radiosensitive region) at high IR doses (Hall, 2006). Moreover, this radioresistant region at low IR doses is specifically observed in S phase (Hall, 2006). In radiation therapy, therapeutic doses are divided into individual fractions delivering low radiation doses, typically 2 Gy (Joiner, 2009). Normal human cells exposed to low doses within the radioresistant region are more likely to recovery from IR-induced DNA damage through a currently unknown mechanism. The present results show that this recovery might be attributed to HRR in S phase, while it disappears with increasing IR doses. If this dose-limited radioresistance could be maximized in normal cells or minimized in tumor cells by the regulation of RIF1, radiation therapy might be more effective for controlling tumors in some tissues. Further studies on the IR dose-dependence of RIF1-mediated HRR inhibition will provide insights into the basic mechanisms involved in HRR and contribute to advances in clinical development.
Materials and methods

Cell culture and transfection.

HeLa and U2OS cells were maintained in DMEM supplemented with 10% FBS. HCT116 cells were maintained in McCoy’s 5A medium supplemented with 10% FBS, 2 mM L-glutamine, 100 U/ml penicillin, and 100 mg/ml streptomycin. Genetic engineering in HCT116 cells was undertaken as described previously (Natsume et al., 2016). To induce the degradation of RIF1-mAID, 0.1 μg/ml doxycycline and 100 μM indole-3-acetic acid (IAA; a natural auxin, Nacalai Tesque) were added to the culture medium. For the degradation of MCM2-mAID, 500 μM IAA was added to the culture medium. The DR-GFP or pEJ reporter gene was stably integrated into the genomes of HeLa and U2OS cells. siRNA sequences were as follows: siNBS1, (#1, 5’-GUACGUUGUGAAGGAAA-3’; #2, 5’-GGGAAAGGGAUGAAGAAAA-3’; #3, 5’-GGACACAAAACCAGAGUUA-3’); siRIF1, 5’-GAAUGAGCCCCUAGGGAAA-3’; siMCM2, 5’-UCAUCGGAAUCCUUCACCA-3’; siBRCA1, siGENOME SMART pool, M-003461-02 (Thermo Scientific).

Reporter assay.

A total of 5×10^6 HeLa or U2OS cells containing the reporter genes were transfected with 30 μg of the pCBASce plasmid using a Gene Pulser (BIO-RAD) at 250V and 500 μF. Cells were plated and cultured for 4 hours before irradiation. Cells were irradiated with γ-rays and then cultured for 26 hours (for a total of 30 hours) before being analyzed with a FACSCalibur Flow Cytometer. In the treatment with XL413, transfected cells were plated on a medium containing 10 μM XL413 and cultured for 10 hours. Cells were washed and then cultured for 20 hours (for a total of 30 hours).

Synchronization.

Cells were synchronized as previously described (Zeng et al., 2009). Briefly, HeLa cells were cultured with 2 mM thymidine for 18 hours, washed, and then released into fresh medium for 10 hours. Cells were subsequently treated with 2 mM thymidine for 15 hours, then washed, and placed into fresh medium. HCT116 cells were synchronized as described previously (Natsume et al., 2017).
Immunofluorescence, immunoblotting, and antibodies.

HeLa cells were synchronized on coverslips in S phase as described above and irradiated with γ-rays. Cells were fixed with 2% FA in PBS (for RAD51, RPA2) or 100% methanol (for RIF1, phospho-53BP1) at each post-irradiation period. Aphidicolin or XL413 was added to the culture medium at a concentration of 0.5 μg/ml or 10 μM, respectively 2 hours prior to irradiation. The primary antibodies used for immunofluorescence were RAD51 (B01P; Abnova; 1:1000), RPA2 (Ab-2; Calbiochem; 1:200), RIF1 (A300-569A; Bethyl Laboratories Inc.; 1:1000), 53BP1 (MAB3802; Merck Millipore or A300-273A; Bethyl Laboratories Inc.; 1:1000), phospho-53BP1 T543 (3428; Cell Signaling Technology; 1:1000), MCM2 (D7G11; Cell Signaling Technology; 1:1000), and CENPF (ab5; Abcam; 1:2000). The secondary antibodies were Alexa Fluor-488, -546 (Molecular Probes), and -647 (life technologies) (1:500). DNA was stained with 4',6-diamidino-2-phenylindole (DAPI). Fluorescent images were collected with BZ-9000 (KEYENCE) or DeltaVision deconvolution microscope (GE Healthcare). Data analyses were performed by using the Volocity software (PerkinElmer). The primary antibodies used for immunoblotting were phospho-RPA2 S4/8 (A300-245A; Bethyl Laboratories Inc.; 1:5000), RPA2 (Ab-2; Calbiochem; 1:2000), ATM (GTX70103; GeneTex Inc.; 1:5000), phospho-53BP1 T543 (3428; Cell Signaling Technology; 1:5000), phospho-Chk2 T68 (2661; Cell Signaling Technology; 1:5000), BRCA1 (D-9; Santa Cruz; 1:2000), γ-actin (A5316; Sigma-Aldrich; 1:20000), phospho-MCM2 S53 (A300-756A; Bethyl Laboratories Inc.; 1:5000), MCM2 (D7G11; Cell Signaling Technology; 1:10000), and γ-tubulin (T9026; Sigma-Aldrich; 1:10000).

ChIP assay.

ChIP analyses were performed as previously described (Murr et al., 2005; Rodrigue et al., 2006), with minor modifications. Briefly, 6×10⁶ U2OS-DR-GFP cells were transfected with 50 μg of the pCBASce plasmid and cultured for 6 hours. Cells were irradiated with 10 Gy of γ-rays and cultured for an additional 4 hours. Cells were fixed with 1% formaldehyde for 10 min and then incubated with 0.125 M glycine for 5 min. Cells were left for 10 min in solution I (10 mM HEPES pH 7.4, 10 mM EDTA, 0.5 mM EGTA, 0.75% Triton X-100). Cells were precipitated and left for 5 min in solution II (10 mM HEPES pH 7.4, 200 mM NaCl, 1 mM EDTA, 0.5 mM EGTA). The cell pellet was resuspended in Nuclei Lysis Buffer (50 mM Tris-HCl pH 8.0, 10 mM EDTA, 0.5% SDS) and sonicated to shear genomic DNA at an average length of approximately 1,000 bp. The lysate was centrifuged to remove debris. The supernatant was diluted at a proportion of 1:5 with IP dilution buffer (20 mM Tris-HCl pH 8.0, 150 mM NaCl, 2 mM EDTA, 0.5% Triton X-100).
and pre-cleaned with protein A for 3 hours. Beads were centrifuged and the supernatant was incubated overnight with 1.5 μg of anti-Ku70 (Abcam, N3H10) or anti-RAD51 (Abnova, B01P) antibodies, followed by immunoprecipitation with 30 μl of protein A sepharose for 2 hours. Beads were washed twice with 0.5 M NaCl in RIPA buffer (20 mM Tris-HCl pH 8.0, 0.5 M NaCl, 2 mM EDTA, 0.1% SDS, 0.1% sodium deoxycholate, 1% NP-40), LiCl buffer (10 mM Tris-HCl pH 8.0, 250 mM LiCl, 1 mM EDTA, 0.5% NP-40, 0.5% sodium deoxycholate), and twice with TE buffer. Beads were treated with RNase A and with proteinase K, and were subsequently incubated in elution buffer (10 mM Tris-HCl pH 8.0, 300 mM NaCl, 5 mM EDTA, 0.5% SDS) at 65°C overnight. DNA was purified with the QIAquick Purification Kit (QIAGEN). The amount of precipitated DNA was quantified with real-time PCR using SYBR Premix Ex Taq II (Tli RNaseH Plus) (TaKaRa Bio Inc.). The primer sequences were: 78-239F, 5’-AGAAGCCAGGAGCAGGAG-3’; 78-239R, 5’-CCTCGCCCTTGCTCACCATG-3’; 207-415F, 5’-AAGGACGACGGCAACTACAAGAC-3’; 207-415R, 5’-TTGTGGCGGATCTTGAAGTTCACC-3’; 362-550F, 5’-CATGGCGACAAGCAGAAGAAC-3’; 362-550R, 5’-CGCTTCTCGTGGGGTGCTTTG-3’.

**Replication foci analysis.**

A replication foci analysis was performed as previously described (Ge and Blow, 2010), with minor modifications. HeLa cells were synchronized in S phase as described above. Four hours after the release, cells were cultured for 10 min with 10 μM EdU to label replication foci. After washing with DMEM medium, the cells were immediately exposed to 0.5 or 3 Gy of γ-rays. Cells were permeabilized 1 or 3 hours after irradiation with HLS buffer (10 mM Tris HCl pH 8.0, 0.5% NP-40, 2.5 mM MgCl₂) and fixed in methanol. Cells were stained with Alexa Fluor 488 azide, according to the manufacturer’s instructions (Click-iT EdU Imaging Kits; Invitrogen). Endogenous PCNA was stained with anti-PCNA (Santa Cruz, PC10) antibodies and Alexa Fluor 546. The pulse SIM fluorescence microscope, BZ-X700 (KEYENCE) was used to visualize replication foci in S phase cells. Images were analyzed with BZ-II Analyzer software. More than 50 cells from three sets of experiments were analyzed to calculate the mean focus number.
**Chromatin fractionation.**

Chromatin samples were prepared as described (Chiu et al., 2014). Briefly, HeLa cells were suspended with 0.1% NP-40 in cytoskeleton (CSK) buffer (20 mM HEPES pH 7.4, 50 mM NaCl, 3 mM MgCl₂, 300 mM sucrose) and incubated on ice for 10 min. After low-speed centrifugation at 4,000 rpm, the pellet was rinsed with 0.1% NP-40 in CSK buffer and centrifuged at 4,000 rpm. The supernatant was then clarified by centrifugation at 13,000 rpm and collected as the S fraction. The pellet was resuspended in IP buffer (20 mM HEPES pH 7.4, 150 mM NaCl, 1 mM EDTA, 0.5% NP-40, 10% glycerol) and left on ice for 20 min. After centrifugation at 13,000 rpm, the supernatant was collected as the P1 fraction. The pellet was further resuspended in IP buffer, sonicated, and incubated with 200 units/mL DNase I in the presence of 5 μM MgCl₂ and 1 μM CaCl₂ at 25°C for 30 min. After centrifugation at 15,000 rpm, the supernatant was used as the P2 fraction.

**Immunoprecipitation.**

Immunoprecipitation was performed as previously described (Ismail et al., 2015), with minor modifications. Briefly, HeLa cells were irradiated with γ-rays and cultured for 1 hour. Cells were washed, left for 20 min in IP buffer (20 mM HEPES pH 7.4, 150 mM NaCl, 1 mM EDTA, 0.5% NP-40, 10% glycerol), and sonicated. After centrifugation, the supernatant was incubated for 3 hours with anti-RIF1 (Bethyl Laboratories Inc., A300-569A) antibodies followed by immunoprecipitation with protein A sepharose for 2 hours. Beads were washed 4 times with 0.3 M NaCl in IP buffer. Samples were heated at 95°C in sample buffer and used for western blot analysis.

**Statistical analysis and reproducibility.**

Statistical analyses of reporter assays, focus formation, and ChiP assays were performed with the Student’s *t*-test, two-way ANOVA, followed by Tukey’s multiple comparisons test, or the Mann-Whitney U test. Statistical analyses of the replication foci studies were performed with the chi-squared test. All data from reporter assays and focus formation were pooled from two or three independent experiments. All western blot analyses were performed at least twice.
Acknowledgments

We thank Anne D Donaldson, Shin-ichiro Hiraga for critical reading of manuscript. Thanks to Toyoaki Natsume for discussion. We also thank Maria Jasin, David J Chen for providing the DR-GFP reporter construct, and the BRCA1 expression vector, respectively, and thank Minoru Takata for help with the utilization of a microscope.

Competing interests

The authors declare that no competing interests exist.

Funding

This work was supported by JSPS KAKENHI Grant Number JP26241013.
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Figure 1. Radiation inhibits HRR in a dose-dependent manner. (A) Schematic illustration of the HRR (DR-GFP) and NHEJ (pEJ) assays after irradiation. GFP-positive cells were scored as HRR or NHEJ frequencies. See also B and C. (B) Decrease in HRR after irradiation. HeLa cells were transfected with pCBASce and irradiated with the represented dose of IR 4 hours after the transfection. The GFP+ frequency was normalized to an un-irradiated control. Data represent the means of three independent experiments ± SDs. (C) No variations were seen in the NHEJ frequency after irradiation. (D) Decrease in RAD51 accumulation but not in Ku70 accumulation. ChIP assays were performed with extracts from U2OS cells, which were transfected with pCBASce and then irradiated with 10 Gy. The enrichment of DNA was determined by qPCR using primers that annealed at the indicated bps far from an I-SceI site. Data represent the means of two independent experiments ± SDs. (E) Number of RAD51 foci after irradiation. The irradiated cells were fixed 1 hour after irradiation and were stained with anti-RAD51 antibodies.
Data represent the means of three independent experiments ±SDs. (F) Signal intensity of individual RAD51 foci in Fig. 1E. Each dot in the bottom panel represents a single RAD51 focus (Mann-Whitney U test).
Figure 2. RIF1 inhibits HRR after exposure to high IR doses but not to low IR doses. (A) Restoration of radiation-induced HRR inhibition by RIF1 depletion. HeLa cells transfected with siCtrl or siRIF1 were analyzed with the DR-GFP assay as in Fig. 1B. (B, C) Restoration of 3 Gy-induced RAD51 foci by RIF1 depletion. HeLa cells transfected with siCtrl or siRIF1 were stained for RAD51 focus formation 1 hour after exposure to 0.5 Gy or 3 Gy. CENPF was used as a marker of the S/G2 cell cycle phases. (D) RPA2 focus formation at DSB sites. HeLa cells were fixed 1 hour after irradiation and were stained with anti-RPA2 antibodies. 53BP1 foci were used as markers of DSB sites. (E) Increased phosphorylation of RPA2 at Ser4/8 (pS4/8) after RIF1 depletion. HeLa cells transfected with siCtrl or siRIF1 were irradiated with 3 Gy and
collected 1 hour after irradiation, for sample preparation. RPA2pS4/8 and RPA2 proteins were
detected by western blot. Two-way ANOVA, followed by Tukey’s multiple comparisons
test was used for (A), (C), and (D).
Figure 3. BRCA1 antagonizes ATM-dependent RIF1 accumulation at low IR doses. (A, B) Marginal formation of 0.5 Gy-induced foci of RIF1 and phospho-53BP1 in S phase cells. HeLa cells were synchronized with a double thymidine block in G1 or S phases and were irradiated with 0.5 Gy or 3 Gy of IR. 0.5 hour after irradiation, cells were fixed and stained with anti-RIF1 (A) or anti-phospho-53BP1 (B) antibodies. Data represent the means of two independent experiments ±SDs. (C) Phosphorylation of 53BP1 and Chk2 after irradiation. HeLa cells synchronized in G1 or S phase were irradiated and collected 1 hour after irradiation for western blot with the indicated antibodies. (D, E) Phospho-53BP1 (D) or RIF1 (E) foci formation after irradiation in BRCA1-depleted and BRCA1-overexpressing cells. HeLa cells transfected with siCtrl, siBRCA1 or a BRCA1 expressing vector were stained with anti-phospho-53BP1 or anti-RIF1 antibodies 0.5 hour after exposure of 0.5 Gy or 3 Gy. (F) Enhanced RAD51 focus formation after exposure to 3 Gy with BRCA1 overexpression. HeLa cells transfected with empty or BRCA1 expressing vectors were stained for RAD51 focus formation 1 hour after exposure to 0.5 Gy or 3 Gy. Two-way ANOVA, followed by Tukey’s multiple comparisons test was used for (A), (B), (D), (E), and (F).
Figure 4. RIF1 inhibits replication initiation after exposure to high IR doses but not to low IR doses. (A) DNA syntheses after 3 Gy in siCtrl and siRIF1 cells. Incorporation of EdU was measured 1 hour after irradiation using a flow cytometer. (B) Schematic illustration of pulse-labeling and PCNA staining. HeLa cells synchronized in mid-S phase were pulse-labeled with EdU and stained with anti-PCNA antibodies after exposure to 0.5 Gy or 3 Gy of IR. (C, D) Time course of PCNA and EdU foci formation in non-irradiated HeLa cells. PCNA foci, co-localized with EdU foci (>0.05 µm² overlap area) or not (<0.05 µm² overlap area), were quantified in each nucleus. (E) Transient replication block after exposure to 3 Gy of IR. After pulse-labeling, cells were irradiated with 0.5 Gy or 3 Gy and were fixed 1 or 3 hours after IR exposure. Cells were stained for EdU and PCNA foci formation. PCNA foci without EdU foci were counted. (F) Restoration of the replication block after RIF1 depletion. HeLa cells transfected with siCtrl or siRIF1 were irradiated with 3 Gy and were fixed 1 hour after IR exposure. PCNA foci without EdU foci were counted. Data in E and F represent the means of three independent experiments ±SDs (Chi-squared test). (G, H) DNA syntheses at DSB sites were measured by the colocalization of EdU with 53BP1. Cells were irradiated with 3 Gy and fixed 1 hour after IR exposure. Cells were stained for EdU and 53BP1 foci formation. Frequency of 53BP1 foci colocalized with EdU were measured (Student’s t test).
Figure 5. Dephosphorylation of MCM2 inhibits DNA end resection in HRR. (A, B) Inhibition of DNA end resection by the DDK kinase inhibitor XL413. HeLa cells were treated with XL413 for 2 hours prior to irradiation and collected 1 hour after IR exposure. RPA2 focus formation was observed as in Fig. 2D. RPA2pS4/8 and RPA2 proteins were detected by western blot as in Fig. 2E. (C) Decrease in functional HRR at an I-SceI-induced two-ended DSB in the presence of XL413. After transfection with pCBASce, cells were treated with XL413 for 10 hours. DR-GFP assay was performed as in Fig. 1B (Student’s t test). (D) Inhibition of IR-induced RAD51 focus formation by XL413. HeLa cells, transfected with siCtrl or siRIF1 were treated with XL413 for 2 hours prior to irradiation. RAD51 focus formation was detected using anti-RAD51 antibodies 1 hour after exposure to 3 Gy of IR. Data represent the means of three independent experiments ±SDs. (E) Construction of MCM2 rescue experiment. (F) IR-induced RAD51 focus formation in MCM2 depleted, wt- or 3SA mutant-expressing cells.
HCT116 cells were transfected with MCM2 wild type or MCM2-3SA mutant expressing vectors. Endogenous MCM2 was depleted by AID system and RAD51 focus formation was detected using anti-RAD51 antibodies 1 hour after exposure to 0.5 Gy of IR. Data represent the means of two independent experiments ±SDs. Two-way ANOVA, followed by Tukey’s multiple comparisons test was used for (A), (D), and (F).
Figure 6. Model of RIF1-mediated HRR suppression in S phase. RIF1 accumulates at DSB sites and suppresses DNA replication initiation only after a high dose of IR. DNA replication promotes DNA end resection, RAD51 accumulation, and HRR, while RIF1 inhibits both DNA replication and HRR. This regulatory mechanism is specific for S phase, in which replication is active.
**Figure S1. Activities of HRR and NHEJ after radiation.** (A) No alterations in transfection efficiency after irradiation. HeLa cells transfected with pEGFP-N1 vector (Clontech) and exposed to the indicated doses of IR. The frequencies of GFP-expressing cells were measured as transfection efficiencies after irradiation. (B) Decrease in HRR after irradiation. U2OS cells harboring a DR-GFP reporter gene were irradiated and assayed for HRR activity as in Fig. 1B. Data represent the means of three independent experiments ±SDs (Student’s t test). (C) No significant alterations in NHEJ repair. U2OS cells harboring a pEJ reporter gene were assayed for NHEJ repair as in Fig. 1C. (D) Radiation dose-dependent phosphorylation of NHEJ factors, DNA-PKcs, and XRCC4. HeLa cells irradiated with each represented dose of IR were collected 1 hour after IR and prepared for western blot with phospho-specific antibodies. Ku70 was used as a loading control. (E) Time course of RAD51 focus formation after exposure to 0.5 Gy of IR. The irradiated cells were fixed at the indicated time point after IR and were stained with anti-RAD51 antibodies. Data represent the means of two independent experiments ±SDs.
**Figure S2. Rescue of HRR after high dose of radiation.** (A) Inhibition of DNA-PKcs did not rescue the suppression of HRR by IR. After treatment with a DNA-PKcs specific inhibitor (NU7026 at 2 μM for 2 hours), HeLa cells were transfected with pCBASce and were irradiated with the represented dose of IR 4 hours after the transfection. The GFP+ frequency was normalized to an un-irradiated control. Data represent the means of two independent experiments ±SDs. (B) Confirmation of RIF1 depletion after transfection of siRIF1. HeLa cells were transfected with siCtrl or siRIF1 and cultured for 3 days. Expression of RIF1 was detected by western blot. (C) Refer to Fig. 2D. RPA2 focus formation at DSB sites. HeLa cells were fixed 1 hour after exposure to 0.5 or 3 Gy of IR and stained with anti-RPA2 and anti-53BP1 antibodies. 53BP1 foci were used as a marker for DSB sites. (D) Cell cycle distribution after RIF1 depletion. HeLa cells were transfected with siCtrl or siRIF1 and cultured for 3 days. After fixation, the DNA content was measured using a flow cytometer.
Figure S3. Cell cycle dependence of 53BP1-BRCA1 circuit. (A) Cell cycle distribution after synchronization. Cells were synchronized with a double thymidine block and collected 12 hours (for G1 phase) or 2 hours (for S phase) after release. After fixation, the DNA content was measured using a flow cytometer. (B, C) Focus formation of phosho-53BP1, RIF1, and 53BP1 in G1 or S phase cells. HeLa cells were synchronized in G1 or S phases and irradiated with 0.5 or 3 Gy of IR. Cells were stained with antibodies against RIF1, phospho-53BP1 and 53BP1 0.5 hour after IR. (D) Quantitative analyses of the focus formations in B, C. Data represent the means of three independent experiments ±SDs (Student’s t test). (E) BRCA1 expression in G1 or S phase cells. HeLa cells transfected with siBRCA1 or with a BRCA1 expression vector were synchronized in G1 or S phases and BRCA1 expression levels were measured with western blot using anti-BRCA1 antibodies. The intensity of the BRCA1 band was normalized to that of a band from G1 phase. (F) Enhanced RAD51 focus formation after IR exposure of 3 Gy by
BRCA1 overexpression. HeLa cells transfected with empty or BRCA1-expressing vectors were stained with anti-RAD51 antibodies 1 hour after an IR exposure of 0.5 or 3.0 Gy.
Figure S4. DNA synthesis after radiation is regulated by ATM-NBS1-RIF1 axis. (A) HCT116-RIF1mAID cells were synchronized with lovastatin and released into G1 phase. Depletion of RIF1 was performed during cell cycle synchronization by treating cells with IAA and doxycycline. When a large part of the cells was in mid-S phase, cells were irradiated either with 0.5 Gy or 3 Gy of IR and collected at each represented time point. After fixation, DNA content was measured using a flow cytometer. (B) Refer to Fig. 4A. Incorporation of EdU was measured 1 hour after irradiation using a flow cytometer. (C, D, E) ATM/NBS1-dependency of phospho-53BP1 and RIF1 focus formations. HeLa cells were irradiated in the presence of 10 µM of an ATM inhibitor (KU55933) or after transfection with siNBS1. Cells were fixed and stained with anti-phospho-53BP1 or anti-RIF1 antibodies 0.5 hour after exposure to 3 Gy of IR. S/G2 phase cells were determined by the expression of CENPF. Data represent the means of three independent experiments ±SDs (Student’s t test).
**Figure S5. RIF1 targets MCM helicase to control HRR.**

(A) Cell cycle distribution after aphidicolin treatment for 2 hours. After fixation, the DNA content was measured using a flow cytometer. (B) Inhibition of IR-induced RAD51 focus formation by aphidicolin. HeLa cells were treated with aphidicolin for 2 hours prior to irradiation. RAD51 focus formation was detected using anti-RAD51 antibodies 1 hour after exposure to 0.5 Gy of IR. CENPF was used as a marker of the S/G2 cell cycle phases. Data represent the means of two independent experiments ±SDs. (C) Phosphorylation of MCM2 at Ser53 in HeLa cells treated with siRIF1 or with the Cdc7 inhibitor (XL413). MCM2 antibody, which recognizes MCM2 regardless of its phosphorylation status, was used as a loading control. (D) Distribution of RIF1 and MCM2 proteins. HeLa cells were fractionated into S, P1 and P2 (see Materials and Methods). Protein samples were subjected to western blot to determine RIF1 and MCM2 presences in each fraction. α-tubulin and Histone H3 were used as cytosol and chromatin markers, respectively. (E) Physical interaction between RIF1 and MCM2 in the absence or presence of DSBs. RIF1 was immunoprecipitated with anti-RIF1 antibodies from nuclear extracts of HeLa cells, which were prepared 1 hour after IR exposure. The interaction between RIF1 and MCM2 was detected...
by co-immunoprecipitation of MCM2. The numerals under each band represented the intensities of MCM2 normalized to an untreated control. (F) Inhibition of IR-induced RAD51 focus formation by MCM2 depletion. HeLa cells were transfected with siMCM2. RAD51 focus formation was detected using anti-RAD51 antibodies 1 hour after exposure to 0.5 Gy of IR. Data represent the means of two independent experiments ±SDs. (G) RAD51 foci formation in MCM2-depleted, wt- or 3SA-expressing cells. Endogenous MCM2 was depleted by AID system and wt- or 3SA-MCM2 was added back by transient transfection. RAD51 focus formation was detected using anti-RAD51 antibodies 1 hour after exposure to 0.5 Gy of IR. Two-way ANOVA, followed by Tukey’s multiple comparisons test was used for (B) and (F).
**Figure S6. RAD51 foci formation in G2 phase of cell cycle.** (A) HeLa cells transfected with siCtrl or siRIF1 were cultured in the presence of a CDK1 inhibitor, RO-3306 for 24 hours. Incorporation of EdU was measured using a flow cytometer. (B, C) Restoration of 3 Gy-induced RAD51 foci by RIF1 depletion was not observed in G2 phase cells. HeLa cells transfected with siCtrl or siRIF1 were synchronized with RO-3306 for 24 hours. RAD51 focus formation was detected using anti-RAD51 antibodies 1 hour after exposure to 3 Gy of IR. Data represent the means of two independent experiments ±SDs. Two-way ANOVA, followed by Tukey’s multiple comparisons test was used.