Supporting Information Appendix

Mutant p53 perturbs DNA replication checkpoint control through TopBP1 and Treslin

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SI Materials and Methods:

Establishment of stable cell lines

H1299 cell lines stably transfected with an empty vector or the expression vector of mutp53-R175H, mutp53-R273H, scrambled shRNA or TopBP1 shRNA were established as described previously (1). Mutp53-harboring C33A cells or BT549 cells were transfected with pLKO.1-shScramble (2), shp53 pLKO.1 puro (sh-mutp53 #1) (3) or pLKO-p53-shRNA-941 (sh-mutp53 #2) (4) (Addgene), followed by selection with puromycin (2 mg/ml) to establish stable cell lines expressing a scrambled shRNA (shScr) or a p53 shRNA. The effect of knockdown was confirmed by Western blotting using antibody specific to p53 or TopBP1.

Antibodies for Immunoprecipitation and Western blot analysis

Antibody specific to E2F1 (C-20 or KH-95), c-Myc (A-14), p53 (FL-393), MCM2 (SMP14), Chk1 (G-4), Chk2 (A-11) or GAPDH (6C5) was from Santa Cruz. Anti-TopBP1 monoclonal antibody and Akt antibody were from BD Transduction Laboratories. Anti-TopBP1 (BL893) rabbit polyclonal antibody, anti-phospho-MCM2 (S108) antibody and anti-Treslin antibody were from Bethyl Laboratories. Anti-phospho-TopBP1 (S1159) antibody was from Abgent. Antibody specific to histone H3 (D1H2), ATR, phospho-Akt (S473), phospho-Chk1 (S345) or phospho-Chk2 (T68) was from Cell Signaling. Anti-FLAG (F7425) rabbit antibody was from Sigma. Antibody specific to H3K9me3 (ab8898) or DNA2 (ab96488) was from Abcam. Anti-Histone γ-H2AX (Ab10020) was from Millipore.

Micrococcal Nuclease Assay

Assay was performed using a modified protocol (*Transcriptional regulation in eukaryotes : concepts, strategies, and techniques*, 2nd edn., chapter 10) (5). Briefly, cells (~1×10^8) were harvested in phosphate-buffered saline (PBS). Following centrifugation at 1500 rpm at 4℃ for 5 min, cell pellets were resuspended in NP-40 cell lysis buffer (10 mM Tris-Cl pH 7.4, 10 mM NaCl, 3 mM $MgCl_2$, 0.5% NP-40, 0.15 mM spermine, 0.5 mM spermidine) on ice for 10 min. Nuclei were spun down at 1000 rpm for 10 min at 4℃, and then resuspended in 1 ml digestion buffer (10 mM Tris-Cl pH 7.4, 15 mM NaCl, 60 mM KCl, 0.15 mM spermine, 0.5 mM spermidine) supplemented with 1 mM $CaCl_2$. 100 µl of nuclei

were transferred to each individual tube containing 1 unit of micrococcal nuclease and were incubated at room temperature for the indicated time (0, 2, 5, 10 min), followed by addition of 80 μl of digestion buffer and 20 μl of stop buffer (100 mM EDTA, 10 mM EGTA, adjusted to pH 7.5). Samples were then treated with 3 μl of 25 mg/ml proteinase K and 10 μl of 20% SDS overnight at 37℃. Genomic DNA from each sample was isolated and subjected to 1% agarose gel electrophoresis.

Bromodeoxyuridine Incorporation Assay and Flow Cytometry

Cells were labeled with 5-bromo-2-deoxyuridine (BrdU) for 17 h, followed by fixation with 4% formaldehyde. The incorporated BrdU was detected with anti-BrdU antibody (Ab-3, Calbiochem) followed by Texas Red X-conjugated secondary antibody (Invitrogen). Nuclei were stained with Hoechst 33258 dye (Sigma). Images were captured with a Zeiss fluorescence microscope (Axio Observer Inverted Microscope). At least 300 nuclei per sample were counted by fluorescence microscopy. For BrdU flow cytometry assay, cells were labeled with BrdU for 2 h and then fixed with 70% ethanol. Cells were treated with 2N HCl/Triton X-100 for 30 min and then neutralized in 0.1 M $NaB_4O_7.10H_2O$, pH 8.5. Cells were then stained with anti-BrdU FITC (BD Biosciences) and propidium iodide followed by flow cytometry. At least 10,000 cells were analyzed for each sample. All experiments were performed in at least triplicate. The DNA content profiles of the synchronized H1299 cells were determined by propidium iodide staining followed by flow cytometry and analyzed using FlowJo software (Tree Star).

Immunofluorescence Staining

H1299 cells were transfected with an empty vector or mutant p53 constructs according to experimental design. After 48 h, cells were plated on collagen-coated coverslips in six-well plates. To visualize the TopBP1 foci formation after HU treatment, cells were fixed with 4% paraformaldehyde for 20 min, followed by permeabilization in PBS buffer containing 0.5% Triton X-100 and 0.5% NP-40 for 10 min. Cells were blocked in 2% bovine serum albumin-containing PBS at room temperature for 1 h, followed by incubation with anti-mouse TopBP1 antibody for 1 h, and Texas Red X-conjugated anti-mouse secondary antibody for another hour. Nuclei were stained with Hoescht 33258 dye. To visualize the colocalization between mutant p53 and PCNA during G1/S phase of the cell cycle, fixed cells were incubated with anti-p53 rabbit polyclonal antibody and anti-PCNA mouse monoclonal antibody (Santa Cruz), followed by Alexa Fluor 488-conjugated anti-rabbit secondary antibody (Invitrogen) and Texas Red X anti-mouse secondary antibody, respectively. Images were captured with a Zeiss fluorescence microscope equipped with ApoTome 2 (Axio Observer inverted microscope).

GST pulldown assay

The DBD of mutp53-R175H and R273H was amplified by PCR and cloned into expression vector pGEX6P1 as described (6). The GST fusion proteins in *Escherichia coli* strain BL21 were induced by 0.1 mM IPTG (isopropyl-β-D-thiogalactopyranoside) and purified according to the standard protocol. The GST portion on GST-TopBP1-BRCT7/8 was excised by Pre-Scission protease (Pharmacia). Purified TopBP1-BRCT7/8 was incubated with GST or GST-mutp53(DBD) on glutathione-Sepharose for 4 hr. The beads were washed three times and subjected to SDA-PAGE as described (6).

MTT Assay and clonogenic survival assay

Transfected H1299 cells were seeded on 96-well plates at 3000 cells per well, and were treated with various concentrations of cisplatin or DNA2 inhibitors for 48 h. DNA2 small molecule inhibitors, NSC360177 (C36) and NSC15765 (C5) were obtained from NCI Developmental Therapeutic Program. MTT assay was performed by adding 20 µl of MTT reagent (Thiazolyl blue tetrazolium bromide, 5 mg/ml) (Sigma) to each well, followed by incubation at 37℃ for 2-4 h. The medium and reagent were removed and 100 µl of DMSO was added to each well. After incubation at 37℃ for 0.5 to 1 h, absorbance was read at 490 nm on a plate reader (BioTek Synergy HT). Each experiment was performed at least in triplicates. Clonogenic survival assay was performed as described previously (1).

DNA Fiber Spread Assay

Cells were harvested and resuspended in ice cold PBS at ~ $5X\ 10^6$ cells/ml. 2 µl of the cell suspension were spotted at the upper end of the microscope slide (Silane-Prep slides, S4651-72EA from Sigma) and air-dried for ~ 5 min. Subsequently, 7 µl of lysis buffer (200 mM Tris-HCl, pH 7.5, 50 mM EDTA and 0.5% SDS) were applied on the top of cell suspension. After 2 min, the slides were tilted to 15° to allow the DNA fibers spread. Slides were air-dried, and fixed with methanol/acetic acid (3:1) for 10 min, and refrigerated overnight. DNA was denatured with 2.5 M HCl for 80 min at room temperature. The slides were rinsed three times in PBS and incubated in blocking buffer (5% BSA in PBS) for 30 min. 150 µl of the primary antibodies in blocking solution (1:200 anti-BrdU antibody [mouse, from BD Bio-sciences, 347580], 1: 400 anti-BrdU antibody [rat, from Abcam, ab6326]) were pipetted on each slide, the slides were gently covered with a coverslip and incubated for 2 h at room temperature. Subsequently, coverslips were removed and slides were washed three times with PBS. 150 µl of the secondary antibodies in blocking solution (1: 400 goat anti-mouse Tex-red [from LifeTechnologies, T6390], 1: 400 chicken anti-rat Alexa Fluor 488 [from LifeTechnologies, A21470]) were applied on each slide, and the slides were covered again with coverslip for 1 h. Following the removal of coverslip and three times PBS washing, the slides were applied with coverslips, mounted, and stored at -20℃ until being analyzed.

Statistical analysis

We performed two-tailed *t* test for comparisons of experimental groups. Data are presented as means ± S.D. from at least three biological replicates. *P* values less than 0.05 were considered statistically significant. To analyze DNA2 gene expression in TCGA breast cancer dataset, DNA2 mRNA expression and *TP53* mutation status were retrieved from TCGA database. Box plots were generated using the R program. The P values for the difference in DNA2 expression between different *TP53* status groups were based on two-tailed *t* test.

SI Figures

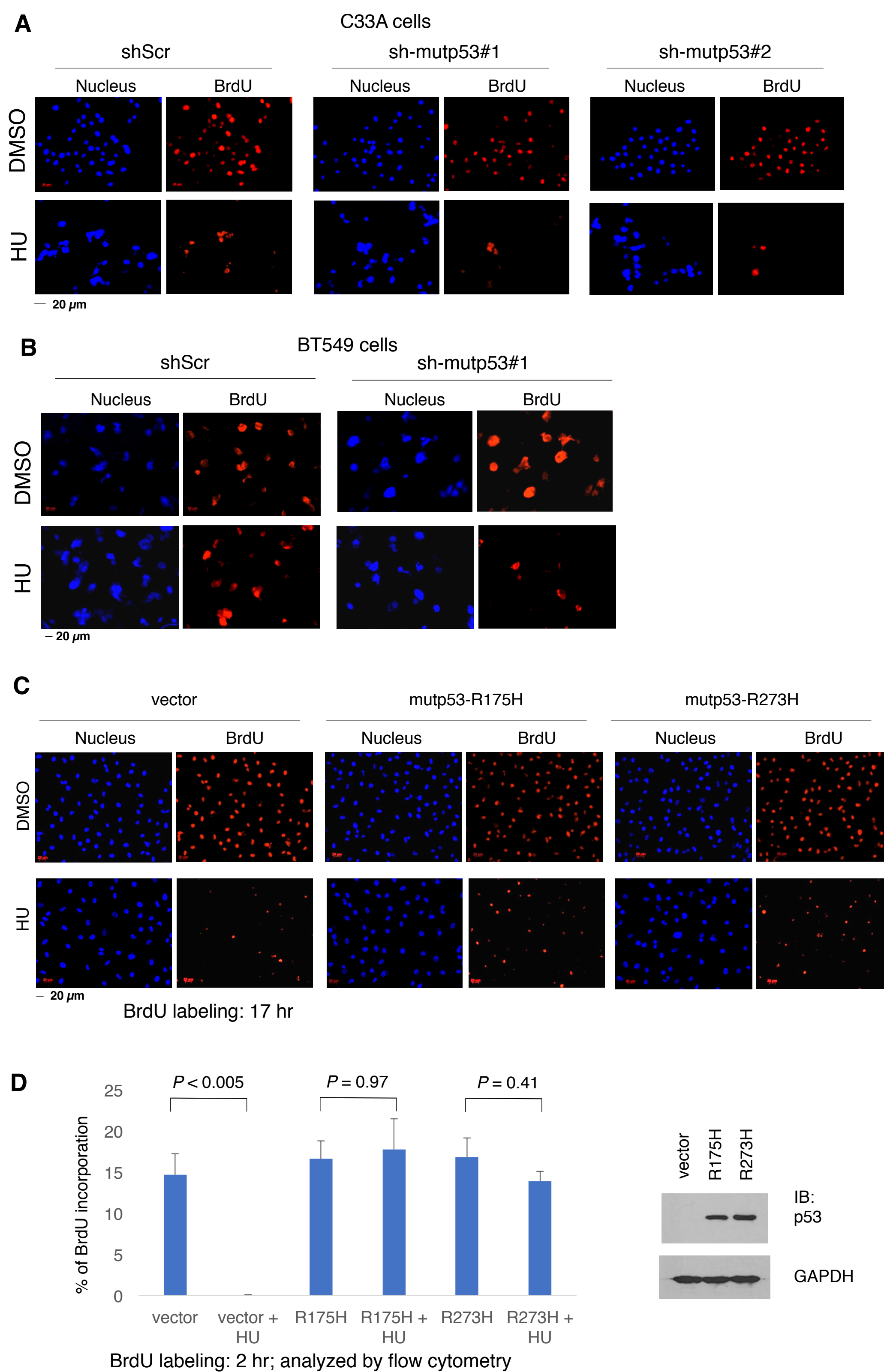

Fig. S1. BrdU incorporation assay

A. Representative images (at 40x magnification) of BrdU incorporation data that are presented in Fig. 1A.
B. Representative images (at 40x magnification) of BrdU incorporation data that are presented in Fig. 1B.
C. Pictures shown are representative images at 20x magnification from each indicated group presented in Fig. 1C.
D. H1299 cells were transfected with either an empty vector or an expression vector of mutp53 (R175H or R273H). 48 h later, cells were treated with vehicle (DMSO) or HU (2 mM) for 16 h, and then labeled with BrdU (10 μM) for 2 h. The cells were fixed for anti-BrdU staining following the manufacturer's instruction. Flow cytometric analysis was performed for quantitation of BrdU incorporation from at least 10,000 cells per sample. The data represent means and standard deviations from three replicates. The *P* values are based on a two-tailed *t* test.

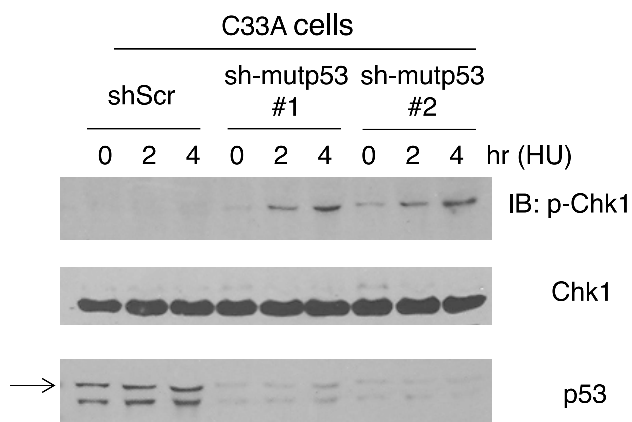

Fig. S2. Depletion of mutp53 in C33A cells enhances HU-induced Chk1 activation. C33A cells stably expressing either a scrambled shRNA (shScr) or a p53 shRNA (sh-mutp53 #1 or #2) were untreated or treated with hydroxyurea (HU, 2 mM) for 2 or 4 h. The whole cell lysates were subjected to Western blot analysis.

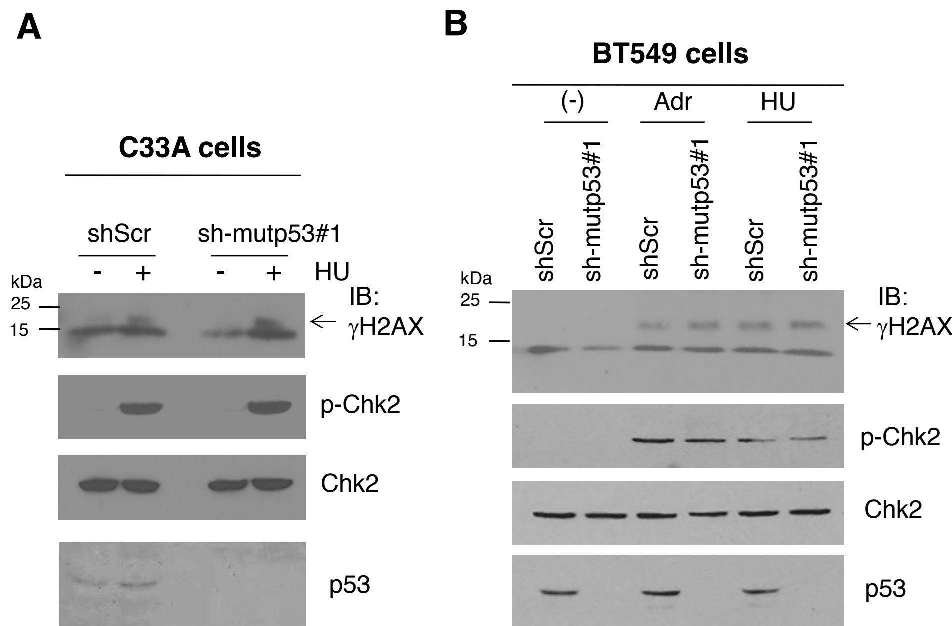

Fig. S3. Cancer cells with mutp53 depletion do not suffer from increased replicative stress nor DNA damage.

A. C33A stable cell lines expressing either a control scrambled shRNA or p53 shRNA were left untreated or treated with HU at 2 mM for 5 h. The cells were harvested, and the cellular lysates were analyzed by Western blot as indicated.

B. BT549 stable cell lines expressing either a control scrambled shRNA or p53 shRNA were left untreated or treated with HU at 2 mM for 5 h or adriamycin (Adr) at 5 µM for 5 h. Cells were then harvested for Western blot analysis.

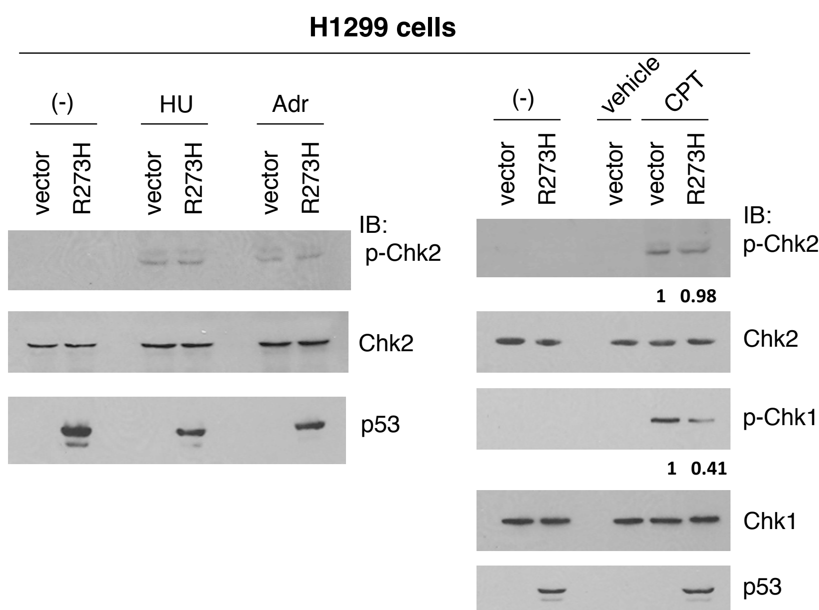

Fig. S4. Mutp53-R273H expression in H1299 cells inhibits ATR activation, but does not significantly affect ATM activation.
Left panel: H1299 cells transfected with either an empty vector or mutp53-R273H were left untreated or treated with HU at 2 mM for 5 h, adriamycin (Adr) at 5 μM for 5 h. The cells were harvested, and the cellular lysates were analyzed by Western blot as indicated.
Right panel: H1299 cells transfected with either an empty vector or mutp53-R273H were left untreated or treated with camptothecin (CPT) at 5 μM for 5 h, and then harvested for Western blot analysis. Since CPT is dissolved in chloroform/methanol (4:1), we included an extra vehicle control for CPT treatment. The signals of p-Chk2 and p-Chk1 were quantified using ImageJ software. The relative intensity is shown at the bottom of each panel.

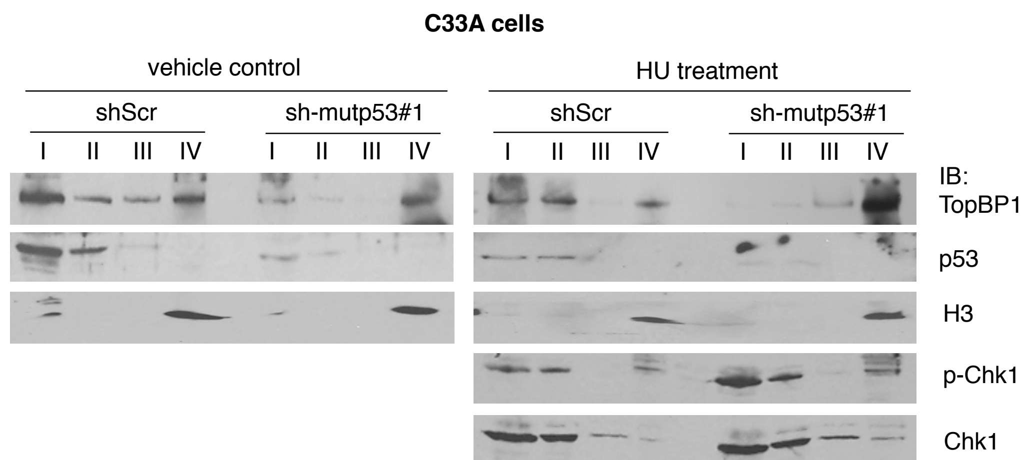

Fig. S5. Depletion of mutp53 enhances TopBP1 chromatin binding in C33A cells.
Figures shown are a representative set of immunoblots for the data shown in Fig. 3A.

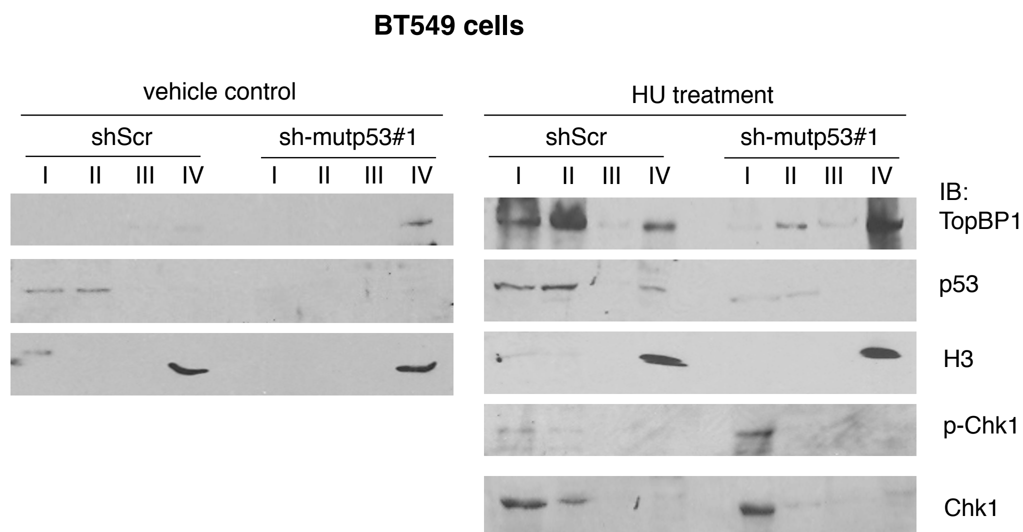

Fig. S6. Depletion of mutp53 enhances HU-induced TopBP1 chromatin binding in BT549 cells.

Figures shown are a representative set of immunoblots for the data presented in Fig. 3B.

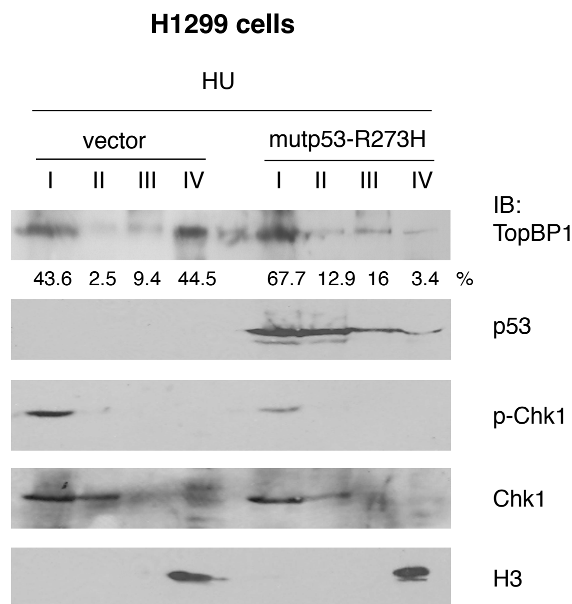

Fig. S7. Expression of mutp53-R273H inhibits HU-induced TopBP1 chromatin binding in H1299 cells.

Figures shown are a representative set of immunoblots for the data shown in Fig. 3D.

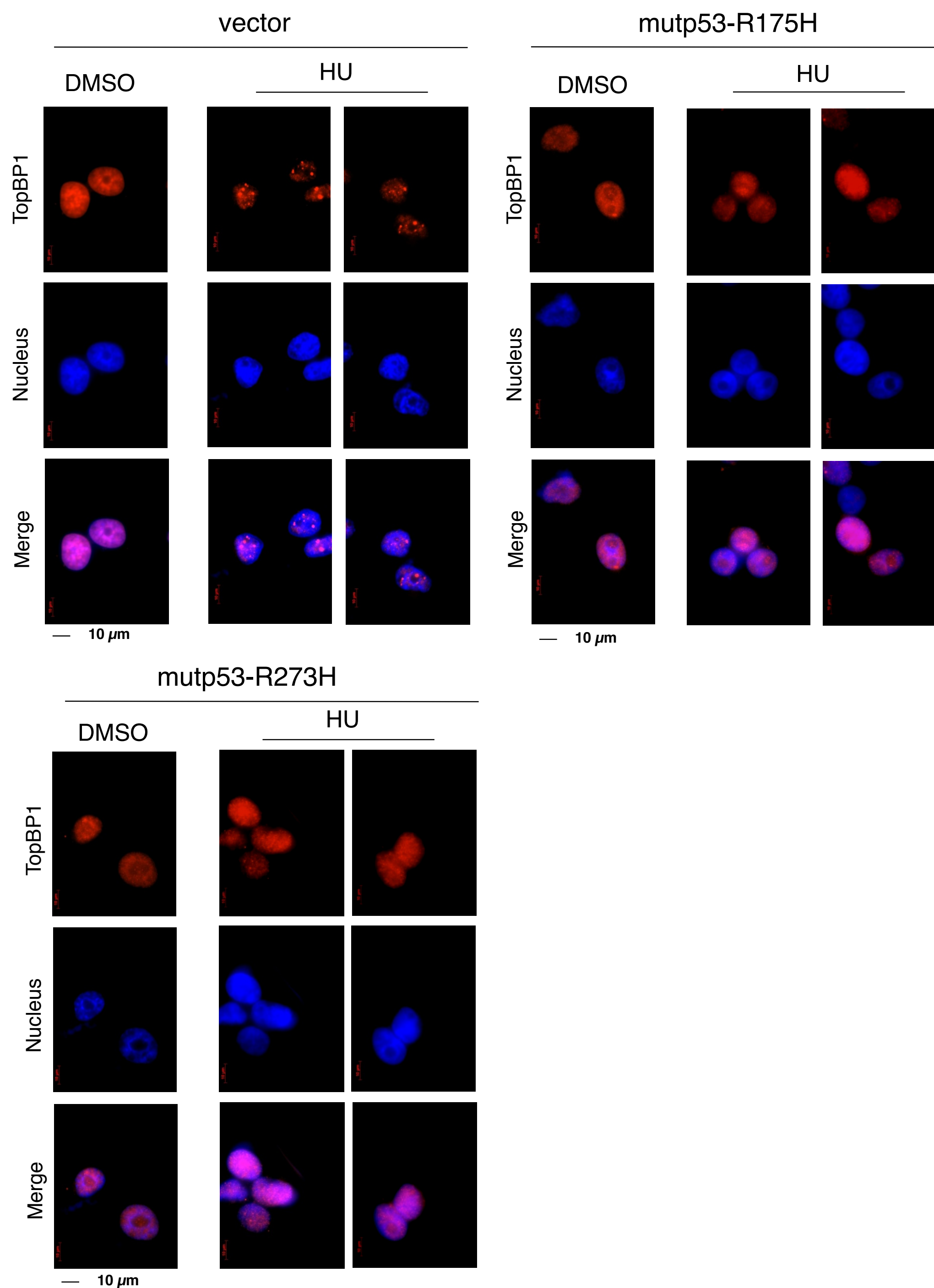

Fig. S8. Mutant p53 inhibits HU-induced TopBP1 foci formation.
Pictures shown are a whole set of representative images as described in Fig. 3E.

Fig. S9. R273H mutp53 induces oligomerization of TopBP1 mutant proteins which are defective in binding to pS1159.

Mutp53-R273H was co-transfected with FLAG-TopBP1-BRCT6/7/8 and either Myc-TopBP1-WT, Myc-TopBP1-K1317M (labeled as K), or Myc-TopBP1-S1273A (labeled as S) into H1299 cells. Co-immunoprecipitation was performed as described in Fig. 3F.

Fig. S10. A tetramerization domain-deleted mutant p53 fails to inhibit Chk1 activation after HU treatment.

H1299 cells transfected with an empty vector or the expression vector of mutp53-R273H or mutp53-R273HΔCT were treated with vehicle (DMSO) or hydroxyurea (HU, 2 mM) for 16 h. The cell lysates were analyzed by Western blotting as indicated.

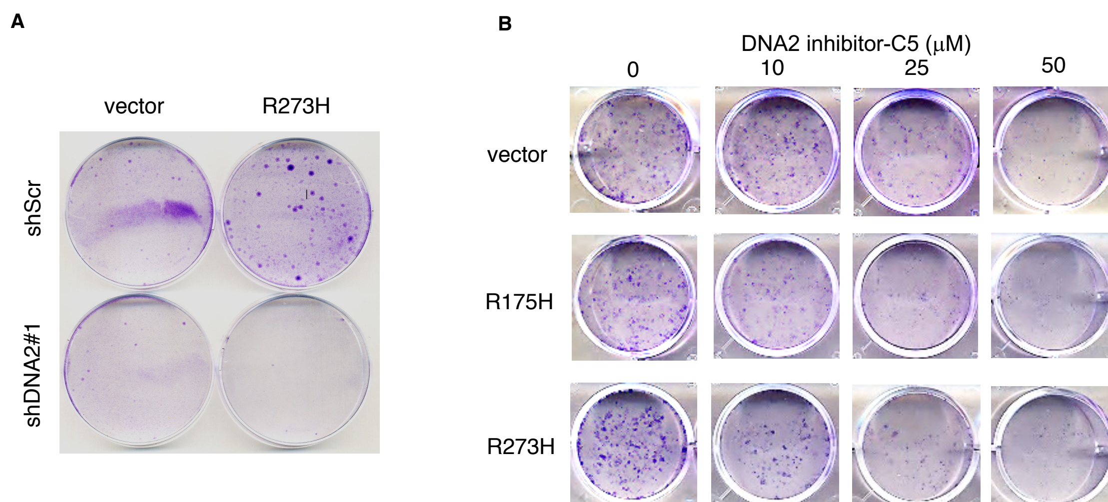

Fig. 11. Clonogenic survival assay
A. Representative images of clonogenic survival assay presented in Fig. 4B.
B. Representative images of clonogenic survival assay presented in Fig. 4E.

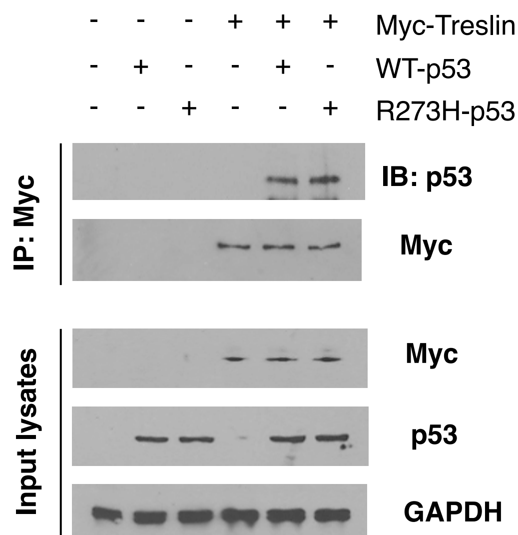

Fig. S12. Treslin interacts with both wild-type p53 and mutp53-R273H.
Wild type p53 or mutp53-R273H was cotransfected with Myc-Treslin into H1299 cells. After 48 h, co-immunoprecipitation was performed using anti-Myc antibody-conjugated agarose beads, followed by immunoblotting as indicated. One tenth of the cell lysates were subjected to Western blot analysis.

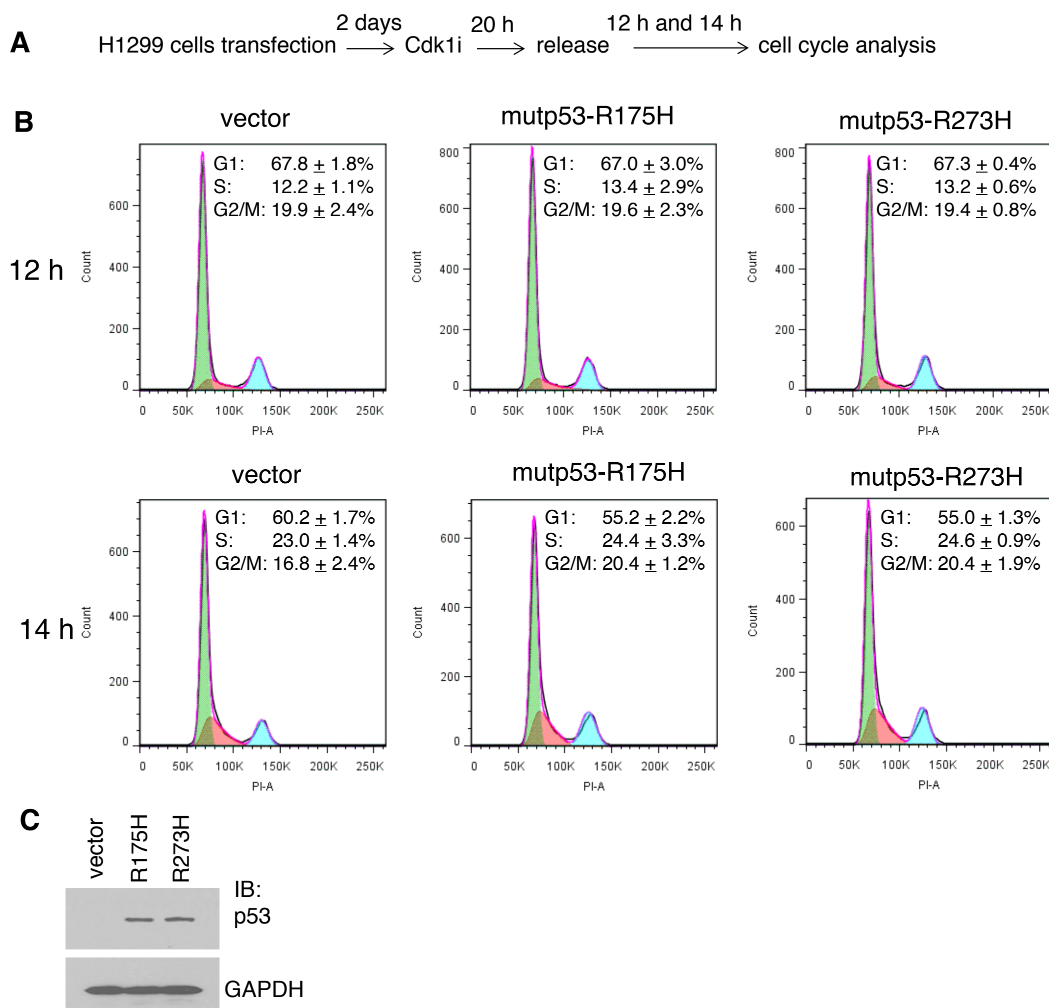

Fig. S13. Cell cycle analysis verifies synchronization of mutp53-expressing H1299 cells.

H1299 cells were transfected with mutp53-R175H or mutp53-R273H. After 48 h, the cells were synchronized by adding a Cdk1 inhibitor (Ro 3306, 2.5 μM) for 20 h, and then were released to enter G1 phase by incubating cells in serum-containing medium without Ro 3306 for 12 h or 14 h. Cells were collected, fixed, and propidium iodide staining of DNA for flow cytometry was performed. 10,000 cells were analyzed for each sample. Data shown represent means and standard deviations from three biological replicates. Expression of mutp53 was verified by immunoblotting (Panel C).

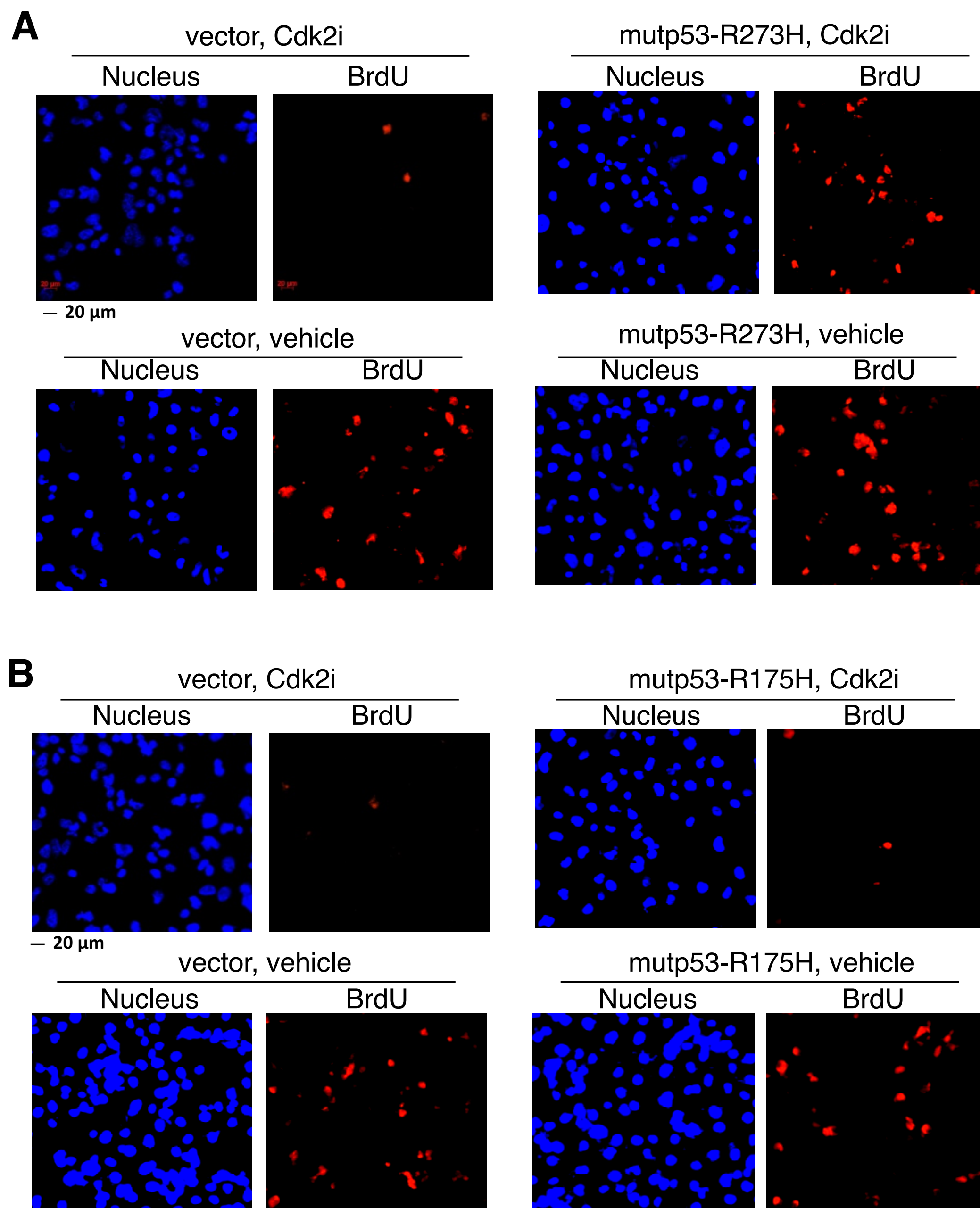

Fig. S14. Mutp53-R273H bypasses the control of Cdk2 and promotes G1/S phase progression during cell cycle.
Pictures shown are representative immunofluorescence images (at 20x magnification) of BrdU incorporation assay described in Fig. 5G.

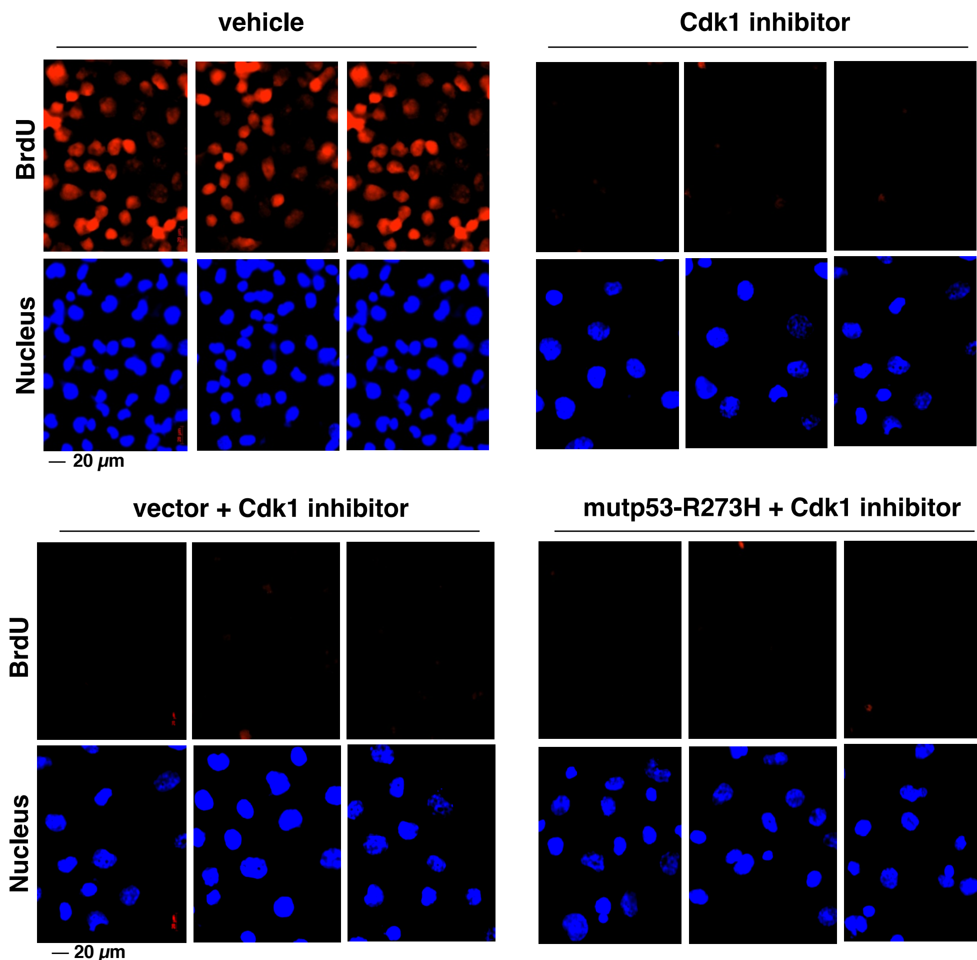

Fig. S15. The expression of mutp53-R273H does not overcome the cell cycle arrest caused by a Cdk1 inhibitor.

H1299 cells were untransfected or tranfected with an empty vector or mutp53-R273H. After 48 h, cells were treated with DMSO vehicle or a Cdk1 inhibitor (Ro 3306, 2.5 μM) for 20 h, and then subjected to BrdU incorporation assay. Pictures shown are representative immunofluorescence images at 40x magnification from each indicated group.

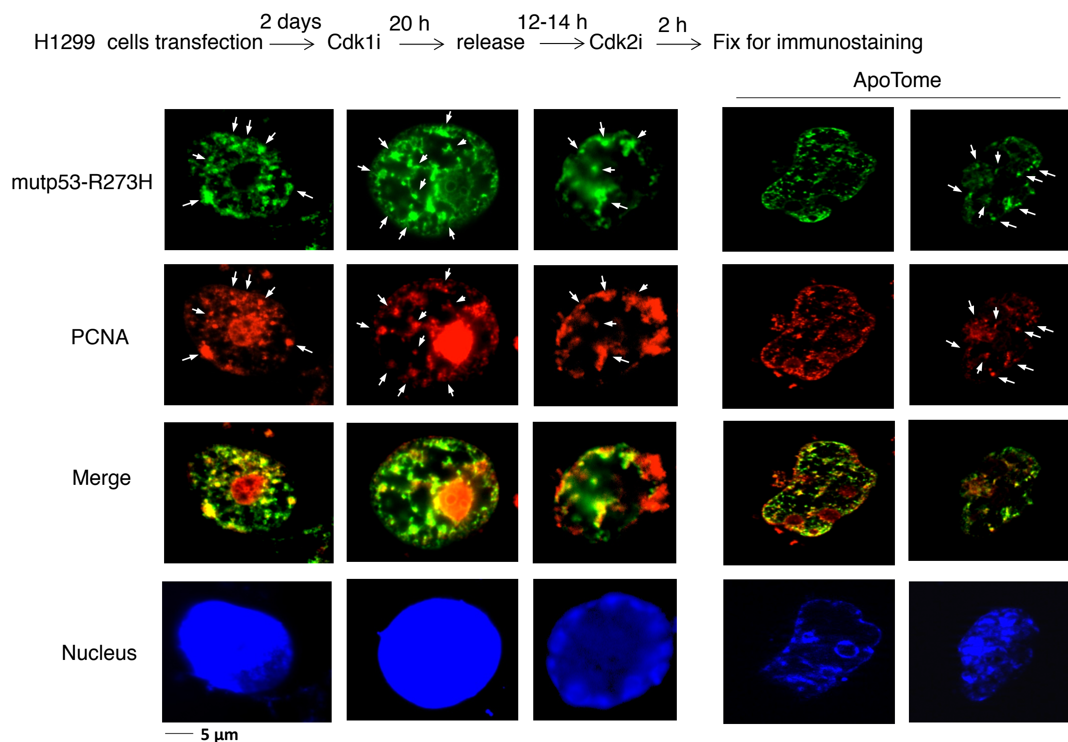

Fig. S16. Localization of mutp53-R273H to the replication forks during DNA replication

Mutp53-R273H colocalizes with PCNA during G1/S transition of the cell cycle. H1299 cells expressing Mutp53-R273H were synchronized with Cdk1i (Ro 3306) as described above. After removal of Cdk1i for 12-14 h, cells were treated with Cdk2i (Cdk2 inhibitor II, 1 µM) for 2 h, and then were fixed and stained with anti-p53 rabbit antibody and anti-PCNA mouse monoclonal antibody, followed by Alexa Fluor 488-conjugated anti-rabbit secondary antibody and Texas Red X-conjugated anti-mouse secondary antibody, respectively. Nuclei were stained with Hoechst 33258. Images were acquired using Zeiss Axiovert fluorescence microscope without or with an ApoTome 2 system. Pictures shown are representative images at 100x magnification from each indicated group. Arrows indicate representative colocalized mutp53 and PCNA signals.

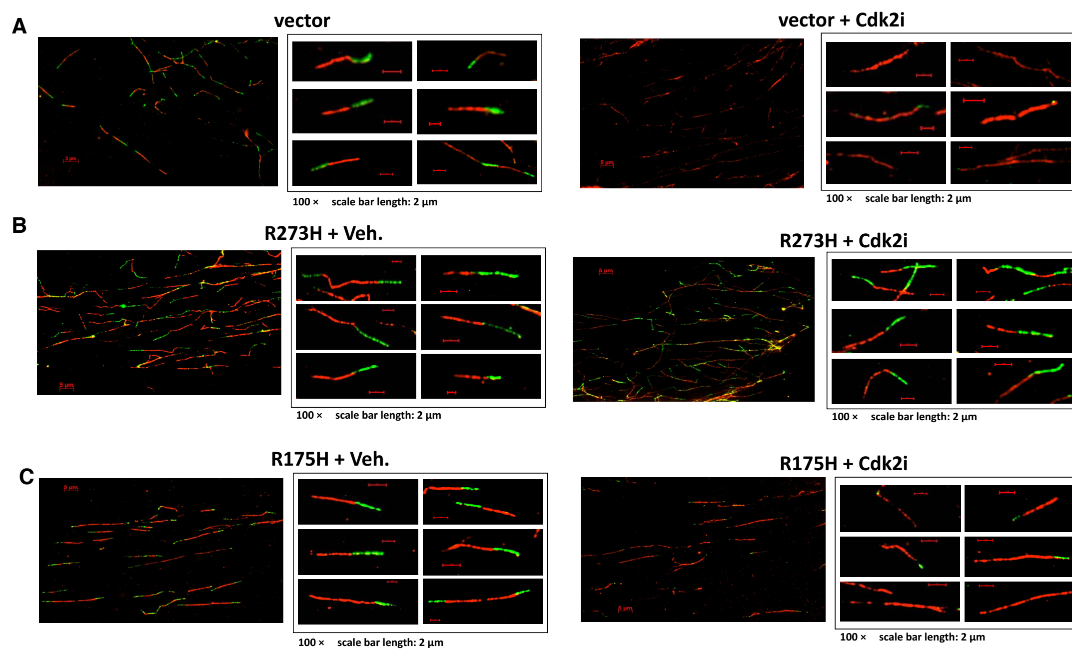

Fig. S17. Representative images of DNA fiber assay presented in Fig. 6A-C.

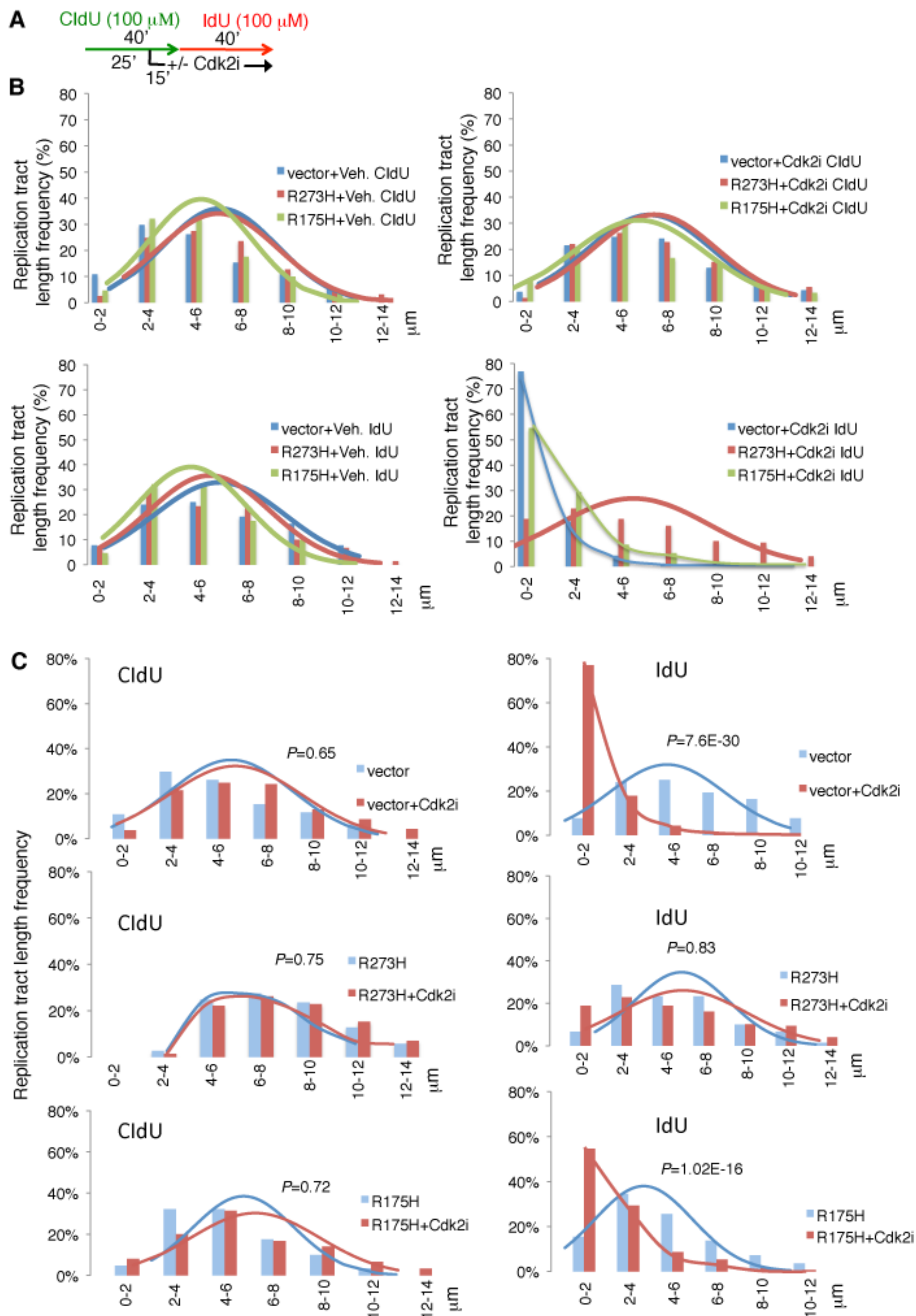
A
CldU (100 μM)
40'
IdU (100 μM)
40'
25'
15'
+/- Cdk2i
B
Replication tract length frequency (%)
vector+Veh. CldU
R273H+Veh. CldU
R175H+Veh. CldU
vector+Cdk2i CldU
R273H+Cdk2i CldU
R175H+Cdk2i CldU
vector+Veh. IdU
R273H+Veh. IdU
R175H+Veh. IdU
vector+Cdk2i IdU
R273H+Cdk2i IdU
R175H+Cdk2i IdU
0-2
2-4
4-6
6-8
8-10
10-12
12-14
μm
C
Replication tract length frequency
CldU
IdU
P=0.65
vector
vector+Cdk2i
P=7.6E-30
P=0.75
R273H
R273H+Cdk2i
P=0.83
P=0.72
R175H
R175H+Cdk2i
P=1.02E-16

Fig. S18. Mutp53-R273H expression renders the DNA replication in cancer cells resistant to Cdk2i treatment.

A. H1299 cells were transfected with either an empty vector, mutp53-R273H or mutp53-R175H. Cells were then sequentially labeled with CIdU 40 min and then IdU 40 min. Some samples were treated with Cdk2i (2 μM) 15 min before IdU labeling. The experimental scheme for DNA fiber analysis is shown.

B. The lengths of CIdU and IdU tracts from DNA fibers were measured. Shown are tract length distributions of CIdU (upper graphs) and IdU (lower graphs) in vehicle (Veh.) control groups (left graphs) and Cdk2i-treated groups (right graphs). About 150-200 DNA fibers in each group were analyzed. P <0.0001, comparing CIdU tract lengths between vector+Cdk2i and R273H+Cdk2i groups (two-tailed t test).

C. To compare the tract length distributions between vehicle control and Cdk2i treatment for each group, both distributions of each group are compared together. P values are based on two-tailed t test. B and C are derived from the same set of data, but are grouped differently for different statistical comparison analyses.

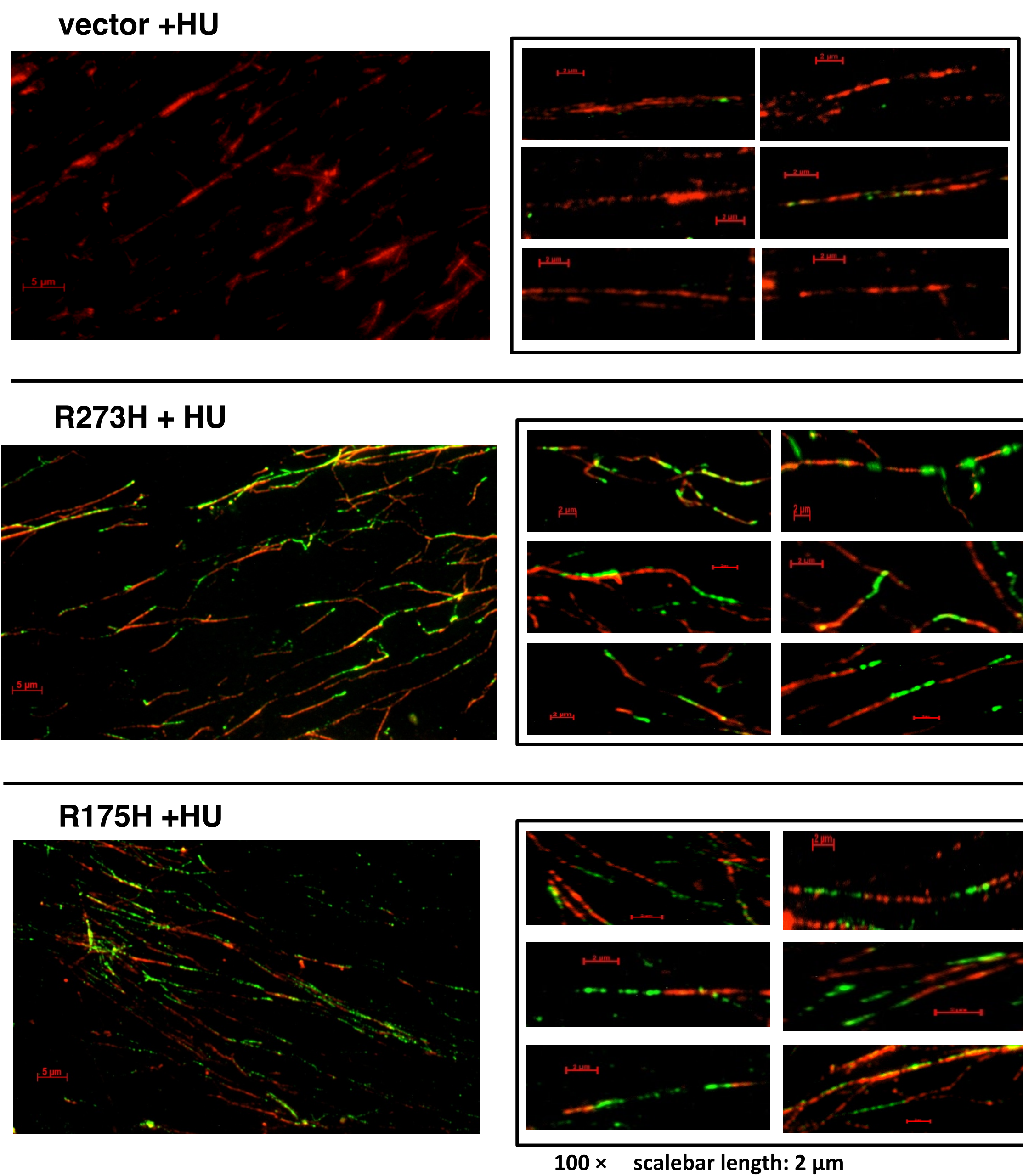

Fig. S19. Representative images of DNA fiber assay presented in Fig. 6D-E.

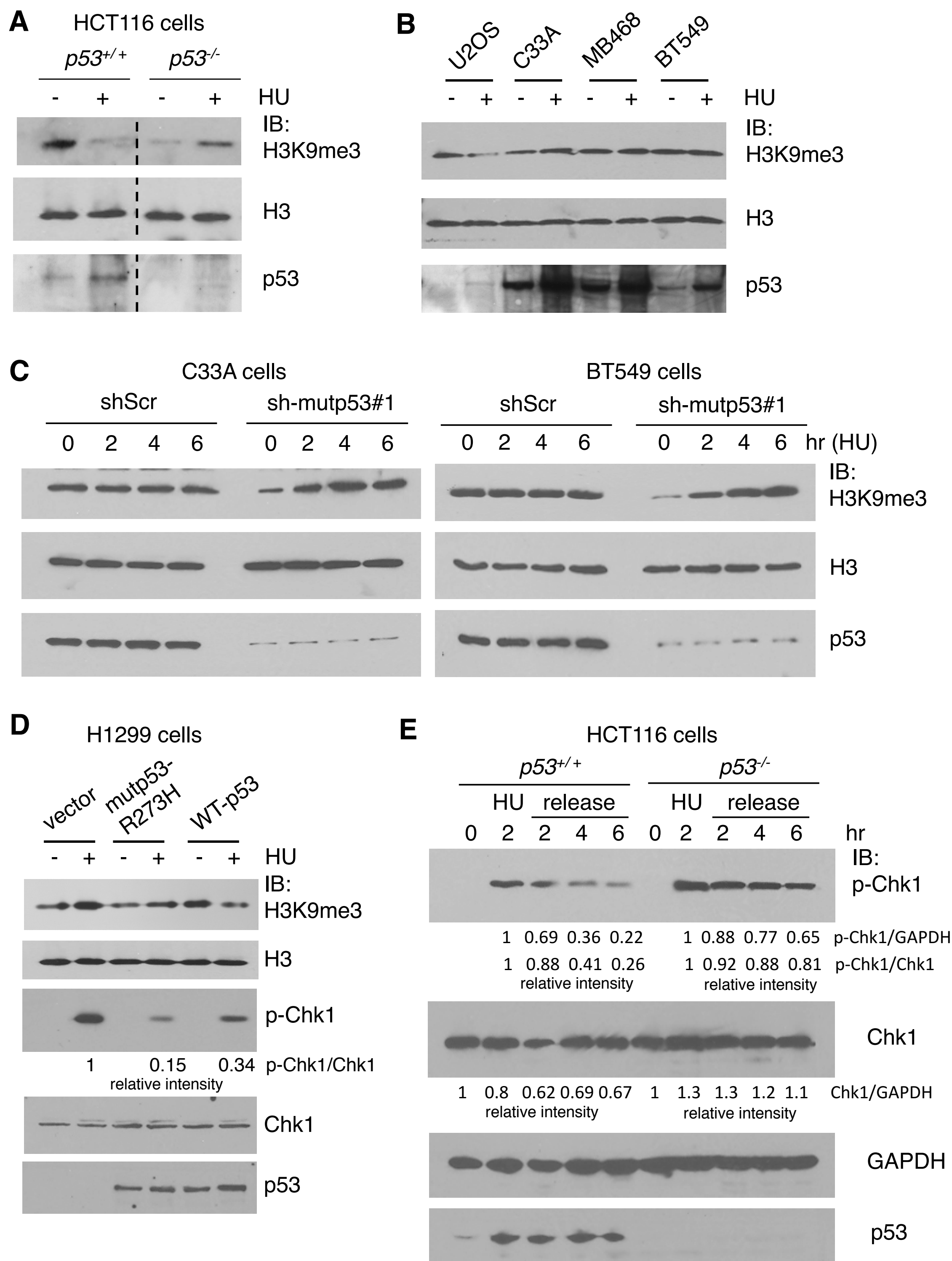

Fig. S20. Wild-type and mutant p53 differentially regulate histone H3K9 trimethylation upon HU treatment. A role for wild-type p53 in down-regulating Chk1 activity once stress is relieved.

A. HCT-116 cells harboring wild-type p53 (*p53*$^{+/+}$) or lacking p53 (*p53*$^{-/-}$) were untreated or treated with HU (2 mM) for 2 h. Cells were harvested for Western blot analysis. The space between *p53*$^{+/+}$ and *p53*$^{-/-}$ lances was excised for brevity (dashed lines).
B. U2OS cells that harbor wild-type p53 or C33A, MDA-MB468, and BT549 cells that harbor mutp53 were untreated or treated with HU (2 mM) for 5 h. Western blot analysis was performed as indicated.
C. Knockdown of mutp53 affects histone H3K9 trimethylation during HU treatment. C33A (left panel) or BT549 (right panel) cells stably expressing either shScr or sh-mutp53 were treated or not with HU (2 mM) for 2, 4, or 6 h. The whole cell lysates were subjected to Western blot analysis as indicated.
D. H1299 cells transfected with an empty vector, mutp53-R273H or wild-type (WT) p53 were untreated or treated with HU (2 mM) for 5 h, followed by immunoblotting using the indicated antibodies. P-Chk1 and Chk1 signals were quantified using ImageJ software and the relative p-Chk1/Chk1 ratios are shown below the panel.
E. HCT-116 cells harboring wild-type p53 (*p53*$^{+/+}$) or lacking p53 (*p53*$^{-/-}$) were untreated or treated with HU (2 mM) for 2 h, and then HU was removed. The cells were harvested at the indicated time points. The whole cell lysates were subjected to Western blot analysis as indicated. GAPDH serves as a loading control. Signals were quantified using ImageJ software and normalized by GAPDH or Chk1. The relative normalized intensities (p-Chk1/GAPDH, p-Chk1/Chk1 and Chk1/GAPDH) are shown below each corresponding panel.

Supporting Information References:

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