Cell Biology

WRNIP1 prevents transcription-associated genomic instability

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Abstract

R-loops are non-canonical DNA structures that form during transcription and play diverse roles in various physiological processes. Disruption of R-loop homeostasis can lead to genomic instability and replication impairment, contributing to several human diseases, including cancer. Although the molecular mechanisms that protect cells against such events are not fully understood, recent research has identified the fork protection factors and the DNA damage response proteins as regulators of R-loop dynamics. Here, we identify the Werner helicase-interacting protein 1 (WRNIP1) as a novel factor that counteracts transcription-associated DNA damage upon replication perturbation. Loss of WRNIP1 leads to R-loop accumulation, resulting in collisions between the replisome and transcription machinery. We observe co-localization of WRNIP1 with transcription/replication complexes and R-loops after replication perturbation, suggesting its involvement in resolving transcription-replication conflicts. Moreover, WRNIP1-deficient cells show impaired replication restart from transcription-induced fork stalling. Notably, transcription inhibition and RNase H1 overexpression rescue all the defects caused by loss of WRNIP1. Importantly, our findings highlight the critical role of WRNIP1 ubiquitin-binding zinc finger (UBZ) domain in preventing pathological persistence of R-loops and limiting DNA damage, thereby safeguarding genome integrity.

eLife assessment

This **valuable** paper examines the role of the WRNIP1 AAA+ ATPase in regulating R-loop formation, which induces a conflict with active replication forks and transcription. The authors provide **convincing** evidence to support a role of the ubiquitin-binding UBZ domain of WRNIP1 in R-loop suppression generated by this conflict. The work is of interest to researchers who work on genome stability/instability.

Introduction

Maintenance of genome integrity is essential for cell viability and relies on complete and accurate DNA replication. However, genome is continuously threatened by several types of DNA lesions that hinder replication fork progression, leading to fork stalling and replication stress and ultimately to genomic instability, a hallmark of cancer cells. Different molecular mechanisms

underlie replication stress in cells, and understanding how they occur has always been of interest for targeting them for cancer therapy. Among known mechanisms that perturb genome duplication, the transcription process plays a crucial role in inducing stalling of replication forks. Indeed, transcription can not only hamper replication fork progression itself but can also act as an enhancer of replication impediments, such as protein-DNA complexes, DNA damage and non-B-form DNA secondary structures (Gómez-González and Aguilera 2019). Among the latter, R-loops have emerged as critical determinants of transcription-associated replication stress. R-loops can cause genomic instability and may promote cancer and other human diseases (Gómez-González and Aguilera 2019; García-Muse and Aguilera 2019; Crossley and Cimprich 2019). R-loops are three-stranded structures that consist of an DNA/RNA hybrid and a displaced single-stranded DNA. They are involved in various physiological processes, including transcription termination, regulation of gene expression, and DNA repair. Under physiological conditions, R-loops are transient structures that are resolved by enzymes (Sanz et al. 2016; Wahba et al. 2011). However, if they persist, they can become pathological and provoke the formation of transcription-replication conflicts (TRCs). Given the high frequency at which replication and transcription processes occur in cells, TRCs are particularly likely to happen. Additionally, the interference between transcription and replication at very large human genes, whose transcription continues into S-phase, may contribute to the instability of common fragile sites (CFS), the most replication stress-sensitive regions in the human genome (Helmrich and Tora 2011). Notably, the idea that R-loops cause fork stalling, promoting TRCs and leading to transcription-associated DNA damage, is supported by the fact that many strategies aimed at minimizing collisions and facilitating replication fork progression following unscheduled R-loop accumulation rely on replication fork protection factors and DNA damage response (DDR) proteins (Shivji et al. 2018; Hatchi et al. 2015; Schwab et al. 2015; García-Rubio et al. 2015; Bhatia et al. 2014; Liang et al. 2019; Urban et al. 2016; Chang et al. 2017; Marabitti et al. 2019; Tresini et al. 2015). The precise mechanisms by which cells cope with TRCs resulting from persistent R-loops are still largely unknown.

Human WRN-interacting protein 1 (WRNIP1) is a genome maintenance factor (Yoshimura and Enomoto 2017). WRNIP1 binds to forked DNA that resembles stalled forks (Yoshimura et al. 2009) and its foci overlap with replication factories (Crosetto et al. 2008), suggesting a role at replication forks. Consistent with this, WRNIP1 protects stalled forks from MRE11-mediated degradation and promotes fork restart upon replication stress (Leuzzi et al. 2016; Porebski et al. 2019). Alongside its role at forks, WRNIP1 is implicated in ATM-dependent checkpoint activation in response to mild replication stress (Kanu et al. 2016; Marabitti et al. 2020). Notably, in cells with defective ATR-dependent checkpoint activation, such as Werner syndrome cells (Basile et al. 2014), WRNIP1 mediates ATM-dependent CHK1 phosphorylation to counteract pathological R-loop accumulation (Marabitti et al. 2020). In addition, WRNIP1 is found to be enriched at CFS, suggesting a role in maintaining their stability (Pladevall-Morera et al. 2019). However, data concerning a role of WRNIP1 in restricting transcription-associated genomic instability are not available.

Here, we uncover that loss of WRNIP1 and the activity of WRNIP1 ubiquitin-binding zinc finger (UBZ) domain are required for limiting unscheduled R-loops that give rise to TRCs. Furthermore, we establish that loss of WRNIP1 functions is accountable for the increased genomic instability observed in WRNIP1-deficient and WRNIP1 UBZ mutant cells upon mild replication perturbation.

Results

Transcription-dependent DNA damage occurs in WRNIP1-deficient and WRNIP1 UBZ mutant cells upon MRS

To investigate the contribution of WRNIP1 in maintaining genome integrity, we evaluated DNA damage accumulation in response to mild replication stress (MRS) induced by nanomolar dose of the DNA polymerase inhibitor aphidicolin (Aph). In addition to its ATPase activity, which is involved in fork restart (Leuzzi et al. 2016), WRNIP1 also contains an ubiquitin-binding zinc finger (UBZ) domain. Although this domain has been implicated in fork-related functions (Yoshimura et al., 2017), its function is still poorly defined.

In our experiments, we used the SV40-transformed MRC5 fibroblast cell line (MRC5SV), MRC5SV cells stably expressing WRNIP1-targeting shRNA (shWRNIP1) and isogenic cell lines stably expressing the RNAi-resistant full-length wild-type WRNIP1 (shWRNIP1^{WT}), its ATPase-dead mutant form (shWRNIP1^{T294A}) (Leuzzi et al. 2016) or the UBZ-dead mutant form of WRNIP1 (shWRNIP1^{D37A}) that abolishes the ubiquitin-binding activity (Bish and Myers 2007; Nomura et al. 2012) (**Figure 1A**). Initially, we measured DNA damage by a single-cell electrophoresis in WRNIP1-deficient and WRNIP1 mutant cells. In agreement with previous experiments (Marabitti et al. 2020), we found that loss of WRNIP1 resulted in higher spontaneous levels of DNA damage compared to wild-type cells (MRC5SV), and that Aph exacerbated this phenotype (**Figure 1B**). Interestingly, in WRNIP1 mutant cells, spontaneous genomic damage was similar to that observed in WRNIP1-deficient cells but, after MRS, it was significantly enhanced only in WRNIP1 UBZ mutant cells (**Figure 1B**).

In parallel experiments, we assessed the presence of chromosomal damage. As shown in **Figure 1C**, shWRNIP1 and WRNIP1 mutant cells exhibited a greater number of chromosomal aberrations under unperturbed conditions, compared to their corrected isogenic counterparts (shWRNIP1^{WT}). However, while loss of WRNIP1 or mutation of its UBZ domain heightened the average number of gaps and breaks after Aph than their corrected counterparts (shWRNIP1^{WT}), that of ATPase activity did not (**Figure 1C**). Therefore, loss of WRNIP1 or its ubiquitin-binding function renders cells highly sensitive to Aph-induced MRS, affecting genomic integrity.

We next wondered whether DNA damage accumulated in a transcription-dependent manner. To test this possibility, we performed a Comet assay using MRC5SV, shWRNIP1, shWRNIP1^{D37A} and shWRNIP1^{T294A} cells incubated with Aph and/or the 5,6-dichloro-1-ß-D-ribofurosylbenzimidazole (DRB), a strong inhibitor of RNA synthesis, as described (Marabitti et al. 2019). Our analysis showed that DNA damage was not sensitive to transcription inhibition at any of the tested conditions in wild-type and WRNIP1 ATPase mutant cells (**Figure 1D**). In contrast, DRB significantly suppressed the amount of DNA damage in both shWRNIP1 and shWRNIP1^{D37A} cells that were either untreated or treated with Aph (**Figure 1D**). Similar results were obtained using another transcription inhibitor, Cordycepin (3'-deoxyadenosine) ((Müller et al. 1977); Suppl Figure 1A). Additionally, anti-phospho-H2AX immunostaining, which is considered an early sign of DNA damage caused by replication fork stalling (Ward and Chen 2001), confirmed a transcription-dependent DNA damage accumulation in shWRNIP1 and shWRNIP1^{D37A} cells (Suppl Figure 1B).

Altogether these findings suggest that WRNIP1 and the activity of its UBZ domain are required to prevent transcription-dependent DNA damage.

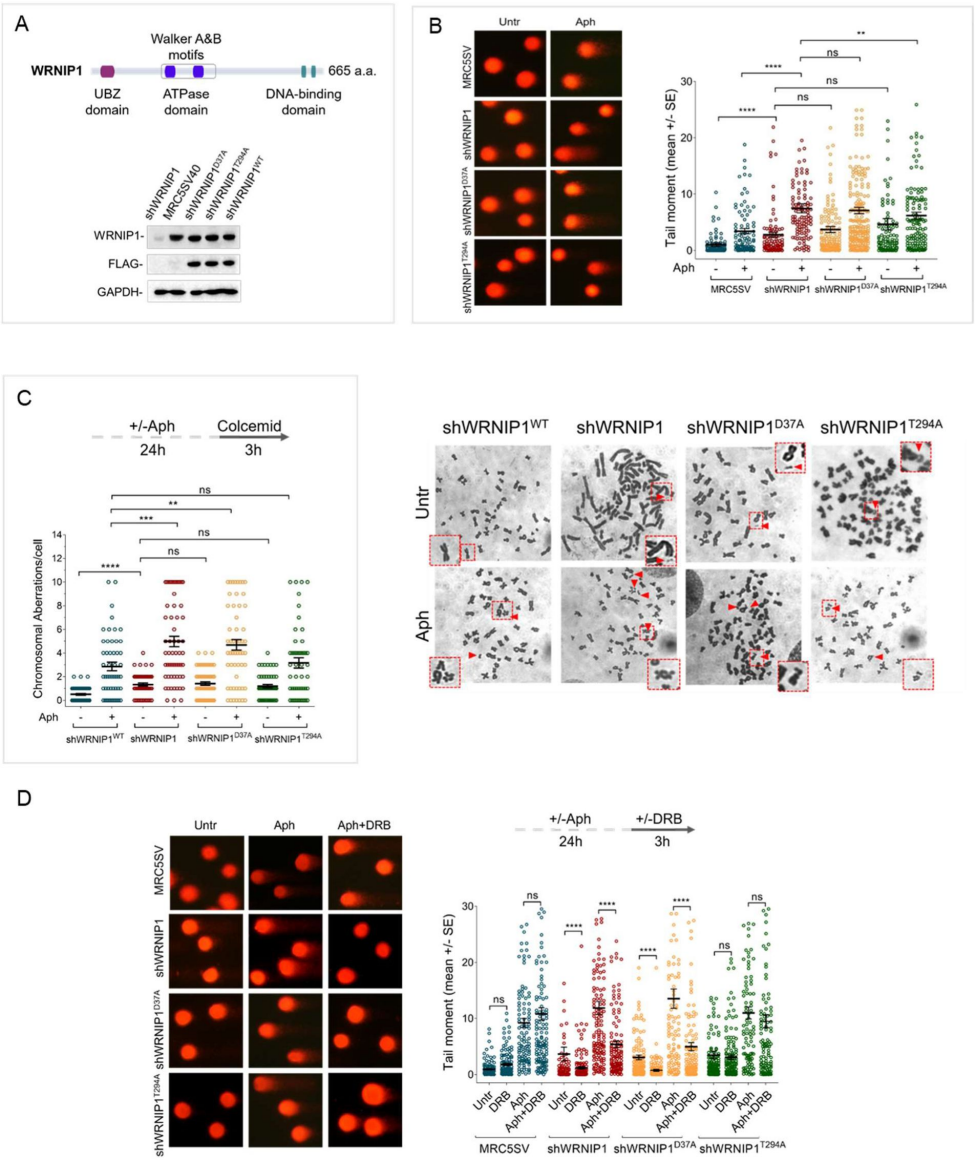

Figure 1.

Loss of WRNIP1 or its UBZ domain results in DNA damage accumulation and enhanced chromosomal instability upon MRS

(A) Schematic representation of human WRNIP1 protein structure. Western blot analysis showing the expression of the WRNIP1 protein in wild-type cells (shWRNIP1^{WT}), WRNIP1-deficient cells (shWRNIP1) and WRNIP1 ATPase mutant (shWRNIP1^{T294A}) or WRNIP1 UBZ mutant (shWRNIP1^{D37A}) cells. MRC5SV40 fibroblasts were used as a positive control. The membrane was probed with an anti-FLAG or anti-WRNIP1 antibody. GAPDH was used as a loading control.

(B) Analysis of DNA damage accumulation evaluated by alkaline Comet assay. MRC5SV, shWRNIP1, shWRNIP1^{D37A} and shWRNIP1^{T294A} cells were treated or not with 0.4 μM Aph for 24 h, then subjected to Comet assay. The graph shows data presented as mean tail moment ± SE from three independent experiments. Horizontal black lines represent the mean (ns, not significant; **, $P < 0.01$; ****, $P < 0.0001$; two-tailed Student's t test). Representative images are given.

(C) Analysis of chromosomal aberrations in the indicated cell lines treated as shown in the experimental scheme. Dot plot shows the number of chromosomal aberrations per cell ± SE from three independent experiments. Horizontal black lines represent the mean (ns, not significant; **, $P < 0.01$; ***, $P < 0.001$; ****, $P < 0.0001$; two-tailed Student's t test). Representative images are given. Insets show enlarged metaphases for a better visualization of chromosomal aberrations.

(D) Evaluation of DNA damage accumulation by alkaline Comet assay. Cells were treated as shown in the experimental scheme, then subjected to Comet assay. Dot plot shows data presented as mean tail moment ± SE from three independent experiments. Horizontal black lines represent the mean (ns, not significant; ****, $P < 0.0001$; two-tailed Student's t test). Representative images are given.

Enhanced R-loop accumulation is observed in WRNIP1-deficient cells

The main structures associated with transcription that can impede fork movement and result in transcription-replication conflicts (TRCs) are R-loops (Gan et al. 2011; Allison and Wang 2019; Gaillard and Aguilera 2016). As WRNIP1 may counteract R-loop-mediated TRCs by reducing transcription-dependent DNA damage accumulation, we first assessed R-loops levels in our cells using the well-established anti-RNA-DNA hybrid S9.6 antibody (Boguslawski et al. 1986; Hamperl et al. 2017; Marabitti et al. 2019). We observed a significant increase in nuclear S9.6 intensity in unperturbed WRNIP1-deficient cells, but not in their corrected counterparts (shWRNIP1^{WT}; **Figure 2A**). Furthermore, while Aph treatment caused increased R-loop levels in both cell lines, the values were considerably higher in shWRNIP1 cells (**Figure 2A**). Importantly, the S9.6 staining was strongly suppressed upon overexpression of RNaseH1 that removes R-loops (Cerritelli et al. 2003) (**Figure 2A**).

To further confirm the accumulation of R-loop within nuclear DNA, we isolated genomic DNA from shWRNIP1^{WT} and shWRNIP1 cells and performed a dot blot analysis. Consistent with fluorescence analysis, the S9.6 signal was higher in shWRNIP1 cells than in shWRNIP1^{WT} cells and was abolished by RNaseH treatment (**Figure 2B**; (Morales et al. 2016)). This result suggest that DNA damage observed in WRNIP1-deficient cells may be correlated with the elevated accumulation of R-loops.

Loss of WRNIP1 or its UBZ domain results in R-loop-dependent DNA damage upon MRS

After demonstrating that WRNIP1-deficient cells accumulate high levels of R-loops, we investigated the risks to genome integrity by comparing different replication stress conditions. As observed above, loss of WRNIP1 slightly increased the amount of spontaneous DNA damage, while Aph enhanced the comet tail moment to a greater extent in shWRNIP1 than in MRC5SV cells (**Figure 3A**). A similar trend was observed using hydroxyurea (HU) as a replication-perturbing agent (**Figure 3A**). It is noteworthy that overexpression of RNaseH1 was able to strongly reduce the tail moment only in shWRNIP1 cells and more efficiently after Aph-induced replication slowdown than arrest by HU (**Figure 3A**). This confirms R-loops as a driver of genome instability in WRNIP1-deficient cells and suggests that R-loop-mediated DNA damage is more pronounced after a replication stress that does not completely block replication fork progression.

Next, we asked which of the WRNIP1 activities could prevent the formation of aberrant R-loops upon MRS. We observed a similar level of spontaneous R-loops in cells with mutations in either ATPase or UBZ domain. However, following MRS, shWRNIP1^{D37A} cells exhibited a more pronounced S9.6 signal than shWRNIP1^{T294A} cells (**Figure 3B**), indicating a role for the UBZ domain of WRNIP1 in counteracting R-loop accumulation upon MRS.

Finally, we wanted to determine whether R-loop-mediated DNA damage in WRNIP1-deficient cells was due to the loss of the UBZ domain of WRNIP1. Notably, we found that suppression of DNA damage in shWRNIP1^{D37A} cells upon RNaseH1 overexpression was similar to that seen in shWRNIP1 cells (**Figure 3C**), suggesting that the ubiquitin-binding activity of WRNIP1 is necessary to prevent R-loop-mediated DNA damage upon MRS.

The UBZ domain of WRNIP1 is required to attenuate TRCs upon MRS

Since TRCs play a crucial role in promoting R-loop-mediated genomic instability (García-Muse and Aguilera 2016), we investigated the occurrence of such conflicts under our conditions. Hence, we performed a proximity ligation assay (PLA), a well-established method for detecting physical interactions (Söderberg et al. 2008). In this assay we utilized antibodies against proliferating cell

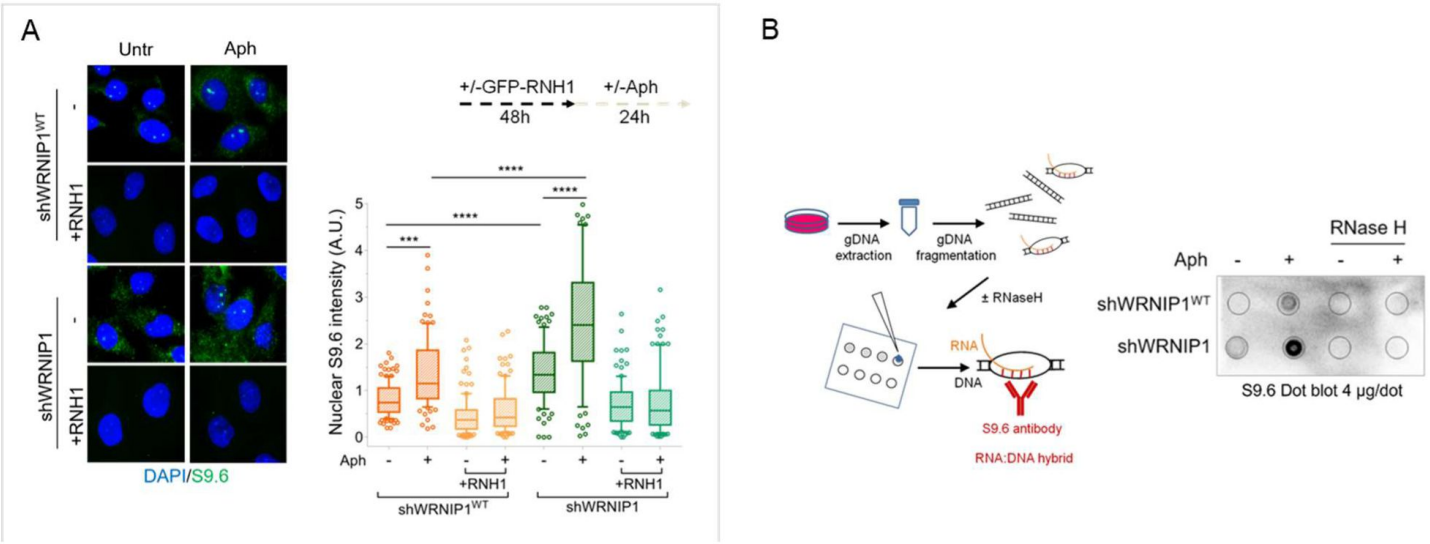

Figure 2.

Loss of WRNIP1 or its UBZ domain results in R-loop accumulation upon MRS

(A) Evaluation of R-loop accumulation by immunofluorescence analysis in shWRNIP1^{WT} and shWRNIP1 cells treated as reported in the experimental design after transfection with GFP-tagged RNaseH1 or empty vector. Cells were fixed and stained with anti-RNA-DNA hybrid S9.6 monoclonal antibody. Representative images are given. Nuclei were counterstained with DAPI. Box plot shows nuclear S9.6 fluorescence intensity. Box and whiskers represent 20-75 and 10-90 percentiles, respectively. The line represents the median value. Data are presented as means of three independent experiments. Horizontal black lines represent the mean. Error bars represent standard error (***, $P < 0.001$; ****, $P < 0.0001$; two-tailed Student's t test).
(B) Dot blot to confirm R-loop accumulation. Genomic DNA isolated from shWRNIP1WT and shWRNIP1 cells, treated as reported in the experimental design and processed as described in "Materials and Methods", was spotted onto nitrocellulose membrane. The membrane was probed with anti-RNA-DNA hybrid S9.6 monoclonal antibody. Treatment with RNase H was used as a negative control. Representative gel images of at least three replicates are shown.

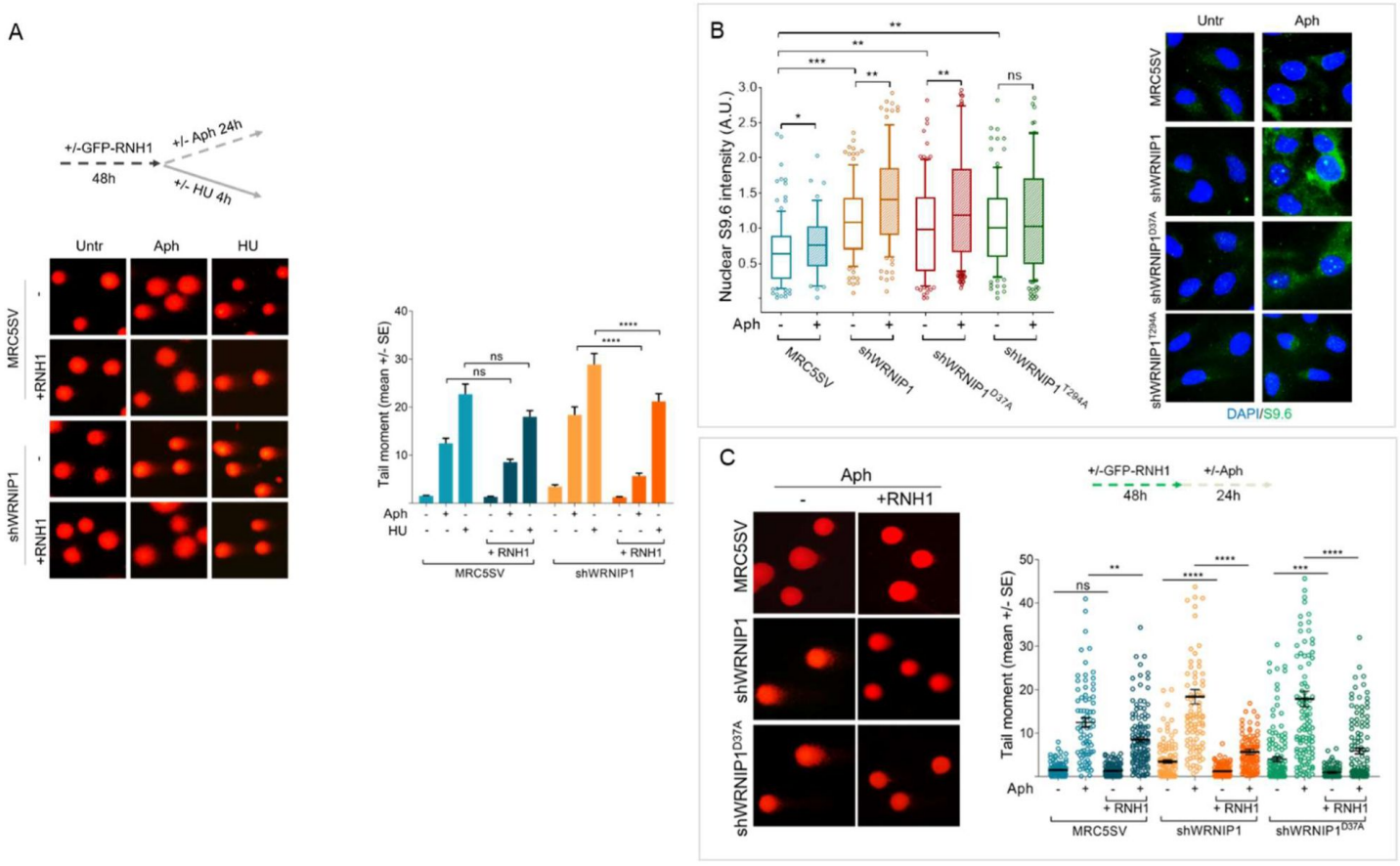

Figure 3.

Loss of WRNIP1 or its UBZ domain leads to R-loop-dependent accumulation upon MRS

(A) Analysis of DNA damage accumulation by alkaline Comet assay. MRC5SV and shWRNIP1 cells were treated or not with Aph or HU, as reported by the experimental scheme after transfection with GFP-tagged RNaseH1 or empty vector (-),then subjected to Comet assay. The graph shows data presented as mean tail moment ± SE from three independent experiments (ns, not significant; **** $P < 0.0001$; two-tailed Student's t test). Representative images are given.

(B) Immunofluorescence analysis to determine R-loop levels in MRC5SV, shWRNIP1, shWRNIP1^{D37A} and shWRNIP1^{T294A} cells treated or not with 0.4 μM Aph for 24 h. Cells were fixed and stained with anti-RNA-DNA hybrid S9.6 monoclonal antibody. Representative images are given. Nuclei were counterstained with DAPI. Box plot shows nuclear S9.6 fluorescence intensity. Box and whiskers represent 20-75 and 10-90 percentiles, respectively. The line represents the median value. Data are presented as means of three independent experiments. Horizontal black lines represent the mean. Error bars represent SE (ns, not significant; * $P < 0.05$; ** $P < 0.01$; ***, $P < 0.001$; two-tailed Student's t test).

(C) Analysis of the effect of R-loop resolution on DNA damage accumulation evaluated by alkaline Comet assay. Cells were treated as reported in the experimental scheme after transfection with GFP-tagged RNaseH1 or empty vector, then subjected to Comet assay. Dot plot shows data presented as mean tail moment ± SE from three independent experiments. Horizontal black lines represent the mean (ns, not significant; ** $P < 0.01$; *** $P < 0.001$; **** $P < 0.0001$; two-tailed Student's t test). Representative images are given.

nuclear antigen (PCNA) and RNA polymerase II (RNA pol II) to label replication forks and transcription complexes, respectively, as previously described (Hamperl et al. 2017). Our analysis revealed a higher number of spontaneous PLA signals (indicated by red spots) in WRNIP1-deficient and WRNIP1 UBZ mutant cells compared to wild-type cells (**Figure 4A**). Importantly, although Aph increased the co-localization of PCNA and RNA pol II in all cell lines, the number of PLA spots was significantly greater in shWRNIP1 and shWRNIP1^{D37A} cells than in shWRNIP1^{WT} cells (**Figure 4A**). Interestingly, this phenotype was substantially reduced by overexpressing RNaseH1 (**Figure 4A**), suggesting that the UBZ domain of WRNIP1 may play a role in attenuating R-loop-induced TRCs upon MRS.

Next, we examined the localization of WRNIP1 near/at transcription and replication machineries by conducting PLA assays in shWRNIP1^{WT} and shWRNIP1^{D37A} cells. For this purpose, we used antibodies against WRNIP1 and RNA pol II or PCNA, respectively. Our findings support the notion that WRNIP1 is indeed localized at the sites of TRCs following treatment with Aph in wild-type cells, as evidenced by an increased number of PLA spots between WRNIP1 and RNA pol II (**Figure 4B**), as well as between WRNIP1 and PCNA (**Figure 4C**). Surprisingly, a similar, but more pronounced, phenotype was observed in WRNIP1 UBZ mutant cells compared to wild-type cells, both under normal conditions and after treatment (**Figures 4B and C**).

Furthermore, we explored the localization of WRNIP1 in proximity to R-loops. Through the PLA assay we noticed a growing number of spots, indicating the presence of interactions between WRNIP1 and R-loops (anti-S9.6) (**Figure 4D**). Interestingly, this phenomenon was more evident in WRNIP1 UBZ mutant cells (**Figure 4D**), suggesting that WRNIP1, along with its UBZ domain, plays a role in the response to R-loop accumulation.

One of the initial steps in resolving R-loops involves the recruitment of RAD51 to stalled forks (Chappidi et al. 2020). Our PLA assay revealed that RAD51-R-loops nuclear spots were clearly observed in both MRC5SV and shWRNIP1^{D37A} cell lines following Aph treatment, but their presence was reduced in WRNIP1 UBZ mutant cells compared to wild-type cells (**Figure 4E**).

Taken together, these findings suggest that WRNIP1 co-localizes with transcription/replication complexes and R-loops following MRS. Additionally, they indicate that the ubiquitin-binding function of WRNIP1 helps to mitigate R-loop-mediated TRCs.

Transcription-mediated R-loop formation can act as a barrier to DNA replication in cells lacking WRNIP1 or its UBZ domain activity

R-loops are transcription-associated structures that can impede the progression of replication forks (Belotserkovskii et al. 2018). To assess the direct impact of R-loops on fork progression, we performed a DNA fiber assay to examine replication fork dynamics at the single molecule level in Aph-treated MRC5SV, shWRNIP1 and WRNIP1 UBZ mutant cells. Cells were labelled sequentially with the thymidine analogues 5-chloro-2'-deoxyuridine (CldU) and 5-iodo-2'-deoxyuridine (IdU), as described in the scheme (**Figure 5A**). Under normal growth conditions, and in agreement with previous data (Leuzzi et al. 2016), MRC5SV and shWRNIP1 cells exhibited nearly identical fork velocities, while shWRNIP1^{D37A} cells showed a significantly reduced velocity (**Figure 5A**). Following Aph treatment, fork velocity decreased in all cell lines, but values were markedly lower in cells lacking WRNIP1 or its UBZ domain (**Figure 5B**). Importantly, RNaseH1 overexpression led to a significant increase in the rate of fork progression only in shWRNIP1 and shWRNIP1^{D37A} cells (**Figure 5A and B**). DNA fiber analysis also revealed that loss of WRNIP1 or its UBZ domain resulted in a greater percentage of stalled forks induced by Aph compared to control cells (**Figure 5C**). Moreover, when comparing the percentage of restarting forks in all cell lines, we observed that the absence of WRNIP1 reduced the ability of cells to resume replication after release from Aph to the same extent as the loss of its UBZ domain (**Figure 5D**). Suppressing R-loop formation

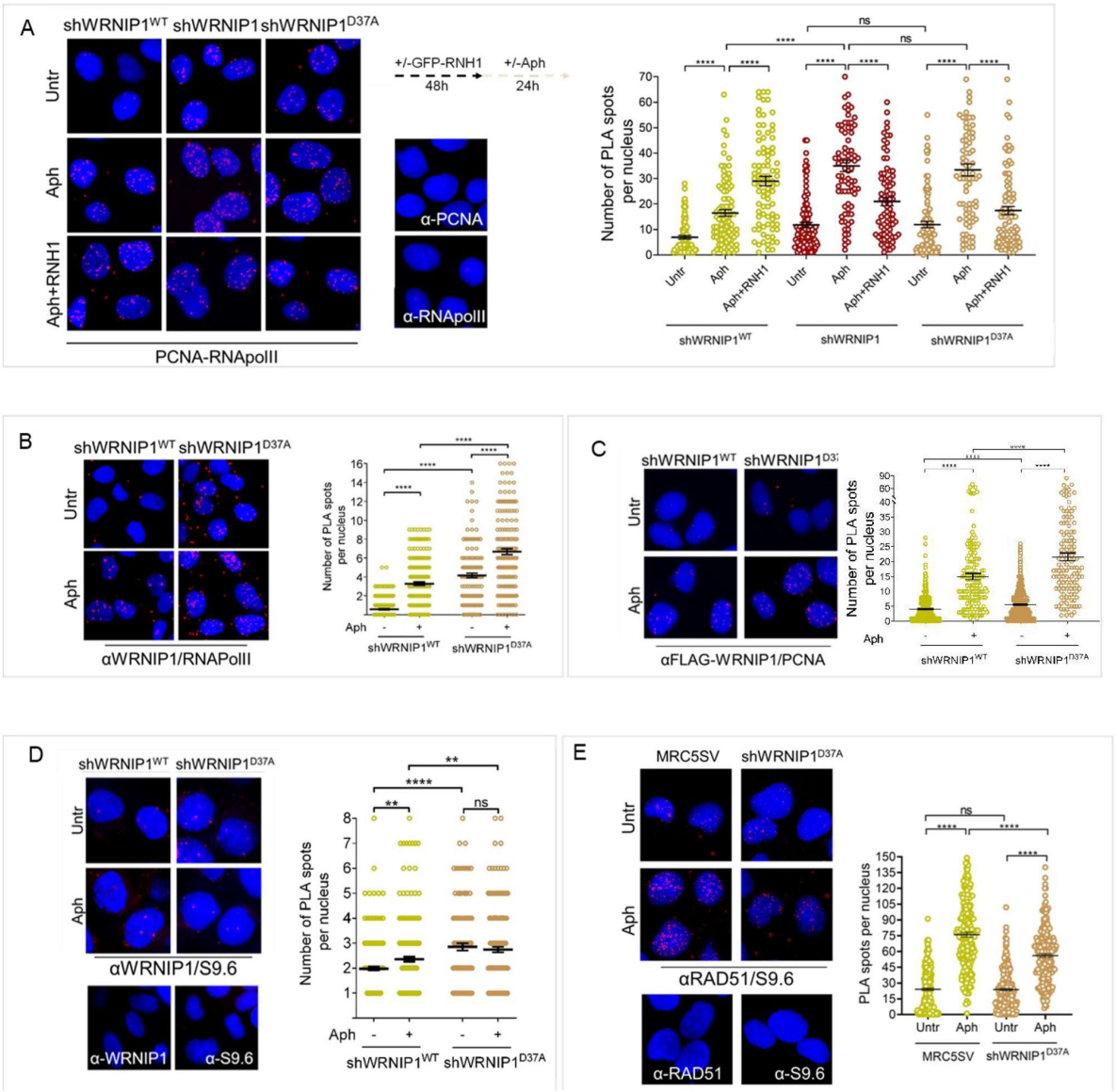

Figure 4.

Loss of WRNIP1 or its UBZ domain promotes R-loop-dependent TRCs accumulation

(A) Detection of TRCs by fluorescence-based PLA assay in MRC5SV, shWRNIP1 and shWRNIP1^{D37A} cells treated as reported in the experimental scheme after transfection with GFP-tagged RNaseH1 or empty vector. Cells were fixed and stained with antibodies against PCNA and RNA pol II. Representative images are given. Each red spot represents a single interaction between proteins. No spot has been revealed in cells stained with each single antibody (negative control). Nuclei were counterstained with DAPI. Dot plot shows the number of PLA spots per nucleus. Data are presented as means of three independent experiments. Horizontal black lines represent the mean ± SE (ns, not significant; ****, $P < 0.0001$; Mann-Whitney test).
(**B** and **C**) Analysis of localization of WRNIP1 near/at the transcription and replication machineries by PLA. Cells were treated or not with 0.4 μM Aph for 24 h, fixed and stained with antibodies against WRNIP1 and RNA pol II (**B**) or WRNIP1 and PCNA (**C**) to visualize the interaction between WRNIP1 and replication or transcription machinery, respectively. Each red spot represents a single interaction between proteins. Representative images are given. Nuclei were counterstained with DAPI. Dot plots show the number of PLA spots per nucleus. Data are presented as means of three independent experiments. Horizontal black lines represent the mean ± SE (****, $P < 0.0001$; Anova one-way test).
(**D** and **E**) Detection of physical interaction between WRNIP1 and R-loops (**D**) or R-loops and RAD51(**E**). Cells were treated or not with 0.4 μM Aph for 24 h, followed by the PLA assay described in "Supplementary Materials and Methods". Cells were stained with antibodies against RNA-DNA hybrid (anti-S9.6) and WRNIP1 (**D**) or RNA-DNA hybrid (anti-S9.6) and RAD51 (**E**). Representative images are given. Each red spot represents a single interaction between R-loops and the respective proteins (WRNIP1 or RAD51). No spot has been revealed in cells stained with each single antibody (negative control). Nuclei were counterstained with DAPI. Dot plot shows the number of PLA spots per nucleus. Horizontal black lines represent the mean ± SE (ns, not significant; ** $P < 0.01$****, $P < 0.0001$; Anova one-way test).

lowered the percentage of stalled forks and, consistently, increased that of restarting forks in both shWRNIP1 and shWRNIP1^{D37A} cells (**Figure 5** C and D). Similar results were obtained by treating cells with DRB (Suppl. Figure 2A-D).

Therefore, we concluded that in cells lacking WRNIP1 or its UBZ domain, R-loops can pose a significant impediment to replication. Additionally, the ubiquitin-binding activity of WRNIP1 may play a crucial role in restarting replication when transcription-induced fork stalling occurs.

FA pathway is correctly activated in WRNIP1-deficient or WRNIP1 UBZ mutant cells

Previous studies have demonstrated that a functional Fanconi anaemia (FA) pathway prevents the unscheduled accumulation of transcription-associated R-loops, with FANCD2 monoubiquitination playing a critical role (Liang et al. 2019). Additionally, it has been reported that the FANCD2 protein complex contains WRNIP1, which, under certain conditions, facilitates the binding of the monoubiquitinated FANCD2/FANCI complex to DNA through its UBZ domain (Socha et al. 2020). Hence, to gain insight into defective R-loop resolution, we assessed the functional status of the FA pathway in our cells by examining FANCD2 monoubiquitination, a well-established readout of FA pathway activation (Taniguchi et al. 2002). To this end, we treated MRC5SV, shWRNIP1 and shWRNIP1^{D37A} cells with Aph and/or DRB and performed Western blotting analysis (**Figure 6A**). Our results showed that loss of WRNIP1 or its UBZ domain did not affect FANCD2 ubiquitination after treatments, indicating that the FA pathway was properly activated under our conditions (**Figure 6A**). Consistently, we observed comparable FANCD2 relocalization during R-loop-associated replication stress in all cell lines tested, and it was reduced by transcription inhibition, providing further evidence of proper FA pathway activation (Suppl. Figure 3A). In agreement with previous findings (Wells et al. 2022), we found that RAD18 plays a role in FANCD2 recruitment upon MRS (Suppl. Figure 3B). Moreover, PLA assay demonstrated that, under our conditions, FANCD2 was localized near/at R-loops (anti S9.6) after Aph with a greater number of spots in shWRNIP1^{D37A} cells compared to control cells (**Figure 6B**).

We then conducted a Comet assay in MRC5SV, shWRNIP1 and shWRNIP1^{D37A} cells depleted of FANCD2 to investigate the potential association between WRNIP1 and the FANCD2 pathway under replication stress. Our results revealed increased spontaneous DNA damage in both the shWRNIP1 and shWRNIP1^{D37A} cells in which FANCD2 has been depleted (**Figure 6C**). Interestingly, disruption of FANCD2 led to a higher degree of DNA damage upon replication stress induced by Aph in the shWRNIP1 and shWRNIP1^{D37A} cells compared to control cells (**Figure 6C**).

These findings collectively suggest that FANCD2 and WRNIP1 may not be inherently interconnected.

Discussion

Discovered as one of the interactors of the WRN helicase (Kawabe et al. 2001; Kawabe et al. 2006), the precise function of WRNIP1 in human cells is largely unknown. Previous results from our lab and others have revealed that WRNIP1 is crucial for the protection of HU-stalled replication forks (Leuzzi et al. 2016; Porebski et al. 2019). More recently, it has been shown that checkpoint defects elicit a WRNIP1-mediated response to limit R-loop-associated genomic instability (Marabitti et al. 2020). However, it remains unclear whether WRNIP1 is directly involved in the mechanisms of R-loop removal/resolution. In this study, we establish a function for WRNIP1 and its ubiquitin-binding zinc finger (UBZ) domain in counteracting DNA damage and genome instability resulting from transcription-replication conflicts (TRCs).

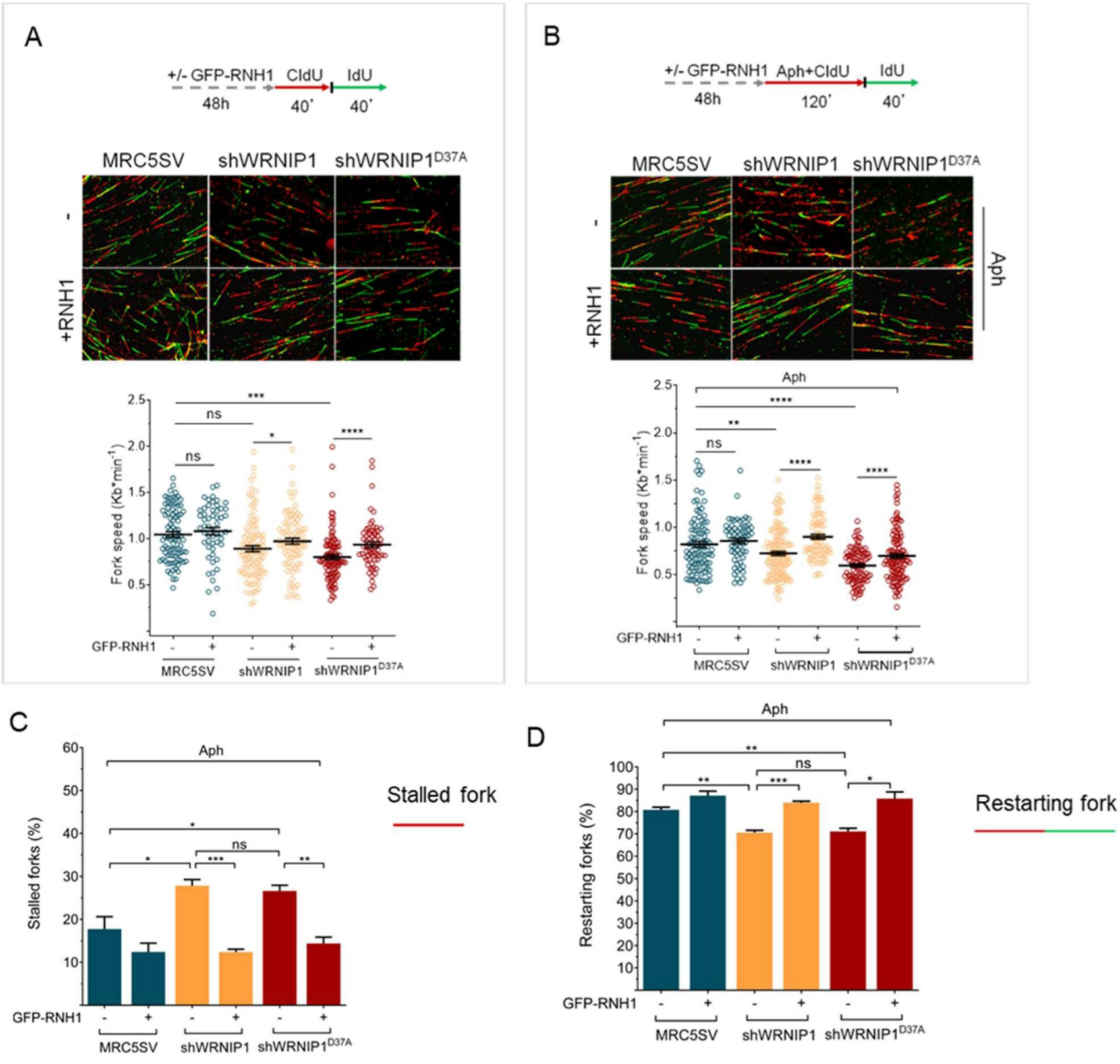

Figure 5.

R-loops affects DNA replication in cells lacking WRNIP1 or its UBZ domain upon MRS

Experimental scheme of dual labelling of DNA fibers in MRC5SV, shWRNIP1 and shWRNIP1^{D37A} cells under unperturbed conditions (**A**) or upon MRS (**B**). After transfection with GFP-tagged RNaseH1 or empty vector (-), cells were pulse-labelled with CldU, treated or not with 0.4 μM Aph, then subjected to a pulse-labelling with IdU. Representative DNA fiber images are shown. The graph shows the analysis of replication fork velocity (fork speed) in the cells. The length of the green tracks was measured. Mean values are represented as horizontal black lines (ns, not significant; *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$; ****, $P < 0.0001$; Mann-Whitney test). (**C** and **D**) The graph shows the percentage of red (CldU) tracts (stalled forks) or green (IdU) tracts (restarting forks) in the cells. Error bars represent standard error (ns, not significant; *, $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$; two tailed Student t-test).

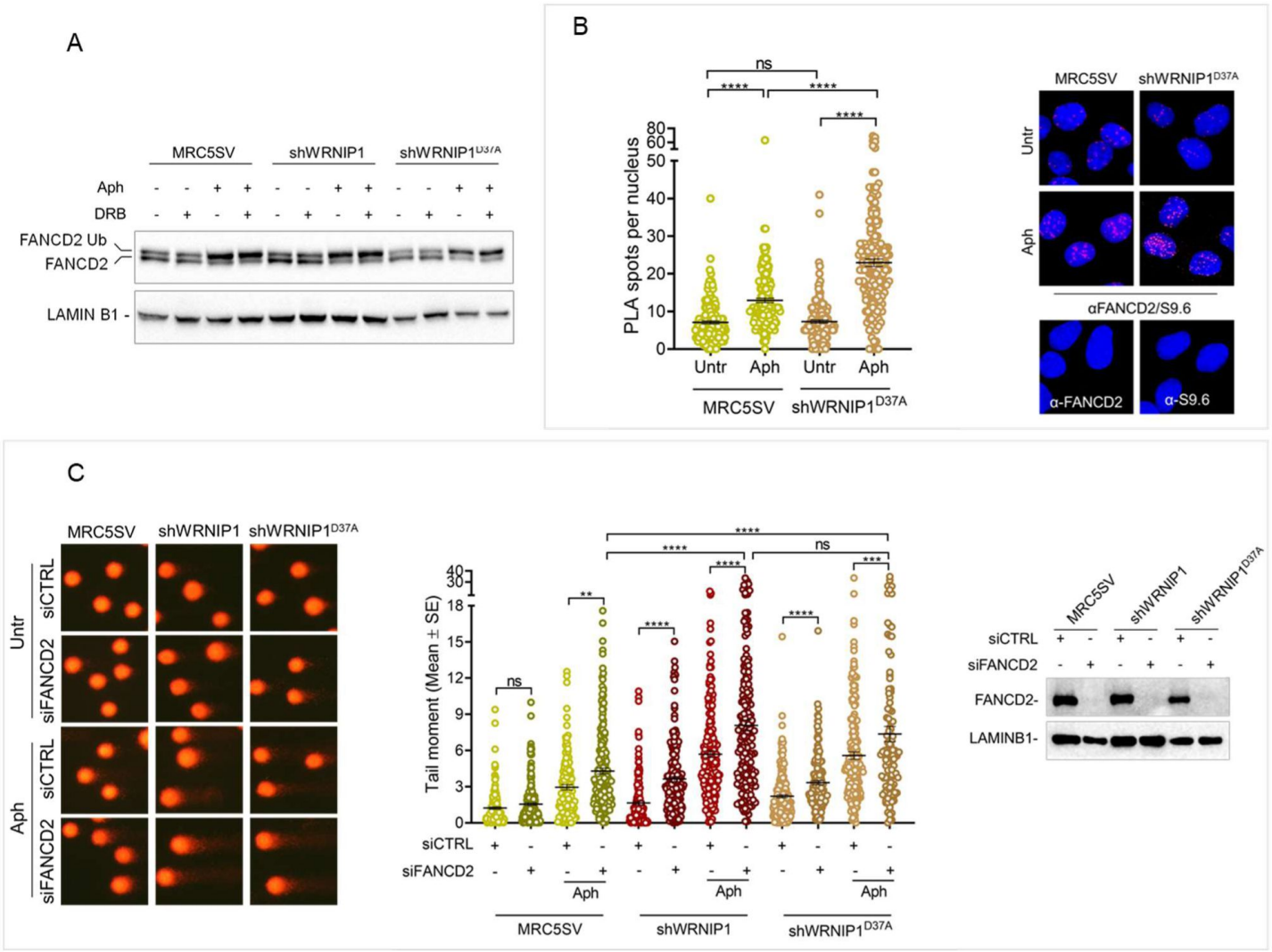

Figure 6.

FANCD2 pathway activation in cells lacking WRNIP1 or its UBZ domain upon MRS

(**A**) Western blot analysis showing FANCD2 ubiquitination in MRC5SV, shWRNIP1 and shWRNIP1^{D37A} cells. The membrane was probed with an anti-FANCD2 antibody. LAMIN B1 was used as a loading control.

(B) Detection of physical interaction between FANCD2 and R-loops by PLA. MRC5SV and shWRNIP1^{D37A} cells treated with or not with 0.4 μM Aph for 24h. Cells were stained with antibodies against RNA-DNA hybrid (anti-S9.6) and FANCD2. Representative images are given. Each red spot represents a single interaction between R-loops and FANCD2. No spot has been revealed in cells stained with each single antibody (negative control). Nuclei were counterstained with DAPI. Dot plot shows the number of PLA spots per nucleus. Data are presented as means of three independent experiments. Horizontal black lines represent the mean ± SE (ns, not significant; ****, $P < 0.0001$; Anova one-way test).

(C) Evaluation of DNA damage accumulation by alkaline comet assay in MRC5SV, shWRNIP1 and shWRNIP1^{D37A} transfected with control siRNAs (siCTRL) or siRNAs targeting FANCD2 (siFANCD2). After 48 h, cells were treated with 0.4 μM Aph for 24h, then subjected to Comet assay. Dot plot shows data presented as the mean tail moment ± SE from three independent experiments.

Horizontal black lines represent the mean (ns, not significant; ** $P < 0.01$; *** $P < 0.001$; **** $P < 0.0001$; two-tailed Student's t test). Representative images are given. Western blot shows FANCD2 depletion in the cells. The membrane was probed with an anti-FANCD2 and LAMIN B1 was used as a loading control.

We have demonstrated that WRNIP1 contributes to fork restart, and that this function requires its ATPase activity (Leuzzi et al. 2016). It is noteworthy that although the ATPase activity of WRNIP1 is important for the restart of stalled forks (Leuzzi et al. 2016), it is dispensable to cope with TRCs. In sharp contrast, a mutation that disrupts the WRNIP1 UBZ domain (Bish and Myers 2007; Nomura et al. 2012) is sufficient to stimulate TRCs and DNA damage at levels comparable to, if not higher than, those detected in WRNIP1-deficient cells. The persistence of R-loops is deleterious because they may interfere with DNA replication, and R-loop-mediated fork stalling is a major feature of TRCs (Gan et al. 2011; Boubakri et al. 2010; Wellinger et al., 2006; Tuduri et al. 2009). In WRNIP1-deficient or WRNIP1 UBZ mutant cells, the elevated DNA damage and reduced fork speed are reverted by RNase H1-GFP overexpression, which degrades RNA/DNA hybrids and wipes out R-loops (Cerritelli et al. 2003). In line with this, many more R-loops are detected in WRNIP1-deficient or WRNIP1 UBZ mutant cells. This suggests that unscheduled R-loops are likely responsible for the increased levels of TRCs, the marked replication defects, and genomic instability observed in the absence of WRNIP1.

R-loops are the main DNA secondary structures formed during transcription and, although they regulate several physiological processes, they can also act as potent replication fork barriers (Crossley et al., 2019; Gómez-González and Aguilera 2019). Consequently, the unscheduled accumulation of R-loops leads to TRCs, creating transcription-associated DNA damage in both yeast and human cells (Huertas and Aguilera 2003; Li and Manley 2005; Santos-Pereira and Aguilera 2015; Sollier et al. 2014; Paulsen et al. 2009). To prevent TRCs, several replication fork protection factors are essential (Chang and Stirling 2017). Disrupting any of these proteins can affect the ability of cells to overcome or resolve R-loops, leading to DNA damage.

Our previous research has established that WRNIP1, although not through its ATPase activity, can protect stalled forks from nucleolytic degradation upon replication stress (Leuzzi et al. 2016). In addition, WRNIP1 has been shown to immunoprecipitate with BRCA2 and RAD51, and loss of WRNIP1 has been found to destabilize RAD51 on ssDNA (Leuzzi et al. 2016) Notably, when R-loop is formed, it can expose a potentially vulnerable single-stranded DNA (ssDNA) region on the non-template strand (Aguilera and García-Muse 2012). Since BRCA2 is kwon to play an important role in mitigating R-loop-induced DNA damage (Shivji et al. 2018; Bhatia et al. 2014; Wang et al. 2022), it is possible that WRNIP1, through its association with the BRCA2/RAD51 complex, could promote RAD51 recruitment or stabilization to protect the ssDNA of R-loops. In support of this, RAD51 co-localizes with R-loops after MRS and this co-localization is dependent on the WRNIP1 UBZ domain. Therefore, WRNIP1 may protect R-loops to ensure their safe handling and promote the successful restart of stalled forks. Consistent with this hypothesis, we observe WRNIP1 co-localizing with transcription/replication complexes and R-loops after MRS. It has been proposed that R-loop incision, which promotes fork progression and restart at TRCs (Chappidi et al. 2020), requires high accuracy to avoid fork collapse and chromosomal damage, putting genome integrity at risk (Sollier et al. 2014; Promonet et al. 2020). Our findings show that R-loop-dependent accumulation of DNA damage and genome instability, which may indicate preferential engagement of error-prone mechanisms upon WRNIP1 depletion. Alternatively, WRNIP1 may stabilize RAD51 on the ssDNA of DNA/RNA hybrids and promote fork restart through fork reversal. Indeed, one proposed mechanism for processing DNA/RNA hybrids involves exposing ssDNA and loading RAD51, which mediates fork reversal (Stoy et al. 2023). While these hybrids are resolved under physiological conditions, allowing stalled fork restart (Stoy et al. 2023; Chappidi et al. 2020), post-replicative DNA/RNA hybrids would represent pathological TRC intermediates that can impair replication fork progression and stimulate fork reversal.

The phenotype of WRNIP1 UBZ mutant cells and WRNIP1-depleted cells is similar, suggesting that the ubiquitin-binding function of WRNIP1 could help mitigate R-loop-induced TRCs. Although the UBZ domain of WRNIP1 still has an undefined function after replication perturbation (Yoshimura et al., 2017), human WRNIP1 binds to both forked DNA, which resembles stalled replication forks, and to template/primer DNA in an ATP-dependent fashion (Yoshimura et al. 2009).

However, our findings suggest that WRNIP1 does not use its ATPase activity to counteract TRC-induced R-loops accumulation. Nevertheless, WRNIP1's ability to bind ubiquitin indicates that it may be implicated in the metabolic processes of ubiquitinated proteins (Bish and Myers 2007). The UBZ domain of WRNIP1 is involved in the physical association with RAD18 (Kanu et al. 2016) RAD18-deficient cells exhibit high levels of TRCs and accumulate DNA/RNA hybrids, which induce DNA double strand breaks and replication stress (Wells et al. 2022). These effects are, in part, dependent on the inability to recruit the Fanconi anaemia protein FANCD2 to R-loop prone genomic sites (Wells et al. 2022). Notably, in our context, FANCD2 localizes with R-loops after MRS, and this localization is more pronounced in cells lacking the WRNIP1 UBZ domain. Additionally, in our cell lines, FA pathway may serve as a backup system to counteract TRCs. Therefore, it is tempting to speculate that the UBZ domain might contribute to direct WRNIP1 to DNA at TRC sites through RAD18 and that the elevated levels of TRCs observed in RAD18-deficient cells are due to the loss of both WRNIP1 and FANCD2 functions. This interesting point deserves further and detailed investigation.

Altogether our findings reveal a previously unappreciated role for WRNIP1 in counteracting the pathological accumulation of transcription-associated R-loops, which ultimately leads to heightened genomic instability following MRS in human cells. A growing body of evidence suggests that R-loops may underlie the genomic instability observed in human cancer cells (Crossley et al., 2019; García-Muse and Aguilera 2019) Moreover, given that WRNIP1 is found to be overexpressed in the most common human cancer types, such as lung and breast cancers, our discoveries expand our comprehension of the mechanisms employed by cells to avert TRCs. These results pave the way for exploring novel pathways that contribute to genomic instability in cancer.

Materials and methods

Cell lines and culture conditions

The SV40-transformed MRC5 fibroblast cell line (MRC5SV) was a generous gift from Patricia Kannouche (IGR, Villejuif, France). MRC5SV cells stably expressing WRNIP1-targeting shRNA (shWRNIP1) and isogenic cell lines stably expressing the RNAi-resistant full-length wild-type WRNIP1 (shWRNIP1^{WT}), its ATPase-dead mutant form (shWRNIP1^{T294A}) were generated as previous reported (Leuzzi et al. 2016). By using the Neon^{TM} Transfection System Kit (Invitrogen) according to the manufacturer's instructions, shWRNIP1 cells were stably transfected with a plasmid expressing a FLAG-tagged full-length WRNIP1 plasmid carrying Ala substitution at Asp37 site missense-mutant form of WRNIP1 with dead form of ubiquitin-binding zinc finger (UBZ) domain (WRNIP1^{D37A}) (Yoshimura et al., 2017), Cells were cultured in the presence of neomycin and puromycin (1 mg/ml and 100 ng/ml, respectively) to maintain selective pressure for expression.

All cell lines were maintained in DMEM media supplemented with 10% FBS, 100 U/mL penicillin and 100 mg/mL streptomycin and incubated at 37°C in an humified 5% CO2 atmosphere.

Site-directed mutagenesis and cloning

Site-directed mutagenesis of the WRNIP1 full-lenght cDNA (Open Biosystems) was performed on the pCMV-FLAGWRNIP1 plasmid that contains the wild-type ORF sequence of WRNIP1. Substitution of Asp37 to Ala in pCMV-FLAGWRNIP1 was introduced by the Quick-change XL kit (Stratagene) using mutagenic primer pairs designed, according to the manufacturer's instructions. Each mutated plasmid was verified by full sequencing of the WRNIP1 ORF.

Chemicals

Chemicals used were commercially obtained for the replication stress-inducing drug: aphidicolin (Aph, Sigma-Aldrich), hydroxyurea (HU, Sigma-Aldrich) and the transcription elongation inhibitor 5,6-dichloro-1-ß-D-ribofurosylbenzimidazole (DRB, Sigma-Aldrich). The final concentrations of the drugs used were: 0.4 aphidicolin, 4 mM HU and 50 µM DRB. Stock solutions for all the chemicals were prepared in DMSO at a concentration of >1000. The final concentration of DMSO in the culture medium was always < 0.1%.

Plasmids and RNA interference

The construct used to perform RNaseH1 overexpression experiments is a generous gift from Prof. R.J. Crouch (National Institutes of Health, Bethesda, USA). As previously described (Cerritelli et al. 2003), the GFP-tagged RNaseH1 plasmid was generated by introducing a mutation on Met27 abrogating mitochondrial localization signal (RNaseH1-M27). To express the plasmids, cells were transfected using the Neon™ Transfection System Kit (Invitrogen), according to the manufacturer's instructions.

FANCD2 genetic knockdown experiment was performed by Interferin (Polyplus), according to the manufacturer's instructions with Silencer Select siRNA (Thermo Fischer) targeting the following region of the mRNA (5'-CAGCCUACCUGAGAUCCUAtt-3'). siRNA was used at 2.5 nM. As a control, a siRNA duplex directed against GFP was used. Depletion was confirmed by Western blot using the relevant antibodies (*see below*).

Western blot analysis

The proteins were resolved on polyacrylamide gels and transferred onto nitrocellulose membrane using the Trans-Blot Turbo Transfer System (Bio-Rad). The membranes were blocked using 5% NFDM in TBST (50 mM Tris/HCl pH 8, 150 mM NaCl, 0.1% Tween-20), and incubated with primary antibody for 1 h at RT. The primary antibodies used for WB were: rabbit-polyclonal anti-WRNIP1 (Bethyl Laboratories, 1:2500), mouse-monoclonal anti-FLAG (Sigma-Aldrich, 1:1000), mouse-polyclonal anti-GAPDH (Millipore, 1:5000), mouse-monoclonal anti-FANCD2 (Santacruz; 1:500) and rabbit-polyclonal anti-LAMIN B1 (Abcam, 1:30000).

The membranes were incubated with horseradish peroxidase-conjugated goat specie-specific secondary antibodies (Santa Cruz Biotechnology, 1:20000), for 1 h at RT. Visualisation of the signal was accomplished using Western Bright ECL HRP substrate (Advansta) and imaged using Chemidoc XSR+ (Chemidoc Imaging Systems, Bio-Rad).

Chromosomal aberrations

Cells for metaphase preparations were collected according to standard procedure and as previously reported (Pirzio et al. 2008). Cell suspension was dropped onto cold, wet slides to make chromosome preparations. The slides were air dried overnight, then for each condition of treatment, the number of breaks and gaps was observed on Giemsa-stained metaphases. For each time point, at least 50 chromosome metaphases were examined by two independent investigators, and chromosomal damage was scored at 100× magnification with an Olympus fluorescence microscope.

Alkaline Comet assay

DNA breakage induction was examined by alkaline Comet assay (single-cell gel electrophoresis) in denaturing conditions as described (Pichierri et al. 2001). Cell DNA was stained with a fluorescent dye GelRed (Biotium) and examined at 40× magnification with an Olympus fluorescence microscope and examined at 40× magnification with an Olympus fluorescence microscope. Slides were analysed by a computerized image analysis system (CometScore, Tritek

Corp). To assess the amount of DNA damage, computer-generated tail moment values (tail length × fraction of total DNA in the tail) were used. A minimum of 200 cells was analysed for each experimental point. Apoptotic cells (smaller comet head and extremely larger comet tail) were excluded from the analysis to avoid artificial enhancement of the tail moment.

Immunofluorescence

Immunostaining for RNA-DNA hybrids was performed as described (Marabitti et al. 2019). Briefly, cells were fixed in 100% methanol for 10 min at −20°C, washed three times in PBS, pre-treated with 6 µg/ml of RNase A for 45 min at 37°C in 10 mM Tris-HCl pH 7.5 supplemented with 0.5 M NaCl, before blocking in 2% BSA/PBS overnight at 4°C. Cells were then incubated with the anti-DNA-RNA hybrid [S9.6] antibody (Kerafast, 1:100) overnight at 4°C. After each primary antibody, cells were washed twice with PBS, and incubated with the specific secondary antibody: goat anti-mouse Alexa Fluor-488 or goat anti-rabbit Alexa Fluor-594 (Molecular Probes). The incubation with secondary antibodies were accomplished in a humidified chamber for 1 h at RT. DNA was counterstained with 0.5 µg/ml DAPI.

Images were acquired randomly using Eclipse 80i Nikon Fluorescence Microscope, equipped with a VideoConfocal (ViCo) system. For each time point, at least 200 nuclei were examined. Nuclear foci were scored at a 40× magnification and only nuclei showing more than five bright foci were counted as positive. Intensity per nucleus was calculated using ImageJ. Parallel samples incubated with either the appropriate normal serum or only with the secondary antibody confirmed that the observed fluorescence pattern was not attributable to artefacts.

Dot blot analysis

Dot blot analysis was performed according to the protocol reported elsewhere (Morales et al. 2016). Genomic DNA was isolated by standard extraction with Phenol/Clorophorm/Isoamylic Alcohol (pH 8.0) followed by precipitation with 3 M NaOAc and 70% Ethanol. Isolated gDNA was randomly fragmented overnight at 37°C with a cocktail of restriction enzymes (BsrgI, EcoRI, HindIII, XbaI) supplemented with 1 M Spermidin. After incubation, digested DNA was cleaned up with Phenol/Clorophorm extraction and standard Ethanol precipitation. After sample quantification, 5 µg of digested DNA were incubated with RNaseH overnight at 37°C as a negative control. Five micrograms of each sample were spotted onto a nitrocellulose membrane, blocked in 5% non-fat dry milk and incubated with the anti-DNA-RNA hybrid [S9.6] antibody (Kerafast, 1:1000) overnight at 4°C. Horseradish peroxidase-conjugated goat specie-specific secondary antibody (Santa Cruz Biotechnology, Inc.) was used. Quantification on scanned image of blot was performed using Image Lab software.

In situ PLA assay

The in situ proximity-ligation assay (PLA; Sigma-Aldrich) was performed according to the manufacturer's instructions. Exponential growing cells were seeded into 8-well chamber slides (Lab-Tek, Sigma Aldrich) at a density of $1.5\text{-}2.5\times10^4$ cells/well. After the indicated treatment, cells were permeabilized with 0.5% Triton X-100 for 10 min at 4°C, fixed with 3% formaldehyde/ 2% sucrose solution for 10 min, and then blocked in 3% BSA/PBS for 15 min. After washing with PBS, cells were incubated with the two relevant primary antibodies. For the detection of R-loops using PLA with the anti-S9.6 antibody, cells were fixed with ice-cold methanol for 10 min and blocked in 2% BSA/PBS for 1h at 37°C.

The primary antibodies used were: anti-FLAG (mouse-monoclonal; Sigma-Aldrich, 1:250), anti-WRNIP1 (rabbit-polyclonal; Bethyl 1:500), anti-S9.6 (mouse-monoclonal; Kerafast, 1:100), anti-PCNA (rabbit-polyclonal; Abcam 1:500), anti-RNA polII (mouse-monoclonal; Santa Cruz, 1:200), recombinant anti-S9.6 (mouse-monoclonal; Kerafast, 1:100), anti-RAD51 (rabbit-polyclonal, Abcam 1:300) and anti-FANCD2 (mouse-monoclonal, Santacruz 1:100). The negative control consisted of

using only one primary antibody. Samples were incubated with secondary antibodies conjugated with PLA probes MINUS and PLUS: the PLA Probe anti-Mouse PLUS and anti-Rabbit Minus (Sigma-Aldrich). The incubation with all antibodies was accomplished in a humidified chamber for 1 h at 37°C. Next, the PLA probes MINUS and PLUS were ligated using their connecting oligonucleotides to produce a template for rolling-cycle amplification. During amplification, the products were hybridized with red fluorescence-labelled oligonucleotide. Samples were mounted in Prolong Gold antifade reagent with DAPI (blue). Images were acquired randomly using Eclipse 80i Nikon Fluorescence Microscope, equipped with a Video Confocal (ViCo) system.

DNA fiber analysis

Cells were pulse-labelled with 50 µM 5-chloro-2'-deoxyuridine (CldU) and 250 µM 5-iodo-2'-deoxyuridine (IdU) at specified times, with or without treatment as reported in the experimental schemes. DNA fibres were prepared and spread out as previously reported (Leuzzi et al. 2016). For immunodetection of labelled tracks the following primary antibodies were used: anti-CldU (rat-monoclonal anti-BrdU/CldU; BU1/75 ICR1 Abcam, 1:100) and anti-IdU (mouse-monoclonal anti-BrdU/IdU; clone b44 Becton Dickinson, 1:10). The secondary antibodies were goat anti-mouse Alexa Fluor 488 or goat anti-rabbit Alexa Fluor 594 (Molecular Probes, 1:200). The incubation with antibodies was accomplished in a humidified chamber for 1 h at RT.

Images were acquired randomly from fields with untangled fibres using Eclipse 80i Nikon Fluorescence Microscope, equipped with a Video Confocal (ViCo) system. The length of green labelled tracks was measured using the Image-J software, and values were converted into kilobases using the conversion factor 1µm = 2.59 kb as reported (Basile et al. 2014). A minimum of 100 individual fibres were analysed for each experiment and the mean of at least three independent experiments presented. Statistics were calculated using Graph Pad Prism Software.

Statistical analysis

Statistical analysis was performed using Prism 8 (GraphPad Software). Details of the individual statistical tests are indicated in the figure legends and results. Statistical differences in all case were determined by Student's t-test, Mann-Whitney test or Anova one way test. In all cases, not significant: $P > 0.05$; * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$; **** $P < 0.0001$. All experiments were repeated at least three times unless otherwise noted.

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Reviewer #1 (Public Review):

This paper describes the role of WRNIP1 AAA+ ATPase, particularly its UBZ domain for ubiquitin-binding, but not ATPase, to prevent the formation of the R-loop when DNA replication is mildly perturbated. By combining cytological analysis for DNA damage, R-loop, and chromosome aberration with the proximity ligation assay for colocalization of various proteins involved in DNA replication and transcription, the authors provide solid evidence to support the claim. The authors also revealed a distinct role of WRNIP1 in the prevention of R-loop-induced DNA damage from FANCD2, which is inconsistent with the known relationship between WRNIP1 and FANCD2 in the repair of crosslinks.

Reviewer #2 (Public Review):

This paper aims at establishing the role of WRN-interacting protein 1 (WRNIP1) and its UBZ domain (an N-terminal ubiquitin-binding zinc finger domain) on genome instability caused by mild inhibition of DNA synthesis by aphidicolin. The authors used human MRC5 fibroblasts investigated with standard methods in the field. The results clearly showed that WRNIP1 silencing and UBZ-mutation (D37A) increased DNA damage, chromosome aberrations, and transcription-replication conflicts caused by aphidicolin.

The conclusions of the paper are overall well supported by results, however, aspects of some data analyses would need to be clarified and/or extended.

1 The methods (immunofluorescence microscopy and dot-blots) to determine R-loop levels can lack sensitivity and specificity. In particular, since the S9.6 antibody can bind to other structures besides heteroduplex, dot-blot analyses only grossly assess R-loop levels in cellular samples of purified nucleic acids, which are constituted by many different types of DNA/RNA structures.

2 Experimental plan has analyzed the impact of WRNIP1 lack or mutations at steady-state conditions. Thus, the possible role of WRNIP1 at an early step of the mechanism would require some sort of kinetics analysis of the molecular process, therefore not at steady-state conditions. The findings of a co-localization of R-loops and WRNIP1 have been obtained with the S9.6 antibody, which recognizes DNA-RNA heteroduplexes. Since WRNIP1 is known to be recruited at stalled forks and DNA cleavage sites, it is not surprising that WRNIP1 is very close to heteroduplexes, abundant structures at replication forks and cleavage sites. Similar interpretations may also be valid for Rad51/S9.6 co-localization findings.

3 Determination of DNA damage, chromosome aberration, and co-localization data are reported as means of measurements with appropriate statistics. However, the fold-change values relative to corresponding untreated samples are not reported. In some instances, it seems that WRNIP1 silencing or mutations actually reduce or do not affect aphidicolin effects. That leaves open the interpretation of specific results.

Reviewer #3 (Public Review):

Summary:
In the manuscript by Valenzisi et al., the authors report on the role of WRNIP1 to prevent R-loop and TRC-associated DNA damage. The authors claim WRNIP1 localizes to TRCs in response to replication stress and prevents R-loop accumulation, TRC formation, replication fork stalling, and subsequent DNA damage. While the findings are of potential significance to the field, the strength of evidence in support of the conclusions is lacking.

Weaknesses:

1. The authors fail to utilize the proper controls throughout the manuscript in regard to the shWRNIP1, WT, and mutant cell lines. It is unclear why the authors failed to use the shWRNIP1WT line in the comet assay, DNA fiber assay, and the FANCD2 assays. This is a key control for i) the use of only a single shRNA (most studies will use at least 2 different shRNAs) and ii) the use of the mutant WRNIP1 lines. In several figures, the authors only show the effect of the UBZ mutant, but don't include the ATPase mutant or WT for comparison. Including these is essential.

2. The authors use the S9.6 antibody to conclude the loss of WRNIP1 causes more R-loops; however, it has been shown that this antibody detects dsRNA in addition to RNA-DNA hybrids. Accordingly, it cannot be ruled out that the increased S9.6 signal is due to increased dsRNA.

3. Multiple pieces of data do not support the conclusions. For example, Figure 1D shows shWRNIP1 to reduce damage in Aph+DRB cells compared to MRC5SV cells with Aph+DRB. This result suggests that WRNIP1 actually increases DNA damage in stressed cells with transcription blocked. Another result is seen in Figure 4a, where the number of PLA spots (presumably TRCs) increases in the shWRNIP1WT cells with Aph+RNH1 compared to Aph alone. If R-loops are required for TRC accumulation, then the RNH1 should decrease the PLA foci. This result instead suggests that WRNIP leads to increased TRCs in stressed cells with R-loops cleared by RNH1.

4. The data are mostly phenomenological and fail to yield mechanistic insight. For example, the authors state that "it remains unclear whether WRNIP1 is directly involved in the mechanisms of R-loop removal/resolution". Unfortunately, the data presented in this manuscript do not provide new insights into this unresolved question.

5. The authors only show merged images making it impossible to visualize differences in PLA foci.