

Original Article

R-loop formation contributes to mTORC1 activation-dependent DNA replication stress induced by p53 deficiency

Xiaolei Li^{1,4,*}, Cheng Yang^{1,2}, Xiaohui Zhang^{1,3}, Feiyang Wang², Longhua Sun^{1,4}, Wei Zhang^{1,4}, and Xinping Xu^{1,4,*}

¹Jiangxi Provincial Key Laboratory of Respiratory Diseases, Jiangxi Institute of Respiratory Disease, Department of Respiratory and Critical Care Medicine, the First Affiliated Hospital, Jiangxi Medical College, Nanchang University, Nanchang 330006, China, ²Jiangxi Medical College, First Clinical Medical College, Nanchang University, Nanchang 330006, China, ³Department of Respiratory and Critical Care Medicine, Renmin Hospital of Shangrao, Shangrao 334000, China, and ⁴China-Japan Friendship Jiangxi Hospital, National Regional Center for Respiratory Medicine, Nanchang 330200, China

*Correspondence address. Tel: +86-791-86319890; E-mail: xiaoleili@ncu.edu.cn (X.L.) / Tel: +86-791-86319889; E-mail: xinpingxu@ncu.edu.cn (X.X.)

Received 20 October 2023 Accepted 30 July 2024 Published 4 November 2024

Abstract

DNA replication stress is a significant contributor to spontaneous DNA damage and genome instability. While the impact of p53 deficiency on increasing DNA replication stress is known, the specific molecular mechanism underlying this phenomenon remains poorly understood. This study explores how p53 deficiency induces DNA replication stress by activating mTORC1 through R-loop formation, which is facilitated by the upregulation of RNR. Research has shown that p53 deficiency results in increased γ H2AX expression and a higher mutation rate in the *HPRT* gene. Interestingly, these effects can be alleviated by rapamycin, an mTORC1 inhibitor. Additionally, rapamycin reduces the abundance of R-loop structures in p53KO cells, which is linked to mTORC1's regulation of ribonucleotide reductase (RNR) level. These findings suggest that p53 deficiency-induced DNA replication stress relies on mTORC1 activation, with the upregulation of RNR expression and R-loop formation. Overall, this study underscores the importance of R-loops in mTORC1 activation-dependent DNA replication stress triggered by p53 deficiency.

Key words p53, mTORC1, DNA replication stress, R loops, RNR

Introduction

In mammalian cells, accurate replication of genetic information before cell division is essential to maintain the integrity and stability of the genome. The delay or blockage of replication progress due to various cellular stresses is referred to as replication stress [1–3]. DNA replication stress is a significant factor contributing to genome instability and is considered a characteristic feature of cancer cells [4]. Replication stress can arise from multiple sources, including DNA template lesions, DNA secondary structures, nucleotide imbalances, and conflicts between transcription and replication [3], as well as dysregulated DNA repair processes [1].

p53 is a critical tumor suppressor in mammals. Its classical function is to regulate gene transcription during the cell cycle and cell apoptosis, but recent research has shown that this regulation is

not necessary for tumor suppression [5]. p53 has also been found to have transcription-independent functions that contribute to its tumor suppressive capabilities, which have garnered attention [6]. Specifically, p53 directly participates in replication and helps prevent replication stress by promoting DNA resection or DNA damage tolerance or bypass [7]. At stalled forks, p53 plays a critical role in suppressing the usage of error-prone DNA damage repair pathways, thereby ensuring replication fidelity [8]. Another study elucidated the function of p53 in maintaining genome integrity by avoiding transcription–replication conflicts [9]. These findings indicate that p53 plays a key role in relieving replication stress, which may be the main contributor to its ability to suppress tumors. Consistent with published results, our study revealed increased expression of γ H2AX and an increased spontaneous gene mutation

rate in *p53*-knockout (KO) cells. Furthermore, our present study revealed that rapamycin can relieve this replication stress, which triggered our interest.

Rapamycin is an inhibitor of mTORC1 (mechanistic target of rapamycin complex 1) [10]. mTORC1 is a serine/threonine protein kinase that regulates cell glucose and lipid metabolism, cell survival and autophagy [11]. Crosstalk between mTORC1 and p53 exists. On the one hand, p53 suppresses/negatively regulates mTORC1 [12,13]; on the other hand, mTORC1 promotes p53 induction via S6K1-MDM2 axis activation [14]. Our data showed that rapamycin relieves p53 deficiency-induced DNA replication stress. However, it is still unclear whether the increased replication stress in *p53*-KO cells is due to mTORC1 activation. If so, what causes the activation of mTORC1 in *p53*-KO cells? These questions have not yet been answered.

Therefore, this study aims to clarify the underlying molecular mechanisms involved in mTORC1 activation and p53 deficiency-induced replication stress, which is essential for understanding tumorigenesis caused by p53 loss.

Materials and Methods

Cell culture and generation of a *p53*-knockout cell line

The colorectal carcinoma cell line HCT116 from ATCC (CCL-247, a gift from Dr SangEun Lee at the UT Health Science Center of San Antonio) was used in this study and maintained in DMEM (C11995500BT; Gibco, Grand Island, USA) supplemented with 10% fetal bovine serum (FBS) (S711-001; Lonsera, Coast, Uruguay) and antibiotics (100 U/mL penicillin and 100 µg/mL streptomycin) (P1400; Transgene, Beijing, China). The cells were incubated at 37°C in a 5% CO₂ incubator (BB 150; Thermo Fisher Scientific, Waltham, USA) and subcultured with trypsin-EDTA (FG301-1; Transgene) before they reached confluence.

The HCT116 *p53*-KO cell line was constructed via the CRISPR-Cas9 technique [15]. The guide RNA sequence was 5'-GGGAG-GAGCTCTGACACTCGGA-3'. pSpCas9 (BB)-2A-GFP (PX458) (Addgene plasmid # 48138, <http://n2t.net/addgene:48138>) was a gift from Feng Zhang (Broad Institute of MIT and Harvard). The PX458 plasmid was transfected into cells using a Neotransfection system (MPK5000; Thermo Fisher Scientific). The *p53*-KO clones were confirmed by immunostaining and western blot analysis.

Nucleus size measurement

HCT116 *p53*-KO and *p53* WT cells were seeded on cover slides placed in 6-well plates (1×10⁵ cells/well). After the cells were allowed to attach, they were washed twice with 1× DPBS and fixed with 100% ice-cold methanol at -20°C for 10 min. Next, the cells were permeabilized with 0.2% Triton X-100 for 10 min at RT. After being washed with 1× DPBS three times, the cells were stained with DAPI (1 µg/mL) at room temperature for 10 min. Well-stained cells were mounted with one drop of mounting media, observed and imaged under a fluorescence microscope (EVOS M5000; Thermo Fisher Scientific).

Nucleus size was analyzed on the basis of the area of DAPI-stained cells via the MATLAB program. Approximately 60 cell nuclei were observed in each image, and 100 images/group were included.

Immunofluorescence staining

The cells (2.5×10⁴ cells/well) were seeded on 4-well cover slides

(Lab-Tek II Chamber Slide;154526; Thermo Fisher Scientific). After the cells had attached, they were synchronized with culture media containing 0.1% FBS, and after 24 h the medium was changed to 10% FBS culture media. EdU (10 µM) was added, and the mixture was incubated for 2 h. Then, the cells were fixed and permeabilized. The cells were first subjected to EdU detection via a BeyoClick EdU-488 kit (C0071S; Beyotime Biotechnology, Shanghai, China) according to the manufacturer's instructions. After the click chemistry was completed, the cells were further subjected to γH2AX staining. The cells were blocked with 3% BSA for 30 min at room temperature. Anti-γH2AX antibody (9718, 1:400; Cell Signaling Technology, Danvers, USA) was incubated with the cells at 4°C overnight. The sections were then incubated with Alexa Fluor 594-conjugated goat anti-rabbit IgG (H+L) (E-AB-1060, 1:100; Elabscience, Wuhan, China) as secondary fluorescent antibodies at room temperature for 1 h. The nuclei were stained with Hoechst 33342 and mounted with Fluorescent Antifading Mounting Buffer (BD5014; Bioworld Technology, Shanghai, China). Images were captured on a STELLARIS 5 confocal microscope (Leica, Wetzlar, Germany). Z-stack line scanning was performed for each image, the stacked images were processed via LAS X software, and the degree of EdU/γH2AX colocalization was quantified via the Quantify Tools of LAS X.

Flow cytometric analysis

The cells (5×10⁵ cells/well) were seeded in 6-well plates. EdU solution (working concentration of 10 µM) was added, and the mixture was incubated for 2 h before the cells were harvested. γH2AX (red) was stained with primary antibody and then labeled with the secondary fluorescent antibody-AF594, and EdU (green) was detected using the BeyoClick EdU-488 kit. The stained cells were analyzed with the CytoFLEX S flow cytometer (Beckman, Brea, USA), and 10,000 cells were counted per sample. The data were analyzed with FlowJo 10.6.2.

HPRT assay

For the hypoxanthine-guanine phosphoribosyl transferase (HPRT) mutagenesis assay, cells were first cultured in HAT medium (H0262; Sigma-Aldrich, St Louis, USA) for 3 days to eliminate spontaneous HPRT mutants and subsequently allowed to recover for 1 day in HT medium (H0137; Sigma-Aldrich). Then, the cells were washed with 1× DPBS and allowed to grow in normal media to obtain enough cells. A total of 1×10⁶ cells were seeded in duplicate in 100-mm dishes and cultured in medium containing 20 µM 6-thioguanine (6-TG, A4660; Sigma-Aldrich) to promote the proliferation of mutated HPRT cells. Moreover, 500 cells were plated in complete medium in duplicate in 60-mm dishes to determine clonal efficiency. After 14 days of selection, the colonies were fixed, stained and counted. The HPRT mutation frequency (MF) was calculated via the following equation: MF=(Number of HPRT colonies/10⁶)×Plating efficiency×100%; Plating efficiency=(Number of colonies/Number of seeding)×100%.

For rapamycin treatment, after HAT selection, the cells were treated with rapamycin (20 nM, S1039; Selleck, San Diego, USA) for 3 days, trypsinized and washed with 1× DPBS. Then, the cells were plated and selected as previously described.

Colony formation assay

The cells were seeded onto 100-mm tissue culture dishes or 6-well

plates depending on the aims of the experiments and incubated for 24 h to allow attachment. The cells were treated with drugs according to the experimental requirements, and the medium was changed every three days. The cells were subsequently cultured for 14 days to form colonies. The colonies were fixed with 6% glutaraldehyde (v/v) and stained with 0.5% (w/v) crystal violet. Colonies containing >50 cells were counted, and the *HPRT* gene mutation frequency was calculated, considering the plating efficiency of the control dish. At least three independent experiments were performed. Each experiment was performed in duplicate.

Plasmids and transfection

The pcDNA3.1 (+) vector was used to construct a p53 expression plasmid. pcDNA3.1 (+) was kindly provided by Professor Jianbin Wang (Nanchang University, Nanchang, China). The p53 coding sequence was PCR-amplified via Pfu Turbo DNA polymerase (P505-d1; Vazyme, Nanjing, China). The PCR conditions were as follows: initial denaturation at 95°C for 3 min, followed by 35 cycles of denaturation at 95°C for 15 s, annealing at 60°C for 15 s, and extension at 72°C for 1 min. A final extension was carried out at 72°C for 5 min. The primer sequences as following: forward primer (F): 5'-atcgGGTACCGtgacacgctccctggat-3' (29 bp) and reverse primer (R): 5'-atcgCTCGAGgtggggaacaagaagtgag-3' (30 bp). The PCR products were verified by sequencing (Sangon Biotech, Shanghai, China) and ligated into the pcDNA3.1 (+) vector between the *XhoI* and *HpaI* sites. pcDNA3.1-p53 was transfected into HCT116 p53-KO cells via Lipofectamine 3000 (Thermo Fisher Scientific) according to the manufacturer's instructions. A stable cell line was created via neomycin selection, and the p53 expression level was determined via western blot analysis.

Western blot analysis

Cell extracts were prepared on ice with RIPA lysis buffer (C1053; Applygen Technologies, Beijing, China) supplemented with protease inhibitors (GK10014; GLP BIO, Hyattsville, USA), phosphatase inhibitors (GK10011; GLP BIO) and 1 mM PMSF (P0100; Solarbio, Beijing, China), followed by pulse sonication for 10 s. The cell extracts were boiled for 5 min at 95°C, and the same amount of protein was resolved via SDS-PAGE and transferred onto PVDF membranes (HybondTM P 0.45 PVDF 10600023; Amersham, Chicago, USA). After being blocked with 5% nonfat milk or 5% BSA (depending on the primary antibody used) in 1× TBST (Tris-buffered saline and Tween 20) at room temperature for 1 h, the membrane was incubated with the primary antibodies (Supplementary Table S1) overnight at 4 °C, followed by incubation with peroxidase-conjugated secondary antibodies AffiniPure goat anti-mouse IgG (H+L) (150976) or goat anti-rabbit IgG (H+L) (151083; Jackson ImmunoResearch Laboratories, West Grove, USA). Western blot detection was performed with an enhanced chemiluminescence system (ChemiDocTM Imaging System; Bio-Rad, Hercules, USA).

Immunofluorescence analysis of the R-loop structure

Cells were treated with/without rapamycin. After treatment, the cells were washed with 1× DPBS and fixed with 100% ice-cold methanol at -20°C for 10 min. Then, the cells were permeabilized with 0.2% Triton X-100 at room temperature for 10 min. The control group was treated with RNase H (60 U/mL, M0297S; NEB, Ipswich,

USA) at 37°C for 30 min. Next, the cells were blocked with 3% BSA/0.2% Triton X-100 at room temperature for 30 min and then incubated with S9.6 (1:100, #ENH001; Kerafast, Boston, USA) at 4°C overnight. The cells were washed with 1× PBS three times and then incubated with secondary antibody (1:100, Alexa 488; Abway Biotechnology, Shanghai, China) in 3% BSA/0.2% Triton X-100 at room temperature for 1 h. After being washed three times with 1× DPBS, the nuclei were stained with DAPI (1 µg/mL, BD5010; Bioworld Technology) and mounted with fluorescent antifading mounting buffer (BD5014; Bioworld Technology). Images were captured via a TCS SPE confocal microscope (Leica).

Statistical analysis

Data are expressed as the mean±standard deviation. Statistical significance was determined via a two-tailed unpaired *t* test or Mann-Whitney test, as appropriate. All data are from three or at least two independent experiments. All tests were performed via GraphPad Prism 8.0.1 (GraphPad Software, La Jolla, USA). *P*<0.05 was considered statistically significant.

Results

p53 deficiency is associated with increased DNA damage and genome instability

We successfully established p53-knockout cell lines in HCT116 and mNS-5 cell lines using CRISPR-Cas9, the knockout efficiency of p53 was verified by western blot analysis and immunofluorescence staining (Figure 1). Our observations indicate that the nucleus size of p53-knockout (KO) cells is heterogeneous, with some nuclei being larger than those of p53 wild-type (WT) cells (Figure 2A). The average size of the p53 WT nucleus was 82.37 µm², whereas the average size of the p53-KO cell nucleus was 120.78 µm². This difference in nucleus size between p53-KO and WT cells was statistically significant (Figure 2B). We propose that the enlarged nuclei observed in p53-KO cells may be attributed to replication. Our investigation of spontaneous DNA damage and genome stability revealed a notable increase in γH2AX foci in p53-KO cells, especially within the enlarged nuclei (Figure 3B). Additionally, our observation of colocalization between γH2AX foci and EdU when the cells were incubated with EdU suggested that this DNA damage occurred during the S phase of the cell cycle (Figures 2C,D, Supplementary Figure S1). Genome stability was also evaluated via the *HRPT* reporter gene system. The mutation frequency of *HPRT* in p53-KO cells was measured to be 1.16/10⁶ cells, whereas in p53 WT cells, it was 0.31/10⁶ cells. This elevated mutation frequency indicates a significantly increased mutation burden in p53-KO cells (Figure 2E and Supplementary Figure S2). To confirm that the increase in the γH2AX level was indeed caused by p53 deficiency, we reintroduced p53 by transfecting the pcDNA3.1-p53 plasmid into HCT116 p53-KO cells. The results demonstrated that restoring appropriate p53 level decreased γH2AX level (Figure 2F). Overall, these results suggest that p53 deficiency induces DNA replication stress, leading to increased DNA damage and genome instability in p53-KO cells.

p53 deficiency-induced DNA replication stress is associated with mTORC1 activation

Activated mTORC1 leads to the phosphorylation of p70S6 kinase 1 (p70S6K1) and eIF4E binding protein (4EBP) [16]. Furthermore,

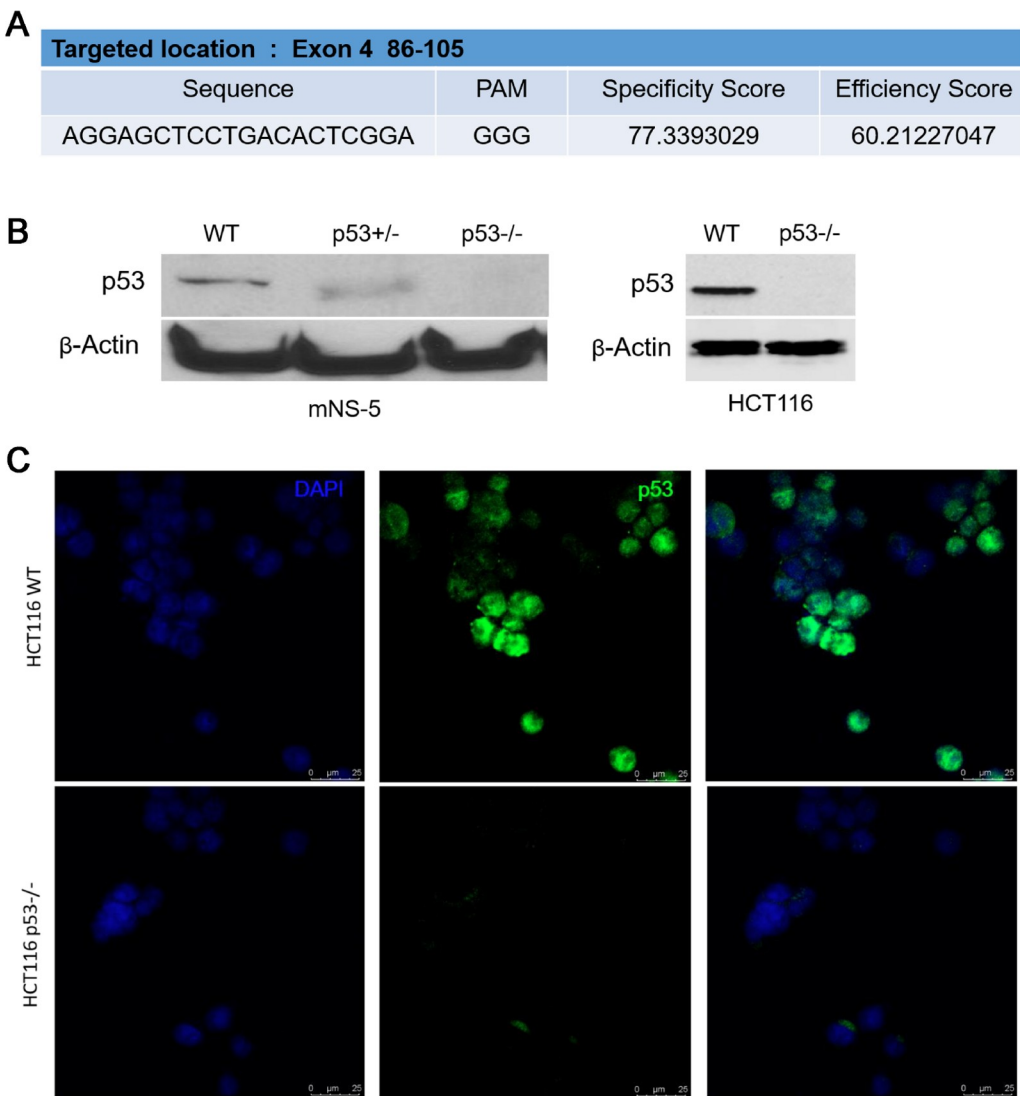


Figure 1. Establishment of *p53*-knockout cell line by CRISPR-Cas 9 (A) Guide-RNA targeted location is *p53* gene Exon 4 (86–105), the specificity and efficiency score of gRNA sequence were evaluated via the Benchling web site. (B) *p53* knockout was done in mNS-5 cell line and HCT 116 cell line. *p53* protein level was confirmed by western blot analysis. (C) *p53* knockout efficiency was confirmed in HCT116 cells by immunostaining using P53(DO-7) mouse mAb (CST #48818) and Alex 488 (green) for *p53* labeling, and DAPI staining for nucleus (blue). Scale bar: 25 μ m.

p70S6K phosphorylates ribosomal protein S6 (S6RP). In our study, we observed elevated levels of p70S6K1 and pS6RP along with decreased level of 4EBP1 in *p53*-KO cells compared with those in *p53* WT cells (Figure 4A and Supplementary Figure S3). Upon re-expression of *p53* in *p53*-KO cells, p70S6K1 and pS6RP levels were inhibited (Figures 4A and Figure 5B). These findings suggest that mTORC1 is overactivated in *p53*-KO cells because of the absence of *p53*. We subsequently explored whether this overactivation of mTORC1 is linked to increased DNA replication stress in *p53*-KO cells. HCT116 *p53* WT and *p53*-KO cells were treated with varying concentrations of rapamycin (Supplementary Figure S4). The results revealed that rapamycin alleviated the enlarged nuclei of *p53*-KO cells (Figure 4C) and decreased γ H2AX expression (Figure 4B). Additionally, the level of γ H2AX decreased with prolonged rapamycin treatment (Figure 4B). After 48 h of rapamycin treatment, the γ H2AX and EdU colocalization rate decreased from 65.66% to 26.85% in *p53*-KO cells (Figure 4F,G). Furthermore,

rapamycin reduced the frequency of *HPRT* gene mutations in *p53*-KO cells (Figure 4D,E). These findings indicate that inhibiting mTORC1 can mitigate *p53* KO-induced DNA damage and genome instability, suggesting a link between *p53* deficiency-induced DNA replication stress and mTORC1 overactivation.

DNA replication stress in *p53*-KO cells is associated with increased transcription-replication conflicts

To investigate the reasons behind the increased DNA damage in *p53*-KO cells, we initially examined the DNA damage response pathway (DDR) in both *p53* WT and *p53*-KO HCT116 cells. Our data revealed the activation of ATM/CHK2 and ATR/CHK1 phosphorylation in *p53*-KO cells (Figure 6A), indicating that the DDR pathway functions properly and is not deficient in *p53*-KO cells. Furthermore, we evaluated the integrity of the DNA repair pathway by treating *p53* WT and *p53*-KO HCT116 cells with doxorubicin (0.1 μ M) for 6 h and 24 h. Western blot analysis of Rad51, Ku70,

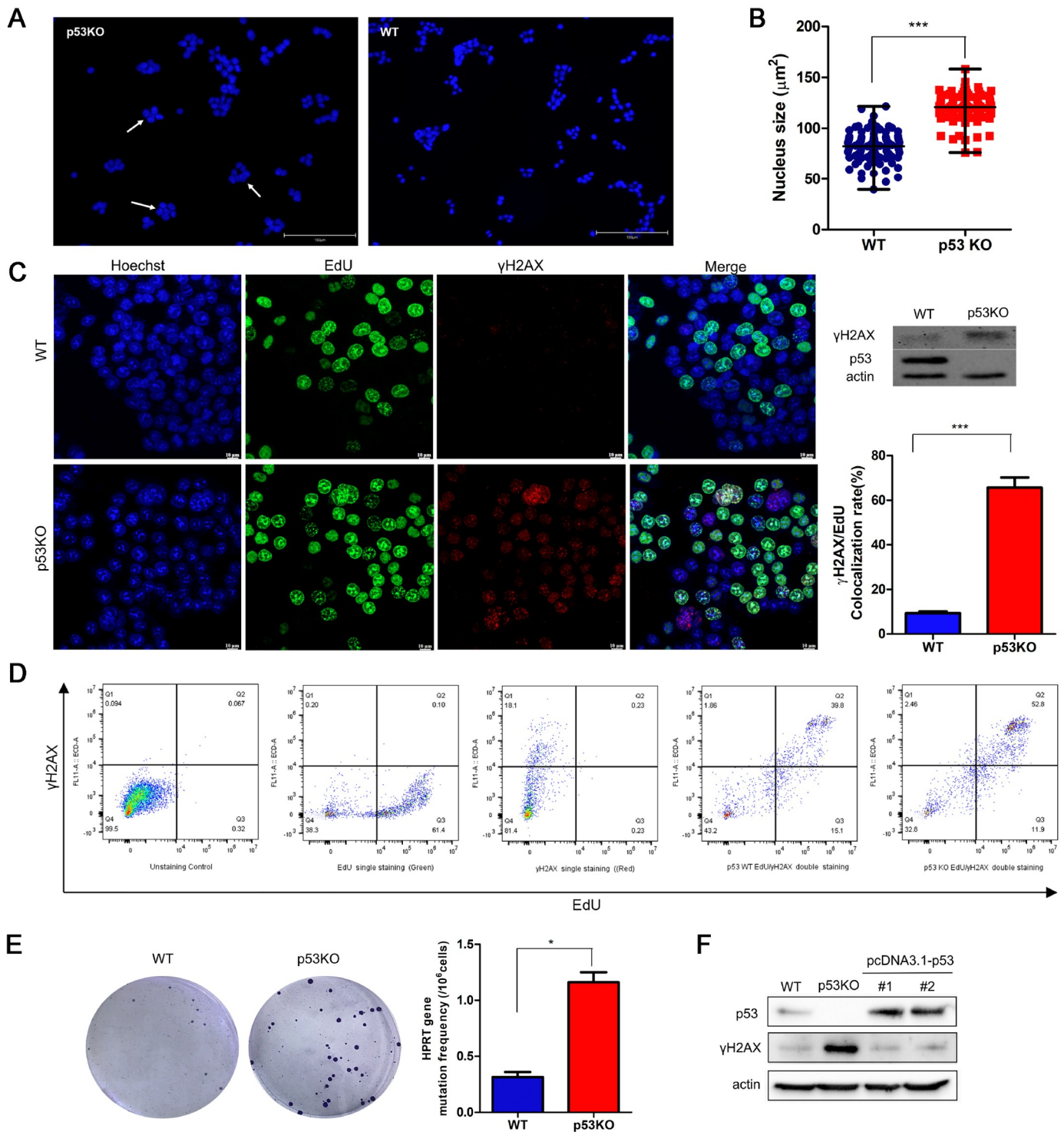


Figure 2. p53 deficiency induced DNA replication stress (A,B) p53-KO cells have enlarged nucleus (white arrow indicated). Nucleus was stained by DAPI, the size was analyzed by Matlab program. Scale bar: 150 μm . About 100 pictures in each group were analyzed. (C) γH2AX is increased in p53-KO cells. Level of γH2AX was evaluated by western blot analysis and immunofluorescence staining. γH2AX (red) was stained with its primary antibody and then labeled by secondary fluorescent antibody AF594, EdU (green) was stained by the BeyoClick EdU-488 kit, nucleus was stained by Hoechst 33342 (blue). Z-stack line scan images were captured on Leica STELLARIS 5 confocal microscope and processed by LAS X software. Scale bar: 10 μm . EdU and γH2AX colocalization was quantified by analyzing the Z-stack line scan images. (D) Representative of dot-plots of anti- γH2AX (Y axis) and anti-EdU (X axis). (E) HPRT mutation frequency was calculated based on the number of colonies formed by 6-TG selection. (F) Re-expression of p53 in HCT116 p53-KO cells; γH2AX and p53 protein levels were analyzed by western blot analysis. Data were from three independent experiments. Data were analyzed by Mann-Whitney test. Data are expressed as the mean $\pm$ standard deviation. * $P < 0.05$, *** $P < 0.001$.

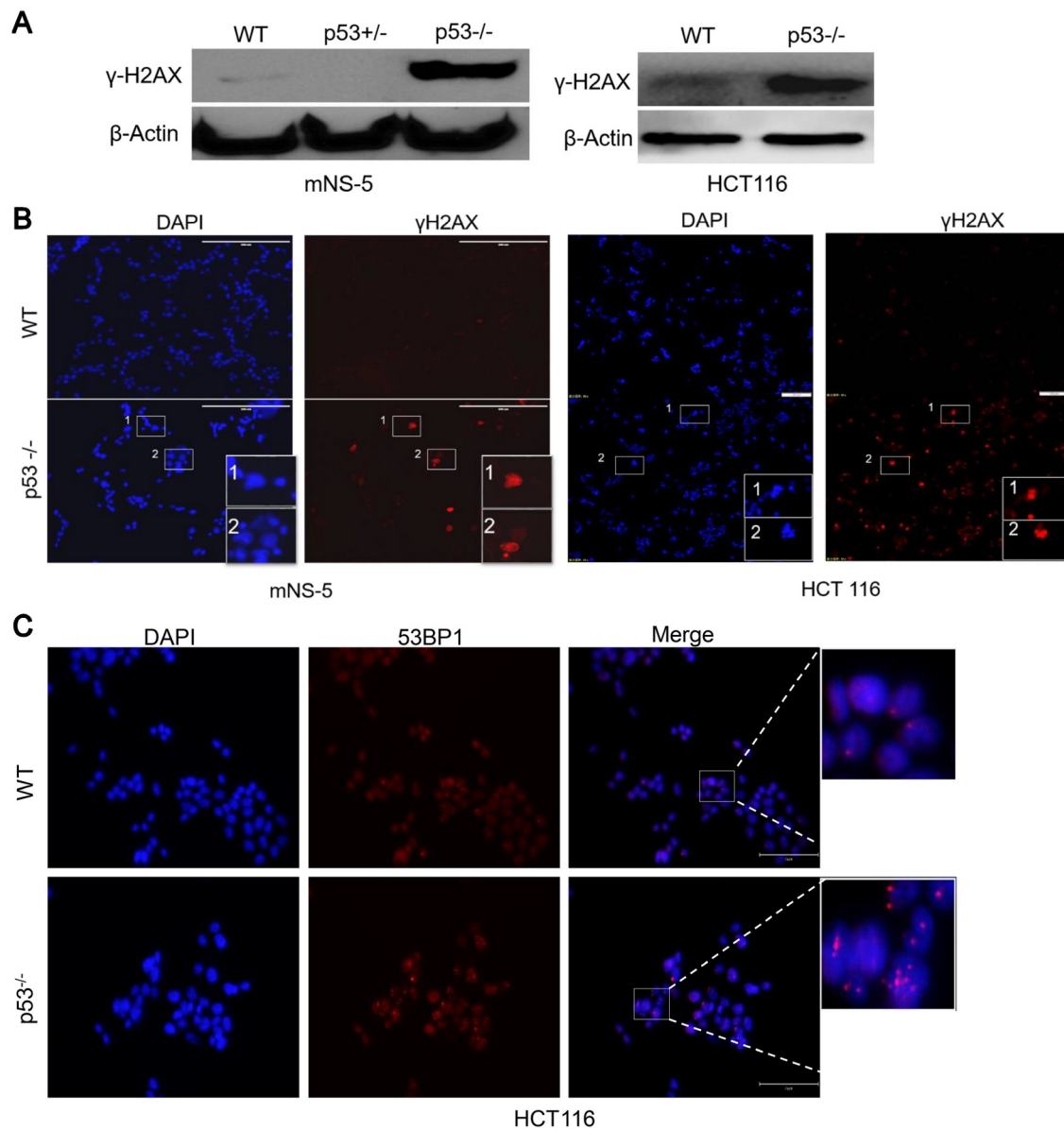


Figure 3. p53 deficiency increased γ H2AX and 53BP1 levels (A) γ H2AX level was evaluated in *p53*-KO cells by western blot analysis. (B) γ H2AX foci was evaluated in *p53*-KO cells by immunostaining, anti- γ H2AX (Ser139) (CST # 9718) and Alex 594 (red) for γ H2AX labeling, DAPI staining for the nucleus (blue), γ H2AX foci appears in enlarged nucleus (white rectangle). Scale bar: 200 μ m. (C) 53BP1 foci was evaluated in *p53*-KO cells by immunostaining, anti-53BP1 (CST # 9718) and Alex 594 (red) for γ H2AX labeling, DAPI staining for the nucleus (blue), Scale bar: 75 μ m.

and Lig4 revealed an increase in the level of Rad51 at 24 h in response to doxorubicin treatment (Figure 6B). Moreover, Ku70 and Lig4 were slightly enriched in *p53*-KO cells at 24 h, whereas no significant changes were observed in *p53* WT cells (Figure 6B). These findings indicate that the homologous recombination (HR) pathway was activated by Dox treatment in both *p53* WT and *p53*-KO cells. The nonhomologous end joining (NHEJ) pathway was not affected in *p53* WT cells but was slightly affected in *p53*-KO cells. Taken together, these results suggest that both the DNA damage response (DDR) and DNA repair pathways remain intact in *p53*-KO cells and are not affected by *p53* deficiency.

Since *p53* deficiency does not affect the DDR or subsequent repair pathway, we further investigated the function of *p53* during replication-related transcription. Research has shown that *p53* loss

impairs normal replication fork progression and increases transcription-replication conflicts [9]. We observed that the expression levels of TOPII α (TOP2A) [17], BRD4 [18], RNaseH1 [19] and RPA [20] were greater in *p53*-KO cells than in *p53* WT cells (Figure 6C). These findings led us to speculate that *p53* deficiency in *p53*-KO cells may result in greater requirements of these proteins for these cells. To test this hypothesis, we treated cells with doxorubicin (0.1 μ M) for 3 h, 6 h or 24 h. The results demonstrated that treatment with Dox increased the levels of Top2A, BRD4, RNase H1 and pRPA/PRA in both HCT116 *p53* WT and *p53*-KO cells. However, these increases occurred earlier in *p53*-KO cells than in *p53* WT cells (Figure 6C). These findings suggest that *p53*-KO cells may experience more topological conflicts, leading to increased DNA damage and replication stress.

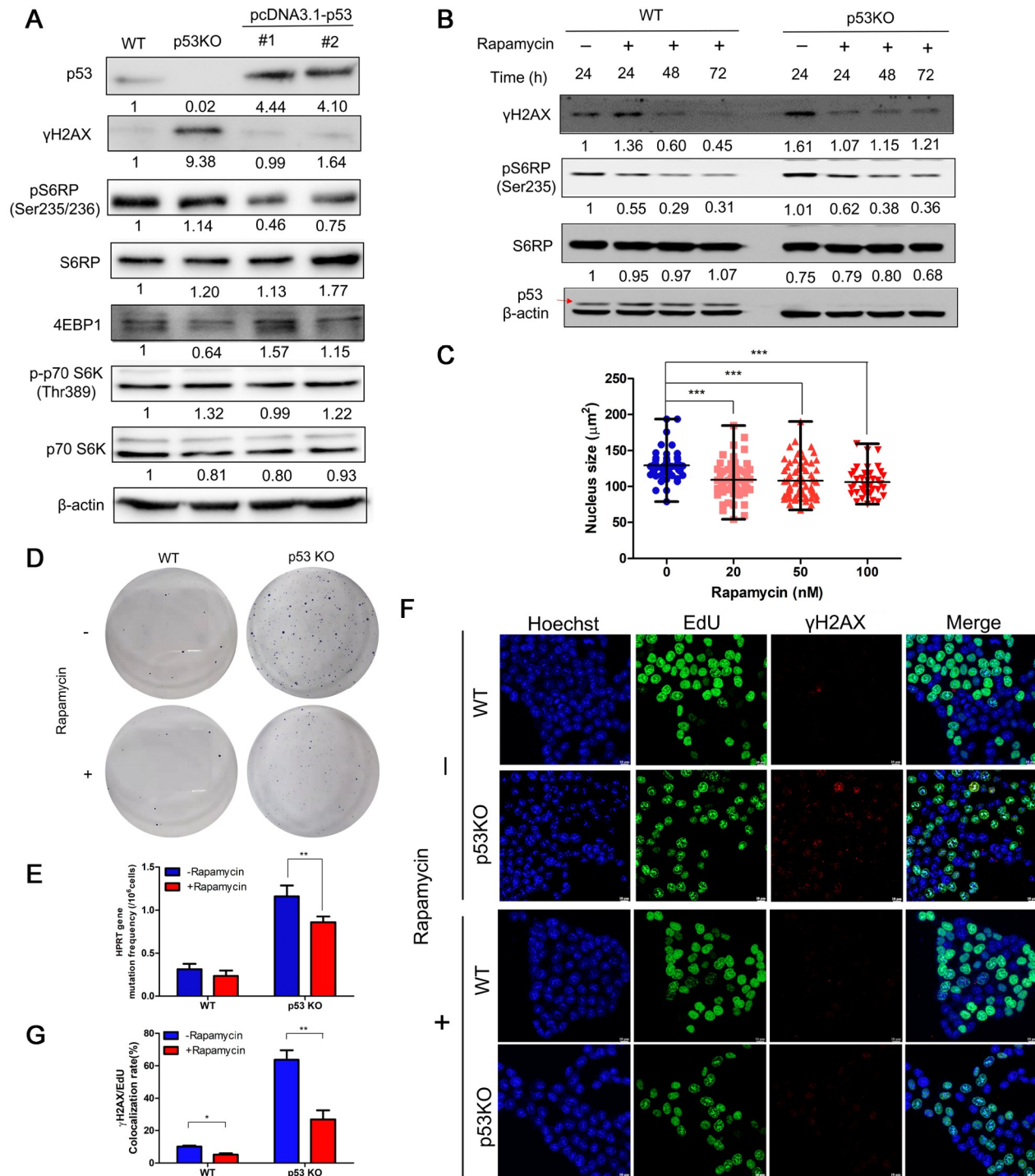


Figure 4. p53 deficiency induced DNA replication stress associated with activation of mTORC1 (A) mTORC1 pathway was overactivated in p53-KO cells. We re-introduce p53 in p53-KO cells by transfecting pcDNA3.1-p53 plasmid and constructed p53 stable expression cell lines. pP70S6K, pS6RP and 4EBP1 protein expression levels were analyzed by western blot analysis. (B) Rapamycin decrease γH2AX expression. HCT116 p53 WT and p53-KO cells were treated with 20 nM rapamycin for 24 h, 48 h and 72 h, γH2AX, pS6RP and S6RP levels were analyzed by western blot analysis. (C) Rapamycin relieves enlarged nucleus of p53-KO cells. HCT 116 p53-KO cells were treated with rapamycin (0, 20 nM, 50 nM and 100 nM) for 72 h. The nucleus was stained by DAPI and the size was analyzed by Matlab program. Scale bar: 150 μm. About 100 pictures in each group were analyzed. (D,E) Rapamycin reduced HPRT gene mutation frequency. (F,G) Rapamycin decrease γH2AX and EdU foci. HCT116 p53 WT and p53-KO cells were treated with 20 nM rapamycin for 48 h, γH2AX (red) was stained with its primary antibody and labeled by AF594. EdU (green) was stained by the BeyoClick EdU-488 kit, the nucleus was stained by Hoechst 33342 (blue). Z-stack line scan images were captured with Leica STELLARIS 5 confocal microscope and processed by LAS X software. Scale bar: 10 μm. EdU and γH2AX colocalization rate was quantified by analyzing the Z-stack line scan images. Data are expressed as the mean±standard deviation. Data were analyzed by Two tailed unpaired t test, *P<0.05, **P<0.01, ***P<0.001.

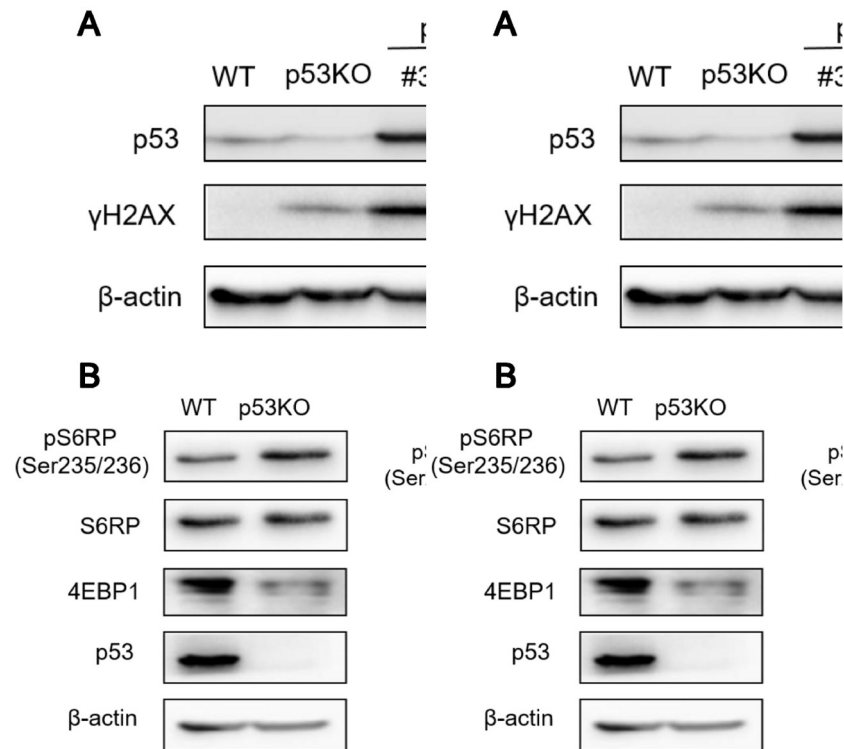


Figure 5. Re-expression of p53 in HCT 116 p53-KO cells repressed mTORC1 signaling (A) We constructed p53 expression stable cell lines and got different clones. Here we show clones #3, #4, and #5 cause γH2AX accumulation. (B) Clones #1 and #2 relieve γH2AX and repress mTORC1 signaling.

The R-loop structure, which is a result of transcription-replication conflicts (TRCs) [21], was assessed via S9.6 staining. We further investigated R-loops in p53-KO cells. Our results revealed a significant increase in S9.6 foci in p53-KO cells compared with those in p53 WT cells (Figure 6D,E and Supplementary Figure S5). The bright green foci were located in the nucleus, and these foci were digested upon RNase H treatment. Moreover, we did not observe obvious S9.6 foci in the nucleus of p53 WT cells (Figure 6D and Supplementary Figure S5B). These data suggest that there is an increased presence of R-loop structures in p53-KO cells, which may be the reason for the increased replication stress.

Inhibiting mTORC1 relieves R-loop structure in p53-KO cells via the downregulation of RNRs

Therefore, we investigated the association between R-loops and mTORC1 overactivation. We found that upon Dox treatment, the requirements for Top2A, RNase H1, BRD4 and RPA were increased in both p53 WT and p53-KO cells (Figure 6C). Additionally, pS6RP level was increased in p53-KO cells, whereas there was no obvious change in p53 WT cells (Figure 7A). These findings suggest that mTORC1 is activated as the topological conflicts increase.

Ribonucleotide reductase (RNR) controls the production speed of dNTPs. In mammalian cells, RNR is composed of two identical ribonucleotide reductase large subunits (RRM1) and two small subunits of RRM2 (p53R2) [22]. The level of RRM2 is critical in controlling the activity of RNR. Our results demonstrated that RRM1 and RRM2 are upregulated in p53-KO cells (Figure 7B), which is consistent with the increased phosphorylation of S6RP. Treatment with rapamycin downregulated the levels of RRM1 and RRM2

(Figure 7B). On the basis of these results, we hypothesize that the increased R-loops in p53-KO cells may be caused by imbalanced dNTPs, which could be a result of upregulated RNRs. The upregulation of RNRs is associated with mTORC1 activation. To further investigate this, we examined R-loop structures in rapamycin-treated p53-KO HCT116 cells. S9.6 staining revealed a decrease in S9.6 foci upon rapamycin treatment (Figure 7C,D and Supplementary Figure S5), suggesting that rapamycin relieves R-loops induced by p53 deficiency. These findings suggest that increased R loops in p53-KO cells are associated with the activation of mTORC1, which may be caused by the upregulation of RNRs. However, direct evidence linking R-loops increase to mTORC1 activation still needs further investigation.

Discussion

As a guardian of the genome, p53 was identified 30 years ago [23]. Despite extensive studies conducted since its discovery, many aspects of its multifaceted role in tumorigenesis are still unknown.

DNA replication stress (RS) is considered one of the hallmarks of cancer [2] and plays a significant role in tumorigenesis. Recent studies have indicated that p53 is helpful in mitigating replication stress [9]. During the normal S phase, p53 prevents conflicts between transcription and replication to avoid RS. When replication fork stalls, p53 recruits repair proteins to facilitate fork restart [8]. Our data also support the involvement of p53 in RS regulation. In p53-KO cells, the expression/foci level of γH2AX and the frequency of gene mutations are significantly increased (Figure 2C–E), indicating increased RS [1]. To further confirm that p53 loss is the cause of this RS, we expressed p53 in p53-KO cells and observed

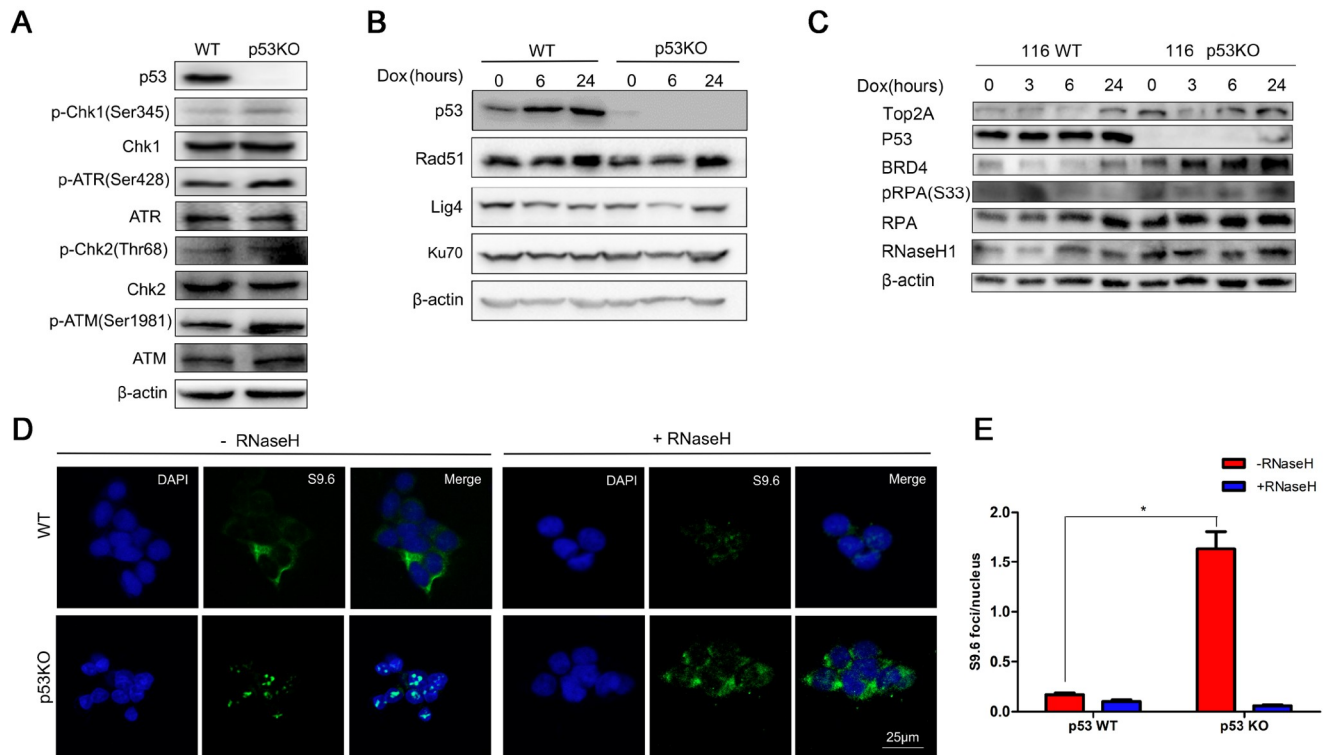


Figure 6. p53 deficiency induced DNA replication stress due to increased transcription-replication conflicts (A) DNA damage response pathway activation status were checked by western blot analysis. (B) HR and NHEJ repair pathways were checked by western blot analysis. Cells were treated with Doxorubicin (0.1 μ M) for 6 h and 24 h. (C) Top2A, BRD4, pRPA/RPA and RNaseH1 protein levels in HCT116 p53 WT and p53-KO cells. (D) S9.6 staining of HCT116 cells. R-loop structures were stained by anti-S9.6 antibody and labeled by fluorescent antibody AF488 (Green), the nucleus was stained by DAPI (blue). R-loops were digested by RNaseH, scale bar: 25 μ m. (E) Quantification of S9.6 staining by counting the green foci using ImageJ software, S9.6 foci per nucleus was calculated as: Total number of green foci/total nucleus. About 1000 nuclei were counted. Data were from two independent experiments. Data were analyzed by Mann-Whitney test. * $P < 0.05$.

that p53 expression indeed relieved the increase in γ H2AX (Figure 2F). However, we found that achieving the 'deal' level of p53 during re-expression was not easy. It is crucial to carefully adjust the quality of the transfected plasmid to obtain an appropriate p53 level to relieve DNA damage (Figure 2F). Different protein levels of p53 resulted in different γ H2AX levels, and excessive p53 expression not only failed to relieve RS but also caused further accumulation of γ H2AX (Figure 5A). These findings indicate the existence of a precise regulatory mechanism by which p53 level mitigates RS, but the molecular basis of this mechanism, which is likely associated with the oncogene function of p53 in tumors, remains unclear.

mTORC1 plays a central role in coordinating cell growth and metabolism [16]. Deregulation of mTORC1 has been implicated in the progression of many diseases, including cancer, diabetes and aging. Our results demonstrate that p53 contributes to the maintenance of replication stress (RS) by inhibiting the activation of mTORC1 (Figure 4D,F). Specifically, the activation of mTORC1 induces RS in p53-KO cells. However, the reason why activation of mTORC1 increases RS has not yet been determined. A recent study reported that mTORC1 activation leads to the degradation of RNF168, a DNA damage response protein. The degradation of RNF168 leads to the accumulation of DNA damage, resulting in genome instability [24]. Importantly, unlike p53 loss, RNF168 is not a tumor suppressor, so the consequences of its degradation may differ. Our results also revealed that mTORC1 activation is required in p53-KO cells, along with an increased requirement for Top2A,

BRD4, RPA and RNaseH1, upon Dox treatment (Figures 6C and Figure 7A). We aimed to investigate whether mTORC1 activation is necessary for increased R-loop formation in p53-KO cells or whether increased R-loop structures require mTORC1 activation for resolution. R-loop structures are a consequence of transcription-replication conflicts (TRCs) [25], which occur when the replication and transcription directions are head-on. R-loops consist of three-strand nucleic acids, with a nascent RNA strand hybridized with the DNA template strand, leaving the nontemplate DNA single strand. R-loops pose a major threat to genome stability [26]. Top2A, BRD4, RPA, and RNase H1 are crucial regulators of various stages of R-loop formation, including both prevention and resolution. Top2A, a topoisomerase, plays a preventative role by unwinding the supercoils that form during replication and transcription, thereby preventing the formation of R-loops [17]. RNase H1, an RNA hybrid endonuclease, is key in resolving R-loops by cleaving the RNA within DNA:RNA hybrids, thus addressing existing R-loop structures [19]. RPA (replication protein A), a single-stranded DNA (ssDNA)-binding protein, detects the presence of R-loops and facilitates the binding of RNase H1 to these structures. This interaction activates the enzymatic activity of RNase H1, enabling it to cleave DNA:RNA hybrids and inhibit further R-loop formation [20]. BRD4 serves as a regulatory molecule during transcription elongation. It activates CDK9 through its C-terminal domain (CTD), which in turn promotes the phosphorylation of RNAPII and enhances transcription elongation. This activity helps avoid

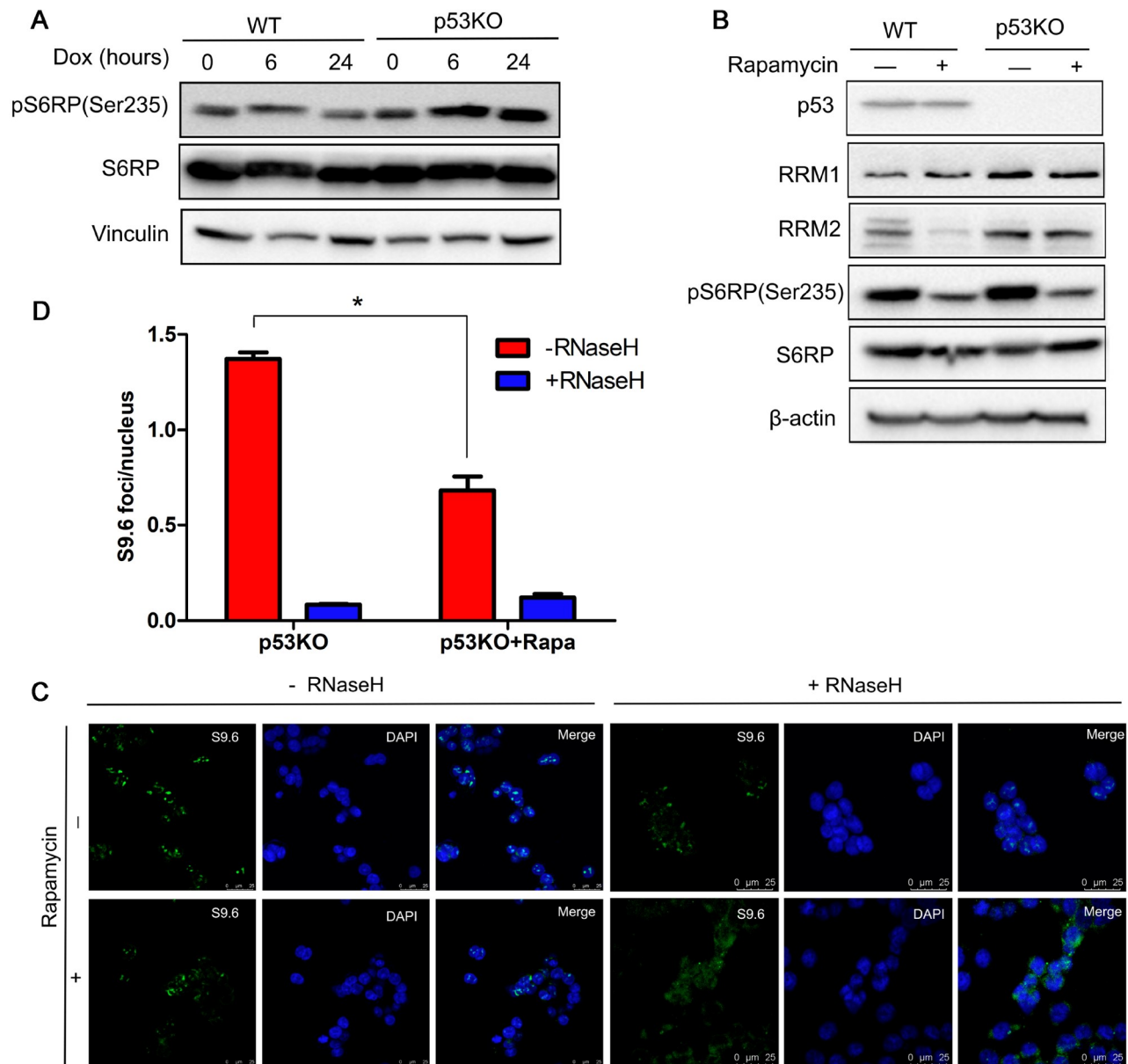


Figure 7. Rapamycin alleviated p53 deficiency-caused R-loop structure due to downregulation of RNRs (A) mTORC1 protein level in HCT116 p53 WT and p53-KO cells, cells were treated with doxorubicin (0.1 μ M) for 6 h and 24 h. (B) RRM1 and RRM2 protein levels were measured by western blot analysis. Cells were treated with rapamycin (20 nM) for 24 h. (C) S9.6 staining of HCT116 p53-KO cells. HCT 116 p53-KO cells were treated with rapamycin (20 nM) for 24h, R-loop structures were stained by anti-S9.6 antibody and labeled by fluorescent antibody AF488 (green), the nucleus was stained by DAPI (blue). Scale bar: 25 μ m. (D) Quantification of S9.6 foci, around 1000 cells were counted in each group. Data were from two independent experiments and analyzed by Mann-Whitney test. Data are expressed as the mean $\pm$ standard deviation. * P <0.05.

transcription–replication conflicts (TRCs) and prevents the formation of R loops [18]. In p53-KO cells, we observed elevated expression levels of Top2A, BRD4, RPA, and RNase H1. Additionally, p53-KO cells exhibit an increase in R-loops. When the cells were treated with Dox, the expressions of Top2A, BRD4, RPA, and RNase H1 continued to increase with prolonged exposure to the drug (Figure 6C). We hypothesize that p53-KO cells upregulate the expressions of Top2A, BRD4, RPA, and RNase H1 in response to increased R-loop structures. This upregulation is likely a mechanism to inhibit R-loop formation and stabilize the genome. This observation also confirms the presence of increased TRCs and R-loops in p53-KO cells. We also observed that activation of mTORC1

increases with increasing TRCs (Figure 7A). This raises the following question: could the overactivation of mTORC1 be responsible for the increased R-loops in p53-KO cells? By exploring the potential link between mTORC1 activation and increased R-loop formation, we may uncover the underlying mechanism through which mTORC1 activation induces RS in p53-KO cells.

p53, a negative regulator of mTORC1 [27], suppresses ribonucleotide reductase (RNR) by inhibiting mTORC1 [13]. We observed increased expression of RNR in p53-KO cells (Figure 7B). Treatment with rapamycin inhibited both RNR (Figure 7B) and R-loops in p53-KO cells (Figure 7C,D). Inhibiting mTORC1 alleviated the increase in R-loops. On the basis of our results, we speculate that

overactivation of mTORC1 may lead to an increase in RNR, which in turn causes an imbalance in dNTP levels, ultimately favoring R-loop formation. RNR is a key regulator of dNTP biosynthesis in mammals [22]. dNTP are the materials required for replication, and upregulation of RNR activity increases elongation [28], thereby potentially increasing the risk of R-loop formation. Increased RNR activity also induces an imbalance in dNTP levels [29], which promotes transcription. Excessive transcription may further favor R-loop formation. The connection between RNR and R-loops requires further investigation.

In conclusion, our study demonstrated that p53 deficiency induces replication stress through mTORC1 activation. This activation leads to an increase in R-loop formation, which is further exacerbated by RNR upregulation. Our findings provide valuable insights into the mechanisms by which p53 inhibits tumor development and support the potential use of rapamycin treatment in cancer treatment.

Supplementary Data

Supplementary data is available at *Acta Biochimica et Biophysica Sinica* online.

Acknowledgements

We would like to thank Professor Lu Yin from the First Affiliated Hospital of Nanchang University for his assistance in image analysis via the MATLAB program.

Funding

This work was supported by the grants from Jiangxi Provincial National Natural Science Foundation (Nos. 20192BAB215032 and 20212BAB206034) and the National Natural Science Foundation of China (No. 82360474).

Conflict of Interest

The authors declare that they have no conflict of interest.

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