Supporting Information

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SI Materials and Methods

Cell Culture. hESCs (H1) (obtained from the WiCell Research Institute) were cultured under feeder-free conditions on growth factor reduced Matrigel (356231; Corning) coated tissue culture plates in hESC media (PeproGrow hESC, BM-hESC-500; Peprotech) and passaged by microdissection every 4–6 d. For experiments, hESCs were passaged using StemPro Accutase Cell Dissociation Reagent (A11105-01; Gibco) (37 °C, 5–7 min), and equal cell numbers were seeded on Matrigel coated plates in media containing ROCK inhibitor (S1049, 10 μM; Selleck Chemicals). On the next day, media was replaced with fresh media without ROCK inhibitor. Cells treatments were performed 3–4 d after passaging. HeLa cells were grown in DMEM (Invitrogen) containing 10% FBS (Gibco).

Gene Targeting. MSH2 knockout hESCs were derived using CRISPR/Cas9 gene targeting. A guide RNA targeting the first exon of *MSH2* (GCCGAAGGAGACGCTGCAGT), designed using the online resource crispr.mit.edu, was cloned into PX330 (plasmid 42230; Addgene). A targeting vector containing MSH2 homology arms PCR amplified from H1 genomic DNA was cloned into vector pLCA.66/2272 (plasmid 22733; Addgene), which contains a loxed cassette acceptor with two tandem heteromeric lox sites flanking PGK-puΔtk and EM7-neoR selection cassettes. Although the acceptor cassette was not utilized in this study, it was used to disrupt exon 1 of *MSH2*. The acceptor cassette was subcloned into a PGK-DTA backbone to provide a negative selection marker (plasmid 13440; Addgene). Ten micrograms of guide RNA and 40 μg of targeting vector were electroporated into H1 hESCs using the Bio-Rad Genepulser xcell. Cells were plated on DR4 mouse embryonic fibroblasts, and after 3 d, underwent puromycin (1 μg/mL) selection. Isolated individual surviving colonies were genotyped by long-range PCR, and targeting was confirmed by Sanger sequencing. Sequencing indicated that both alleles were targeted by Cas9, and the acceptor cassette was introduced within one allele while the second allele contained insertions and/or deletions that disrupted the reading frame due to repair by nonhomologous end joining.

To generate the MSH2 knockout HeLa cells using CRISPR/Cas9 gene, the above-mentioned guide RNA sequence was cloned into the vector Px459V2.0 (plasmid 62988; Addgene). HeLa cells were transfected with 4 μg of the vector DNA expressing the guide RNA, the Cas9 cDNA, and a puromycin resistance gene 3 d after seeding at a density of ∼70% using Lipofectamine 2000 Reagent (11668019; Invitrogen) as per manufactures instructions. The DNA–lipid complexes were incubated with cells for 6 h after which 1 mL of DMEM containing 10% FBS, Gibco was added. Next day, cells were transferred to a 10-cm dish and underwent selection in media containing 1 μg/mL puromycin. Hotshot PCR was performed on each of the surviving clones (Fwd primer: AGTAGCTAAAGTCACCAGCGTGC; Rev primer: CATGTACTTGATCACCCCCTGG), and positive clones were identified using a restriction enzyme screen to determine formation of insertions or deletions at the Cas9 cleavage site. Clones identified with a positive PCR screen were further screened by Western blot to check for loss of MSH2 protein expression.

Chemicals. MNNG (CAS: 70-25-7; National Cancer Institute Chemical Carcinogen Reference Standard Repository) and O^6BG (B2292; Sigma) were dissolved in DMSO and stored at −20 °C. Inhibitors to ATR (ATRi) (VE-821), ATM (ATMi) (KU-5593) and DNA-PK (DNA-Pkcsi) (NU-7026), and small molecule inhibitor, pifithrin-μ (PFT-μ), purchased from Selleck Chemicals were dissolved in DMSO and stored at −80 °C. These were used at the following concentrations: MNNG: 2 μM, except in case of MTT assay; O^6BG: 25 μM; ATR (VE-821): 10 μM; ATM (KU-5593): 100 μM; DNA-PK (NU-7026): 50 μM. MNNG treatments involved a 2-h pretreatment and continual maintenance in O^6BG containing media until point of harvest.

Cell Synchronization, Cell Cycle Analysis, and MTT Assay. hESCs pretreated with O^6BG for 2 h were treated with MNNG for 4 h. Cells were washed with PBS, and fresh medium containing O^6BG was added. Twenty hours later, hESCs were harvested for cell cycle analysis. For cell synchronization at G_2/M, HeLa cells were grown in 300 ng/mL nocodazole for 16 h, detached by mechanical shake-off, washed three times with PBS, and replated in fresh medium without nocodazole. After 5 h, 25 μM O^6BG was added. After 2 h, cells were treated with 2 μM MNNG and/or ATRi and harvested at different time points for cell cycle analysis. hESCs and HeLa cells were harvested using StemPro Accutase and Trypsin-EDTA (Gibco), respectively. The cells were washed with PBS and fixed overnight with chilled 70% ethanol at −20 °C. Fixed cells were washed with PBS and incubated with propidium iodide (20 μg/mL) and RNase A (200 μg/mL) for 30 min at 37 °C. After filtration, cell suspensions were analyzed using a LSRII flow cytometer (BD Biosciences).

For studies in hESCs, cells were pretreated with O^6BG and incubated with increasing concentrations of MNNG (0.1, 0.2, 0.5, 1, and 2 μM) and corresponding inhibitor in the presence of O^6BG for 4 h. Cell viability was assessed 20 h later following continued incubation in media containing the corresponding inhibitors. For studies in HeLa cells, cell survival was assessed at 72 h in cells treated with 2 μM MNNG and/or ATRi in the presence of O^6BG for 16 h. Cell viability was determined using Vybrant MTT Cell Proliferation Assay Kit (V-13154) as per manufacturer's instructions.

Total Cell Extracts and Western Blotting. Total cell extracts were prepared as described in ref. 15. Briefly, harvested cells were lysed in ice-cold RIPA buffer containing protease inhibitors for 45 min at 4 °C and centrifuged at 16,000 × *g* for 10 min. Supernatants were collected as total cell extracts. Equal protein concentrations of cell extracts were separated by denaturing SDS/PAGE, transferred onto a PVDF membrane, and Western blot analysis was performed. Antibodies included Cell Signaling: ATR (2790), p-ATR (2853), p53 (9282), p-p53-Ser-15 (9284), p-Chk-Ser-345 (2341), Chk1 (2345), pChk2-Thr-68 (2661), Chk2 (2662); Bethyl: MLH1 (550838), MSH6 (A300-023A), PMS2 (556415), RPA-S4/S8 (A300-245A), RPA-S33 (A300-246A) p-Chk1-Ser-317 (A304-673A); Sigma-Aldrich: Actin (A5060); Calbiochem: RPA (NA19L), MSH2 (NA27); EMD Millipore: γ-H2AX (05-636).

Immunofluorescence and Image Analysis. hESCs were plated on Matrigel-coated Thermanox Cell Culture Coverslips (174985; Nunc). To detect DNA synthesis, cells were treated with or without MNNG in the presence of EdU (10 μM). After 4 h, EdU incorporated into DNA was detected using the Click-iT Plus EdU Alexa Fluor 647 Imaging Kit (C10640; Molecular Probes) and further processed for immunofluorescence analysis. To assess 53BP1 and Rad51 foci formation in hESCs, cells were treated with MNNG for 4 h and processed for immunofluorescence analysis. To assess cleaved-caspase-3, hESCs were treated with MNNG for 4 h and fixed immediately, or 20 h later.

For detection of ssDNA formation, cells were grown in BrdU (10 μM) containing medium for 16 h and then in BrdU-free

media for 2 h. Subsequently, they were treated with O^6BG for 2 h, MNNG for 2 h, and then fixed 6 h later using chilled methanol for 10 min at −20 °C. Cells were then processed for immunofluorescence analysis as described below.

To assess 53BP1 foci formation in HeLa cells, cells were treated with MNNG and/or ATRi for 14 h and fixed for immunofluorescence analysis. For cleaved-caspase-3 staining, HeLa cells were treated with MNNG and/or ATRi for 16 h, washed three times with PBS, and maintained in fresh medium containing O^6BG. Cells were processed for immunofluorescence analysis after 72 h from start of MNNG exposure. To examine replication origin patterns, HeLa cells were synchronized in G_2/M and harvested as mentioned above. Cells were seeded on glass coverslips. After 5 h, cells were treated with O^6BG for 2 h and then treated with MNNG. Fifteen minutes before harvest, cells were grown in EdU-containing medium to label active origins of replication, followed by fixation and processing as described below.

Following MNNG treatment, cells were fixed with 4% paraformaldehyde (10 min), permeabilized with 1% Triton X-100 (10 min) and blocked with 3% goat serum in PBS for 1–2 h at room temperature (RT). Cells were incubated with primary antibodies (diluted in 3% goat serum in PBS) and then with Alexa Fluor 488 or Alexa Fluor 568 secondary antibodies (Molecular Probes) for 1 h each at RT. Antibodies included γ-H2AX (EMD Millipore; 05–636), BrdU (sc-32323; Santa Cruz Biotechnology), Rad51 (PC130; EMD Millipore), 53BP1 (NB100-304; Novus Biologicals) cyclin A (sc-271645; Santa Cruz Biotechnology), cleaved-caspase-3 (559565; BD Biosciences). Nuclei were counterstained with 4′,6-diamidino-2-phenylindole (DAPI) or Hoechst-33342 (3570; Life Technologies), mounted, and images were captured using a Zeiss LSM 780 confocal microscope equipped with a Zeiss Plan-apochromat 63×/1.4 N.A. oil immersion objective or a Nikon Eclipse Inverted Fluorescent microscope. Image analysis was performed using MetaMorph.

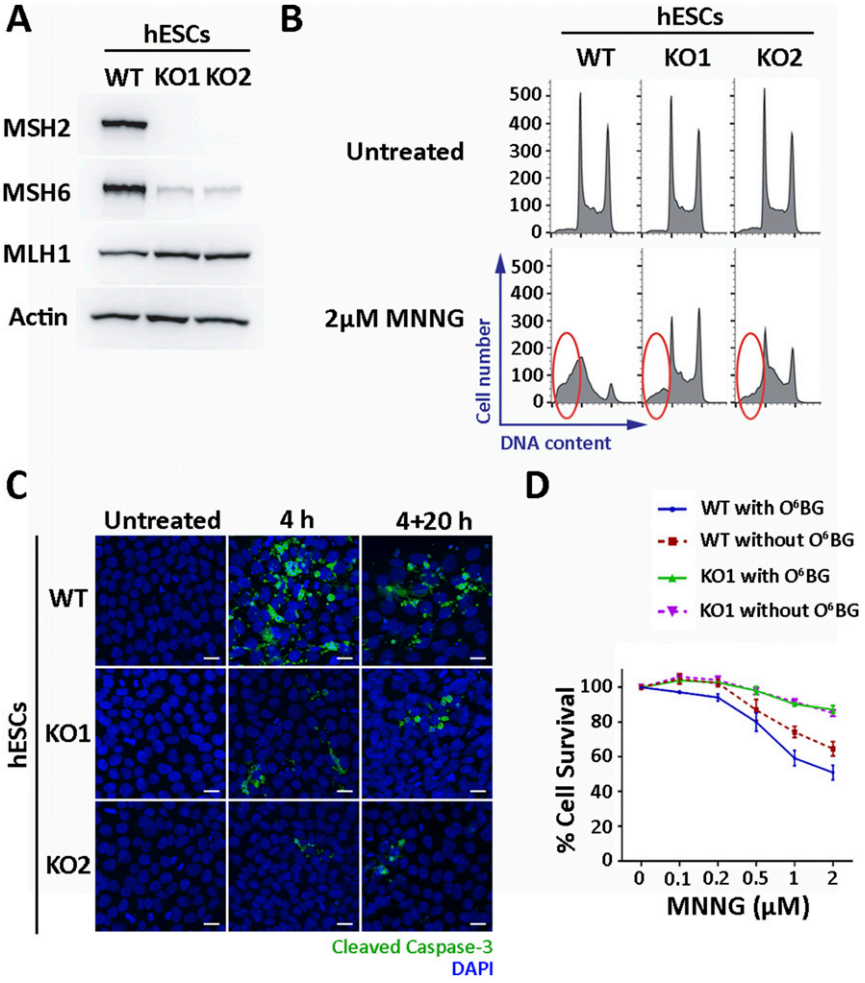

Fig. S1. Recognition of MNNG-induced ^{Me}G lesions by MMR proteins triggers a rapid apoptotic response in hESCs. (*A*) Immunoblot of mismatch repair proteins MSH2, MSH6, and MLH1 confirming knockout of both endogenous *MSH2* alleles in two independent MSH2 KO clones, KO1 and KO2, created by CRISPR/Cas9 gene targeting. Actin is included as a loading control. (*B*) Representative cell cycle profiles as measured by flow cytometry at 24 h of WT, KO1, and KO2 hESCs treated with 2 μM MNNG for first 4 h. Encircled are cells with sub-G_1 DNA content indicative of apoptosis. (*C*) Immunostaining and confocal imaging for cleaved-caspase-3 of WT, KO1, and KO2 hESCs fixed immediately (0 h) or 20 h after treatment with 2 μM MNNG for 4 h. Nuclei are counterstained with DAPI. (Scale bars: 20 μm.) (*D*) Percentage of cell survival after 24 h of WT and KO1 hESCs treated with increasing concentrations of MNNG for the first 4 h and in the presence or absence of O^6BG using the MTT assay. All experiments were performed in triplicate.

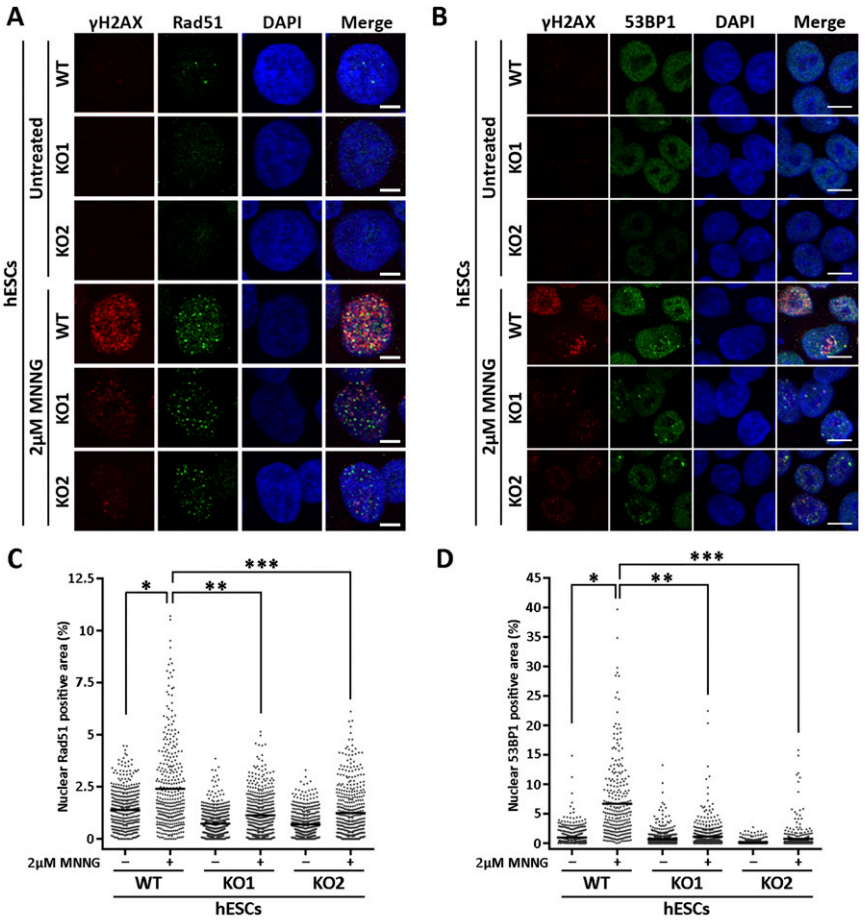

Fig. S2. Upon MNNG treatment, homologous recombination (HR) and nonhomologous end joining (NHEJ) repair factors are recruited to sites of MMR-induced double-strand breaks. (*A*) Immunofluorescence and confocal imaging of γH2AX and HR repair factor Rad51 in WT, KO1, and KO2 hESCs 4 h after treatment with 2 μM MNNG. Nuclei are counterstained with DAPI. (*B*) Immunofluorescence and confocal imaging of γH2AX and NHEJ repair factor 53BP1 in WT, KO1, and KO2 hESCs 4 h after treatment with 2 μM MNNG. Nuclei are counterstained with DAPI. (Scale bars: 10 μm.) (*C*) Quantitation of percentage of nuclear area positive for Rad51 signal. Results from one representative experiment as described in *A* ($n > 300$); *, **, ***, $P < 0.0001$, Mann–Whitney test. (*D*) Quantitation of percentage of nuclear area positive for 53BP1 signal. Results from one representative experiment as described in *B* ($n > 250$); *, **, ***, $P < 0.0001$, Mann–Whitney test.

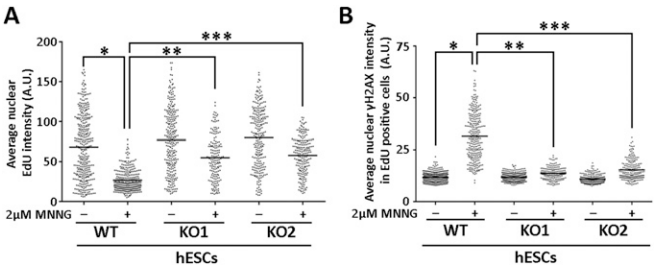

Fig. S3. MMR-dependent processing of ^{Me}G/T lesions in hESCs compromises DNA replication and promotes accumulation of double-strand breaks. (*A*) Quantitation of average nuclear EdU from one representative experiment as performed in Fig. 2*B* ($n > 150$); *, **, ***, $P < 0.0001$, Mann–Whitney test. (*B*) Quantitation of average nuclear γH2AX intensities in EdU-positive cells from one representative experiment as performed in Fig. 2*B* ($n > 150$); *, **, ***, $P < 0.0001$, Mann–Whitney test.

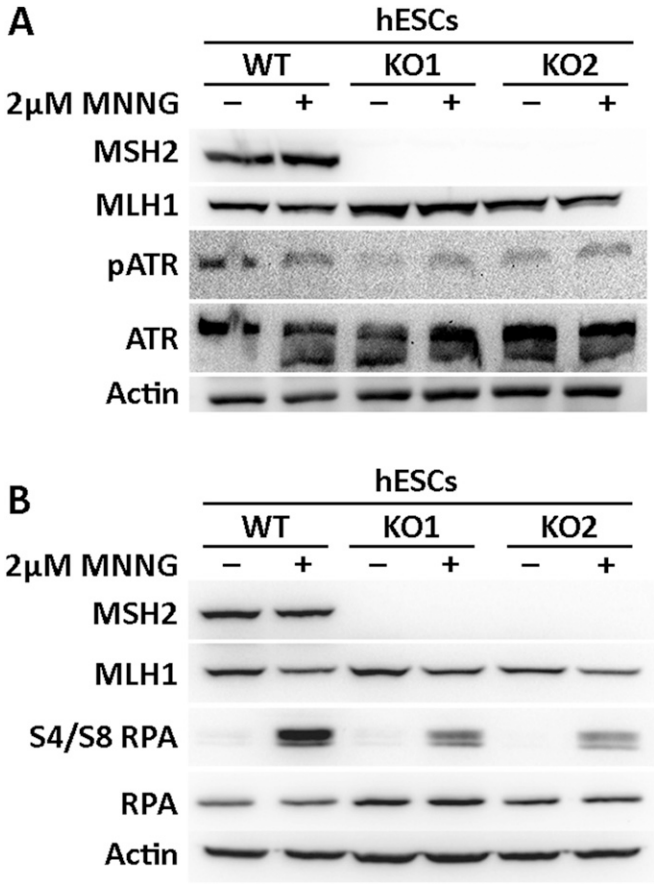

Fig. S4. MNNG-induced replication stress in hESCs triggers fork collapse without activating ATR kinase in a MMR-dependent manner. (*A*) Immunoblot of pATR (Ser-428), ATR, MSH2, and MLH1, in WT, KO1, and KO2 hESCs treated with 2 μM MNNG for 4 h. Actin used as a loading control. (*B*) Immunoblot of MSH2, MLH1, pRPA (S4/S8), and RPA in WT, KO1, and KO2 hESCs treated with or without 2 μM MNNG for 4 h. Actin is included as loading control.

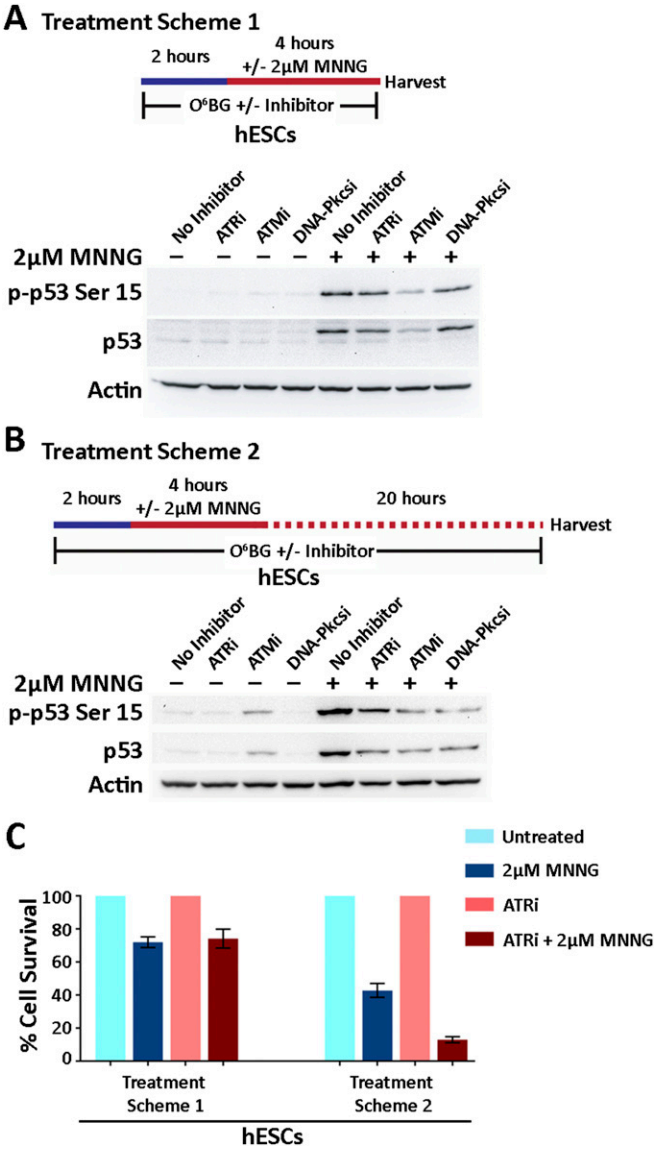

Fig. S5. ATR kinase activity does not contribute to early apoptotic induction in MNNG-treated WT hESCs. (*A*) Immunoblot of p-p53 (Ser-15) and p53 in WT hESCs treated with or without 2 μM MNNG for 4 h in the presence of inhibitors to ATR (VE-821; 10 μM), ATM (KU-5593; 100 μM), or DNA-PKcs (NU-7026; 50 μM). Actin is included as loading control. (*B*) Immunoblot of p-p53 (Ser-15) and p53 in WT hESCs treated with or without 2 μM MNNG for 4 h and harvested 20 h later in the presence of inhibitors to ATR (VE-821; 10 μM), ATM (KU-5593; 100 μM), or DNA-PKcs (NU-7026; 50 μM). Actin is included as loading control. (*C*) Percentage cell survival of WT hESCs treated with 2 μM MNNG for 4 h in the presence or absence of inhibitors to ATR (VE-821; 10 μM). Cell survival was measured by MTT assay immediately or 20 h following treatment as described in schemes for *A* and *B*. All experiments were performed in triplicate.

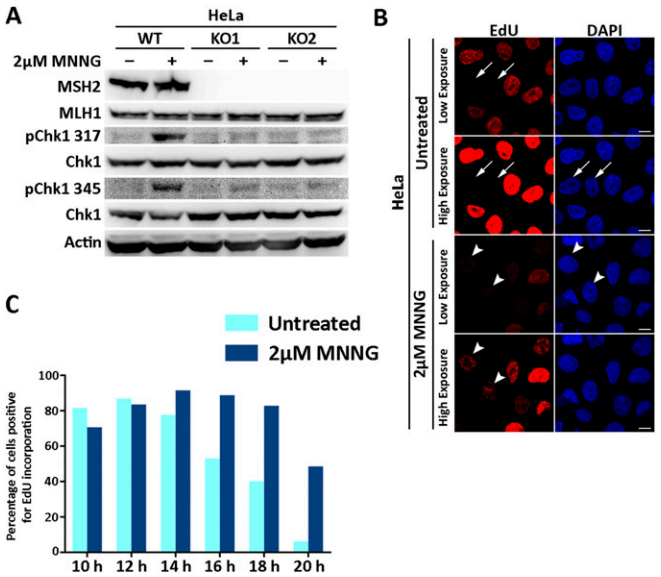

Fig. S6. MMR processing of MNNG-induced lesions in HeLa cells activates a ATR-Chk1–dependent replication stress checkpoint. (*A*) Immunoblot of pChk1 (Ser-345), pChk1 (Ser-317), Chk1, MSH2, and MLH1 in MSH2 WT and MSH2 KO (KO1 and KO2) HeLa cells treated with 2 μM MNNG for 14 h. Actin used as a loading control. Experiments were performed in triplicate. (*B*) Overexposure of 10-h time point images in Fig. 3*C* to visualize percentage of nuclei positive for EdU incorporation. Arrows point to nuclei that are truly negative for EdU incorporation in untreated cells harvested at 10 h even at high exposures. Arrowheads point to nuclei that appear negative for EdU incorporation at low exposure in MNNG-treated cells harvested at 10 h, but are actually positive for EdU at high exposure. (Scale bars: 10 μm.) (*C*) Quantitation of EdU-positive cells from one representative experiment as described in Fig. 3*C* visualized at high exposure.

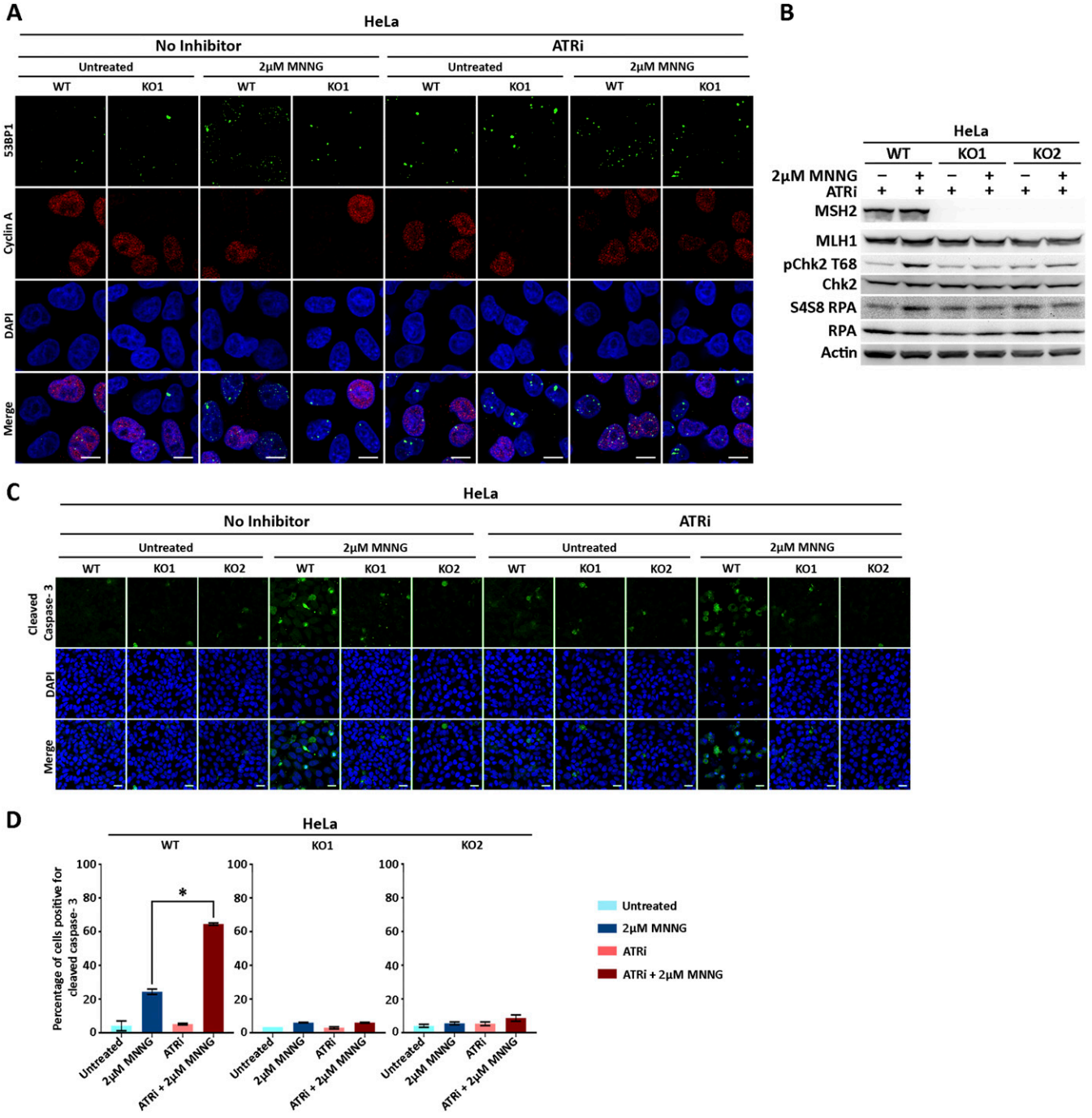

Fig. S7. DNA damage accumulation upon inhibition of ATR-Chk1 signaling in the first S phase of MNNG-treated HeLa cells is dependent on functional MMR. (*A*) Immunostaining for 53BP1 and cyclin A, a S/G_2 phase marker, in WT and MSH2 KO1 HeLa cells treated with 2 μM MNNG in the presence or absence of ATRi (10 μM) for 14 h. (*B*) Immunoblot of pChk2 (Thr-68), Chk2, pRPA (S4/S8), and RPA in WT and MSH2 KO (KO1 and KO2) HeLa cells treated with 2 μM MNNG in the presence or absence of ATRi (10 μM) for 14 h. Actin used as a loading control. (*C*) Immunostaining for cleaved-caspase-3 at 72 h in WT and MSH2 KO (KO1 and KO2) HeLa cells treated with 2 μM MNNG for the first 16 h in the presence or absence of ATRi (10 μM). Nuclei are counterstained with DAPI. (Scale bars: 20 μm.) (*D*) Quantitation of percentage of cells positive for cleaved-caspase-3 staining ($P \leq 0.01$, Student's *t* test).