Rif1 Is required for resolution of ultrafine DNA bridges in anaphase to ensure genomic stability

RCC Hengeveld*, HR de Boer*, PM Schoonen, EGE de Vries, SMA Lens* and MATM van Vugt*
*equal contribution

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ABSTRACT

Sister-chromatid disjunction in anaphase requires the resolution of DNA catenanes by topoisomerase II together with PICH (Plk1-interacting checkpoint helicase) and BLM (Bloom’s helicase). We here identify Rif1 as a novel factor involved in the resolution of DNA catenanes that are visible as ultrafine DNA bridges (UFBs) in anaphase to which PICH and BLM localize. Rif1, which during interphase functions downstream of 53BP1 in DNA repair, is recruited to UFBs in a PICH-dependent fashion, but independently of 53BP1 or BLM. Similar to PICH and BLM, Rif1 promotes the resolution of UFBs; its depletion increases the frequency of nucleoplasmic bridges and RPA70-positive UFBs in late anaphase. Moreover, in the absence of Rif1, PICH or BLM more nuclear bodies with damaged DNA arise in ensuing G1 cells, when chromosome decatenation is impaired. Our data reveal a thus far unrecognized function for Rif1 in the resolution of UFBs during anaphase to protect genomic integrity.

Proper chromosome segregation in mitosis requires that chromosomes correctly attach to microtubules of the mitotic spindle. Upon silencing of the mitotic checkpoint, the cohesin complexes that hold sister chromatids together are cleaved by separase allowing sister chromatid separation in anaphase.\(^{(1)}\) Besides linkage by cohesin, sister chromatids are also physically connected by DNA catenanes.\(^{(2)}\)

Sister chromatid catenation is a direct and physiological consequence of DNA replication in S-phase.\(^{(3)}\) DNA catenanes require topoisomerase II activity for their resolution,\(^{(4)}\) a process which at chromosome arms is completed prior to metaphase.\(^{(5)}\) However, at centromeric regions catenanes persist until anaphase and are visible as ultrafine DNA bridges (UFBs).\(^{(6-8)}\) Alternatively, UFBs can also arise between common fragile sites (CFSs) at chromosome arms after induction of replication stress in the previous S-phase.\(^{(9)}\) UFBs differ from canonical bulky chromatin bridges in that they are devoid of histones and cannot be stained with conventional DNA dyes. Their presence can thus far only be demonstrated by immunofluorescence (IF) staining of proteins that bind to these DNA bridges, such as PICH, BLM and Replication Protein A 70 (RPA70).\(^{(7)}\) UFB resolution must be completed by the end of anaphase to ensure sister-chromatid disjunction.\(^{(6-8,10,11)}\) Exactly how UFBs are resolved, the factors required for UFB resolution and the consequences of defective UFB resolution for genome integrity are not completely understood.

PICH, a DNA translocase from the Swi/SNF family, and BLM, a RecQ family helicase, are thought to act in conjunction with topoisomerases (II α and III) to resolve UFBs.\(^{(6,12)}\) Here, we present Rif1 as a novel UFB binding protein. Originally identified as an interactor of the telomere-binding protein Rap1 in budding yeast,\(^{(13)}\) Rif1 was recently shown to function in DNA break repair downstream of ATM and 53BP1\(^{(14-19)}\) and in controlling replication.

Figure 1. Rif1 is localized to DNA double strand breaks and to UFBs in anaphase

A) Representative images of Rif1 and γ-H2AX during interphase and anaphase in non-transformed RPE-1 cells, 30 min. after 5 Gy irradiation. B) Quantification of average numbers of Rif1 foci per cell, with or without 5 Gy irradiation (IR) in RPE-1 cells (n=3). Error bars indicate standard deviations (SD, n>25 cells/condition). ** p<0.01, unpaired Student’s t-test.
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C) Synchronization protocol: RPE-1 cells were arrested in G2 phase using the reversible Cdk1 inhibitor RO-3306. Wash-out of RO-3306 allowed synchronous mitotic entry. Fifteen min. later, cells were treated with ICRF-193 (160nM). D, E) RPE-1 cells were treated as in C) and subsequently stained with Rif1 and CREST antibodies and DAPI. DMSO-treated or ICRF-193-treated anaphase cells were categorized based on the distance between chromosome packs. Number of Rif1-positive bridges per anaphase were scored. Error bars indicate standard deviation (SD, n>25 cells/condition). F, G) RPE-1 cells were treated as in C) and cells were stained for Rif1 and PICH (F) or Rif1 and BLM (G). See also Figure S1.
timing in situations of stress. We demonstrate that Rif1 plays a thus far unrecognized role in protecting the genome from damage through resolution of UFBs during anaphase.

RESULTS

Rif1 localizes to ultra-fine DNA bridges during anaphase

The cellular response to DNA damage is rewired during mitosis. While DNA double-strand breaks (DSBs) are normally detected in mitosis, downstream effectors, including 53BP1, are no longer recruited, most likely to prevent unwanted telomere fusions. In analogy to 53BP1, we found that Rif1 cannot be recruited to DNA DSBs during mitosis in untransformed RPE-1 cells (Figure 1A,B) and in MCF-7 and HeLa cells (Figures S1A,B). However, we noticed that in anaphase Rif1 localized to thread-like structures that bridged segregating chromosomes, irrespective of earlier inflicted DNA damage (Figure 1A). Although previous work suggested that Rif1 co-localizes with midzone microtubules, cold-induced depolymerization of midzone microtubules did not significantly affect Rif1 localization during anaphase, indicating that the majority of these thread-like structures does not reflect microtubules (Figures S1C,D).

Rif1-positive thread-like structures were present in high numbers at anaphase onset but progressively disappeared upon sister-chromatid segregation (Figure 1D,E). This localization pattern of Rif1 resembles that of PICH and BLM, which localize to ultrafine DNA bridges (UFBs) in early anaphase. In non-transformed and non-stressed cells, UFBs are mainly caused by catenated centromeric DNA that requires topoisomerase activity for its decatenation during anaphase. Since Rif1-positive threads appeared between centromeres in unperturbed RPE-1 cells (Figure 1D), it suggested that these UFBs reflected persistent DNA catenanes, rather than under-replicated fragile sites at chromosome arms that arise as a consequence of replication stress and that can be distinguished from centromeric UFBs by the presence of FANCD2 foci.

To investigate this, RPE-1 cells were released from a G2 arrest imposed by the Cdk1 inhibitor RO-3306 (Figure 1C). Fifteen min. after the release, cells were treated with a low concentration of the topoisomerase II inhibitor ICRF-193 to delay decatenation at anaphase onset (Figure 1C). This resulted in a significant increase in the number of Rif1-positive threads during early anaphase (Figure 1D,E). Moreover, these Rif1-positive threads were not flanked by FANCD2-positive foci (Figure S1E), suggesting that in both unperturbed and ICRF-193-treated cells Rif1 is indeed predominantly recruited to UFBs that reflect DNA catenanes. To further confirm that Rif1 associates with UFBs, we analyzed its co-localization with PICH and BLM. Indeed, Rif1 showed overlapping localization at anaphase bridges with both PICH and BLM (Figure 1F,G). The specificity of Rif1 localization at UFBs was verified by siRNA-mediated Rif1 depletion (Figure 2C-E), and by using GFP-tagged Rif1 (Figure S1E,F). Finally, although the centromeric UFBs we detected in unperturbed and ICRF-193-treated cells reflected catenated DNA, when we induced replication stress by treatment with aphidicolin (APH), we observed occasional UFBs that connected FANCD2 foci. Also to these UFBs Rif1 was recruited, suggesting that Rif1 is a common component of UFBs, irrespective of their origin (Figure S1E).

Rif1 recruitment to UFBs occurs independently of 53BP1, ATM and BLM but requires PICH
We next investigated the molecular requirements for Rif1 localization to UFBs. In mitosis the recruitment of 53BP1, and hence Rif1, to DSBs is suppressed by Cdk1-dependent phosphorylation of 53BP1 and RNF8 (Figure 1A and S1A,B). Interestingly, depletion of 53BP1 did not affect Rif1 localization at UFBs in anaphase (Figure 2A and S2A,G), while it did perturb Rif1 recruitment to irradiation-induced foci (IRIF) in interphase (Figure S2B,C). In fact, Rif1 recruitment to UFBs was independent of ATM signaling altogether as ATM inhibition did not prevent Rif1 recruitment to PICH-
positive UFBs (Figure 2B, S2D-F, S2I). Rif1 was previously shown to reside in a complex with BLM during S-phase, and its recruitment to stalled replication forks was delayed in BLM-deficient cells.\(^6,12,28,29\) BLM was therefore considered a likely candidate to mediate localization of Rif1 to UFBs. However, when we delayed UFB resolution by ICRF-193 treatment at anaphase onset, we found that Rif1 normally localized to UFBs in BLM-depleted cells (Figure 2C, E and S2L). In contrast, when we depleted PICH, Rif1 recruitment to UFBs was completely blocked (Figure 2D, E and S2K). Neither the localization of PICH nor BLM depended on the presence of Rif1 (Figure 2C-E and S2J,K). This demonstrates that BLM and Rif1 localize to UFBs independently of each other. However, Rif1 requires the presence of PICH to localize to UFBs, similar to the requirement of PICH for BLM recruitment to UFBs.

To investigate whether Rif1 and PICH are part of the same protein complex, we transfected GFP-Rif1 and FLAG-PICH into HEK293T cells and performed co-immunoprecipitation experiments. Precipitation of GFP-Rif1 pulled down FLAG-tagged PICH in HEK293T cells (Figure S2L), showing that Rif1 and PICH can form a complex in cells. This interaction depended on the N- and C-terminal TPR domains of PICH, since deletion of either the N-terminal 76 amino acids, or C-terminal 160 amino acids spanning these domains partially affected the interaction with Rif1, whereas deletion of both the N- and C-termini (PICH 76-1090) fully abrogated the interaction between Rif1 and PICH (Figures S2L). Of note, we were unable to detect endogenous Rif1 by western blot after PICH immunoprecipitation in either interphase, or anaphase cells suggesting that only a small fraction of Rif1 is associated with PICH. Deletion of the PICH TPR domains impaired kinetochore localization of PICH in mitosis, but did not affect PICH localization to UFBs in anaphase (Figure S2M). Surprisingly, however, PICH 76-1090 was still able to restore Rif1 localization to UFBs in PICH-depleted cells, suggesting that PICH does not recruit Rif1 to UFBs through direct or indirect protein interaction (Figure S2M).

### Rif1 recruitment to UFBs is suppressed by Cdk1 activity before anaphase

Before anaphase, cohesin is thought to shield centromeric DNA from topoisomerase II-mediated decatenation. In line with this notion, premature removal of centromeric cohesin in (pro)metaphase after depletion of the cohesin protector Shugoshin1 (Sgo1), resulted in the visualization of PICH-positive UFBs in prometaphase cells (Figure 2F-H). Remarkably, these UFBs did not contain Rif1 (Figure 2G,H), suggesting the recruitment of Rif1 to UFBs is somehow prevented before anaphase. Since cyclin B-Cdk1 activity is high until anaphase onset, we hypothesized that Cdk1 could prevent the recruitment of Rif1 to UFBs in (pro)metaphase. Indeed, after chemical Cdk1 inhibition, Rif1 was recruited to PICH-positive UFBs in Sgo1-depleted prometaphase cells (Figures 2G,H). From these data it can be inferred that Rif1 recruitment to UFBs, and most likely centromeric UFB resolution altogether, is inhibited by Cdk1 and as such restricted to anaphase.

### Rif1 is required for timely UFB resolution

PICH and BLM are thought to promote UFB resolution during anaphase and absence of these proteins leads to an increased frequency of histone-containing anaphase bridges.\(^6,11,12,23\) To understand the relevance of Rif1 at UFBs in anaphase, we depleted Rif1 with two independent siRNAs in H2B-YFP-
expressing HeLa cells, and monitored chromosome behavior using time-lapse microscopy. Whereas chromatin bridges were observed in approximately 10% of anaphases in control-depleted cells, ~30% of Rif1-depleted cells showed thin chromatin bridges during anaphase (Figures 3A,B, Movies S1, S2). Although sometimes hard to detect with H2B-YFP, these DNA bridges appeared

**Figure 3. Rif1 is required for proper sister-chromatid disjunction**

A) HeLa cells stably expressing YFP-H2B were transfected with Rif1 siRNAs. After a thymidine release the cells were analyzed by live cell video microscopy. Representative DIC and YFP stills of Movie S2 are shown. Arrowheads indicate nucleoplasmic bridges. B) HeLa-YFP-H2B cells were transfected with the indicated siRNAs and anaphases were quantified for nucleoplasmic bridges using live cell video microscopy (for examples see Movies S1-S4). Error bars indicate SD (n = 3 experiments, 30 cells/condition, ** p<0.01, unpaired Student’s t-test). C) RPA70 is recruited to persistent UFBs. HeLa cells were released from a RO-3306-inflicted G2 arrest and fixed 45 min. later. Cells were stained for RPA70. Representative late anaphase cell is shown. D) Cells were transfected with indicated siRNAs and treated as in (C). Anaphase cells were scored for the presence of RPA70 positive bridges. >100 cells/condition were analyzed. ** p<0.01; *** p<0.001 (unpaired Student’s t-test). E) HeLa cells were transfected with indicated siRNAs and treated as in (1C). Cells were fixed and stained for PICH and RPA70. Representative early and late anaphases are depicted. F) HeLa cells treated as in (E). Anaphase cells were categorized based on the distance between chromosome packs and the numbers of PICH and RPA70-positive bridges per anaphase were scored. Error bars indicate SD (n>25 cells/condition). See also Figure S3.
to persist during telophase given the presence of cytokinetic bridges (Figure 3A). Importantly, comparable increases of nucleoplasmic bridges were observed after PICH or BLM depletion (Figures 3B, Movies S3, S4), suggesting that PICH, BLM and Rif1 act together in resolving these DNA bridges.

To further characterize the DNA bridges that persisted in Rif1-depleted cells, we analyzed the presence of the ssDNA-binding protein RPA70, which was previously shown to be recruited to UFBs. Overall, depletion of Rif1 increased the frequency of cells with persistent RPA70-positive bridges in late anaphase (Figures 3C,D). In marked contrast, we failed to detect RPA70-positive UFBs in late anaphases of BLM-depleted cells (Figures 3B), despite the persistence of nucleoplasmic bridges (Figure 3B). This implies that BLM is (in)directly required for RPA70 recruitment to UFBs.

Because RPA70-positive UFBs have been described in cancer cell lines in which replication stress was induced (28), we tested whether the increased frequency of RPA70-positive UFBs after Rif1 depletion in otherwise unchallenged HeLa cells were an indirect consequence of stalled DNA replication. We therefore analyzed DNA replication in single DNA fibers after sequential CldU and IdU incorporation (Figure S3A). Whereas treatment with hydroxyurea (HU) clearly blocked ongoing replication, depletion of Rif1, PICH or BLM did not significantly alter replication progression (Figure S3A,B). Although indirect effects cannot be fully excluded, we deemed it more likely that the increased frequency of RPA70-positive UFBs in Rif1-depleted cells were not caused by replication stress. To assess whether RPA70 recruitment to UFBs in Rif1-depleted cells could thus be a consequence of impaired UFB resolution in anaphase, we inhibited topoisomerase II \( \alpha \) activity at anaphase onset to delay DNA decatenation (Figure 1C). Strikingly, this resulted in a dramatic increase in the appearance of RPA70-positive UFBs in anaphase (Figure 3E,F). In contrast to the decrease in PICH-positive threads upon anaphase progression, RPA70 recruitment to UFBs initially increased upon chromosome segregation, reaching a maximum when separating sister-chromatid packs attained a distance of \( \sim 10 \) \( \mu \)m (Figure 3F). At later stages of anaphase, RPA70 disappeared along with the resolution of PICH-positive fibers. Interestingly, also under these conditions we were unable detect RPA70 on UFBs when BLM was depleted (Figure 3F). Taken together, these data

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**Figure 4. Impaired UFB resolution increases frequency of micronuclei and 53BP1 nuclear body formation**

A) PICH, BLM, Rif1 and actin levels in the parental or indicated HAP1 knock-out cell lines determined by immunoblotting, (*) aspecific band. B) Parental HAP1 cells, or HAP1 cell lines harboring frame shift mutations in Rif1, BLM or PICH were analyzed for micronuclei (arrow in image). Mean ± SD of 3 experiments (>1,000 cells condition in each experiment). **p<0.01; ***p<0.001 (unpaired Student’s t-test). C, D) MCF-7 cells were transfected with indicated siRNAs and labeled with CldU and IdU according to the indicated scheme. Where indicated, cells were treated with ICRF-193 during IdU incubation, or with HU as a positive control. DNA was spread into single fibers and IdU track length was determined for 300 fibers per condition. Representative fibers are shown in (C), actual and average fiber lengths are plotted in (D). *p<0.05; ***p<0.001, n.s. = not significant (unpaired Student's t-test). E-G) MCF-7 cells were transfected with indicated siRNAs and treated for 24h with ICRF-193. 48 hours after transfection cells were fixed and stained for 53BP1. Nuclear 53BP1 bodies per cell were scored. Percentages are mean ± SD of 3 experiments with >400 cells per condition. Representative images of 53BP1 bodies in siRNA transfected MCF-7 cells are shown in (E). H) During anaphase, Rif1 and BLM are recruited to UFBs in a PICH-dependent fashion. In the absence of Rif1 UFB resolution is impaired. This gives rise to nucleoplasmic bridges in anaphase/ telophase, and to micronuclei and nuclear bodies with damaged DNA in G1. See also Figure S4.
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A

B

C

D

E

F

G

H

1.53µm
11.08µm
11.48µm
11.64µm
11.67µm
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**
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n.s.
n.s.

RIF1
PICH
BLM
Actin

Parental
ΔERCC6L
ΔBLM
ΔRIF1

HAP1 cell line

siRNA
+/- HU or ICRF

siLUC
siRif1 #1
siRif1 #2
siP ICH
siBLM

DAPI 53BP1 merge

siLUC
siRif1 #1
siRif1 #2
siP ICH
siBLM

ICRF-193

siLUC
siRif1 #1
siRif1 #2

G1 phase
53BP1 bodies
Micronuclei

TOPO

Anaphase, lack of RIF1

Telophase
Nucleoplasmic bridge

G1 phase
53BP1 bodies
Micronuclei
demonstrate that RPA70 is recruited to UFBs in a BLM-dependent manner when DNA decatenation is delayed, and that Rif1 is required for timely resolution of these UFBs.

**Rif1 depletion increases the frequency of micronuclei formation**

We next assessed whether impaired UFB resolution due to loss of Rif1 could have consequences for genomic integrity. Since knock-down of PICH and BLM was associated with micronuclei formation,\(^{(11)}\) we tested whether Rif1 inactivation would also give rise to micronuclei. In our hands, transient knock-down of Rif1, BLM or PICH in either RPE-1 or HeLa cells only induced a minor increase in micronuclei formation, compared to control cells. We therefore analyzed Rif1, BLM and PICH knock-out cells obtained through CRISPR/Cas9-mediated gene editing of HAP1 cells (Figure 4A).\(^{(34)}\) Prolonged inactivation of Rif1 significantly increased the frequency of HAP1 cells with micronuclei to a similar extend as PICH or BLM gene mutation (Figure 4B).

**Impaired UFB resolution gives rise to nuclear bodies with damaged DNA in G1**

Unresolved late-stage replication intermediates lead to the formation of nuclear bodies in ensuing G1 cells. These nuclear bodies consist of Mdc1 and 53BP1 among others, and shield sites of damaged DNA in nuclear compartments until recombination-mediated repair is available in the following S/G2 phase.\(^{(33,35)}\) Currently it is unclear whether these nuclear bodies can in fact originate from unresolved UFBs.

We therefore tested whether delayed UFB resolution per se, without prior DNA replication defects, gives rise to nuclear bodies in G1. To delay UFB resolution, we again used a low concentration of ICRF-193. To reassure that this treatment does not cause significant replication defects, especially when combined with Rif1, PICH or BLM depletion, we analyzed replication dynamics in MCF-7 cells using three independent assays. Firstly, global replication analysis by flow cytometry was used to show that low dose ICRF-193 treatment did not notably alter Edu incorporation, even when Rif1, BLM or PICH were depleted (Figure S4A,B). Secondly, mitotic cells were analyzed immediately after a 15' pulse of EdU to demonstrate that ICRF-193 treatment of control-depleted or Rif1-depleted cells did not result in any EdU incorporation in mitotic cells (Figure S4C,D). This indicated that active replication in these cells has finished well before mitotic entry.\(^{(10)}\) Thirdly, DNA replication speed measured at single DNA fiber resolution was also not significantly affected by the low dose of ICRF-193 that we used to increase the number of UFBs (Figure 4C,D). Importantly, depletion of neither Rif1, BLM nor PICH caused a decrease in replication speed in ICRF-193-treated cells (Figure 4C,D).

Having established that a low dose of ICRF-193 in combination with knock-down of Rif1, BLM or PICH did not notably delay replication progression, we used MCF-7 cell lines, stably expressing GFP-Mdc1 or GFP-53BP1, in combination with cyclin A staining to discriminate S/G2 cells from G1 cells to assess if impaired DNA decatenation would result in nuclear body formation in G1 (Figures S4E,F). Treatment with ICRF-193 alone resulted in the formation of Mdc1-GFP and GFP-53BP1 nuclear bodies in G1 phase (Figures S4E,F), and also resulted in nuclear bodies consisting of endogenous 53BP1 (Figure 4E). Importantly, we found that depletion of Rif1, PICH or BLM significantly increased the number of these 53BP1 nuclear bodies in ICRF-193-treated cells (Figure 4E,F). Of note, the increase in
53BP1 nuclear bodies after Rif1 depletion was comparable to the increase in PICH or BLM-depleted cells. Since PICH was not previously reported to play a role during S-phase, and even localizes to the cytoplasm during interphase, our data suggest that the observed nuclear 53BP1 bodies are due to an inability to resolve UFBs by a pathway comprising PICH, BLM and Rif1. To further strengthen this notion, we co-depleted PICH with Rif1 or PICH with BLM (Figure S4G). This did not lead to the formation of additional 53BP1 nuclear bodies compared to PICH-depleted cells (Figure 4G), supporting our findings that the localization of both Rif1 and BLM to UFBs is dependent on PICH (Figure 2), and strengthening the model that Rif1, PICH and BLM function in a similar pathway to resolve DNA catenanes during anaphase to ensure genomic integrity (Figure 4H).

DISCUSSION

We here uncovered a role for Rif1 in UFB resolution in anaphase. During interphase, Rif1 functions downstream of 53BP1 in controlling DNA double strand break repair choice, and timing of DNA replication. We here show that the recruitment of Rif1 to UFBs in anaphase is 53BP1 independent. Interestingly, while the cellular response to DNA damage is re-wired during the cell cycle, and mitosis specifically, also the here described role for Rif1 at UFBs appears to be subject to cell cycle regulation. In line with Cdk1-mediated inactivation of the 53BP1-Rif1 signaling axis during mitosis, also Rif1 recruitment to UFBs is inhibited by Cdk1 activity. These data point at a generic role for Cdk1 in suppressing the cellular response to DNA lesions during mitosis, both in response to DNA double-strand breaks as well as unresolved DNA catenanes.

Rif1 is recruited to UFBs in anaphase together with the BLM DNA helicase. Besides DNA helicase activity, also topoisomerase activity and regulatory factors including TopBP1 and RM1 are recruited to UFBs. This complex resembles the BTRR (BLM-Topoisomerase IIIα-RMI1-RMI2) complex that is recruited to resolve recombination intermediates and promote stalled replication recovery during S-phase. Our data show that the recruitment of BLM to UFBs in anaphase differs from recruitment of BLM to replication intermediates during S-phase. Whereas during S-phase, Rif1 appears to be the DNA binding interface mediating BLM recruitment, BLM recruitment to UFBs is independent of Rif1 but depends on PICH. These differential requirements may be necessitated by the fundamentally different chromatin state during anaphase, with elevated levels of tension and the absence of histones. Although PICH and Rif1 can be found in the same protein complex, this interaction does not appear to be required for the PICH-dependent loading of Rif1 on UFBs, implying an alternative mode of Rif1 UFB recruitment regulation. Since PICH functions as DNA translocase, it suggests a DNA remodeling role for PICH at UFBs. We propose this may enhance the accessibility of DNA for Rif1, without PICH directly recruiting Rif1.

We found that the ssDNA-binding protein RPA70 was recruited to UFBs especially when UFB resolution was delayed by topoisomerase II inhibition, and the localization of RPA70 to UFBs was completely dependent on the presence of BLM. RPA70 recruitment to UFBs most likely reflects ssDNA generation given that RPA70 only binds ssDNA efficiently. As such, RPA70 recruitment may reflect BLM DNA helicase activity, with Rif1 having an inhibitory effect on BLM activity.
at UFBs. This idea is in line with a previously reported genetic interaction between Rif1 and BLM, in which Rif1 inhibits BLM function. This latter observation, however, was made in the context of eroded telomere processing, and it is unclear whether BLM and Rif1 interact similarly at UFBs. Since RPA showed preferential recruitment to longer UFBs when compared to optimal PICH recruitment, we cannot formally exclude the possibility that DNA under high tension may adopt alternative confirmations in which bases are exposed that allow interaction with RPA70. Clearly, future studies are required to uncover how Rif1, BLM and PICH act at the molecular level to resolve UFBs.

Finally, we demonstrated that impaired UFB resolution gives rise to nuclear bodies with damaged DNA in G1. The inability to properly resolve DNA catenanes or other late-stage replication intermediates that lead to UFBs in anaphase could thus lead to accumulation of genomic lesions and may as such contribute to tumorigenesis.

**EXPERIMENTAL PROCEDURES**

**Synchronization and treatment of cell lines.** The following cell lines were used: HeLa, MCF-7, HAP1, RPE-1, 293T. HeLa and RPE-1 cells were blocked in G2 phase using RO-3306 (5 μM and 7.5 μM respectively, Calbiochem) for 18 hours. Fifteen min. after release from the RO-3306 block, ICRF-193 was added (160nM, Sigma). Where indicated, cells were irradiated using a Cesium137 source (CIS international/IBL 637), transfected with 20nM of the indicated siRNAs using HyPerfect or treated with the indicated inhibitors.

**Microscopy.** Immunofluorescence microscopy was done with a Leica DM-6000 microscope, equipped with a DFC360FX camera, a CTR6000 Xenon light source, 63x objective and LAS-AF software (Leica). Alternatively, a DeltaVision Elite microscope, equipped with a CoolSNAP HQ2 camera and 100X objective was used to analyze HeLa cells, expressing YFP-tagged Histone-H2B. Live cell immunofluorescence microscopy was done using a Zeiss Axiovert 200M microscope, equipped with a 40x objective.

**DNA replication and nuclear body formation.** At 48 hours after siRNA transfection, MCF-7 cells were incubated with Edu (10 μM), CldU (25 μM) or IdU (250 μM), and fixed in 70% ethanol for flow cytometry, in formaldehyde (3.7%) for microscopy, or processed for single DNA fiber analysis. At least 300 fibers were analyzed per condition. Nuclear body formation was assessed in MCF-7 cells expressing Mdc1-GFP or GFP-53BP1, or through staining of formaldehyde-fixed cells for endogenous 53BP1.

**Flow cytometry.** Cells were fixed in 70% ethanol and stained with propidium Iodide (50 μg/ml)/RNAse (100 μg/ml). Incorporated Edu was labeled with Alexa-488 for 30 min. using click chemistry (Molecular Probes). At least 5,000 events were analyzed per sample on a FACS-Calibur (Becton Dickinson) using Cell Quest software (Becton Dickinson).

**Statistical analysis.** Data are shown as mean ± SD where indicated. An unpaired Student’s t-test or Mann-Whitney U test was performed using GraphPad statistical analysis, and p-values ≤0.05 were considered significant.

**Supplemental Movies.** Supplemental Movies 1–4 can be found online at Developmental Cell.
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SUPPLEMENTAL EXPERIMENTAL PROCEDURES

Cell lines and treatment. Human cervical cancer HeLa cells were cultured in a 1:1 mixture of DMEM (Gibco) and Ham’s F12 (Gibco) medium, supplemented with penicillin (100 units/mL), streptomycin (100 μg/mL) (Gibco) and 10% fetal calf serum (Bodino Bio). MCF-7 human breast cancer cells were cultured in RPMI-1640 (Gibco) medium supplemented with penicillin (100 units/mL), streptomycin (100 μg/mL) and 10% FCS. Human retinal pigment epithelium (RPE-1) cells and human embryonic kidney (HEK) 293T cells were cultured in DMEM medium supplemented with penicillin (100 units/mL), streptomycin (100 μg/mL) and 10% FCS. HEK293T cells were obtained from Haplogen GmbH (Vienna, Austria) and maintained in IMDM medium (Gibco), supplemented with penicillin (100 units/mL), streptomycin (100 μg/mL) (Gibco) and 10% FCS. CRISRP/Cas9-mediated gene targeting was used to generate ΔRif1 (Guide RNA sequence: ACTCAGCTCCGAGTTTTGAC, causing a 7-bp deletion in exon 4, creating a frameshift), ΔPICH (Guide RNA sequence: GGGCTCAAGGCCTCGGCTTC, causing a 2 bp deletion in exon 1, creating a frameshift) and ΔBLM (Guide RNA sequence: AGATTTCGATGACGATGC, causing a 5 bp deletion in exon 3, creating a frameshift).

For retroviral short-hairpin shRNA delivery, MCF-7 or HeLa cells were retrovirally infected with VSV-G pseudotyped viruses containing control pRetrosuper (scrambled: 5'-TTCTCCGAACGGTGCACGT-3') or pRetrosuper-53BP1 (53BP1-targeting sequence: 5'-GAACGAGGAGACGGTAATA-3') as described previously. In short, HEK293T cells were transfected with indicated pRetrosuper plasmids along with pMDG and pMDG/p in a 3:2:1 ratio. Twenty-four hours after transfection, medium was replaced. Subsequently, virus-containing medium was collected, filtered using a 0.45 μm PVDF syringe filter (Millipore) and used for three consecutive 12 hour periods to infected target cells. At 24 hours after the last infection, cells were selected with 2μg/ml puromycin. For expression of GFP-Rif1, the pDEST pcDNA5/FRT/TO-eGFP plasmid containing human Rif1 was kindly provided by Dr. Daniel Durocher (University of Toronto, Canada).

Full length human PICH and indicated fragments were generated by PCR on a human cDNA library and ligated into the pCR3 vector (Invitrogen), containing an N-terminal FLAG tag or into pcDNA5/FRT/TO (Invitrogen), containing an N-terminal AcGFP-tag. HeLa FLP-In cells (Life Technologies) were transfected with pcDNA5/FRT/TO containing eGFP-Rif1, AcGFP-PICH or AcGFP-PICH 76-1090 along with pOG44, encoding the Flp recombinase, (Invitrogen) using Xtreme Gene 9 DNA Transfection Reagent (Roche). At 48 hours after transfection, cells with successful integration were selected with 400μg/ml hygromycin (Invitrogen) and expanded as polyclonal cell lines. GFP-Rif1 expression was induced for 48 hours with 2μg/ml doxycycline (Sigma), and GFP-Rif1 positive cells were FACS-sorted and expanded as monoclonal cell lines for further use.

Where indicated, cells were gamma-irradiated using CIS Bio international/IBL 637 irradiator, equipped with a Cesium137 source (dose rate: 0.01083 Gy/s), or treated with 10μM ATM inhibitor KU-55933 (Axon Medchem, Groningen, the Netherlands), 160nM ICRF-193 (Sigma Aldrich), 5 or 7.5μM RO-3306 (Axon Medchem), 0.15, 0.2 or 2μM Aphidicolin (Sigma Aldrich), 2.5 μM thymidine, 2 or 5mM hydroxyurea (Sigma Aldrich), or 5μM MG132 (Sigma Aldrich).

RNA interference. MCF-7, HeLa, or RPE-1 cells were transfected at approximately 80% confluence with the following siRNA’s targeting Rif1: 5’-GACTCACATTTCGATCAA-3’ (Rif1#1), and 5’-CCAGUGACUUUGGGCAUAAUUCUUU-3’ (HSS124069, Rif1#2). For BLM depletion we used either 5’-ACAGGGAUUCAUGAGAGAUGUUAA-3’ (HSS101023) or 5’-GGAGCACATCTGTAAT-
Rif1 positive cells were FACS-sorted and expanded as monoclonal cell lines for further use. Full length human PICH and indicated fragments were generated by PCR on cDNA and ligated into pCR3 vector (Invitrogen), containing an N-terminal FLAG tag.

**Antibodies.** The following antibodies were used: BLM pAb (#ab2179) and FANCD2 mAb (#ab108928) were from Abcam. PICH mAb (H00054821, Abnova, Figure 2A, B). Rif1 pAb (A300-568A, Bethyl Laboratories). FLAG mAb (F425) was from Sigma. Phospho-Thr68-Chk2 pAb (2197), phospho-Ser139-H2AX pAb (9718) and RPA70 pAb (2267) were from Cell Signaling. 53BP1 pAb (sc22760), BLM pAb (sc1611), CDK4 pAb (sc-260-g) and Chk2 pAb (sc56297) and GFP mAb (sc9996) were from Santa Cruz. Phospho-Ser139-H2AX (05-636), PICH mAb (142-26-3) and MPM2 mAb (05-368) were from Millipore and mouse anti-Actin was from MP Biomedicals (#69100). CREST pAb (cs-1058) was from Cortex Biochem. For immunoblotting, HRP-conjugated secondary antibodies (DAKO P044801; P026002) were used in combination with enhanced chemiluminescence (ECL) using Lumi-Light (Roche). Membranes were visualized using a ChemiDoc in combination with Quantity One 4.5.0 software (Bio-Rad).

Alexa-488, Alexa-568, and Alexa-647-conjugated secondary antibodies (Invitrogen A11008; A11001; A21134; A21235; A21244) were used for immunofluorescence microscopy and flow cytometry.

**Immunoprecipitation.** HEK293T cells were transfected with pRetrosuper plasmids along with pMDG/p in a (3:2:1) ratio. Twenty-four hours after transfection, medium was replaced. Subsequently, virus-containing medium was collected, filtered using a 0.45 μm PVDF syringe filter (Millipore) and used for three consecutive 12 hour periods to infected target cells. At 24 hours after the last infection, cells were selected with 2μg/ml puromycin. For expression of GFP-Rif1, the pDEST pcDNA5/FRT/TO-eGFP plasmid containing human Rif1 was kindly provided by Dr. Daniel Durocher (University of Toronto, Canada). (16) HeLa FLP-In cells were transfected with pcDNA5/FRT/TO-eGFP-Rif1 along with pOG44, which encodes the Flp recombinase, (Invitrogen) using Xtreme Gene 9 DNA Transfection Reagent (Roche). 48 hours after transfection, cells with successful integration of GFP-RIF1 were selected with 400μg/ml hygromycin (Invitrogen) and expanded as polyclonal cell line. GFP-Rif1 expression was induced for 48 hours with 2μg/ml doxycyclin (Sigma), and GFP-Rif1 positive cells were FACS-sorted and expanded as monoclonal cell lines for further use. Full length human PICH and indicated fragments were generated by PCR on cDNA and ligated into pCR3 vector (Invitrogen), containing an N-terminal FLAG tag.

Plasmids and transfections. For retrovirial short-hairpin shRNA delivery, MCF-7 or HeLa cells were retrovirally infected with VSV-G pseudotyped viruses containing control pRetrosuper (scrambled: 5'-TTCTCCGACGGTACGT-3') or pRetrosuper-53BP1 (53BP1-targeting sequence: 5'-GAACGAGGAGACGGTACGT-3') as described previously.(46) In short, HEK293T cells were transfected with indicated pRetrosuper plasmids along with pMDG and pMDG/p in a (3:2:1) ratio. Twenty-four hours after transfection, medium was replaced. Subsequently, virus-containing medium was collected, filtered using a 0.45 μm PVDF syringe filter (Millipore) and used for three consecutive 12 hour periods to infected target cells. At 24 hours after the last infection, cells were selected with 2μg/ml puromycin. For expression of GFP-Rif1, the pDEST pcDNA5/FRT/TO-eGFP plasmid containing human Rif1 was kindly provided by Dr. Daniel Durocher (University of Toronto, Canada). (16) HeLa FLP-In cells were transfected with pcDNA5/FRT/TO-eGFP-Rif1 along with pOG44, which encodes the Flp recombinase, (Invitrogen) using Xtreme Gene 9 DNA Transfection Reagent (Roche). 48 hours after transfection, cells with successful integration of GFP-RIF1 were selected with 400μg/ml hygromycin (Invitrogen) and expanded as polyclonal cell line. GFP-Rif1 expression was induced for 48 hours with 2μg/ml doxycyclin (Sigma), and GFP-Rif1 positive cells were FACS-sorted and expanded as monoclonal cell lines for further use. Full length human PICH and indicated fragments were generated by PCR on cDNA and ligated into pCR3 vector (Invitrogen), containing an N-terminal FLAG tag.

Antibodies. The following antibodies were used: BLM pAb (#ab2179) and FANCD2 mAb (#ab108928) were from Abcam. PICH mAb (H00054821, Bethyl Laboratories). FLAG mAb (F425) was from Sigma. Phospho-Thr68-Chk2 pAb (2197), phospho-Ser139-H2AX pAb (9718) and RPA70 pAb (2267) were from Cell Signaling. 53BP1 pAb (sc22760), BLM pAb (sc1611), CDK4 pAb (sc-260-g) and Chk2 pAb (sc56297) and GFP mAb (sc9996) were from Santa Cruz. Phospho-Ser139-H2AX (05-636), PICH mAb (142-26-3) and MPM2 mAb (05-368) were from Millipore and mouse anti-Actin was from MP Biomedicals (#69100). CREST pAb (cs-1058) was from Cortex Biochem. For immunoblotting, HRP-conjugated secondary antibodies (DAKO P044801; P026002) were used in combination with enhanced chemiluminescence (ECL) using Lumi-Light (Roche). Membranes were visualized using a ChemiDoc in combination with Quantity One 4.5.0 software (Bio-Rad).

Alexa-488, Alexa-568, and Alexa-647-conjugated secondary antibodies (Invitrogen A11008; A11001; A21134; A21235; A21244) were used for immunofluorescence microscopy and flow cytometry.

**Immunoprecipitation.** HEK293T cells were transfected with plasmids encoding human Rif1 or GFP-Rif1 under control of a tetracycline-inducible promoter. After 48 hours, medium was replaced and cells were harvested in lysis buffer (50 mM Tris–HCl pH 7.7; 150 mM NaCl; 0.5% (v/v) Nonidet P-40 (Sigma), supplemented with protease inhibitor cocktail (Roche). After sonification, GFP-Rif1 was immunoprecipitated using GFP-Trap beads (ChromoTek). After extensive
RIF1 is recruited to UFBs and promotes their resolution in anaphase.

Flow cytometry. For flow cytometry, at least 5,000 events were analyzed per sample on a FACS-Calibur (Becton Dickinson) using Cell Quest software (Becton Dickinson).

Immunofluorescence and flow cytometry. HeLa, MCF-7, HAP1 or RPE-1 cells were grown on glass cover slips for at least 48 hours to a maximum confluence of 80%. Cells were then fixed with 3.75% formaldehyde (Sigma) or paraformaldehyde (Sigma) in phosphate-buffered saline (PBS) for 5 minutes. Cells were permeabilized for 5 minutes in 0.1% Triton-X100 (Sigma) in PBS or PBS containing 0.5% NP40 (Sigma). Cells were subsequently blocked in PBS containing 0.05% Tween-20 (Sigma) and 2.5% bovine serum albumin (BSA; GE Healthcare). Cells were incubated with primary antibodies in PBS/Tween-20/BSA for 16 hours, followed by extensive washing and incubation with secondary antibodies. Subsequently, cells were incubated with Hoechst 33342 (Sigma) or 500 ng/mL DAPI (Sigma) before mounting slides with Kaiser's glycerol/gelatine (Sigma) or with ProLong Gold antifade reagent (Life Technologies). Micronuclei analysis in HAP1 cell lines was performed in three independent experiments, with at least 1,000 cells analyzed per experiment per cell line. In order to visualize DNA replication, cells were incubated with 5-Ethynyl-2′-deoxyuridine (EdU; final concentration 10μM) for 30 minutes. Cells were subsequently harvested by trypsinization and fixed in 70% ice-cold ethanol for flow cytometry analysis, or alternatively in 3.7% formaldehyde for microscopy. Incorporated Edu was subsequently labeled with Alexa-488 using click chemistry by incubating in staining buffer (100mM Tris pH8.5, 1mM CuSO4, 100mM L-ascorbic acid), supplemented with 10μM Alexa-488-azide (Invitrogen, A10266) for 30 minutes at room temperature in the dark. Cells were subsequently counterstained with propidium Iodide (50μg/ml)/RNAse (100μg/ml) for flow cytometry or with DAPI for microscopy analysis. Statistical analysis was performed using two-sided Mann-Witney tests with 95% confidence intervals. For flow cytometry, at least 5,000 events were analyzed per sample on a FACS-Calibur (Becton Dickinson) using Cell Quest software (Becton Dickinson).
Figure S1, related to Figure 1: Cell cycle-dependent localization of Rif1 to IRIF.

A) Rif1 localizes to irradiation-induced foci (IRIF) during interphase, but not in mitosis. Representative images of Rif1 and γ-H2AX localization to IRIF in MCF-7 cells during interphase or the various stages of mitosis at 30 minutes after irradiation with 5 Gy. B) Quantification of average numbers and standard deviations of Rif1 foci from a representative experiment in MCF-7 cells before irradiation (interphase n=50, mitosis n=69) or after 5 Gy irradiation (interphase n=48, mitosis n=61) and in HeLa cells before irradiation (interphase n=31, mitosis n=40) or after 5 Gy irradiation (interphase n=25, mitosis n=47). C) Rif1 recruitment to anaphase bridges is independent of microtubules. MCF-7 cells were treated with ICRF-193 (160nM) to induce Rif1-positive anaphase bridges. At 1 hour after ICRF-193 treatment, cells were treated with a cold-shock to destabilize central spindle microtubules, fixed and stained for α-Tubulin and Rif1. D) Quantification of results from panel C. Anaphase cells from untreated (n=42) or cold treated (n=30) conditions were analyzed for the presence of Rif1-positive threads. E) HeLa with stable inducible expression of GFP-Rif1 were treated with doxycycline (DOX) for 24 hours and treated with ICRF-193 (160nM) or Aphidicolin (APH, 150nM). After one hour of ICRF-193 treatment or 24 hours of APH treatment, cells were fixed and stained for FANCD2 and DAPI. F) HeLa cells stably expressing inducible GFP-Rif1 were treated with doxycycline for 24 hours and subsequently stained for PICH and DAPI.
Figure S2, related to Figure 2: Rif1 recruitment to anaphase bridges is independent of 53BP1 and ATM, but dependent of PICH.
Figure S2, related to Figure 2: Rif1 recruitment to anaphase bridges is independent of 53BP1 and ATM, but dependent of PICH
A) HeLa and MCF-7 cells were stably infected with retroviral short hairpins targeting 53BP1 or scrambled sequence (pRS-53BP, pRS-SCR). Levels of 53BP1 were assessed by immunoblotting. B) IR-induced Rif1 foci formation in interphase cells is 53BP1-dependent. MCF-7 cells expressing pRS-SCR or pRS-53BP1 were fixed at 1 hour after irradiation with 5 Gy. Cells were stained for Rif1 and nuclei were stained with DAPI. C) MCF-7 cells and HeLa cells were treated as for panel C). Rif1 foci per interphase cell in HeLa or MCF-7 cells from one representative experiment are plotted. Numbers of analyzed cells per condition are indicated in the graph. *** p<0.001 (unpaired two-sided Student’s t-test). D) ATM inhibition using KU-55933 prevents Chk2 phosphorylation. One hour prior to irradiation (5 Gy) HeLa and MCF-7 cells were treated with KU-55933. Chk2 phosphorylation at Thr-68 was assessed by immunoblotting. E) IR-induced Rif1 foci formation in interphase cells depends on ATM activity. MCF-7 cells were treated with KU-55933 at 1 hour prior to irradiation (5 Gy), and fixed 1 hour after irradiation. Cells were stained for Rif1 and nuclei were stained with DAPI. F) Quantification of numbers of Rif1 foci per cell in HeLa or MCF-7 cells as shown in E). Rif1 foci per interphase cell in HeLa or MCF-7 cells are indicated from one representative experiment. Numbers of analyzed cells per condition are indicated in the graph. *** p<0.001 (unpaired Student’s t-test). G) Rif1 localizes to PICH-positive UFBs independent of 53BP1. MCF-7 cells and HeLa cells infected with pRS-SCR or pRS-53BP1 were co-immunostained for PICH and Rif1. Distance between sister chromatids (µm) was measured and plotted against the number of Rif1/PICH-positive UFBs. Indicated numbers of anaphases from one representative experiment are plotted. Numbers of analyzed cells per condition are indicated in the legend of the graph. H) Rif1 localization to PICH-positive UFBs is independent of ATM. MCF-7 and HeLa cells were treated with KU-55933 and cells were co-immunostained for PICH and Rif1 and analyzed as shown in G. Indicated numbers of anaphases from one representative experiment are plotted. Numbers of analyzed cells per condition are indicated in the legend of the graph. I) RPE-1 cells were transfected with indicated siRNA and levels of Rif1, PICH, BLM and Cdk4 were assessed by immunoblotting at 48 hours after transfection. J, K) HeLa cells were transfected with indicated siRNAs and treated as in Figure 1C. Cells were fixed and stained for Rif1 and PICH (j), or were stained for Rif1 and BLM (K) in combination with DAPI. L) GFP-Rif1 was immunoprecipitated from HEK293T cells expressing full length FLAG-PICH or indicated deletion mutants. Input samples (1%) and immunoprecipitations were immunoblotted for Rif1 and FLAG. Domain organization of PICH is indicated in the lower panel (TPR: tetratricopeptide repeats; SNF2: sucrose non-fermenting-family domain; PFD: PICH family domain). M) HeLa cells stably expressing doxycycline-inducible GFP-tagged PICH or GFP-tagged PICH 76-1090 were transfected with PICH siRNA. Cells were processed to visualize PICH and Rif1 or GFP and Rif1. Note that PICH-Rif1 protein complex formation (shown in L) is not required for PICH-dependent recruitment of Rif1 to UFBs.
RIF1 is recruited to UFBs and promotes their resolution in anaphase

Figure S3, related to Figure 3: Depletion of PICH, Rif1 or BLM does not result in DNA replication delay
A) MCF-7 cells were transfected with the indicated siRNAs and labeled with IdU and CldU according to the indicated time scheme. Where indicated, cells were treated with hydroxyurea (HU, 5mM) during CldU incubation. DNA was spread into single fibers and representative images of IdU/CldU tracks are shown. B) CldU track length was determined of at least 300 fibers per condition. Fiber length is indicated in µm. n.s = not significant; *** p<0.001 (unpaired two-sided Student’s t-test).
Figure S4, related to Figure 4: DNA replication in ICRF-193-treated cells with or without Rif1, PICH or BLM

A, B) MCF-7 cells were transfected with indicated siRNAs and treated with aphidicolin (APH) or ICRF-193 for 24 h incubation. 48 hours after transfection, cells were incubated with EdU for 15 minutes and subsequently fixed. EdU was conjugated to azide-Alexa488 and DNA was stained with PI/RNAse, and analyzed by flow cytometry. B) Quantification of Edu-Alexa488 signal in at least 5,000 cells per condition from panel A. C) Cells were treated as in panel A and EdU incorporation was analyzed by fluorescent microscopy. Edu was conjugated to azide-Alexa488 and DNA was stained with DAPI. Representative images of mitotic and interphase cells are indicated. D) Quantification of Edu-Alexa488 signal in mitotic and interphase cells from panel C. At least 120 cells were quantified per condition. n.s. = not significant; * p<0.05; *** p<0.001 (Mann-Whitney U test). E, F) MCF-7 cells stably expressing Mdc1-GFP (E) or 53BP1-GFP (F) were treated with or without ICRF-193 for 24 hours. Cells were subsequently fixed and stained with anti-cyclin A and DAPI. Representative images of Mdc1-GFP nuclear bodies (E) and 53BP1-GFP nuclear bodies (F) in cyclin A-negative cells are shown. Below microscopy images, quantifications of Mdc1-GFP and 53BP1-GFP bodies per cell are plotted in relation to cyclin A status. G) MCF-7 cells were transfected with indicated siRNAs and levels of Rif1, PICH, BLM and Actin were assessed by immunoblotting at 48 hours after transfection.
RIF1 is recruited to UFBs and promotes their resolution in anaphase