

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 fork protection factors and DNA damage response proteins as regulators of R-loop dynamics. In this study, 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

The maintenance of genome integrity is essential for cell viability, relying on complete and accurate DNA replication. However, the genome is continuously threatened by several types of DNA lesions that impede replication fork progression, leading to fork stalling, replication stress

and ultimately to genomic instability - a hallmark of cancer cells. Different molecular mechanisms underlie replication stress, and understanding their occurrence has been of interest for cancer therapy. Among these mechanisms, the transcription process plays a crucial role in inducing replication fork stalling. Transcription not only hampers replication fork progression but also acts as an enhancer of replication impediments, including protein-DNA complexes, DNA damage, and non-B-form DNA secondary structures (Gómez-González and Aguilera 2019 [↗](#)).

R-loops have emerged as critical determinants of transcription-associated replication stress, capable of causing genomic instability and promoting 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 normal conditions, R-loops are transient structures resolved by enzymes (Sanz et al. 2016 [↗](#); Wahba et al. 2011 [↗](#)). However, persistent R-loops can become pathological, leading to the formation of transcription-replication conflicts (TRCs). Given the high frequency of replication and transcription processes in cells, TRCs are particularly likely to occur. 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).

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 [↗](#)). However, 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 [↗](#)) with its foci overlapping 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 [↗](#)). Additionally, 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 [↗](#)). Furthermore, WRNIP1 is found to be enriched at CFS, suggesting a role in maintaining their stability (Pladevall-Morera et al. 2019 [↗](#)). However, data regarding a role of WRNIP1 in restricting transcription-associated genomic instability are not available.

Here, we reveal that loss of WRNIP1 and the activity of WRNIP1 ubiquitin-binding zinc finger (UBZ) domain are necessary to limit unscheduled R-loops leading to TRCs. Additionally, we establish that loss of WRNIP1 functions is responsible 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 a 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. Consistent 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-β-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, 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.

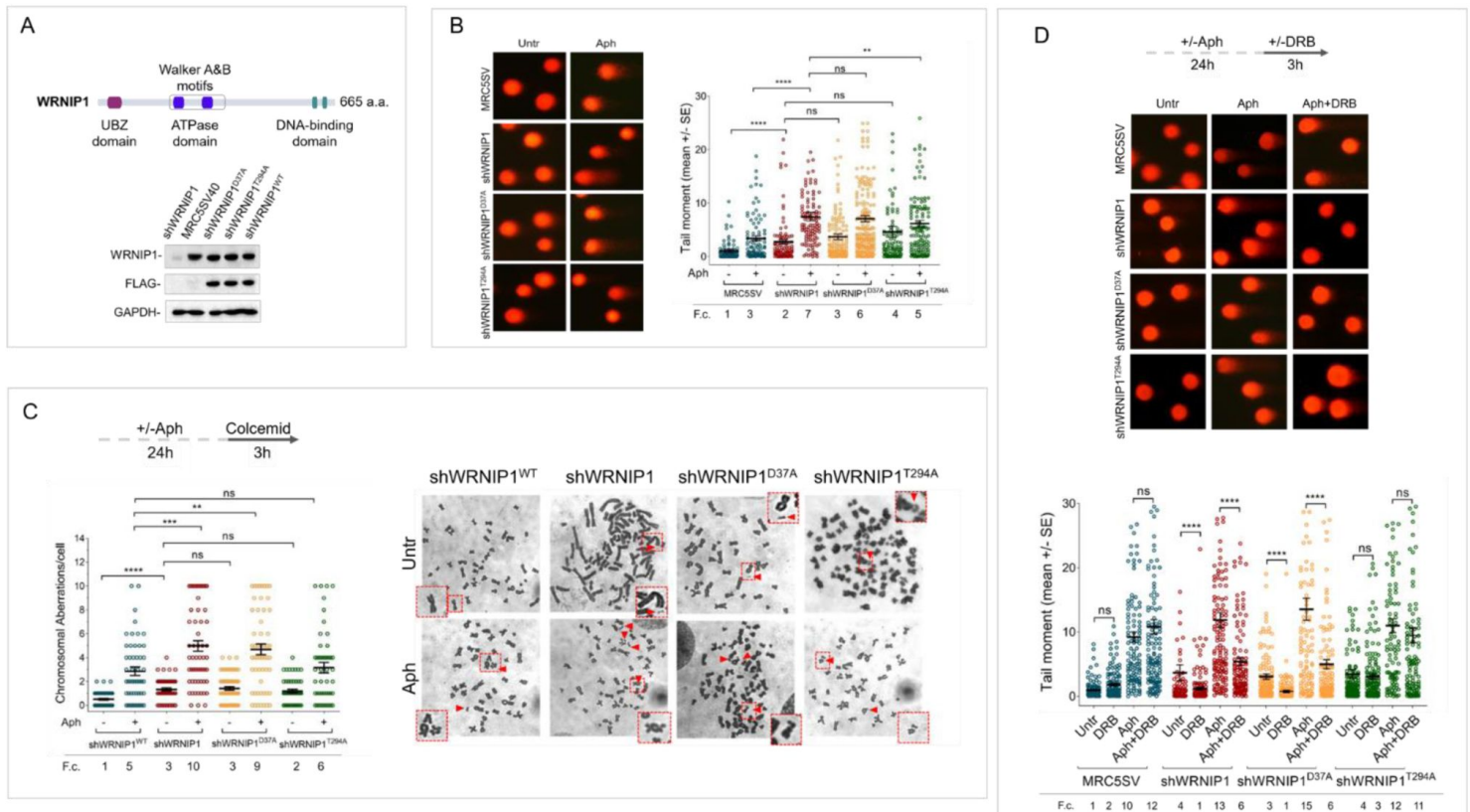


Figure 1.

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 shows WRNIP1 protein expression 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 μ M Aph for 24 h, followed by Comet assay. Data are presented as means 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 provided.

(C) Analysis of chromosomal aberrations in the indicated cell lines treated according to the experimental scheme. Dot plot displays the number of chromosomal aberrations per cell $\pm$ 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, including insets with enlarged metaphases for better visualization of chromosomal aberrations, are included.

(D) Evaluation of DNA damage accumulation by alkaline Comet assay. Cells were treated according to the experimental scheme and subjected to Comet assay. Dot plot presents as means 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 provided.

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 RNase H1 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 RNase H 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** [↗](#)). Overexpression of RNase H1 strongly reduced the tail moment, particularly 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.

To remove contaminant-free RNA, especially dsRNA that might interfere with the capture of RNA-DNA hybrids by the S9.6 antibody (Hartono et al. 2018 [↗](#)), and to enhance the accuracy of R-loop level determination, cells were subjected to RNase III treatment following established protocols (Crossley et al. 2021 [↗](#)). We initially verified the specificity of the enzyme under our conditions. RNase III treatment substantially decreased the level of dsRNA, nearly eliminating it, as confirmed by assessing specific antibody staining against dsRNA (Suppl. Figure 2).

Next, through RNase III treatment, we investigated which of the WRNIP1 activities could prevent the formation of aberrant R-loops upon MRS. We observed similar levels of spontaneous R-loops in cells with mutations in either the 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 during MRS.

Finally, we aimed 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 RNase H1 overexpression was similar to that observed in shWRNIP1 cells (**Figure 3C** [↗](#)), suggesting that the ubiquitin-binding activity of WRNIP1 is necessary to prevent R-loop-mediated DNA damage upon MRS.

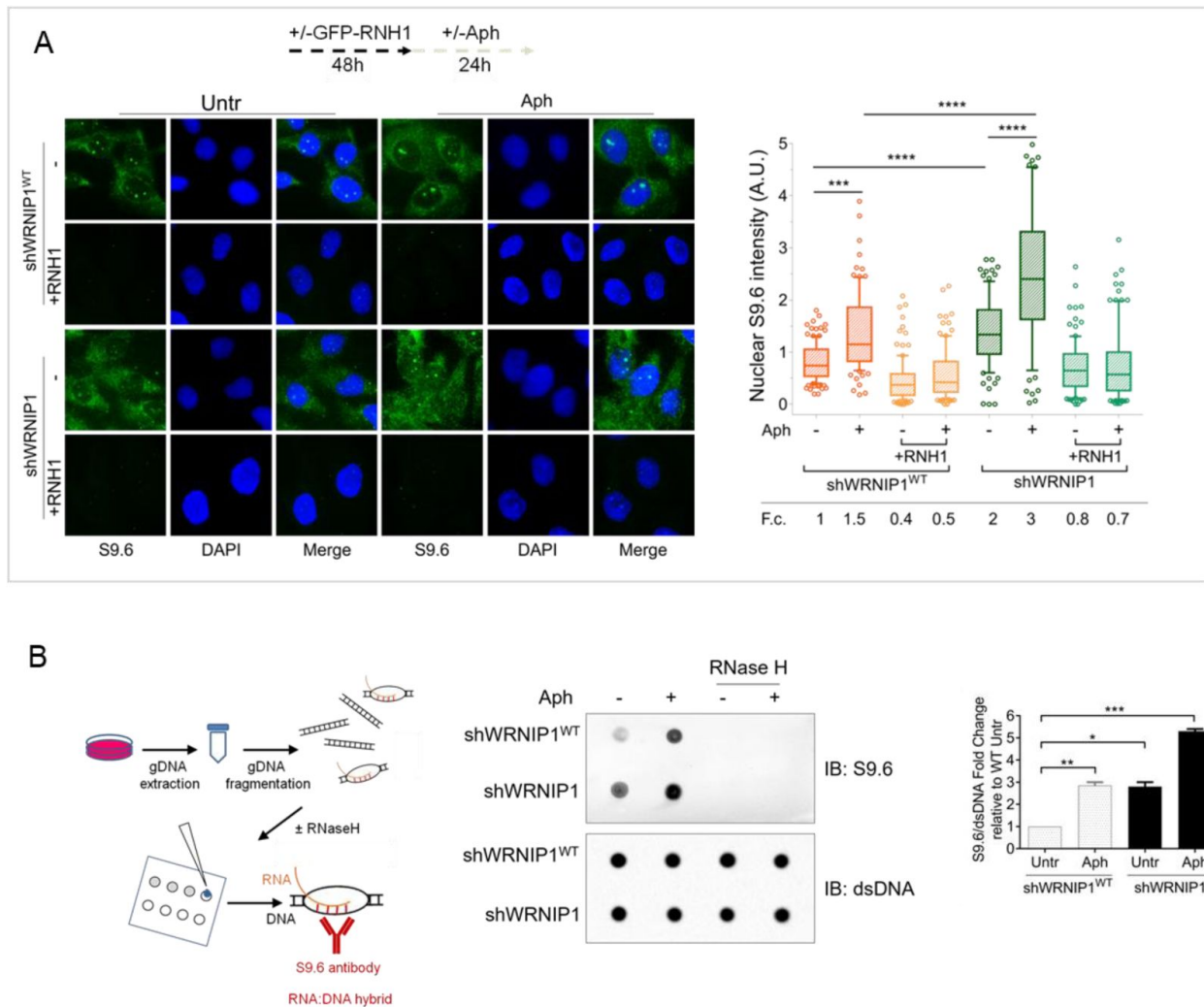


Figure 2.

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, following treatment as reported in the experimental design post-transfection with GFP-tagged RNase H1 or empty vector. Cells were fixed, stained with anti-RNA-DNA hybrid S9.6 antibody, and DNA counterstained with DAPI. Representative images are provided for each single color channel. Box plot displays nuclear S9.6 fluorescence intensity, with boxes and whiskers representing 20-75 and 10-90 percentiles, respectively. The line represents the median value. Data are presented as means from 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 shWRNIP1^{WT} and shWRNIP1 cells, treated as reported in the experimental design, was spotted onto a nitrocellulose membrane. The membranes were probed with anti-RNA-DNA hybrid S9.6 and anti-dsDNA antibodies. Treatment with RNase H was used as a negative control. Representative gel images of at least three replicates are shown (* $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$; two-tailed Student's t test).

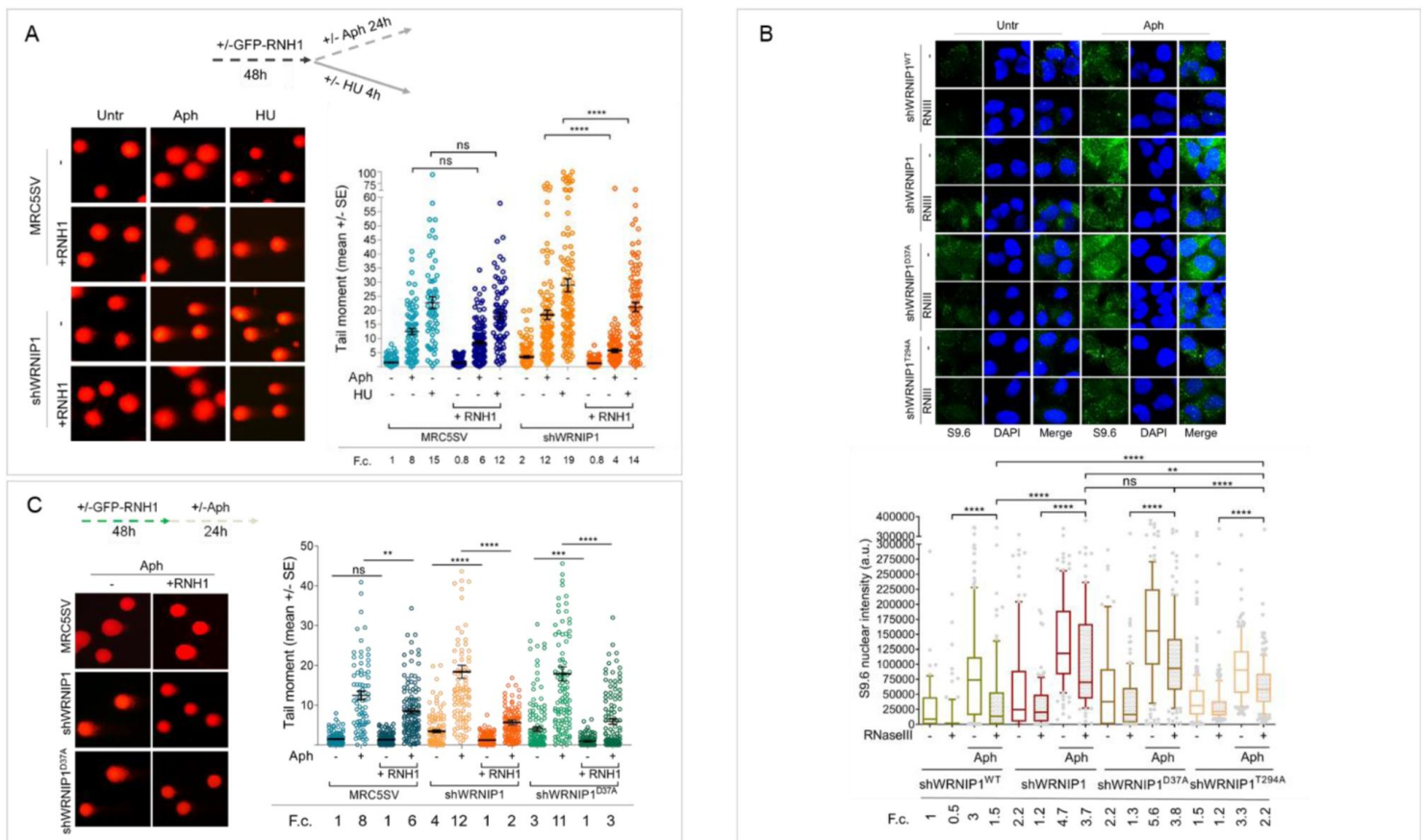


Figure 3.

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, post-transfection with GFP-tagged RNaseH1 or empty vector (-), were treated or not with Aph or HU, following the experimental scheme. Subsequently, they were subjected to Comet assay. Dot plot shows data from three independent experiments (ns, not significant; **** $P < 0.0001$; two-tailed Student's t test). Representative images are provided.

(B) Immunofluorescence analysis to determine R-loop levels in shWRNIP1^{WT}, shWRNIP1, shWRNIP1^{D37A} and shWRNIP1^{T294A} cells, treated or not with 0.4 μ M Aph for 24 h. After fixing, cells were subjected or not to RNase III digestion and stained with anti-RNA-DNA hybrid S9.6 antibody. Representative images are provided for each single color channel. Nuclei were counterstained with DAPI. Box plot displays nuclear S9.6 fluorescence intensity, with boxes and whiskers representing 20-75 and 10-90 percentiles, respectively. The line represents the median value. Horizontal black lines represent the mean. Error bars represent SE (ns, not significant; **, $P < 0.01$; ****, $P < 0.0001$; two-tailed Student's t test).

(C) Analysis of the effect of R-loop resolution on DNA damage accumulation using alkaline Comet assay. Cells, post-transfection with GFP-tagged RNase H1 or empty vector, were treated as reported in the experimental scheme and subjected to Comet assay. Dot plot represents data 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 provided.

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. We employed a proximity ligation assay (PLA), a well-established method for detecting physical interactions (Söderberg et al. 2008 [↗](#)). Antibodies against proliferating cell nuclear antigen (PCNA) and RNA polymerase II (RNA pol II) were utilized 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** [↗](#)). This phenotype was substantially reduced by overexpressing RNase H1 (**Figure 4A** [↗](#)), suggesting that the UBZ domain of WRNIP1 may play a role in attenuating R-loop-induced TRCs upon MRS. The unexpected increase in TRCs upon RNase H1 overexpression in Aph-treated wild-type cells may be explained with the disruption of repair proteins, potentially leading to heightened fork stalling and increased conflicts (Shen et al. 2017 [↗](#)).

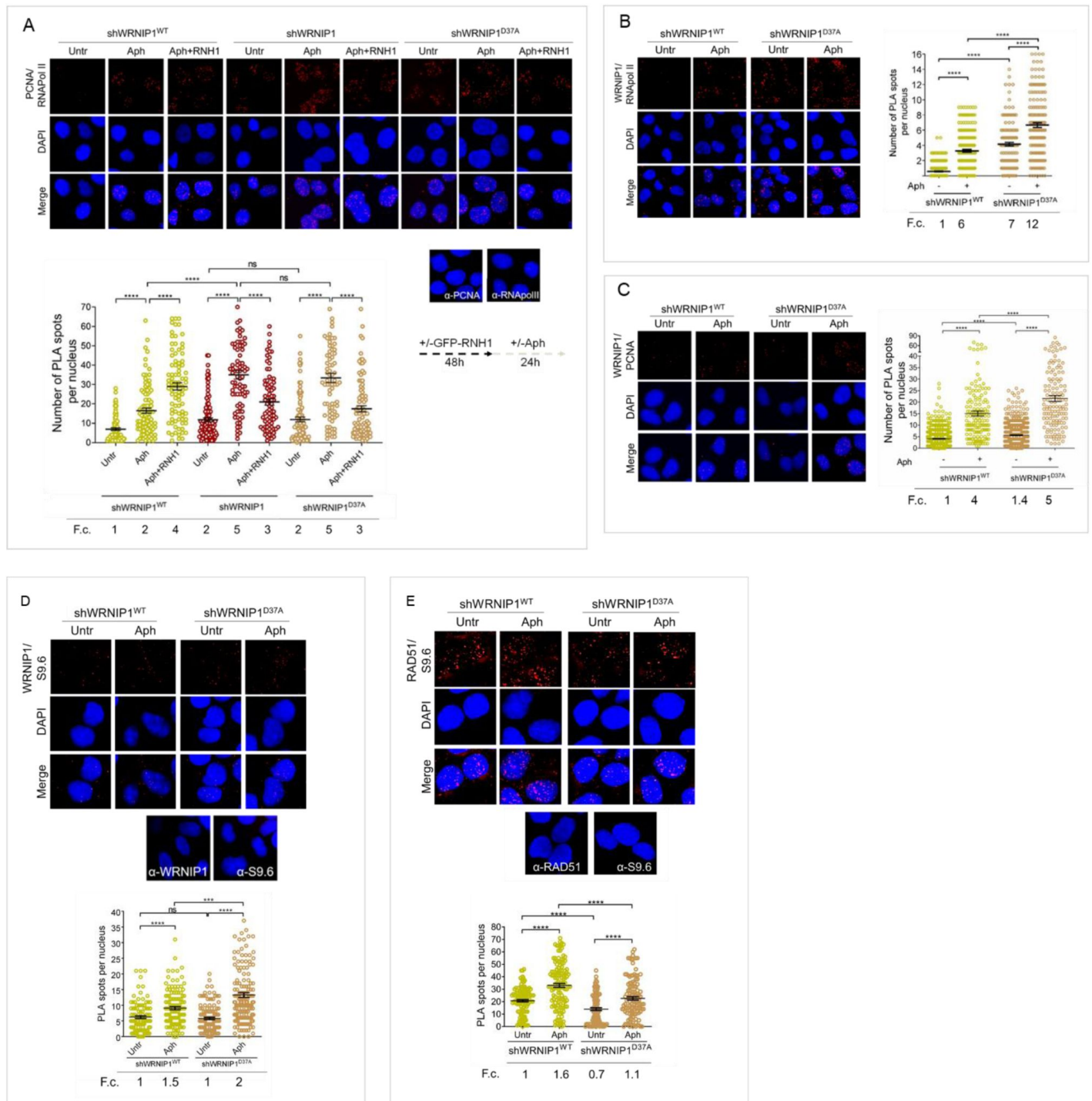


Figure 4.

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. Post-transfection with GFP-tagged RNase H1 or empty vector, cells treated according to the experimental scheme. After fixing, cells were stained with antibodies against PCNA and RNA pol II. Representative images are provided for each single color channel. Each red spot represents a single interaction between proteins. No spots were observed 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 from three independent experiments. Horizontal black lines represent the mean $\pm$ SE (ns, not significant; ****, $P < 0.0001$; one-way ANOVA test).

(B and C) Analysis of the localization of WRNIP1 near/at the transcription and replication machineries by PLA. Cells were treated or not with 0.4 μ M 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 provided for each single color channel. Nuclei were counterstained with DAPI. Dot plots show the number of PLA spots per nucleus. Data are presented as means from three independent experiments. Horizontal black lines represent the mean $\pm$ SE (****, $P < 0.0001$; one-way ANOVA 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 μ M Aph for 24 h, followed by RNase III digestion and the PLA assay. 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 provided for each individual color channel. Each red spot represents a single interaction between R-loops and the respective proteins (WRNIP1 or RAD51). No spots were observed 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 $\pm$ SE (ns, not significant; ***, $P < 0.001$; ****, $P < 0.0001$; one-way ANOVA test).

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, antibodies against WRNIP1 and RNA pol II or PCNA, respectively, were used. 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 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 responding 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 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 [\[1\]](#)). 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 [\[1\]](#)). Under normal growth conditions, and in agreement with previous data (Leuzzi et al. 2016 [\[2\]](#)), MRC5SV and shWRNIP1 cells exhibited nearly identical fork velocities, while shWRNIP1^{D37A} cells showed a significantly reduced velocity (Figure 5A [\[1\]](#)). 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 [\[1\]](#)). Importantly, RNase H1 overexpression led to a significant increase in the rate of fork progression only in shWRNIP1 and shWRNIP1^{D37A} cells (Figure 5A [\[1\]](#) 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 [\[1\]](#)). 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 [\[1\]](#)). Suppressing R-loop formation lowered the percentage of stalled forks and, consistently, increased that of restarting forks in both shWRNIP1 and shWRNIP1^{D37A} cells (Figure 5 [\[1\]](#) C and D). Similar results were obtained by treating cells with DRB (Suppl. Figure 3A-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 [\[3\]](#)). 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 [\[4\]](#)). 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 [\[5\]](#)). To this end, we treated MRC5SV, shWRNIP1 and shWRNIP1^{D37A} cells with Aph and/or DRB and performed Western blot analysis (Figure 6A [\[1\]](#)). 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 [\[1\]](#)). 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 4A). In agreement with previous findings (Wells et al. 2022 [\[6\]](#)), we found that RAD18 plays a role in FANCD2 recruitment upon MRS (Suppl. Figure 4B). 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 and shWRNIP1^{D37A} cells compared to control cells (Figure 6B [\[1\]](#)).

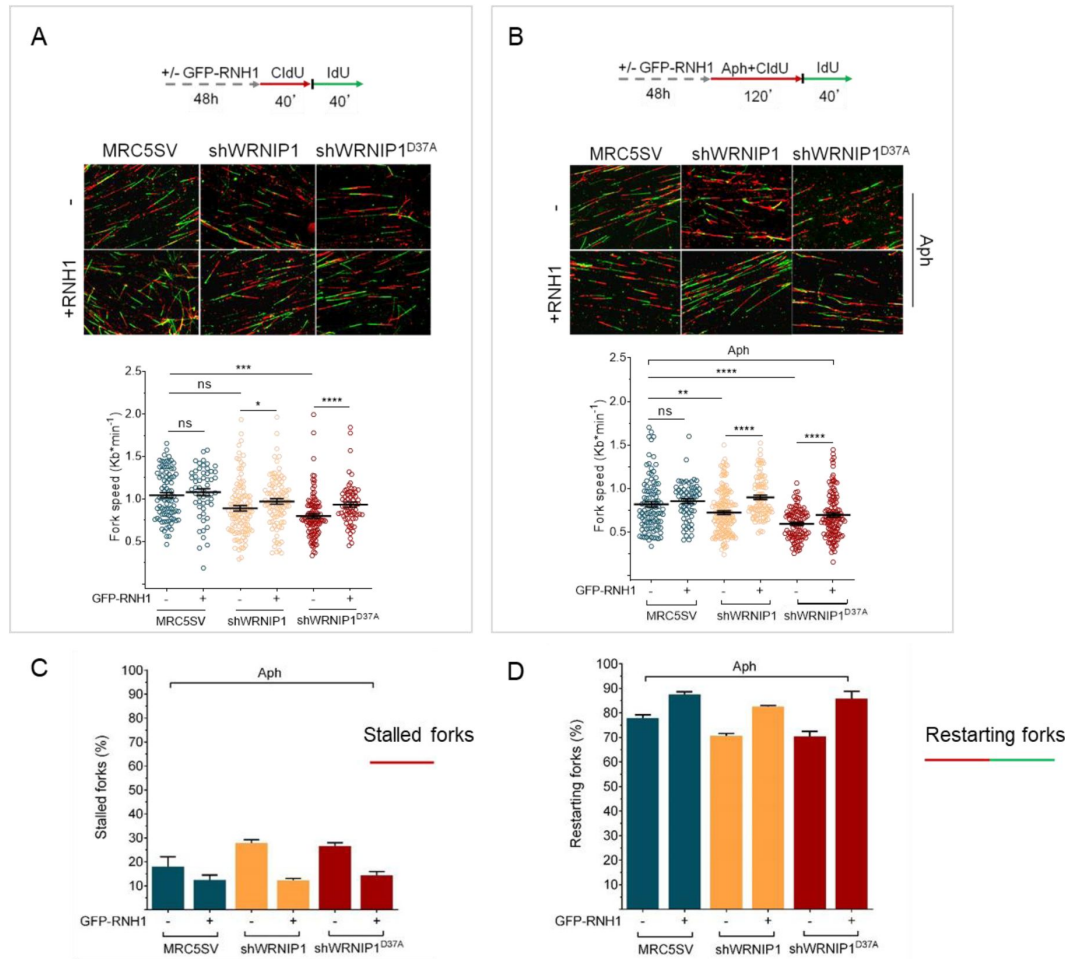


Figure 5.

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

Experimental scheme for 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 μ M Aph, then subjected to a pulse-labelling with IdU. Representative DNA fiber images are provided. Graphs display 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) Graphs represent 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).

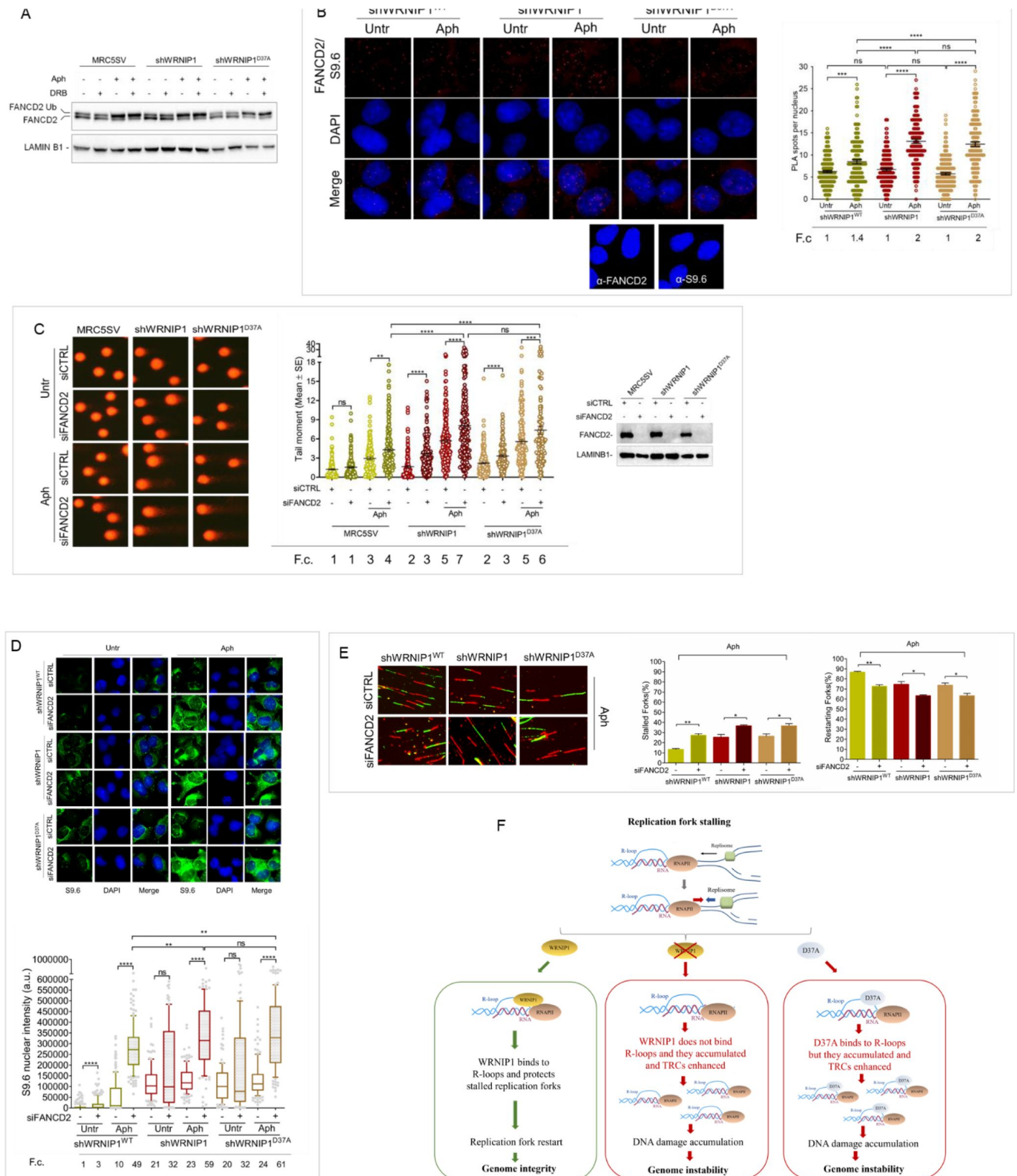


Figure 6.

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 the physical interaction between FANCD2 and R-loops by PLA. shWRNIP1^{WT}, shWRNIP1 and shWRNIP1^{D37A} cells were treated or not with 0.4 μ M Aph for 24 h and subjected to RNase III digestion. Cells were stained with antibodies against RNA-DNA hybrid (anti-S9.6) and FANCD2. Representative images are provided. Each red spot represents a single interaction between R-loops and FANCD2. No spots were 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 from three independent experiments. Horizontal black lines represent the mean $\pm$ SE (ns, not significant; ***, $P < 0.001$; ****, $P < 0.0001$; one-way ANOVA 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 or not with 0.4 μ M Aph for 24 h, then subjected to Comet assay. Dot plot shows data presented as means 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 provided. 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.

(D) Immunofluorescence analysis to determine R-loop levels in shWRNIP1^{WT}, shWRNIP1 and shWRNIP1^{D37A} cells depleted or not of FANCD2 under untreated conditions or after MRS. Cells were fixed, subjected or not to RNase III digestion, and stained with anti-RNA-DNA hybrid S9.6 antibody. Representative images are provided. Nuclei were counterstained with DAPI. Dot plot shows nuclear S9.6 fluorescence intensity. Boxes and whiskers represent 20-75 and 10-90 percentiles, respectively. The line represents the median value. Data are presented as means from three independent experiments. Horizontal black lines represent the mean. Error bars represent SE (ns, not significant; **, $P < 0.01$; ****, $P < 0.0001$; two-tailed Student's *t* test).

(E) Experimental scheme of dual labelling of DNA fibers in shWRNIP1^{WT}, shWRNIP1 and shWRNIP1^{D37A} cells upon MRS **(B)**. After transfection with control siRNAs (siCTRL) or targeting FANCD2 (siFANCD2), cells were pulse-labelled with CldU, treated or not with 0.4 μ M Aph, then subjected to a pulse-labelling with IdU. Representative DNA fiber images are provided. Graphs represent the percentage of red (CldU) tracts (stalled forks) or green (IdU) tracts (restarting forks) in the cells. Error bars represent standard error (* $P < 0.05$; ** $P < 0.01$; two-tailed Student's *t* test).

(F) Working model for the potential role of WRNIP1 in R-loop accumulation. Upon replication fork stalling, WRNIP1 binds to R-loops and protects stalled forks, promoting genomic integrity. In the absence of WRNIP1, R-loops accumulate, and TRCs are enhanced, leading to genome instability. In case of WRNIP1 UBZ mutation, the protein binds to R-loops but they accumulate, increasing TRCs and, thus, contributing to genomic instability.

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 shWRNIP1 and shWRNIP1^{D37A} cells compared to control cells (**Figure 6C**).

Therefore, to explore more in-depth the role of FANCD2 in the regulation of R-loops, we examined the impact of FANCD2 depletion on the accumulation of R-loops in shWRNIP1 and WRNIP1 UBZ mutant cells. In agreement with our observations, the analysis of R-loop formation in WRNIP1-deficient cells depleted of FANCD2 revealed a significantly higher accumulation of R-loops in cells

with a concomitant loss of both WRNIP1 and FANCD2 compared to those with a single deficiency upon MRS (**Figure 6D** [↗](#)). Similar results were observed in the WRNIP1 UBZ mutant cells in which FANCD2 was abrogated (**Figure 6D** [↗](#)).

We also performed a DNA fiber assay to evaluate restarting replication forks in shWRNIP1^{WT}, shWRNIP1 and shWRNIP1^{D37A} cells in which FANCD2 was abrogated. Our results show that FANCD2 depletion slightly decreased the ability of the cells to restart forks from MRS (**Figure 6E** [↗](#)). These findings collectively suggest that FANCD2 pathway 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 demonstrated that checkpoint defects trigger a WRNIP1-mediated response to limit R-loop-associated genomic instability ([Marabitti et al. 2020](#) [↗](#)). Nevertheless, whether WRNIP1 is directly involved in the mechanisms of R-loop removal/resolution remains unclear.

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).

We have demonstrated that WRNIP1 plays a crucial role in facilitating fork restart, and this function specifically relies on its ATPase activity ([Leuzzi et al. 2016](#) [↗](#)). Notably, while the ATPase activity of WRNIP1 is essential for the restart of stalled forks ([Leuzzi et al. 2016](#) [↗](#)), it is dispensable for dealing with TRCs. In sharp contrast, a mutation disrupting the WRNIP1 UBZ domain ([Bish and Myers 2007](#) [↗](#); [Nomura et al. 2012](#) [↗](#)) is sufficient to induce TRCs and DNA damage at levels comparable to, if not higher than, those observed in WRNIP1-deficient cells.

The persistence of R-loops is detrimental as they can 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 cells lacking WRNIP1 or expressing the WRNIP1 UBZ mutant, the elevated DNA damage and reduced fork speed are restored by RNase H1-GFP overexpression, which degrades RNA/DNA hybrids and eliminates R-loops ([Cerritelli et al. 2003](#) [↗](#)). Consistent with this, many more R-loops are detected in WRNIP1-deficient or WRNIP1 UBZ mutant cells. This strongly suggests that unscheduled R-loops likely contribute to 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 results in TRCs, leading to 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 of these proteins can compromise the ability of cells to overcome or resolve R-loops, leading to DNA damage. Our previous research has demonstrated that WRNIP1, not relying on its ATPase activity, protects stalled forks from nucleolytic degradation upon replication stress ([Leuzzi et al. 2016](#) [↗](#)). Additionally, WRNIP1 has been found to immunoprecipitate with BRCA2 and RAD51, and its loss destabilizes RAD51 on ssDNA ([Leuzzi et al. 2016](#) [↗](#)). Notably, R-loop formation exposes a potentially vulnerable ssDNA region on the non-template strand ([Aguilera and García-Muse 2012](#) [↗](#)). Since

BRCA2 is known to mitigate R-loop-induced DNA damage (Shivji et al. 2018 [↗](#); Bhatia et al. 2014 [↗](#); Wang et al. 2022 [↗](#)), it is conceivable that WRNIP1, through its association with the BRCA2/RAD51 complex, could facilitate RAD51 recruitment or stabilization to protect the ssDNA of R-loops. Supporting this, RAD51 co-localizes with R-loops after MRS, and this co-localization depends 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 suggested that the incision of R-loops, a process promoting fork progression and restart at TRCs (Chappidi et al. 2020 [↗](#)), requires high accuracy to prevent fork collapse and chromosomal damage, posing a risk to genome integrity (Sollier et al. 2014 [↗](#); Promonet et al. 2020 [↗](#)). Our findings indicate that the R-loop-dependent accumulation of DNA damage and genome instability may suggest a preference for engaging error-prone mechanisms when WRNIP1 depletion. Alternatively, WRNIP1 may play a role in stabilizing RAD51 on the ssDNA of DNA/RNA hybrids, facilitating fork restart through fork reversal. Indeed, one proposed mechanism for processing DNA/RNA hybrids involves the exposure of ssDNA and the loading of RAD51, which mediates fork reversal (Stoy et al. 2023 [↗](#)). While these hybrids are typically resolved under physiological conditions, allowing stalled fork restart (Stoy et al. 2023 [↗](#); Chappidi et al. 2020 [↗](#)), post-replicative DNA/RNA hybrids could represent pathological TRC intermediates capable of impairing replication fork progression and stimulating fork reversal.

The phenotype observed in WRNIP1 UBZ mutant cells is similar to that of WRNIP1-deficient cells, suggesting that the ubiquitin-binding function of WRNIP1 could play a role in mitigating R-loop-induced TRCs. Despite the UBZ domain of WRNIP1 having an undefined function after replication perturbation (Yoshimura et al., 2017 [↗](#)), human WRNIP1 is known to bind both forked DNA, resembling stalled replication forks, and template/primer DNA in an ATP-dependent fashion (Yoshimura et al. 2009 [↗](#)). However, our findings indicate that WRNIP1 does not use its ATPase activity to counteract TRC-induced R-loop accumulation. Nevertheless, WRNIP1's ability to bind ubiquitin suggests its potential involvement in the metabolic processes of ubiquitinated proteins (Bish and Myers 2007 [↗](#)). The UBZ domain of WRNIP1 is implicated in the physical association with RAD18 (Kanu et al. 2016 [↗](#)). RAD18-deficient cells exhibit high levels of TRCs and accumulate DNA/RNA hybrids, leading to DNA double strand breaks and replication stress (Wells et al. 2022 [↗](#)). These effects are partially dependent on the failure 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 WRNIP1 and its UBZ domain. Additionally, in our cell lines, although R-loop levels are more elevated in FANCD2-depleted cells, DNA damage is more pronounced in WRNIP1-deficient cells, suggesting that the FA pathway may act as a backup system to counteract TRCs. Therefore, it is tempting to speculate that the UBZ domain might contribute to directing 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 intriguing point deserves further and detailed investigation. Altogether our findings unveil a previously unappreciated role for WRNIP1 in counteracting the pathological accumulation of transcription-associated R-loops, thereby preventing heightened genomic instability following MRS in human cells (**Figure 6F** [↗](#)). A growing body of evidence suggests that R-loops may be implicated in the genomic instability observed in human cancer cells (Crossley et al., 2019 [↗](#); García-Muse and Aguilera 2019). Notably, considering the observed overexpression of WRNIP1 in the most common human cancer types, such as lung and breast cancers, our discoveries contribute to a deeper understanding of the mechanisms cells employ to counteract TRCs. These results open avenues for exploring novel pathways that may 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}) and its ATPase-dead mutant form (shWRNIP1^{T294A}) were generated as previously reported (Leuzzi et al. 2016 [DOI](#)).

Using the NeonTM Transfection System Kit (Invitrogen) following 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, a missense-mutant form of WRNIP1 with dead form of ubiquitin-binding zinc finger (UBZ) domain (WRNIP1^{D37A}). 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 cultured in DMEM media supplemented with 10% FBS, 100 U/mL penicillin and 100 mg/mL streptomycin, and incubated at 37°C in an humidified 5% CO₂ atmosphere.

Site-directed mutagenesis and cloning

Site-directed mutagenesis of the WRNIP1 full-length cDNA (Open Biosystems) was performed on the pCMV-FLAGWRNIP1 plasmid, which contains the wild-type ORF sequence of WRNIP1. The substitution of Asp37 to Ala in pCMV-FLAGWRNIP1 was introduced using the Quick-change XL kit (Stratagene) with 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-β-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 for RNase H1 overexpression experiments was generously provided by Prof. R.J. Crouch (National Institutes of Health, Bethesda, USA). As previously described (Cerritelli et al. 2003 [DOI](#)), the GFP-tagged RNase H1 plasmid was generated by introducing a mutation on Met27 to abrogate mitochondrial localization signal (RNase H1-M27). To express the plasmids, cells were transfected using the NeonTM Transfection System Kit (Invitrogen), according to the manufacturer's instructions.

For the FANCD2 genetic knockdown experiment, Interferin (Polyplus) was used according to the manufacturer's instructions with Silencer Select siRNA (Thermo Fischer Scientific) targeting the following region of the mRNA (5'-CAGCCUACCUGAGAUCUAtt-3'). The siRNA was used at a concentration of 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 separated on polyacrylamide gels and transferred onto a nitrocellulose membrane using the Trans-Blot Turbo Transfer System (Bio-Rad). The membranes were blocked with 5% NFDM in TBST (50 mM Tris/HCl pH 8, 150 mM NaCl, 0.1% Tween-20) and incubated with primary antibodies 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 (Santa Cruz Biotechnology; 1:500) and rabbit-polyclonal anti-LAMIN B1 (Abcam; 1:30000).

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

Chromosomal aberrations

Cells for metaphase preparations were collected following standard procedures, as previously reported (Pirzio et al. 2008 [\[1\]](#)). A cell suspension was dropped onto cold, wet slides to make chromosome preparations. After air-drying overnight, the number of breaks and gaps was observed on Giemsa-stained metaphases for each treatment condition. For each time point, a minimum of 50 chromosome metaphases were independently examined by two investigators, and chromosomal damage was scored at 100× magnification using an Olympus fluorescence microscope.

Alkaline Comet assay

DNA breakage induction was examined by alkaline Comet assay (single-cell gel electrophoresis) under denaturing conditions, as described (Pichierri et al. 2001 [\[2\]](#)). Cell DNA was stained with a fluorescent dye GelRed (Biotium) and examined at 40× magnification using an Olympus fluorescence microscope. Slides were analysed with a computerized image analysis system (CometScore, Tritek Corp). To assess the extent of DNA damage, computer-generated tail moment values (tail length × fraction of total DNA in the tail) were used. A minimum of 200 cells were analysed for each experimental point. Apoptotic cells (identified by 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 with minor changes (Marabitti et al. 2019 [\[3\]](#)). 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. Subsequently, cells were treated for 90 min with a 1:150 dilution of RNase III (New England Biolabs) in low-salt buffer (50 mM Tris-HCl pH 7.6, 75 mM KCl, 3 mM MgCl₂, 0.1% BSA) 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. Following each primary antibody incubation, 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). Immunostaining for dsRNA was performed as described with minor changes (Crossley et al. 2021 [\[4\]](#)). Cells were fixed in 4% PFA for 10 min at RT, washed twice with PBS, and then permeabilized in 0.25% Triton X-100 for 10 min. After two washes in PBS, samples were incubated in low-salt buffer (50 mM Tris-HCl pH 7.6, 75 mM KCl, 3 mM MgCl₂, 0.1% BSA) with or without Short Cut RNase III (New England Biolabs; 1:150) for 90 min at 37°C. Coverslips were then washed twice with PBST, followed by one wash in PBS for 5 min, before blocking in 3% BSA in PBS for 30

min. Cells were then incubated with a 1:200 dilution of anti-dsRNA monoclonal antibody J2 (Sigma-Aldrich) overnight at 4°C. After the primary antibody, cells were washed twice with PBS and incubated with 1:200 goat anti-mouse Alexa Fluor-488 (Molecular Probes).

The incubation with secondary antibodies was carried out 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 Video Confocal (ViCo) system at 40x magnification. 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 with minor changes according to the protocol reported elsewhere (Morales et al. 2016 [DOI](#)). Genomic DNA was isolated by standard extraction with Phenol/Chlorophorm/Isoamyl Alcohol (pH 8.0) followed by precipitation with 3 M NaOAc and 70% Ethanol. The isolated genomic DNA was randomly fragmented for 4h at 37°C with a cocktail restriction enzymes (BsrGI, EcoRI, HindIII, XbaI, SspI) supplemented with 1 M spermidine. After incubation, the digested DNA was cleaned up with Phenol/Chlorophorm extraction and standard Ethanol precipitation. After sample quantification, 350 ng of digested DNA was incubated with RNase H overnight at 37°C as a negative control. The same quantity of each sample was 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) or anti-dsDNA antibody (Abcam; 1:2000) overnight at 4°C. A horseradish peroxidase-conjugated goat specie-specific secondary antibody (Santa Cruz Biotechnology) was used. Quantification on the scanned image of the blot was performed using Image Lab software with dsDNA as loading control.

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 (Nun Lab-Tek) at a density of $1.5\text{--}2.5 \times 10^4$ 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 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. Subsequently, cells were treated with a 1:150 dilution of RNase III (New England Biolabs) in low-salt buffer (50 mM Tris-HCl pH 7.6, 75 mM KCl, 3 mM MgCl₂, 0.1% BSA) for 90 min before blocking in 2% BSA/PBS for 1h at 37°C.

The primary antibodies used were as follows: anti-FLAG (mouse-monoclonal, Sigma-Aldrich; 1:250), anti-WRNIP1 (rabbit-polyclonal, Bethyl; 1:500), anti-S9.6 (mouse-monoclonal, Kerafast; 1:200), anti-PCNA (rabbit-polyclonal, Abcam; 1:500), anti-RNA pol II (mouse-monoclonal, Santa Cruz Biotechnology; 1:200), recombinant anti-S9.6 (rabbit-monoclonal, Kerafast; 1:200), anti-RAD51 (rabbit-polyclonal, Abcam; 1:300) and anti-FANCD2 (mouse-monoclonal, Santa Cruz Biotechnology; 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 carried out 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 a red fluorescence-labelled oligonucleotide. Samples were mounted in Prolong Gold antifade reagent with DAPI (blue). Images were acquired randomly using an Eclipse 80i Nikon Fluorescence Microscope equipped with a Video Confocal (ViCo) system.

DNA fiber analysis

Cells were pulse-labelled with 50 μ M 5-chloro-2'-deoxyuridine (CldU) and 250 μ M 5-iodo-2'-deoxyuridine (IdU) at specified times, with or without treatment as outlined in the experimental schemes. DNA fibres were prepared and spread out as previously described (Leuzzi et al. 2016 [\[1\]](#)). For the 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 carried out in a humidified chamber for 1 h at RT.

Images were randomly acquired 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 $1\mu\text{m} = 2.59\text{ kb}$ as reported (Basile et al. 2014 [\[2\]](#)). A minimum of 100 individual fibres were analysed for each experiment, and the mean of at least three independent experiments is 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 one-way ANOVA test. In all cases, not significant: $P > 0.05$; * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$; **** $P < 0.0001$. All the experiments were repeated at least three times unless otherwise noted. Fold changes were calculated as ratio of means between each experimental sample and the relative wild-type untreated.

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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 perturbed. 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.

<https://doi.org/10.7554/eLife.89981.2.sa2>

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-blot) 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.

<https://doi.org/10.7554/eLife.89981.2.sa1>

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.

In the revised submission by Valenzisi et al., the manuscript is still missing the controls that were requested in the original review. One cannot conclude the D37A mutant is unable to rescue DNA damage unless it is shown in the same experiment that the WT is able to rescue it. This is also true for the fork speed, stalled forks, and restarting forks experiments. Below is a list of Figures missing key controls.

Figure 1B -missing the shWRNIP1WT control

Figure 1C - missing the MRC5SV control

Figure 1D - missing the shWRNIP1WT control

Figure 3C - missing the shWRNIP1WT control

Figure 5A - missing the shWRNIP1WT control

Figure 5B - missing the shWRNIP1WT control

Figure 5C - missing the shWRNIP1WT control

Figure 5D - missing the shWRNIP1WT control

Figure 6C - missing the shWRNIP1WT control

Also, the authors did not explain the result showing shWRNIP1 decreases DNA damage compared to MRC5SV in Figure 1D (compare lanes 4 and 8). Again, this suggests WRNIP1 actually increases DNA damage in response to Aph and DRB. This concern was raised in the original peer review, and it remains unaddressed in the revised manuscript.

The use of RNaseHIII increases the specificity of the S9.6 antibody and improves confidence in the DNA-RNA hybrid data throughout the manuscript.

<https://doi.org/10.7554/eLife.89981.2.sa0>

Author Response

The following is the authors' response to the original reviews.

Reviewer #1:

This paper describes the role of WRNIP1 AAA+ ATPase, particularly its UBZ domain for ubiquitinbinding, but not ATPase, to prevent the formation of the R-loop when DNA replication is mildly perturbed. 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.

One concern is the relationship between WRNIP1 and FANCD2 (Figure 6) in the suppression of R-loop-induced DNA damage. This is different from the relationship in inter-crosslink (ICL) repair (Socha et al. 2020), which shows the epistatic relationship between WRNIP1 as well as its UBZ domain and FANCD2 in the ICL repair. The authors need to re-evaluate the role of FANCD2 in R-loop suppression under mild replication stress (MRS) by analyzing R-loop formation in the FANCD2 knockdown (KD) cells as well as colocalization of FANCD2 with PCNA and RNA polymerase II by the PLA method and restarting the forks by the DNA coming.

In this line, it is important to show PLA signal between FANCD2 and R-loop depends on WRNIP1 since WRNIP1 recruits FANCD2 in ICL repair (Socha et al. 2020).

In the study referenced by the reviewer, the authors implicated WRNIP1 in repairing interstrand crosslinks (ICLs) induced by agents, such as TMP/UVA, MMC, and Cisplatin (Socha et al., 2020). For the repair of ICLs, the FANCD2/FANCI complex, the central component of the FA pathway, must be recruited to DNA. The study suggests a potential role for WRNIP1 in loading the FANCD2/FANCI complex onto DNA immediately after ICL formation. However, even in the absence of WRNIP1, a residual recruitment of the FANCD2/FANCI complex to DNA was observed, possibly due to alternative mechanisms, as proposed by the authors. Interestingly, the study did not establish a similar relationship between WRNIP1 and FANCD2 after treatments that does not induce ICLs, demonstrating that WRNIP1 and FANCD2 may also play independent roles. Hence, our data demonstrating a distinct role of WRNIP1 from the FA pathway in response to R-loop-associated replication stress are not inconsistent with prior findings. Additionally, considering the UBZ domain ability to interact with ubiquitin in both its free form and when conjugated to other proteins, thereby regulating protein functions, it is not surprising that the UBZ domain of WRNIP1 may also play a role in the response to R-loop accumulation.

Therefore, to address the reviewer's request for a more in-depth exploration of the role of FANCD2 in the regulation of R-loops, we chose to examine the impact of FANCD2 loss on the accumulation of R-loops in WRNIP1-deficient and WRNIP1 UBZ mutant cells, as well as on the dynamics of stalled forks following aphidicolin-induced MRS. Additionally, we investigated the colocalization between FANCD2 and R-loops in shWRNIP1WT, shWRNIP1 and shWRNIP1D37A cells. Details are provided below.

In agreement with our observations, the analysis of R-loop formation upon MRS, in WRNIP1-deficient cells depleted of FANCD2, revealed a significantly higher accumulation of R-loops in cells with a concomitant loss of both WRNIP1 and FANCD2 compared to those with a single deficiency (see Fig. 6D of the revised manuscript). Similar results were observed in the WRNIP1 UBZ mutant cells in which FANCD2 was abrogated (see Fig. 6D of the revised manuscript). It is important to note that, to eliminate contaminant-free RNA, particularly dsRNA, which could interfere with the binding of RNA-DNA hybrids by the S9.6 antibody (Hartono et al., 2018), and to determine the proximity between FANCD2 and R-loops more accurately, cells were treated with RNase III, following established protocols (Crossley et al., 2020).

Furthermore, we examined the interaction of FANCD2 with R-loops using a proximity ligation assay (PLA). Our findings revealed significant colocalization between FANCD2 and R-loops in the absence of WRNIP1 and in WRNIP1 UBZ mutant cells following low-dose aphidicolin treatment and RNase III exposure, showing a significant increase compared to the control counterpart (shWRNIP1WT cells; see Fig. 6B of the revised manuscript). Consequently, we

conclude that neither WRNIP1 nor its UBZ domain is necessary for FANCD2 recruitment under conditions of MRS.

We also performed a DNA fiber assay to evaluate restarting replication forks in shWRNIP1WT, shWRNIP1 and shWRNIP1D37A cells in which FANCD2 was abrogated. Our results show that FANCD2 depletion slightly decreased the ability of the cells to restart forks from MRS (see Fig. 6E of the revised manuscript).

Given a low number (2-4) of PLA foci for WRNIP1-RNA polymerase II or WRNIP1 and R-loop (Figure 4B and 4D), how does this colocalization reflect the functional significance?

The data from the PLA of Figures 4B and 4D are reported as the mean of three independent experiments. It is important to note that we have introduced a new Figure 4D. To selectively assess R-loop structures, cells were treated with RNase III, a double-stranded RNA-specific endoribonuclease, following established protocols (Crossley et al., 2020). Our PLA analysis confirms the localization of WRNIP1 at/near R-loops in shWRNIP1 and shWRNIP1D37A cells, and this phenomenon is more evident in WRNIP1 UBZ mutant cells (see Fig. 4D of the revised manuscript). Specifically, the new protocol allows us to visualize a higher number of PLA foci, and we observed that Aph increased the spots per nucleus in shWRNIP1D37A cells compared to the previous experiment.

Regarding the Fig. 4B, it is not uncommon for a low number of PLA spots per nucleus to correspond to a phenotypic effect. For instance, a similar low average in the colocalization of PCNA or RNA pol II with FANCD2 has been observed in a prior paper as well, suggesting that transcription-replication collisions occur upon Aph-induced MRS (Okamoto et al., 2019). Also, not all R-loops could be “targeted” by WRNIP1.

It would be helpful to readers if the authors were to provide a summary figure of this paper.

As suggested by the reviewer, we have developed a model to summarize the findings obtained in our study (see Fig. 6F of the revised manuscript).

Minor points:

(1) Most of the cytological images in the paper show only colocalized ones, which makes it hard to see a signal. Please show a single-color image.

For a better visualization of nuclei signals in the figures, single-color images have been provided for Figs. 2A; 3B; 4A, B, C, D and E; 6B and D; Suppl. Fig. 2A and B of the revised manuscript.

(2) In Figure 2A, only one or two S9.6 focus(foci) can be seen. Why 1 or 2? This focus marks a specific chromosomal locus such as the centromere or telomere.

We agree with the reviewer that the observed foci in nuclei may indicate a specific chromosomal locus, such as telomeres or centromeres.

(3) Figure 3A, graph: Why this graph does not use a dot plot like Figure 1B and Figure 3C?

The graph in Figure 3A has been represented as a dot plot, as requested.

(4) Figure 1C: P values between unperturbed conditions should be provided.

In Figure 1C, P values comparing unperturbed conditions were already included. The results showed no significance between shWRNIP1 and shWRNIP1D37A cells when compared to MRC5SV cells and, similarly, to shWRNIP1T294A cells, as indicated in the corresponding legend.

(5) *Figure 2B: Please provide the quantification or show the reproducibility of the data.*

The quantification of R-loops using the S9.6 monoclonal antibody is not accurate, as the specificity for RNA-DNA hybrids is questionable (Hartono et al., 2018). Therefore, to demonstrate the reproducibility of the findings in Fig. 2B, we conducted a repeat of the dot-blot experiment. We treated the samples with RNase H to degrade RNA-DNA hybrids and hybridized the membrane with an anti-dsDNA to quantify R-loop levels more accurately. Our analysis confirms that the S9.6 signal strongly accumulates in shWRNIP1 cells compared to shWRNIP1WT cells (see Fig. 2B of the revised manuscript). Additionally, a graph illustrating the fold-change values of the S9.6/dsDNA signal relative to wild-type untreated cells is provided.

(6) *Figure 4A: the expression of RNaseH under aphidicolin addition increased colocalization of PCNA and RNA pol II. It is important to mention the result and provide an explanation of why it is increasing in the main text.*

Although the result may appear unexpected, and we lack experiments that explain the nature of this phenotype, a previous study reported that overexpression of RNase H1 in mammalian cells may lead to a dose-dependent reduction of certain proteins of the repair pathway, resulting in a significant accumulation of DNA damage (Shen et al., 2017). Consequently, the observed increase in TRCs upon RNase H1 overexpression in wild-type cells may be attributed to the disruption of proteins that, by impairing the repair process, can potentially cause more fork stalling and, consequently, more conflicts. We have introduced a comment in the text.

Reviewer #2:

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.

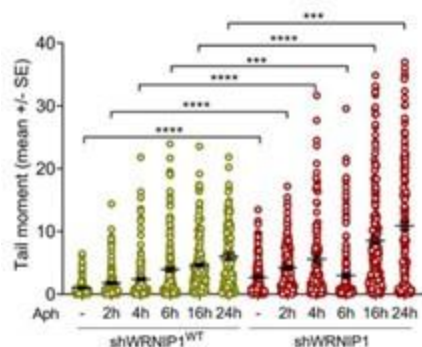
To eliminate contaminant-free RNA, particularly dsRNA, which could interfere with the capture of RNA-DNA hybrids by the S9.6 antibody (Hartono et al., 2018), and to determine R-loop levels more accurately, we treated cells with RNase III, following established protocols (Crossley et al., 2020). Under our experimental conditions, RNase III treatment significantly reduced the amount of dsRNA, nearly eliminating it, as evaluated using a specific antibody against dsRNA (see Suppl Fig 2 of the revised manuscript). To better appreciate the effect of the loss of WRNIP1 or its UBZ domain on Rloop accumulation and the amount of DNA

damage, we have reproduced key data (see Figs 2B; 3B; 4D and E; 6B of the revised manuscript). Our analysis from immunofluorescence experiments, performed using a dsRNA ribonuclease (RNase III), confirms higher R-loop accumulation in WRNIP1-deficient or WRNIP1 UBZ mutant cells compared to control cells (Fig 3B). Additionally, proximity ligation assay (PLA) data are consistent with those previously presented and, in some cases, are more readily interpretable (see Figs 4D and E; 6B of the revised manuscript). Finally, we performed a new dot-blot experiment (see Fig. 2B of the revised manuscript). We treated with RNase H to degrade RNA-DNA hybrids and hybridized the membrane with an anti-dsDNA antibody to quantify R-loop levels more accurately. Our analysis confirms a significant accumulation of the S9.6 signal in shWRNIP1 cells compared to shWRNIP1WT cells. Additionally, a graph illustrating the foldchange values of the S9.6/dsDNA signal relative to wild-type untreated cells is provided.

(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.

Investigating the potential role of WRNIP1 at an early step in the mechanism is undoubtedly very interesting and requires separate investigation. Our decision to explore the relevance of the loss of WRNIP1 or WRNIP1 mutations under steady-state conditions is based on a preliminary alkaline comet assay (provided below). The comet assay, performed at various exposure times of aphidicolin at a concentration of 0.4 micromolar, clearly indicates that the most significant effect on DNA damage accumulation in WRNIP1-deficient cells occurs after 24 hours of treatment. Therefore, we have chosen to study the transcription-associated genomic instability in our cells by treating them with a low-dose of aphidicolin for 24 hours to maximize the effect.

Author response image 1.



We agree that the presence of WRNIP1 or RAD51 in proximity to R-loops is consistent with their roles and may not be surprising. However, these experiments formally demonstrate their proximity to R-loops under our conditions. Notably, the new graphs, obtained from experiments repeated by treating with RNase III to reduce the amount of dsRNA and improve the specificity of the S9.6 antibody, show increased interaction of the mutated form of WRNIP1 in the UBZ domain with R-loops when compared to the wild-type form. Additionally, it is more evident that the presence of RAD51 at/near R-loops is reduced in WRNIP1 UBZ

mutant cells both in untreated conditions and after MRS (see Figs 4D and E of the revised manuscript).

(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.

To better evaluate the significance of the data presented in the study, we have introduced the foldchange values calculated with respect to the untreated samples, as requested by the reviewer. This allowed us to conclude that the loss of WRNIP1 or the expression of the UBZ mutant form of WRNIP1 does not reduce in any case the effects of aphidicolin-induced mild replication stress.

I would suggest some additional experiments or analyses to get more convincing results:

(1) DNA damage should be verified also with other methods, such as DNA damage markers pH2AX and 53BP1.

The quantification of DNA damage was also corroborated by determining the percentage of gammaH2AX-positive cells, as reported in Supplementary Figure 1B. This result is consistent with the findings from the comet assay, confirming transcription-dependent DNA accumulation in shWRNIP1 and shWRNIP1D37A cells. Regarding the 53BP1 marker, we believe that the existing data sufficiently demonstrate DNA damage accumulation in the absence of WRNIP1 or when its UBZ domain is mutated, providing comprehensive support to the study without necessitating additional results.

(2) Repair foci may also be detected with Rad51 foci. That will also provide evidence for increased DNA damage levels under the tested conditions.

Our prior study identified WRNIP1 as a crucial factor for RAD51 function (Leuzzi et al., 2016). Loss of WRNIP1 indeed results in a defective relocalization of RAD51 to chromatin. Consequently, the analysis of RAD51 foci may be not a useful readout to evaluate DNA damage levels under our conditions.

(3) WRNIP1 effects should be presented as FC (fold-changes) of DNA damage, PLA results, chromosomal errors, etc, to provide evidence of the level of effects on the tested phenotypes.

We have introduced the fold-change values calculated with respect to the untreated samples, as requested by the reviewer, for a more comprehensive analysis in the graph of Figs. 1B, C and D; 2A and B; 3A, B and C; 4A, B, C, D and E; 6B, C and D.

(4) R-loop detection ideally should be performed by one of the several types of immunoprecipitation techniques. Alternatively, dot-blot assays should be performed with a 1:2 dilution series of each sample. Then, heteroduplexes should be detected with S9.6 along with a general aspecific dye for DNA quantity in each spot. Next, densitometric analyses of S9.6 signal should be normalized over DNA quantity.

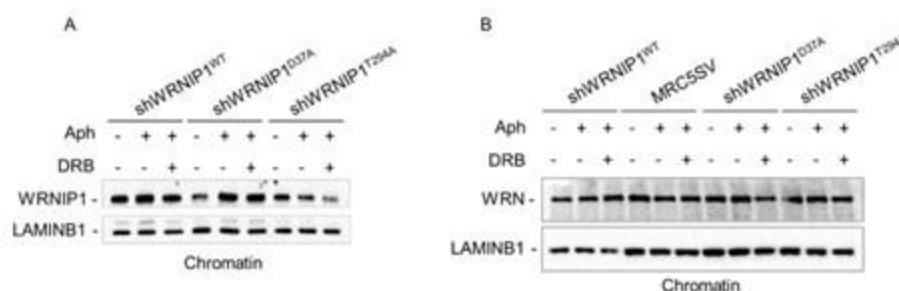
We acknowledge that the quantification of R-loops using the S9.6 monoclonal antibody is not accurate, as the specificity for RNA-DNA hybrids is questionable (Hartono et al., 2018). Therefore, to overcome this issue, we repeated the experiment shown in Fig. 2B. We treated the samples with RNase H to degrade RNA-DNA hybrids and hybridized the membrane with

an anti-dsDNA antibody to quantify R-loop levels more accurately. Our analysis confirms that the S9.6 signal strongly accumulates in shWRNIP1 cells compared to shWRNIP1WT cells (see Fig. 2B of the revised manuscript). Additionally, a graph illustrating the fold-change values of the S9.6/dsDNA signal relative to wild-type untreated cells is provided.

(5) A major focus on WRNIP1 D37A and T294A mutations may also make the paper overall more convincing. For instance: do the mutations affect protein recruitment at damaged chromatin? Do they increase repair foci? Do they affect the recruitment of WRN or BLM helicases or specific nucleases at chromatin under the tested conditions of MRS?

To address this point raised by the reviewer, we performed a chromatin experiment to assess the ability of WRNIP1 and its mutated forms to translocate to chromatin upon MRS. Our analysis shows that the mutated forms of WRNIP1 do not exhibit any defects in recruitment to chromatin, although the levels of the WRNIP1 ATPase mutant appear lower than the others (see Western blotting provided below for the reviewer's use only, Fig. A). Additionally, we tested the presence of WRN helicase, which does not show any difference between cells lines (see Western blot provided below, Author Response image 2B).

Author response image 2.



(6) I suggest revising the text for spelling errors.

The manuscript has been carefully revised to identify and correct any spelling errors that may have occurred.

Reviewer #3:

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.*

We agree with the reviewer's criticism that the use of shWRNIP1WT cells as a control is more appropriate. Therefore, all the new experiments presented in the revised version of the manuscript have been performed using the shWRNIP1WT cells. Notably, new results are in line with those obtained using the MRC5SV cells, rendering us confident that our findings are reliable overall. By contrast, we do not feel that including the WRNIP1 ATPase mutant cells is always essential, since our data clearly demonstrate that the loss of ATPase activity of WRNIP1 does not affect transcription-associated genome instability.

(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.

To eliminate contaminant-free RNA, particularly dsRNA, which could interfere with the capture of RNA-DNA hybrids by the S9.6 antibody (Hartono et al., 2018), and to determine R-loop levels more accurately, we treated cells with RNase III, following established protocols (Crossley et al., 2020). Under our experimental conditions, RNase III treatment significantly reduced the amount of dsRNA, nearly eliminating it, as evaluated using a specific antibody against dsRNA (see Suppl Fig 2 of the revised manuscript). To better appreciate the effect of the loss of WRNIP1 or its UBZ domain on R-loop accumulation and the amount of DNA damage, we have reproduced key data (see Figs 3B; 4D and E; 6B, D and E of the revised manuscript). Our analysis from immunofluorescence experiments, performed using a dsRNA ribonuclease, confirms higher R-loop accumulation in WRNIP1-deficient or UBZ WRNIP1 mutant cells compared to control cells (Fig. 3B). Additionally, proximity ligation assay (PLA) data are consistent with those previously presented and, in some cases, are more readily interpretable (see Figs 4D and E; 6B of the revised manuscript).

(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.

Regarding Figure 1D, in MRC5SV cells, DRB does not significantly increase DNA damage upon Aph treatment. Therefore, it is not correct to conclude that WRNIP1 exacerbates DNA damage in stressed cells with transcription blocked.

Regarding Figure 4A, while the outcome may appear unexpected, and we do not provide data that explain the nature of this phenotype, a previous study demonstrated that overexpression of RNase H1 in mammalian cells may lead to a dose-dependent reduction of certain proteins of the repair pathway, leading to a significant accumulation of DNA damage (Shen et al., 2017). Accordingly, the observed increase in TRCs upon RNase H1 overexpression in wild-type cells may be attributed to the disruption of proteins that, by impairing the repair process, can potentially cause more fork stalling and, consequently, more conflicts. We have introduced a comment in the text.

(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.

We agree with the reviewer that elucidating the mechanism by which WRNIP1 contributes to R-loop suppression would be of interest. Nevertheless, the findings presented here provide compelling evidence of a novel role for WRNIP1 in preventing R-loop accumulation. Investigating how WRNIP1 accomplishes this function will require significant effort, which we are committed to undertaking.

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

For a better visualization of nuclei signals in the PLA panels of Figs 4A, B, C, D and E; 6B, singlecolor images have been provided.

In addition to including the controls I mentioned in the public review, I recommend investigating the mechanism of how WRNIP1 prevents R-loop accumulation. If it is indeed related to its UBZ domain, then does that mean ubiquitination is an important step in R-loop removal? I believe elucidating this would be a novel and significant contribution. If it's not related to ubiquitination, then how does the UBZ domain regulate R-loops?

We agree with the reviewer that investigating the precise role of the UBZ domain of WRNIP1 in R-loop prevention would be of interest, and several experiments are required to adequately address this issue. However, as discussed, we hypothesize that the UBZ domain might contribute to directing WRNIP1 to DNA at TRC sites through RAD18.

I recommend using purified RNH1-dead-GFP to detect R-loops as opposed to the S9.6 antibody. The Cimprich lab has published this recently as a tool for detecting R-loops in fixed cells.

As explained in point 2), to eliminate contaminant-free RNA, particularly dsRNA, which could interfere with the capture of RNA-DNA hybrids by the S9.6 antibody (Hartono et al., 2018), and to determine R-loop levels more accurately, we used treatment with RNase III, following established protocols (Crossley et al., 2020). New experiments are reported in the revised version of the manuscript for R-loops in all cell lines (see Fig. 3B of the revised manuscript).

Additionally, colocalization by PLA of WRNIP1/R-loops, RAD51/R-loops, FANCD2/R-loops, and R-loop accumulation by anti-S9.6 antibody in cells depleted of FANCD2 are presented (see Figs. 4D and E; 6B and D of the revised manuscript).

Furthermore, we repeated the dot-blot experiment (see Fig. 2B of the revised manuscript). We treated the samples with RNase H to degrade RNA-DNA hybrids and hybridized the membrane with an anti-dsDNA antibody to quantify R-loop levels more accurately. Our analysis confirms that the S9.6 signal strongly accumulates in shWRNIP1 cells compared to shWRNIP1WT cells. Additionally, a graph illustrating the fold-change values of the S9.6/dsDNA signal relative to wild-type untreated cells is provided.

Importantly, overall, our findings suggest that treatment with RNase III does not substantially change the results obtained without it, but in some cases, such as in Fig. 4D, makes them more readily interpretable. Specifically, the new protocol allows us to visualize a higher number of PLA foci, and Aph increased the spots per nucleus in shWRNIP1D37A cells compared to the previous experiment (see Fig. 4D of the revised manuscript).