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Supplementary Information for:

# The chromatin modifiers Mdm2 and RNF2 prevent RNA:DNA-hybrid that impair DNA replication

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**This PDF file includes:**
Supplementary Figures 1-7 including legends
References for SI reference citations

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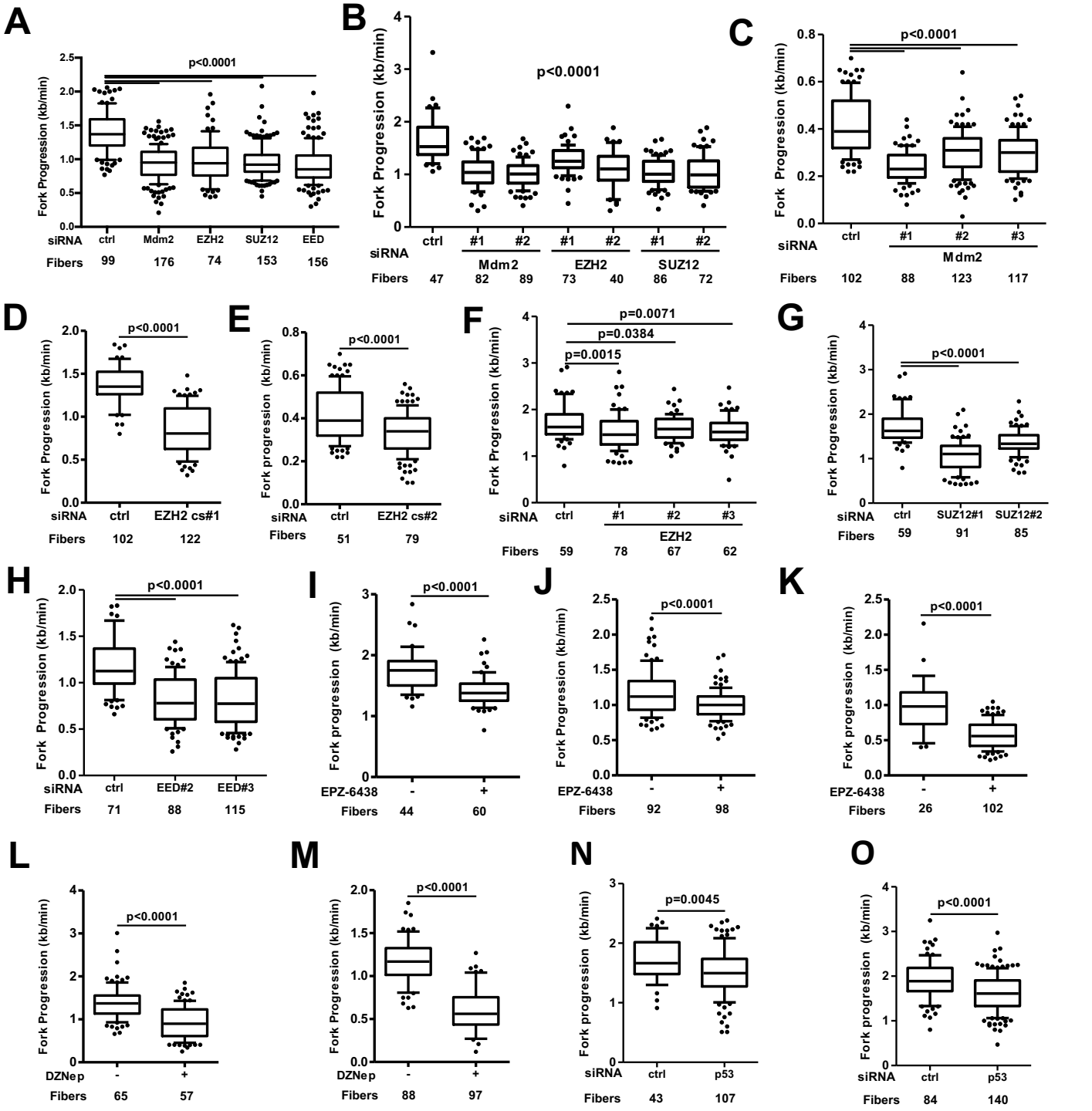
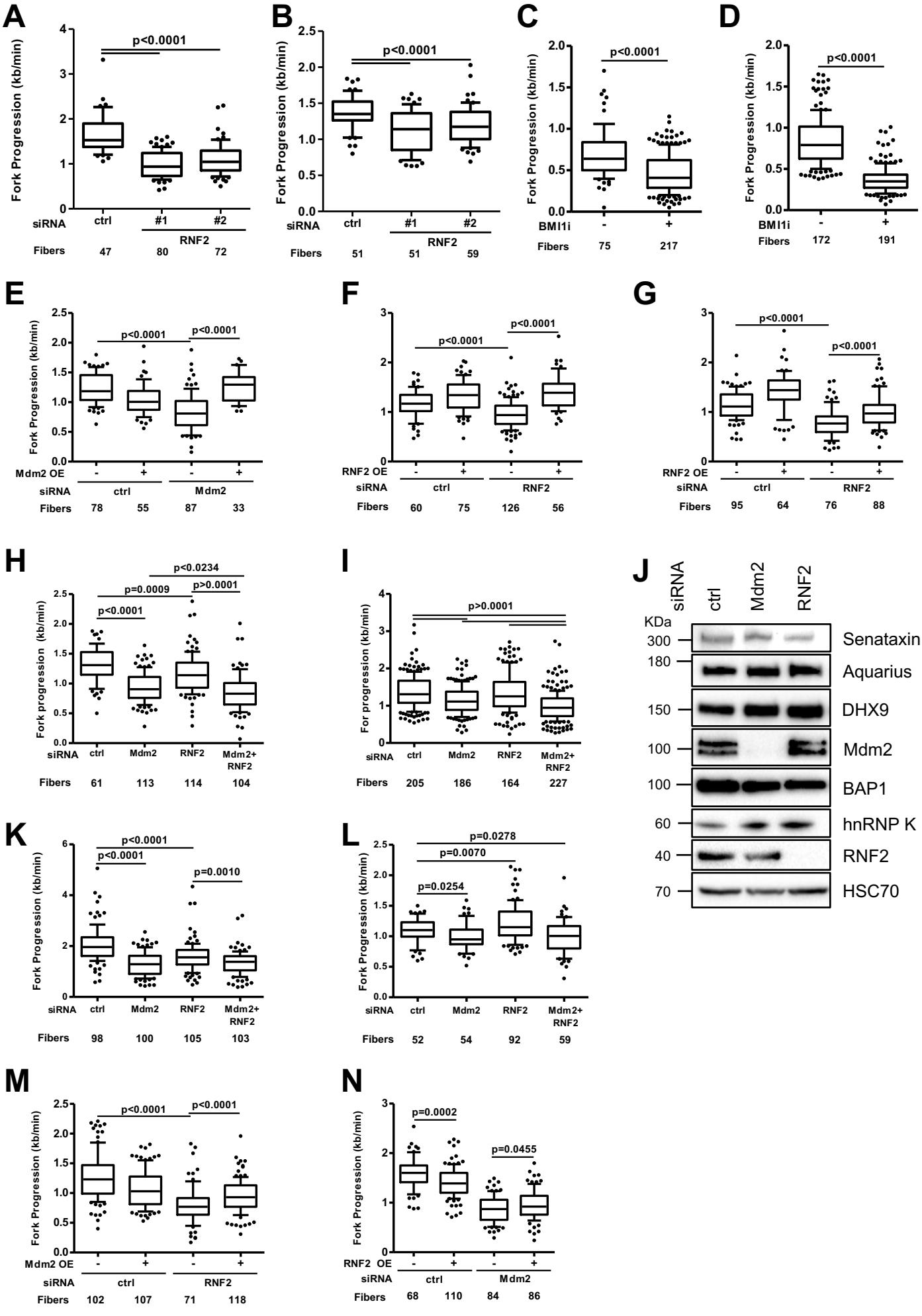
# Supplemental Figure 1




**SUPPLEMENTAL FIGURE 1. Compromised DNA replication fork progression upon depletion of Mdm2 or PRC2 members.** Related to Fig. 1.

**(A)** H1299 cells were transfected with a second siRNA set to Mdm2, EZH2, SUZ12, and EED for 48 hours. The experiment was carried out in parallel to the one in Fig. 1C-E and therefore shares the control sample. **(B)** A biological replicate to the experiment displayed in Fig.1C-E and panel A of this figure. Fiber analysis shows reduced fork progression after all knockdowns compared to scrambled siRNA knockdown. **(C)** Mdm2 depletion in HCT116 p53 $^{-/-}$ reduces fork progression using different siRNAs. Depletion of PRC2 components EZH2 with two siRNAs **(D-F)** and SUZ12 **(G)** reduces fork progression in HCT116 p53 $^{-/-}$ cells. **(H)** siRNA transfection targeting the non-catalytical PRC2 component EED for 48 hours reduces replication fork progression in H1299 cells. **(I-J)** Biological replicates to Fig. 1F shows reduced fork progression in H1299 cells treated with the EZH2 inhibitor EPZ-6438 for 48 hours. **(K)** A similar reduction can be observed in U2OS cells treated with the same conditions as in (I). **(L-M)** HCT116 p53 $^{-/-}$ cells treated with 5 μM DZnep, another EZH2 inhibitor, for 48 hours also reduces fork progression. **(N-O)** SJSA cells transfected with p53 siRNA for 48 hours display reduced replication fork progression as in Fig.1K.

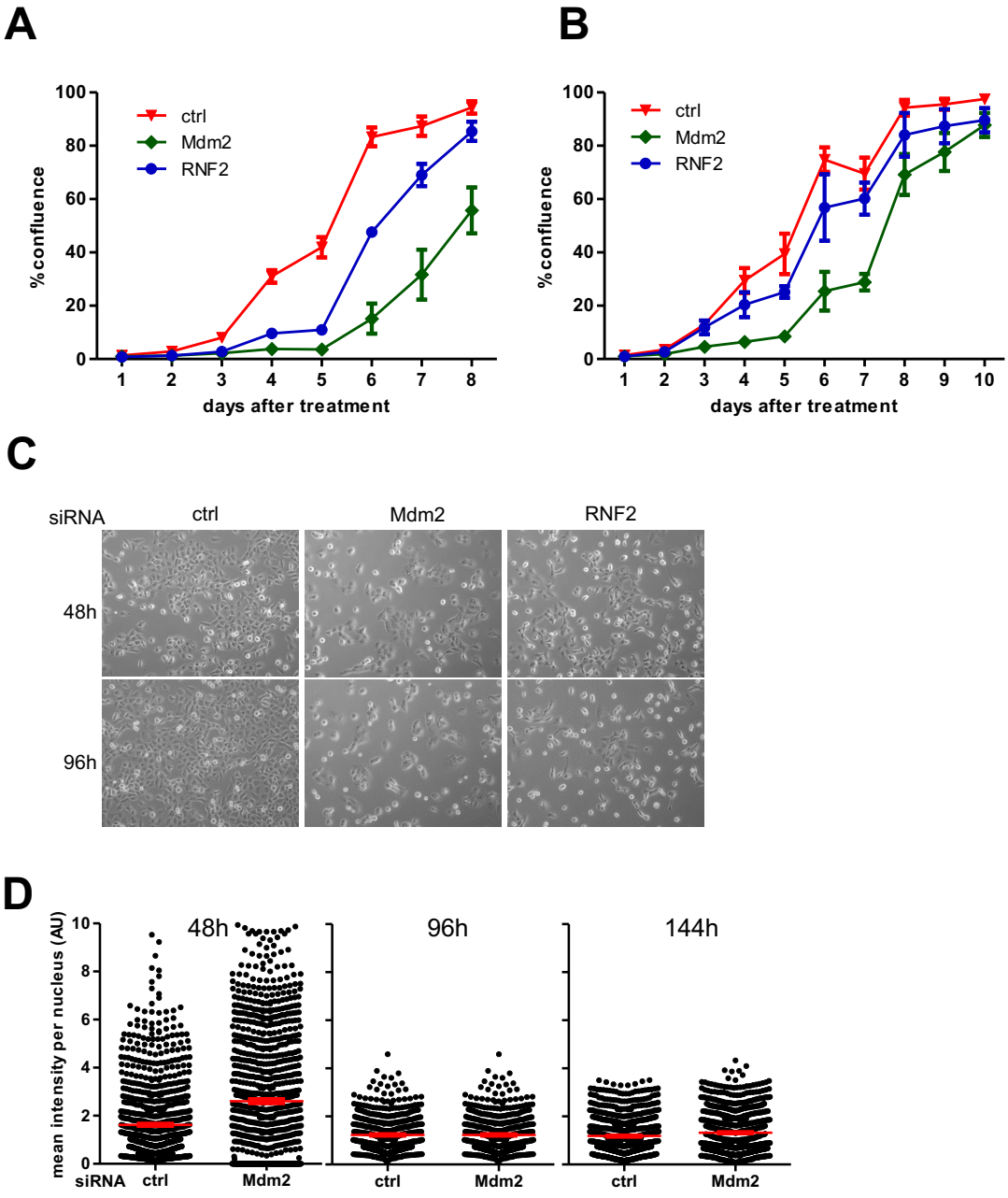
# Supplemental Figure 2



**SUPPLEMENTAL FIGURE 2. Decreased fork progression upon RNF2 depletion or Bmi1 inhibition** Related to Fig. 2.

RNF2 depletion in H1299 cells **(A)** and HCT116 p53 $^{-/-}$ cells **(B)** reduces replication fork progression in the IdU label. **(C-D)** Biological replicates to Fig. 2F shows H1299 cells treated with 1μM of the BMI1 inhibitor PTC-209 for 48 hours reduces fork progression. **(E)** Boxplot analysis of fork progression after Mdm2 depletion and plasmid transfection with pcDNA3 and pCMV-Mdm2**. (F-G)** Fork progression analysis after RNF2 depletion and transfection with RNF2 plasmid. **(H-L)** Biological replicates to Fig. 2I and 2L displaying fork rates of cells with co-depletion of Mdm2 and RNF2 in H1299 cells **(H-I)** and RPE p53-/- cells **(K-L)**. **(J)** Immunoblot analysis of H1299 cells depleted of Mdm2 and RNF2 for proteins involved in R-loop metabolism. **(M-N)** Biological replicates to Fig. 2O and 2R show that impaired replication can be rescued by the overexpression of each other in a cross-fashion.

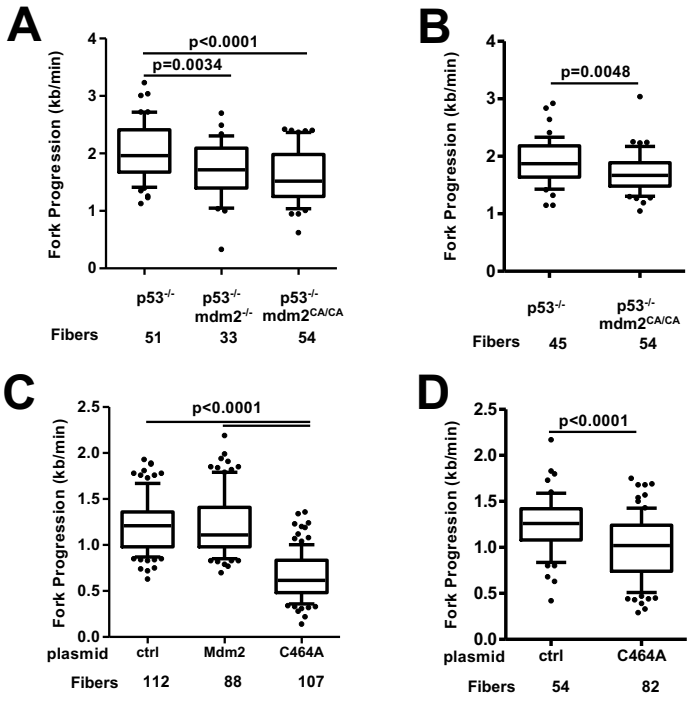
# Supplemental Figure 3




**SUPPLEMENTAL FIGURE 3. Mdm2 depletion impairs genomic stability.** Related to Fig. 3.

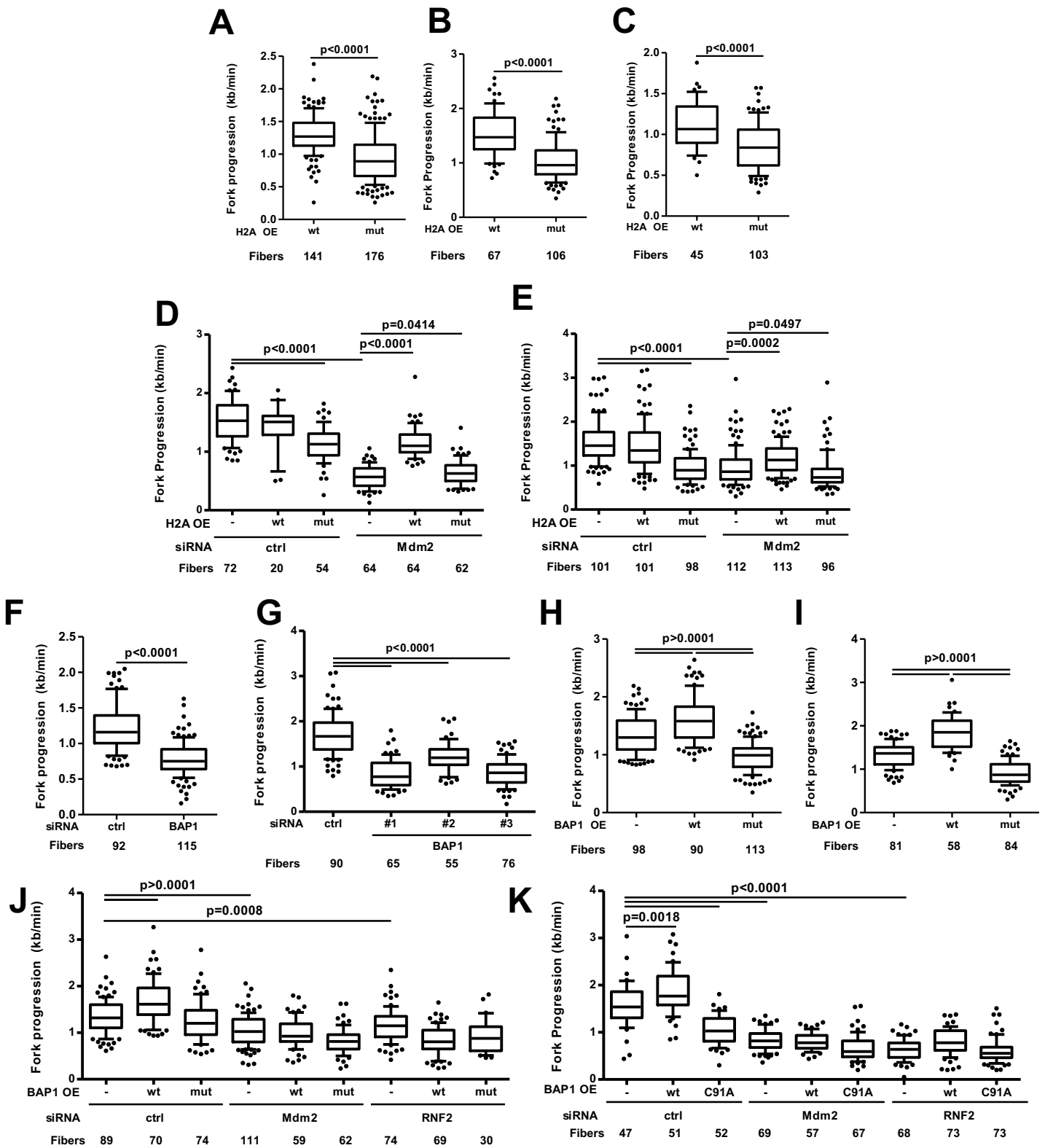
**(A-B)** Two biological replicates of clonogenic growth assay after Mdm2 and RNF2 knockdown shown in Fig. 3F. **(C)** Brightfield microscopy of H1299 transfected with siRNA against Mdm2 and RNF2 for 48 hours and a second transfection for another 48 hours (96h timepoint) shows reduced proliferation compared to control. **(D)** Biological replicate to Fig. 3K showing gH2AX levels 48, 96, and 144 hours post Mdm2 knockdown normalised to the median of control siRNA treated samples.

# Supplemental Figure 4




**SUPPLEMENTAL FIGURE 4. Requirement for the Ring finger domain of Mdm2 to support replication fork progression.** Related to Fig. 4.
**(A-B)** Biological replicates to Fig. 4B shows a reduced fork progression for p53/Mdm2 double knockout MEFs as well as a RING mutant MEF cell line with a p53-null background. **(C-D)** In the human H1299 cell line, an overexpression of a RING mutant Mdm2 reduces fork progression as seen in Fig. 4F.

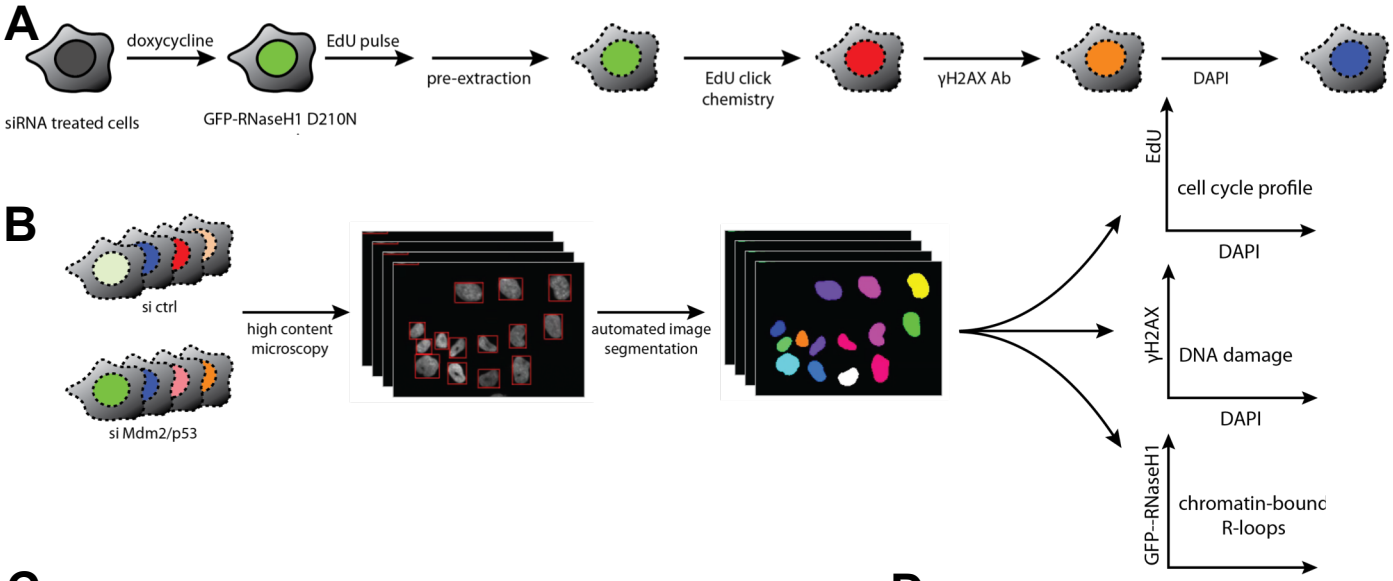
# Supplemental Figure 5

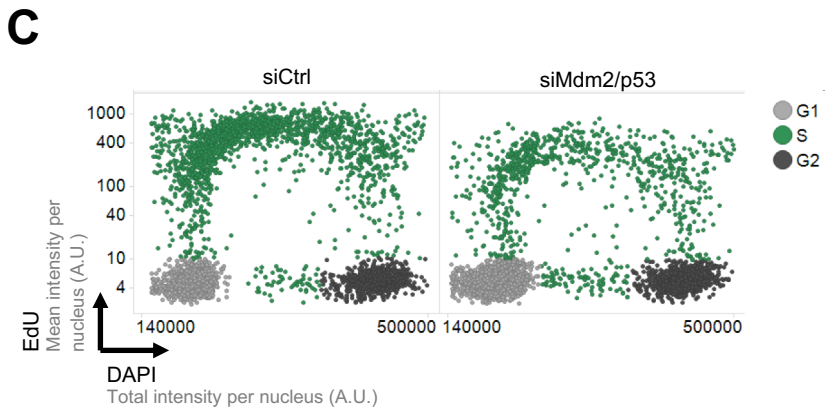


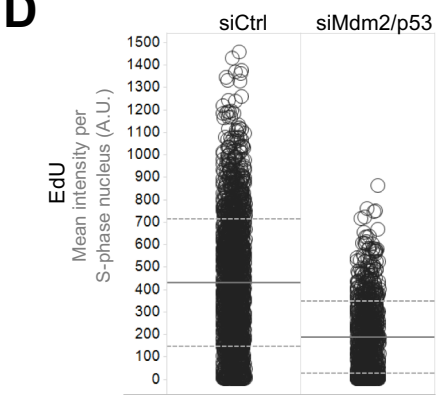

**SUPPLEMENTAL FIGURE 5. Dynamic changes in H2A ubiquitination are required for DNA replication.** Related to Fig. 5.

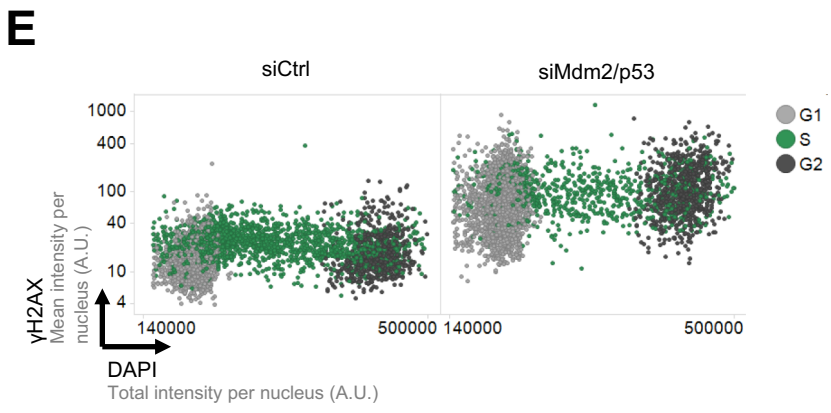
**(A-C)** Three biological replicates of experiment in Fig. 5B showing impaired replication with the ectopic expression of a mutant H2A lacking the ubiquitination site for Mdm2 and RNF2 (K119). **(D-E)** Biological replicates to replication analysis in Fig. 5E. **(F-G)** H1299 cells depleted of BAP1 with different siRNAs impairs replication fork progression as seen in Fig. 5H. **(H-I)** Replicates to Fig. 5K showing increased fork progression with ectopic expression of BAP1 and a decrease with a catalytic mutant version of BAP1. **(J-K)** Replicates confirming results in Fig. 5O showing no rescue with BAP1 overexpression in the absence of Mdm2 and RNF2.

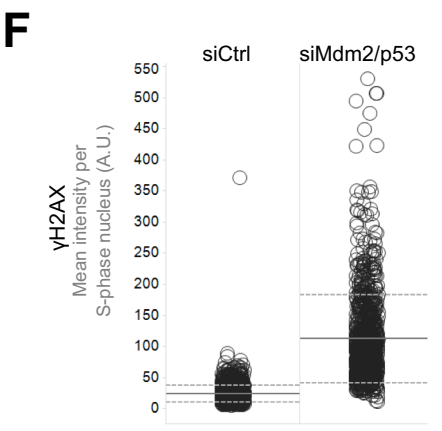
# Supplemental Figure 6

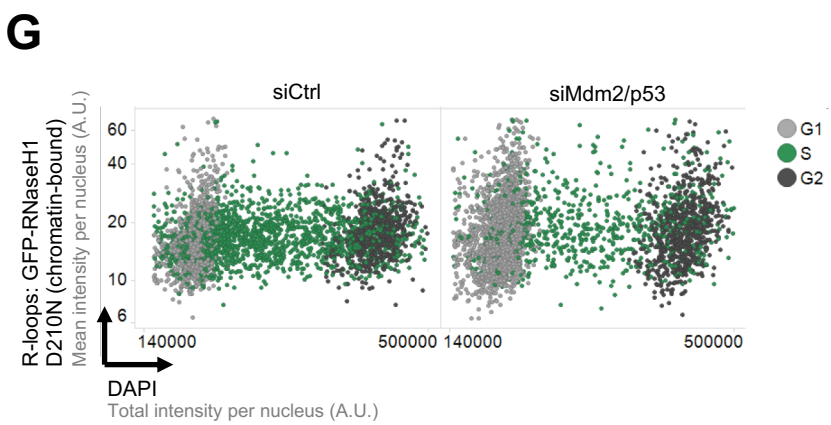


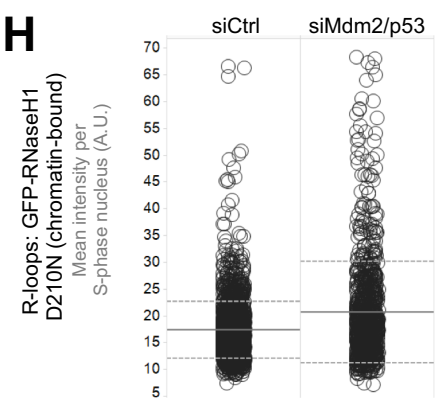



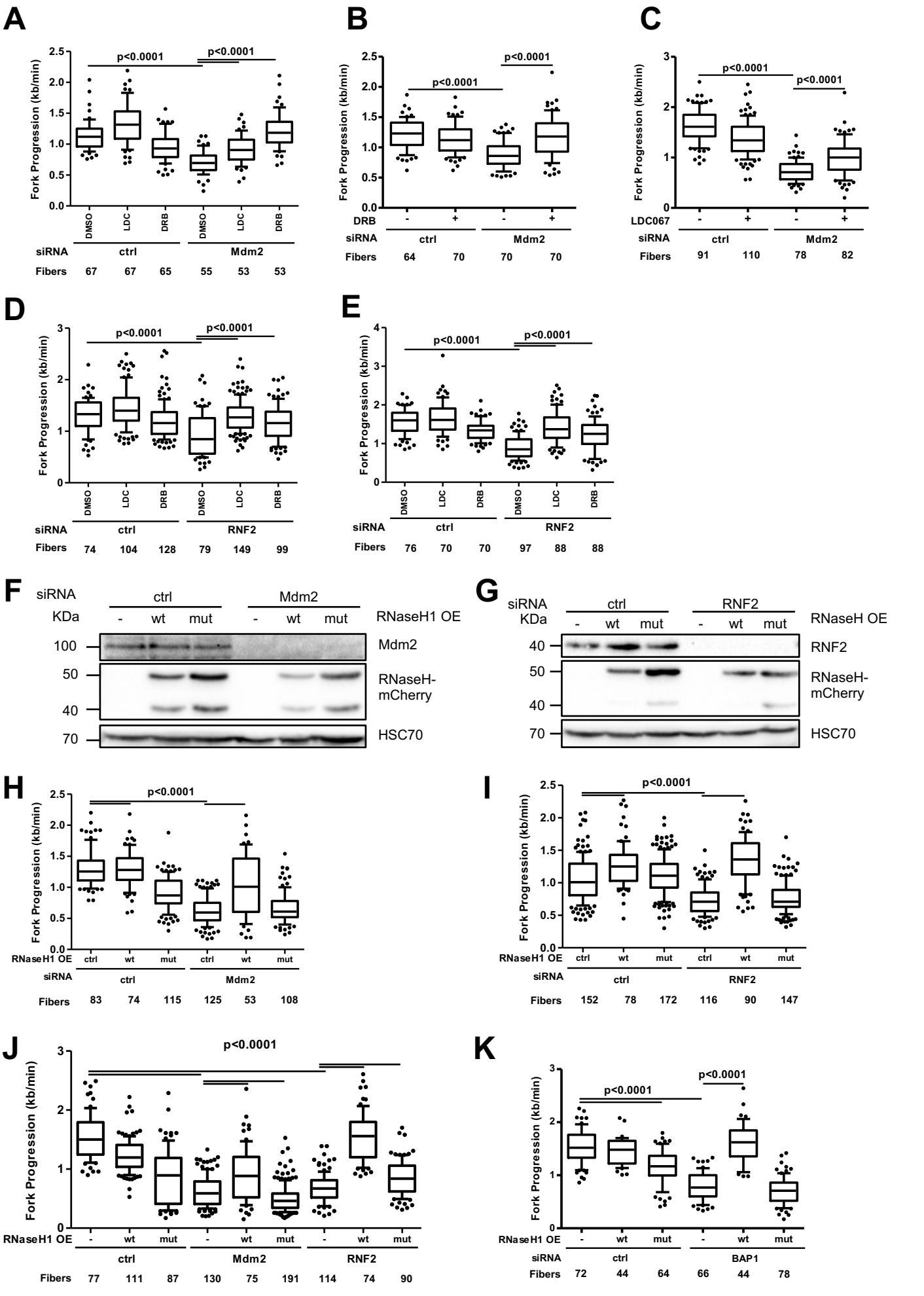



**SUPPLEMENTAL FIGURE 6: Mdm2 depletion increases replicative stress and R-loop formation.** Related to Fig. 6.

As in Figure 6, U2OS cells that stably express GFP-RNaseH1-D210N were transfected with siRNA against p53 and Mdm2 for 48 hours, prior to mutant RNaseH1 induction by doxycycline treatment for another 24 hours. Next, cells were pulsed with EdU to detect DNA synthesis for 20 minutes, pre-extracted, fixed and stained and the signals for DAPI, EdU, γH2AX, and chromatin-bound RNaseH1-D210N were quantified by high-content microscopy. **(A)** Schematic outline of the experimental setup for quantitative image based cytometry used to quantify DNA damage and R-loops upon depletion of Mdm2. **(B)** Two-dimensional analysis of the fluorescent signals of EdU, γH2AX, and GFP-RNaseH1-D210N versus DAPI intensity allows cell cycle staging as well as S-phase-specific analysis of DNA damage and R-loops. **(C**) Two-dimensional cell cycle staging was performed based on the EdU and DAPI signals. **(D)** The S-phase specific EdU signals were plotted for Mdm2/p53-depleted cells versus a control siRNA transfection. Horizontal lines represent averages and standard deviations. **(E)** Levels of the DNA damage marker γH2AX increase upon depletion of Mdm2 and p53 throughout the cell cycle. **(F)** The S-phase specific γH2AX signals were plotted for Mdm2/p53-depleted cells versus a control siRNA transfection. Horizontal lines represent averages and standard deviations. **(G)** Chromatin-bound RNaseH1-D210 marks RNA:DNA hybrids, and this signal increases when Mdm2 and p53 were depleted. **(H)** The S-phase specific signals of chromatin-bound RNaseH1-D210N were plotted for Mdm2/p53-depleted cells versus a control siRNA transfection. Horizontal lines represent averages and standard deviations.

# Supplemental Figure 7



**SUPPLEMENTAL FIGURE 7. CDK9 inhibition and RNaseH1 overexpression each allow processive DNA replication despite the removal of Mdm2 or RNF2.** Related to Fig. 7.

H1299 cells were transfected with siRNA against Mdm2 and RNF2 and treated with two different CDK9 inhibitors (DRB and LDC0067) to inhibit global transcription. **(A-C)** Fork progression in Mdm2-depleted samples could be rescued with a 60 minute inhibitor treatment (c.f. Fig. 7C). **(D-E)** Replication defects due to RNF2 depletion was rescued using CDK9 inhibitor pre-treatment for 30 minutes as seen in Fig. 7E.
H1299 cells were transfected with siRNA against Mdm2 **(F, H, J)**, RNF2 **(G, I, J)** and BAP1 **(K)** for 24 hours and transfected with plasmids containing wildtype and a catalytically inactive mutant version of RNaseH1 for another 30 hours (c.f. Fig. 7F). Immunoblot analysis to confirm the depletion of Mdm2 **(F)** and RNF2 **(G)** after siRNA transfection as well as the overexpression of RNaseH1 constructs carrying and mCherry tag. Impaired replication fork progression displayed after both Mdm2 and RNF2 knockdown was rescued by wildtype but not mutant RNaseH.

## SUPPLEMENTAL MATERIALS AND METHODS

### Cell culture

H1299 (non-small cell lung carcinoma, p53 null; male), U2OS (osteosarcoma, p53 proficient; female), SJSA (osteosarcoma, p53 proficient, Mdm2 amplification: male) and hTert-RPEp53$^{/}$ (retinal epithelial cells, p53 deficient; female) were maintained in Dulbecco's modified Eagle's medium (DMEM). Mouse embryonic fibroblasts of p53$^{-/-}$, p53$^{-/-}$ mdm2$^{-/-}$ as well as p53$^{-/-}$ mdm2 $^{CA/CA}$ animals were obtained from the Zhang lab (1) and cultured in Dulbecco's modified Eagle's medium (DMEM). HCT116 (colon carcinoma, p53 null; male) cells were cultivated in RPMI-1640 medium. Cell culture media were supplemented with 10% fetal calf serum (FCS) and antibiotics (pencillin, streptomycin, and ciprofloxacin). Human U2OS-derived clones (female) for the inducible expression of catalytically inactive GFP-RNAseH1 D210N (kindly provided by Pavel Janscak) were grown in Dulbecco's modified Eagle's medium (DMEM) containing 10% Tet system approved fetal bovine serum (Takara), with penicillin-streptomycin, puromycin and hygromycin antibiotics. Over-expression of GFP-RNAseH1-D210N was induced by addition of 1ng/ml doxycycline (Sigma).

Cells were maintained at 37°C in a humidified atmosphere with 5% $CO_2$. All cell lines were routinely tested for mycoplasma contamination and scored negatively.

### Treatments and transfections

For treatments, EPZ-6438, RG7388, and DZNep stock solutions were prepared in DMSO and aqueous PTC-209, gemcitabine and hydroxyurea stock solutions were used. All were diluted in pre-warmed medium and added to culture medium for times indicated in Figure legends.

For siRNA knockdown in Figures 1-5 and 7, H1299, U2OS and HCT116 p53-/- cells were reverse transfected with 10nM siRNA (Ambion) and a negative control siRNA (scrambled#2), using Lipofectamine 3000 (Invitrogen) according to manufacturer's instructions. Medium was changed after 24 hours followed by further incubation of 24 hours. siRNA transfections in Figure 6 were performed for 72 hours using Lipofectamine RNAiMAX (Thermo Fisher Scientific). Addition of doxycycline (1 ng/ml, D9891, Sigma) for the last 24 hours induced the expression of GFP-RNAseH1 D210N.

Plasmid transfections were carried out using 2µg of plasmid DNA with Lipofectamine3000 and P3000 according to manufacturer's instructions. A reverse protocol was used for plasmid-only experiment and a forward protocol was used following a siRNA transfection the previous day.

**Fiber assays**

DNA fiber assays to analyze replication fork progression and processivity were essentially carried out as in (2). Following transfection with siRNA and/or plasmid DNA, cells were incubated with 5-chloro-2′-deoxyuridine (CldU) for 20 min, followed by 5-iodo-2′-deoxyuridine (IdU; both from Sigma-Aldrich) for 60 min. For processivity assays, cells were pulse labelled with CldU for 1 hour, followed by alternate labels of IdU and CldU for 10 minutes each and a total of 7 labels. Fork restart was assessed by labelling cells with three pulses of CldU, IdU and CldU at the same concentrations for 15 minutes each, followed by a block with 2mM hydroxyurea for 4 hours. The incorporation of a fourth label of IdU for one hour was used as a readout for fork restart. Controls omitting the hydroxyurea treatment and continuous labelling were performed in addition. DNA fibers were spread and lysed on glass slides using a spreading buffer (200mM Tris pH 7.4, 50mM EDTA, 0.5% SDS). After acid treatment (2.5M HCl), CldU- and IdU-labeled tracks were detected by 1 h incubation with rat anti-BrdU antibody (1:400, AbD Serotec; 1:1000, abcam) and mouse anti-BrdU antibody (1:400, detects BrdU and IdU; Becton Dickinson). Slides were fixed in 4% paraformaldehyde/PBS and incubated for 2 h with Alexa Fluor 555-conjugated goat anti-rat antibody (dilution 1:200; Invitrogen) or Alexa Fluor 488-conjugated goat anti-mouse antibody (dilution 1:200; Invitrogen). Representative images of labelled fibers were selected, brightness and contrast changed to +45 in Adobe Photoshop, rotated and cropped.

**Immunoblot analysis**

Cells were harvested in protein lysis buffer (20 mM Tris-HCl pH 7.5, 150 mM NaCl, 1 mM $Na_2$EDTA, 1 mM EGTA, 1 mM beta-glycerophosphate, 2 M Urea, proteinase inhibitors (pepstatin, leupeptin hemisulfate, aprotinin, AppliChem). The samples were briefly sonicated to disrupt DNA-protein complexes. Total protein concentration was measured using a Pierce BCA Protein assay kit (Thermo Scientific Fisher). After boiling the samples in Laemmli buffer at 95°C for 5 min, equal amounts of protein samples were separated by SDS-PAGE, transferred onto nitrocellulose, and visualized with the following antibodies: Mdm2 (OP46, Calbiochem), EZH2 (D2C9 XP, #5246, Cell Signaling), H3K27me3 (pAb-069-050, Diagenode), p53 (DO-1 sc-126, Santa Cruz), RNF2 (D22F2 XP, #5694, Cell Signaling), H2AK119ub1 (ABE569, Millipore), H2AX-Ser139P (#9718, Cell Signaling), Flag (M2, #F3165, Sigma), H2A (ab18255, abcam), BAP1 (#13187, Cell Signaling), Senataxin (A301-105A, Bethyl Laboratories), Aquarius (A302-547A, Bethyl Laboratories), DHX9 (VPA00298, BioRad), hnRNP K (MCA2622GA, BioRad), mCherry (ab125096,abcam)

**Cell proliferation assay (Celigo)**

H1299 cells transfected with siRNAs targeting Mdm2 and RNF2 were treated with gemcitabine 24 hours post knockdown and seeded into 12-well plates at 48 hours after

knockdown. Proliferation of cells was measured using the Celigo Cytometer (Nexcelom, software version 2.0). Cell confluence in triplicate samples was measured every 24 h for up to 10 days.

**Immunofluorescence analysis**

For immunofluorescence analysis, cells on 13mm coverslips were fixed with 4% paraformaldehyde/PBS for 30 minutes followed by permeabilisation with 0.5% Triton-X100/PBS for 30 minutes. After blocking with 5%BSA/PBS-T and incubation with H2AX-Ser139P antibody (1:200; #9718, Cell Signaling) for 1 hour , cells were washed and incubated with Alexa Fluor 546-conjugated secondary antibody (1:250; Invitrogen). Nuclear staining was achieved by staining with DAPI (1:1000; Sigma) for 10 minutes. For analysis, images were acquired using an Axio Scope A1 microscope (Zeiss) equipped with a Axio Cam MRc/503 camer. Analysis was carried out using Fiji software (3).

**EdU incorporation for QIBC**

For pulsed EdU (5-ethynyl-2'-desoxyuridine) (Thermo Fisher Scientific) incorporation, cells were incubated for 20 minutes in medium containing 10 µM EdU. The Click-iT EdU Alexa Fluor Imaging Kit (Thermo Fisher Scientific) was used for EdU detection. EdU-positive cells as detected by QIBC were considered the S-phase population**.**

**Quantitative image-based cytometry (QIBC)**

GFP-RNAseH1 D210N cells were grown on sterile 12 mm glass coverslips, pre-extracted in ice-cold 0.2% Triton X-100 (Sigma-Aldrich) in PBS for 2 minutes on ice to wash out detergent-sensitive, non-chromatin-bound proteins, washed twice in PBS, and fixed in 3% formaldehyde in PBS for 15 minutes at room temperature. Primary and secondary antibodies (Alexa fluorophores, Life Technologies) were diluted in filtered DMEM containing 10% FBS and 0.02% Sodium Azide. Antibody incubations were performed for 2 hours (primary antibodies) or 1 hour (secondary antibodies) at room temperature. After antibody incubations, coverslips were washed once with PBS and incubated for 10 minutes with PBS containing 4',6-Diamidino-2-Phenylindole Dihydrochloride (DAPI, 0.5 µg/ml) at room temperature to stain DNA. Coverslips were mounted on 5 µl Mowiol-based mounting media (Mowiol 4.88 (Calbiochem) in Glycerol/TRIS). H2AX Phospho S139 antibody (mouse, Biolegend 613401, 1:1000) was used to detect DNA damage signaling. Automated multichannel wide-field microscopy for QIBC was performed on an Olympus ScanR Screening System equipped with an inverted motorized Olympus IX83 microscope, a motorized stage, IR-laser hardware autofocus, a fast emission filter wheel with single band emission filters, and a digital monochrome Hamamatsu ORCA-FLASH 4.0 V2 sCMOS camera (2048 x 2048 pixel, 12 bit dynamics) as described previously (4). For each condition, image information of large cohorts of cells was acquired under non-saturating conditions with

a UPLSAPO 20x (NA 0.75) objective. Identical settings were applied to all samples within one experiment. Images were analyzed with the Olympus ScanR Image Analysis Software Version 2.5.1, a dynamic background correction was applied and nuclei segmentation was performed using an integrated intensity-based object detection module using the DAPI signal. All downstream analyses were focused on properly detected interphase nuclei or mitotic chromosomes containing a 2C-4C DNA content as measured by total and mean DAPI intensities. Fluorescence intensities were quantified and are depicted as arbitrary units. Color-coded scatter plots of asynchronous cell populations were generated with Spotfire data visualization software (TIBCO). Within one experiment, similar cell numbers were compared for the different conditions. Representative scatter plots and quantifications of independent experiments, typically containing several thousand cells, are shown.

## QUANTIFICATION AND STATISTICAL ANALYSIS

### DNA Fiber Assay

Fiber images were acquired by fluorescence microscopy and analyzed manually using Image J. The number of fibers measured for each condition is indicated in the graph and biological replicates shown in corresponding supplemental figures. Statistical testing was performed using Graph Pad Prism 6 and 7. A 2-sided unpaired t test was calculated with an assumed significance for p-values ≤ 5%. Analogous statistical analyses were performed when evaluating the phospho-H2AX signal from immunostained cells.

### References to Materials and Methods


1. Wienken M*, et al.* (2016) MDM2 Associates with Polycomb Repressor Complex 2 and Enhances Stemness-Promoting Chromatin Modifications Independent of p53. *Mol Cell* 61(1):68-83.
2. Klusmann I*, et al.* (2016) p53 Activity Results in DNA Replication Fork Processivity. *Cell Rep* 17(7):1845-1857.
3. Schindelin J*, et al.* (2012) Fiji: an open-source platform for biological-image analysis. *Nat Methods* 9(7):676-682.
4. Pellegrino S, Michelena J, Teloni F, Imhof R, & Altmeyer M (2017) Replication-Coupled Dilution of H4K20me2 Guides 53BP1 to Pre-replicative Chromatin. *Cell Rep* 19(9):1819-1831.