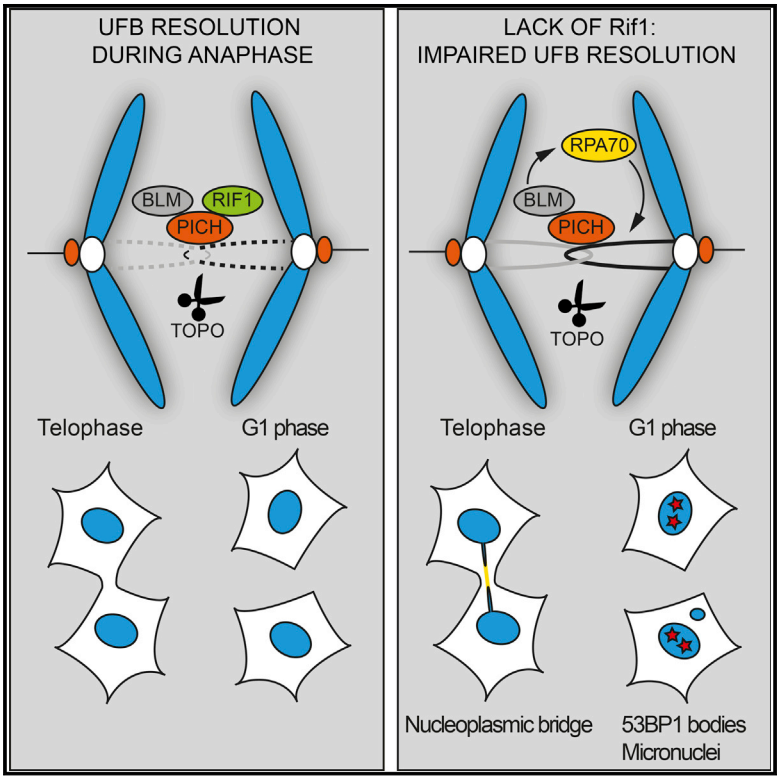
Short Article

Developmental Cell

Rif1 Is Required for Resolution of Ultrafine DNA Bridges in Anaphase to Ensure Genomic Stability

Graphical Abstract



Authors

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In Brief

Rif1 functions downstream of 53BP1 in DNA repair during interphase. Hengeveld et al. show that independent of 53BP1, Rif1 is required during anaphase for resolution of ultrafine DNA bridges (UFBs) that result from persistent DNA catenanes. Impaired UFB resolution gives rise to nuclear bodies with damaged DNA in daughter cells.

Highlights

- Rif1 has a 53BP1-independent function during anaphase
- Rif1 is recruited to anaphase UFBs in a PICH-dependent manner
- Rif1 promotes UFB resolution in anaphase
- Impaired UFB resolution gives rise to nuclear bodies with damaged DNA in G_1

Hengeveld et al., 2015, Developmental Cell *34*, 466–474
August 24, 2015
http://dx.doi.org/10.1016/j.devcel.2015.06.014

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Rif1 Is Required for Resolution of Ultrafine DNA Bridges in Anaphase to Ensure Genomic Stability

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SUMMARY

Sister-chromatid disjunction in anaphase requires the resolution of DNA catenanes by topoisomerase II together with Plk1-interacting checkpoint helicase (PICH) and Bloom's helicase (BLM). We here identify Rif1 as a 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 G_1 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.

INTRODUCTION

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 (Foley and Kapoor, 2013). Besides linkage by cohesin, sister chromatids are also physically connected by DNA catenanes (Mankouri et al., 2013).

Sister chromatid catenation is a direct and physiological consequence of DNA replication in S phase (Sundin and Varshavsky, 1980). DNA catenanes require topoisomerase II activity for their resolution (Holm et al., 1985), a process that at chromosome arms is completed prior to metaphase (Porter and Farr, 2004). However, at centromeric regions, catenanes persist until anaphase and are visible as ultrafine DNA bridges (UFBs) (Chan et al., 2007; Liu et al., 2014; Wang et al., 2010). Alternatively, UFBs can also arise between common fragile sites (CFSs) at chromosome arms after induction of replication stress in the previous S phase (Chan et al., 2009). 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) (Liu et al., 2014). UFB resolution must be completed by the end of anaphase to ensure sister-chromatid disjunction (Chan et al., 2007; Germann et al., 2014; Ke et al., 2011; Liu et al., 2014; Wang et al., 2010). 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 (IIα and III) to resolve UFBs (Baumann et al., 2007; Chan et al., 2007; Wang et al., 2010, 2008). Here, we present Rif1 as an UFB binding protein. Originally identified as an interactor of the telomere-binding protein Rap1 in budding yeast (Hardy et al., 1992), Rif1 was recently shown to function in DNA break repair downstream of ATM and 53BP1 (Chapman et al., 2012; Di Virgilio et al., 2013; Escribano-Díaz et al., 2013; Silverman et al., 2004; Xu and Blackburn, 2004; Zimmermann et al., 2013) and in controlling replication timing in situations of stress (Cornacchia et al., 2012; Hayano et al., 2012; Peace et al., 2014; Yamazaki et al., 2012). 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 UFBs during Anaphase

The cellular response to DNA damage is rewired during mitosis (Heijink et al., 2013). 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 (Giunta et al., 2010; Orthwein et al., 2014). In

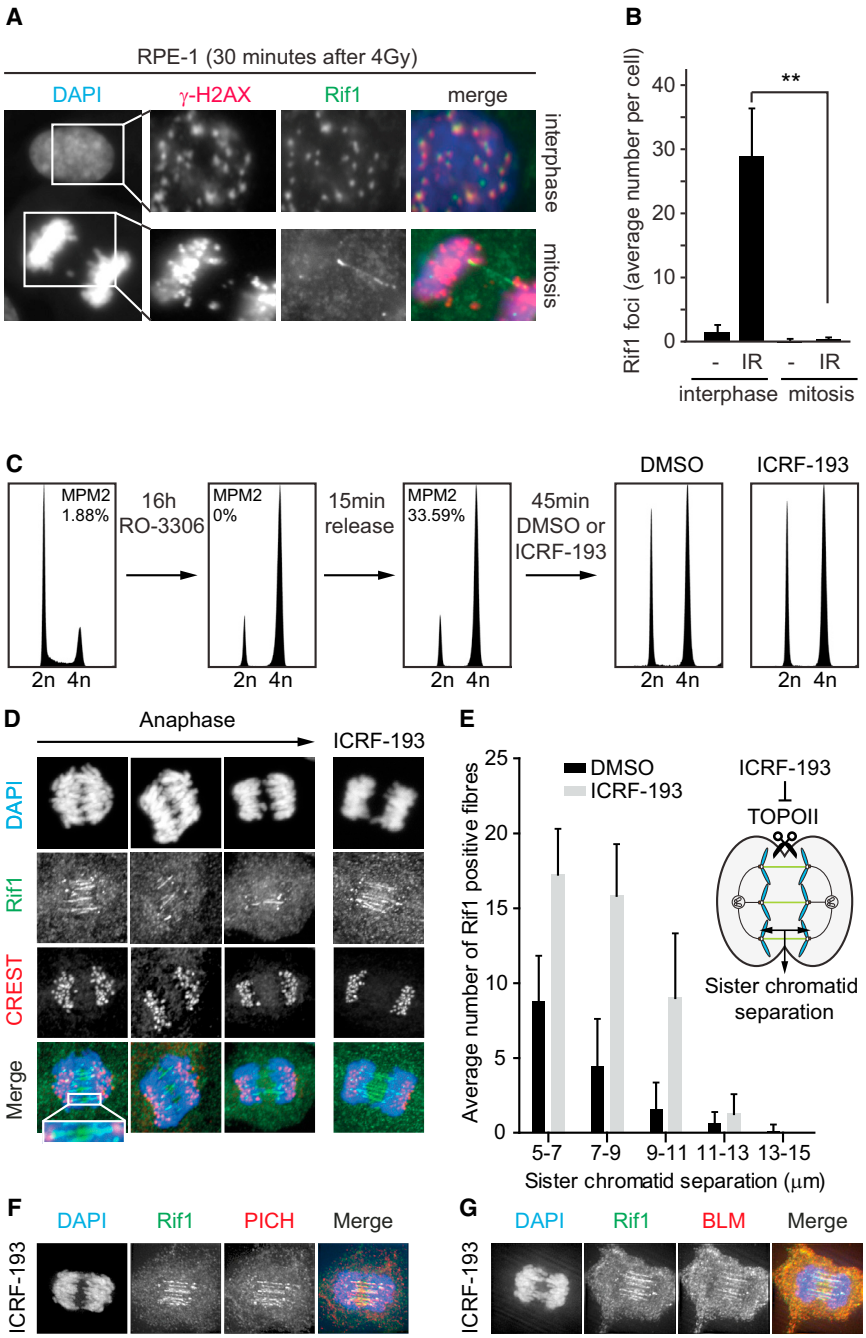

Figure 1. Rif1 Is Localized to DNA DSBs during Interphase and to UFBs in Anaphase

(A) Representative images of Rif1 and γ-H2AX during interphase and anaphase in non-transformed RPE-1 cells, 30 min after 4 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). The error bars indicate SD (n > 25 cells/condition) (**p < 0.01 and unpaired Student's t test).

(C) Synchronization protocol: RPE-1 cells were arrested in G_2 phase using the reversible Cdk1 inhibitor RO-3306. The washout of RO-3306 allowed synchronous mitotic entry. At 15 min later, the cells were treated with ICRF-193 (160 nM).

(D and E) RPE-1 cells were treated as in (C) and subsequently stained with Rif1 and CREST antibodies and DAPI. The DMSO-treated or ICRF-193-treated anaphase cells were categorized based on the distance between chromosome packs. The number of Rif1-positive bridges per anaphase was scored. The error bars indicate SD (n > 25 cells/condition).

(F and 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.

analogy to 53BP1, we found that Rif1 cannot be recruited to DNA DSBs during mitosis in untransformed RPE-1 cells (Figures 1A and 1B) and in MCF-7 and HeLa cells (Figures S1A and S1B). However, we noticed that in anaphase, Rif1 localized to thread-like structures that bridged segregating chromosomes, irrespective of earlier inflicted DNA damage (Figure 1D). Although previous work suggested that Rif1 co-localizes with midzone microtubules (Xu and Blackburn, 2004), 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 and S1D).

Rif1-positive thread-like structures were present in high numbers at anaphase onset, but progressively disappeared upon sister-chromatid segregation (Figures 1D and 1E). This localization pattern of Rif1 resembles that of PICH and BLM, which localize to UFBs in early anaphase (Baumann et al., 2007; Chan et al., 2007). In non-transformed and non-stressed cells, UFBs are mainly caused by catenated centromeric DNA that requires topoisomerase activity for its decatenation during anaphase (Wang et al., 2008). 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 (Chan et al., 2009; Liu et al., 2014). To investigate this, RPE-1 cells were released from a G_2 arrest imposed by the Cdk1 inhibitor RO-3306 (Figure 1C). At 15 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) (Wang et al., 2008). This resulted in a significant increase in the number of Rif1-positive threads during early anaphase (Figures 1D and 1E). 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

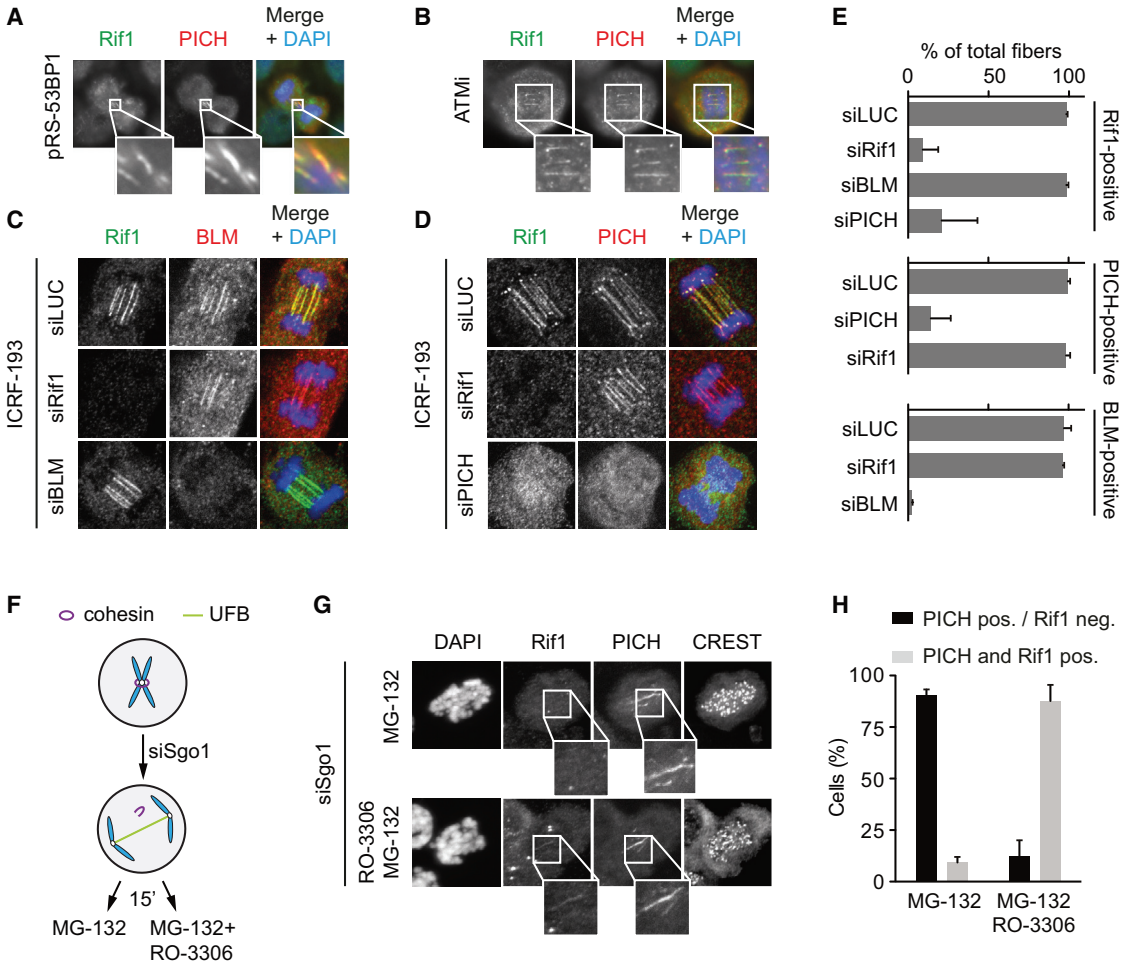

Figure 2. Rif1 Localization to UFBs Is Independent of ATM, 53BP1, and BLM, Requires PICH, and Is Blocked by Cdk1 Activity

(A and B) MCF-7 cells were stably depleted of 53BP1 (A) or treated with ATM inhibitor KU-55933 (B) and co-immunostained for PICH and Rif1.

(C and D) RPE-1 cells were transfected with the indicated siRNAs (siRNA#1 was used for Rif1), treated as in Figure 1C, and fixed and immunostained for Rif1 and BLM (C) or for Rif1 and PICH (D).

(E) Quantification of (C) and (D). The number of cells with Rif1-, PICH-, or BLM-positive bridges positive is depicted. The error bars indicate SD (n = 3 experiments and >50 cells/condition).

(F) Schematic representation of Sgo1-mediated cohesin protection at centromeres and of the experimental setup.

(G) RPE-1 cells were depleted of Sgo1 and treated with or without RO-3306. In both of the conditions, MG-132 was added to prevent mitotic exit. The cells were fixed and stained for Rif1, PICH, and CREST.

(H) Quantification of (G). The percentages of mitotic cells with PICH-positive/Rif1-negative bridges (black) versus cells with PICH-positive/Rif1-positive (gray) are depicted. The error bars indicate SD (n = 3 experiments with at least 50 cells/condition).

See also Figure S2.

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 (Figures 1F and 1G). The specificity of Rif1 localization at UFBs was verified by short interfering (si)RNA-mediated Rif1 depletion (Figures 2C–2E) and by using GFP-tagged Rif1 (Figures S1E and S1F). 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 (Orthwein et al., 2014) (Figures 1A, S1A, and S1B). Interestingly, depletion of 53BP1 did not affect Rif1 localization at UFBs in anaphase (Figures 2A, S2A, and S2G), while it did perturb Rif1 recruitment to irradiation-induced foci (IRIF) in interphase (Figures S2B and S2C). In fact, Rif1 recruitment to UFBs was independent of ATM signaling altogether, as ATM inhibition did not prevent Rif1 recruitment to PICH-positive UFBs (Figures 2B, S2D–S2F, and S2H).

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 (Baumann et al., 2007; Burrell et al., 2013; Chan et al., 2007; Xu et al., 2010). 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 (Figures 2C, 2E, S2I, and S2J). In contrast, when we depleted PICH, Rif1 recruitment to UFBs was completely blocked (Figures 2D, 2E, S2I, and S2K). Neither the localization of PICH nor BLM depended on the presence of Rif1 (Figures 2C–2E, S2J, and S2K). 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 tetratricopeptide repeat (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 (Figure 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 (Gómez et al., 2013; Stanvitch and Moore, 2008; Toyoda and Yanagida, 2006). 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 (Figures 2F–2H) (Wang et al., 2010). Remarkably, these UFBs did not contain Rif1 (Figures 2G and 2H), 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 and 2H). 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 (Baumann et al., 2007; Chan et al., 2007; Ke et al., 2011; Lukas et al., 2011). 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 and 3B; Movies S1 and S2). Although sometimes hard to detect with H2B-YFP, these DNA bridges appeared 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 (Figure 3B; Movies S3 and 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 single-stranded (ss)DNA-binding protein RPA70, which was previously shown to be recruited to UFBs (Germann et al., 2014; Liu et al., 2014). Overall, depletion of Rif1 increased the frequency of cells with persistent RPA70-positive bridges in late anaphase (Figures 3C and 3D). In marked contrast, we failed to detect RPA70-positive UFBs in late anaphases of BLM-depleted cells (Figure 3D), 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 (Burrell et al., 2013), we tested whether the increased frequency of RPA70-positive UFBs after Rif1 depletion in otherwise unchallenged HeLa cells was 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 (Figures S3A and S3B). 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 IIα 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 (Figures 3E and 3F). 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 ~10 μm (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 to detect RPA70 on UFBs when BLM was depleted (Figure 3E). Taken together, these data 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.

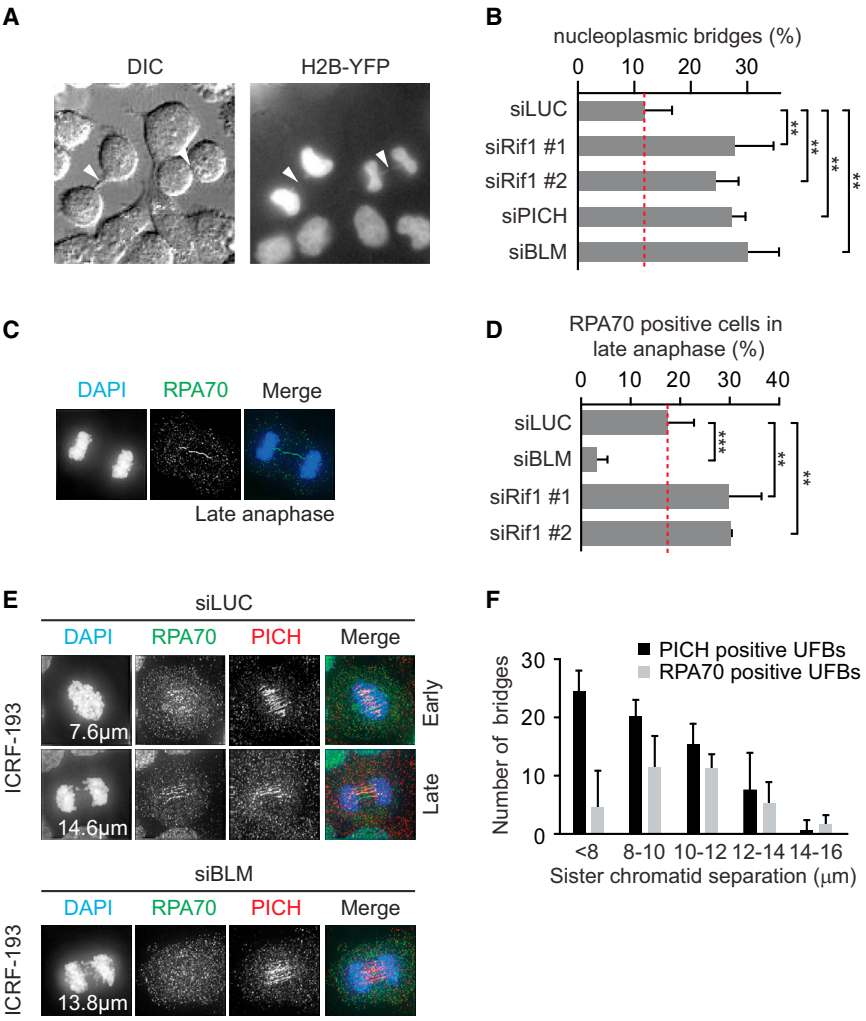

Figure 3. Rif1 Is Required for Proper Sister-Chromatid Disjunction

(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. The representative DIC and YFP stills of Movie S2 are shown. The 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, S2, S3, and S4). The error bars indicate SD (n = 3 experiments, 30 cells/condition, $^{**}p < 0.01$, and unpaired Student's t test).

(C) RPA70 is recruited to persistent UFBs. The HeLa cells were released from a RO-3306-inflicted G_2 arrest and fixed 45 min later. The cells were stained for RPA70. The representative late anaphase cell is shown.

(D) Cells were transfected with indicated siRNAs and treated as in (C). The anaphase cells were scored for the presence of RPA70 positive bridges. There were >100 cells/condition that were analyzed ($^{**}p < 0.01$ and $^{***}p < 0.001$) (unpaired Student's t test).

(E) HeLa cells were transfected with indicated siRNAs and treated as in Figure 1C. The cells were fixed and stained for PICH and RPA70. The representative early and late anaphases are depicted.

(F) HeLa cells treated as in (E). The anaphase cells were categorized based on the distance between chromosome packs and the numbers of PICH and RPA70-positive bridges per anaphase were scored. The error bars indicate SD (n > 25 cells/condition).

See also Figure S3.

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 knockdown of PICH and BLM was associated with micronuclei formation (Ke et al., 2011), we tested whether Rif1 inactivation would also give rise to micronuclei. In our hands, transient knockdown 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 *ERCC6L* (encoding PICH) knockout cells obtained through CRISPR/Cas9-mediated gene editing of HAP1 cells (Bürckstümmer et al., 2013) (Figure 4A). Prolonged inactivation of *RIF1* significantly increased the frequency of HAP1 cells with micronuclei to a similar extent as *ERCC6L* or *BLM* gene mutation (Figure 4B).

Impaired UFB Resolution Gives Rise to Nuclear Bodies with Damaged DNA in G_1

Unresolved late-stage replication intermediates lead to the formation of nuclear bodies in ensuing G_1 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/G_2 phase (Harrigan et al., 2011; Lukas et al., 2011). 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 G_1. 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. First, global replication analysis by flow cytometry was used to show that low dose ICRF1-193 treatment did not notably alter Edu incorporation, even when Rif1, BLM, or PICH were depleted (Figures S4A and S4B). Second, mitotic cells were analyzed immediately after a 15-min pulse of EdU to demonstrate that ICRF-193 treatment of control-depleted or Rif1-depeted cells did not result in any EdU incorporation in mitotic cells (Figures S4C and S4D). This indicated that active replication in these cells has finished well before mitotic entry (Germann et al., 2014). Third, 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

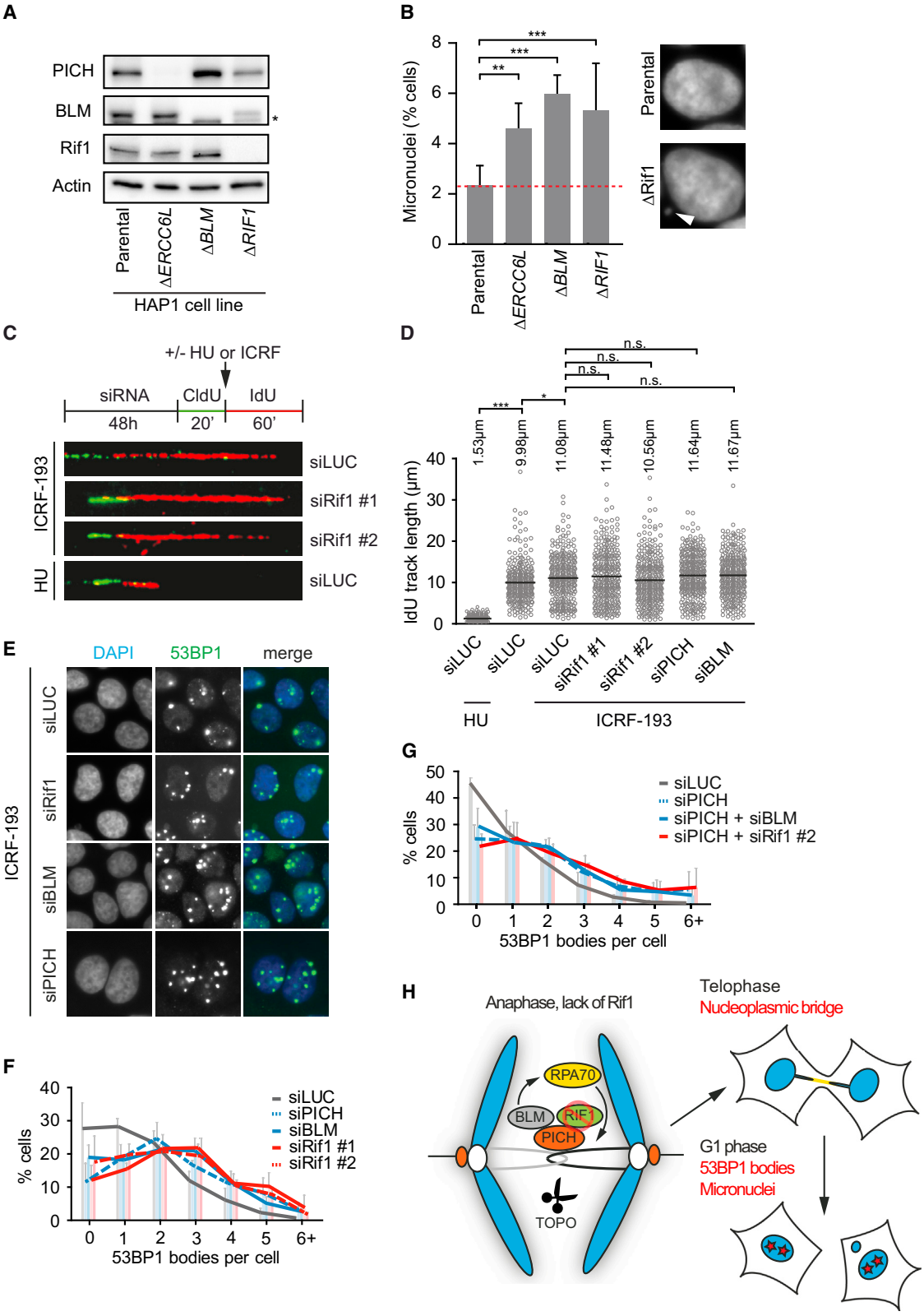

Figure 4. Impaired UFB Resolution Increases Frequency of Micronuclei and 53BP1 Nuclear Body Formation

(A) PICH, BLM, Rif1, and actin levels in the parental or indicated HAP1 knockout cell lines determined by immunoblotting, (*) aspecific band.

(B) Parental HAP1 cells or HAP1 cell lines harboring frame shift mutations in *RIF1*, *BLM*, or *ERCC6L* were analyzed for micronuclei (arrow in image). The mean ± SD of three experiments (>1,000 cells/condition in each experiment) (**p < 0.01, ***p < 0.001, and unpaired Student's t test).

(legend continued on next page)

UFBs (Figures 4C and 4D). Importantly, depletion of neither Rif1, BLM, nor PICH caused a decrease in replication speed in ICRF-193-treated cells (Figures 4C and 4D).

Having established that a low dose of ICRF-193 in combination with knockdown 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/G_2 cells from G_1 cells to assess whether impaired DNA decatenation would result in nuclear body formation in G_1 (Figures S4E and S4F). Treatment with ICRF-193 alone resulted in the formation of Mdc1-GFP and GFP-53BP1 nuclear bodies in G_1 phase (Figures S4E and S4F) 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 (Figures 4E and 4F). 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 (Baumann et al., 2007), 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 DSB repair choice (Chapman et al., 2012; Di Virgilio et al., 2013; Escribano-Díaz et al., 2013; Feng et al., 2013; Zimmermann et al., 2013) and timing of DNA replication (Cornacchia et al., 2012; Hayano et al., 2012; Peace et al., 2014; Yamazaki et al., 2012). 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 (Heijink et al., 2013), 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 (Orthwein et al., 2014), 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 DSBs 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 RMI1 are recruited to UFBs (Chan et al., 2007; Germann et al., 2014). This complex resembles the BLM-Topoisomerase IIIα-RMI1-RMI2 (BTRR) complex that is recruited to resolve recombination intermediates and promote stalled replication recovery during S phase (Manthei and Keck, 2013). 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 (Xu et al., 2010), 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 (Biebricher et al., 2013). 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 (Biebricher et al., 2013), 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 (Wold, 1997). 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 (Zimmermann et al., 2013). 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 (Biebricher et al., 2013). Clearly, future studies are required to uncover how Rif1, BLM, and PICH act at the molecular level to resolve UFBs.

(C and 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. The DNA was spread into single fibers and IdU track length was determined for 300 fibers per condition. The representative fibers are shown in (C) and actual and average fiber lengths are plotted in (D) (*$p < 0.05$, ***$p < 0.001$, n.s. = not significant, and unpaired Student's t test).

(E–G) MCF-7 cells were transfected with indicated siRNAs and treated for 24 hr with ICRF-193. At 48 hr after transfection, the cells were fixed and stained for 53BP1. The nuclear 53BP1 bodies per cell were scored. The percentages are mean ± SD of three experiments with >400 cells per condition. The 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 G_1.

See also Figure S4.

Finally, we demonstrated that impaired UFB resolution gives rise to nuclear bodies with damaged DNA in G_1. 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, and 293T. HeLa and RPE-1 cells were blocked in G_2 phase using RO-3306 (5 μM and 7.5 μM respectively, Calbiochem) for 18 hr. At 15 min after release from the RO-3306 block, ICRF-193 was added (160 nM, Sigma). Where indicated, cells were irradiated using a Cesium^{137} source (CIS international/IBL 637), transfected with 20 nM of the indicated siRNAs using HyperFect or treated with the indicated inhibitors.

Microscopy

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

DNA Replication and Nuclear Body Formation

At 48 hr 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 FACSCalibur (Becton Dickinson) using CellQuest 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.

See Supplemental Information for full experimental details.

SUPPLEMENTAL INFORMATION

Supplemental Information includes Supplemental Experimental Procedures, four figures, and four movies and can be found with this article online at http://dx.doi.org/10.1016/j.devcel.2015.06.014.

AUTHOR CONTRIBUTIONS

M.A.T.M.v.V. and S.M.A.L. conceived the study. R.C.C.H., H.R.d.B., and P.M.S. designed and performed experiments. E.G.E.d.V. contributed to the preparation of the manuscript. R.C.C.H., H.R.d.B., M.A.T.M.v.V., and S.M.A.L. wrote the manuscript.

ACKNOWLEDGMENTS

We thank Dr. A. Duursma for valuable comments on the manuscript and members of the S.M.A.L., Kops, and M.A.T.M.v.V. laboratories for helpful discussions. This work is financially supported by grants from the Netherlands Organization for Scientific Research (NWO-VICI 91812610 to S.M.A.L. and NWO-VIDI 91713334 to M.A.T.M.v.V.), the Dutch Cancer Society (UU2009-4311 to S.M.A.L. and RUG2011-5093 to M.A.T.M.v.V.), and the European Research Council (ERC-Advanced Grant ERC-2011-293-445 to E.G.E.d.V.).

Received: August 14, 2014
Revised: May 21, 2015
Accepted: June 16, 2015
Published: August 6, 2015

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