SUPPLEMENTARY FIGURE LEGENDS

Figure S1. DNA2 associates with replication forks and prevents the accumulation of replication-associated DSBs. A, Endogenous DNA2 was co-immunoprecipitated with replication factors. The whole-cell extracts from U2OS cells were subjected to immunoprecipitation with control, anti-PCNA, or DNA polymerase δ (DNA polyδ) antibodies and then subjected to Western blot analysis with the indicated antibodies. B, Representative images for comet assay in Fig. 1D. (scale bar: 20 μm). C, U2OS cells were transfected with control siRNA (nontargeted; NT) or DNA2 siRNA (SMARTpool). Forty-eight hours later, cells were untreated or treated with aphidicolin (Api) (5 μM) for overnight. Then cells were pulsed with BrdU (10 μM) for 30 min prior to staining with the indicated antibodies. (Left) Representative immunostaining images (scale bar: 5 μm). (Right) Cells were scored as positive for one or both proteins. At least 50 cells were scored in each sample.

Figure S2. DNA2 facilitates HR repair. A, U2OS cells were transfected with control siRNA (nontargeted; NT) or DNA2 siRNA (SMARTpool). Forty-eight hours later, cells were exposed to UV (50 J/m^2) and harvested at the indicated time points. Cell lysates were extracted for Western blot analysis with the indicated antibodies. B, Schematic diagram of HR assay. The DRGFP reporter substrate was integrated into cellular genomic DNA. SceGFP contains an I-*Sce*I endonuclease site within the coding region, which abolishes GFP expression. iGFP is a truncated GFP, which contains homologous sequence for the SceGFP. Expression of I-*Sce*I induces a single DSB in the genome. When this DSB is repaired by HR, the expression of GFP can be restored and analyzed

by flow cytometry to indicate the efficiency of HR repair. C, U2OS cells were transfected with FLAG-DNA2 and FLAG-DNA2 (del734-829). Forty-eight hours later, cells were fixed and co-stained with anti-FLAG antibody and mtHSP70. D, U2OS cells were transfected with control or DNA2 siRNA#9 and reconstituted with indicated DNA constructs. Cell cycle analysis with propidium iodide staining was performed seventy-two hours after transfection.

Figure S3. DNA2's function in HR repair is dependent on its nuclease activity. A, U2OS cells were transfected with the indicated constructs. Twenty-four hours later, cells were split and plated at a very low density for clonogenic assay analysis. B, Nuclease activity of DNA2 promotes HR repair. DRGFP cells were transfected with indicated contrstructs. Twenty-four hours later, cells were subjected to HR repair analysis. C, DNA2 alleviates replication stress-induced by cyclin E activation. DNA2 facilitates BrdU incorporation in the presence of oncogenic stimuli. MCF10A cells stably expressed cyclin E were transfected with the indicated constructs. Forty-eight hours later, cell lysates were subjected to Western blot analysis. Forty-eight hours after transfection, cells were labeled with BrdU (10 μM) for 30 min before fixation. BrdU were co-stained in denatured conditions. (Left) Representative immunostaining images (scale bar: 5 μm). (Right) Quantitative summary for BrdU-positive cells. At least 50 cells were scored in each sample. Each value represents the mean ± SEM from two independent experiments. Results of Western blots demonstrating DNA2 and cyclin E overexpression are shown next to graph.

Figure S4. Biological effects of DNA2 depletion on cancer cells. A, Quantitative

summary for Figure 4D. Each value is relative to the percentage of double-positive cells in cells with control siRNA transfection, which was set to 1 and represents the mean ± SD. dependent on its nuclease activity. B, MCF-7 cells were treated with control or DNA2 siRNAs. Seventy-two hours later, cells were harvested and subjected to comet analysis (Top). (Right) Representative images (Left) Quantitative analysis. (Bottom) cell growth of MCF-7 cells were measured by trypan blue exclusion assay at indicated time points. Western blot analysis was shown next to the graph indicating knockdown efficiency. C, U2OS cells were transfected with control siRNA or two different siRNAs specifically targeting DNA2 (#9 or #11). Seventy-two hours later, cell size was analyzed by Multisizer 3 Coulter counter. Median cell size is indicated in the graphs. (Top) Cells transfected with control siRNAs. (Bottom) Cells transfected with DNA2 siRNAs. D, U2OS cells were transfected with DNA2 siRNA (SMARTpool). Ninety-six hours later, cell lysates were prepared and subjected to Western blot analysis with the indicated antibodies. E, U2OS cells were transfected with DNA2 siRNAs (si#9 or si#11) and then treated with or without aphidicholin (Api). (Top) Images of SA-β-gal-stained cells 96 hr after transfection (scale bar: 50 μm). (Bottom) Mean ± SEM of percentages of SA-β-gal-positive cells. Results of Western blot analyses demonstrating effective DNA2 knockdown are shown next to the graph.

Figure S5. Effects of DNA2 depletion on the generation of ROS

A, There was no dramatic shift in ROS production in DNA2-deficient cells compared to control cells. U2OS cells were transfected with control siRNA (nontargeted; NT) or DNA2 siRNA (SMARTpool). Forty-eight hours later, cells were incubated with DCF-DA

(5 μM) for 1 hr and then analyzed by flow cytometry. (Left) Representative flow cytometry profiles. (Right) Quantitative summary. Each value is relative to the percentage of DCF-positive cells in the control cells, which was set to 1 and represents the mean ± SD. B, The presence of N-acetylcysteine, an antioxidant commonly used to block the effects of ROS failed to reverse senescence in DNA2-knockdown cells, which excluded the possibility that DNA2-depletion-induced senescence was due to DNA2's mitochondrial function. U2OS cells were transfected with control siRNA or DNA2 siRNAs (SMARTpool) and then treated with or without N-acetylcysteine (NAC, 10 mM). (Left) Images of SA-β-gal-stained cells 96 hr after transfection (scale bar: 50 μm). (Right) Mean ± SEM of percentages of SA-β-gal-positive cells. The effect of NAC on blocking ROS is shown at the bottom.

SUPPLEMENTARY METHODS

Plasmids, Small Interfering RNAs (siRNAs), and Transfection

DNA2 cDNA was purchased from Origene with sequence verification. Then DNA2 was cloned into N-terminal 3xFLAG-CMV vector (Sigma) via polymerase chain reaction (PCR) using primers with restriction sites. FLAG-DNA2 (del734-829) was generated by Epoch Biolabs, Inc. FLAG-DNA2 (D363A) and FLAG-DNA2 (K740E) were generated by using a QuickChange II Site-Directed Mutagenesis Kit. On-target smart pool siRNAs against DNA2 were purchased from Dharmacon Research (Lafayette, CO) (#9: AGACAAGGUUCCAGCGCCA; #10: UAACAUUGAAGUCGUGAAA; #11:

AAGCACAGGUGUACCGAAA; #12: GAGUCACAAUCGAAGGAUA). Two siRNAs (#9 and #11) were used specifically target DNA2 were also purchased from Dharmacon. siRNA-resistant constructs against #9 DNA2 sequence were generated by mutagenesis as described above by replacing the original sequence of DNA2 (ACA GAC AAG GTT CCA GCG CCA GAA CAA) with a different sequence (ACA GAC AAG GTA CCT GCG CCA GAA CAA), which maintains the original protein sequence. PCMV-H-RAS (V12) was purchased from Invitrogen. Based on the commercial protocols, transfection of indicated plasmids in U2OS cells was performed with FuGENE 6 reagent from Roche. Transfection of siRNAs into U2OS was performed with Oligofectamine (Invitrogen). Transfection of plasmids into MCF-10A cells was performed with Lipofectamine 2000 (Invitrogen).

Affinity Purification of PCNA Protein Complex

Whole cellular extracts were prepared from MCF-10A and MCF-10AT cells with RIPA buffer (50 mM Tris HCl, pH 7.4, 1% NP-40, 150 mM NaCl, 1 mM EDTA, 10% Na-deoxycholate, freshly added with 1 mM phenylmethylsulfonyl fluoride (PMSF), 1 mM Na_3VO_4, and 1 mM NaF) and precleaned with protein A/G plus-agarose beads (Santa Cruz) for 1 hr at 4°C. Then cellular extracts were subjected to immunoprecipitation with anti-PCNA antibody (Santa Cruz) (2 μg/ 1 mg cell lysis) overnight and then incubated with protein A/G agarose beads for 4 hr at 4°C. The immunocomplex was eluted in loading buffer by boiling at 95°C for 5 min and subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). The silver staining was performed with a SilverSNA kit for mass spectrometry (Pierce). Specific bands were excised and

digested, and the peptides were analyzed by mass spectrometry at the MD Anderson Proteomics Shared Resource.

Co-immunoprecipitation of DNA2 with CIdU at Stalled Replication Forks

The presence of DNA2 at stalled replication forks was assayed as previously described (17). Briefly, a total of $6x10^6$ cells were treated or untreated with 1 μM CPT for 1 hr. CPT was washed away, and cells were labeled with CIdU (100 μM) for 40 min. Cells were cross-linked in 1% formaldehyde for 15 min at room temperature and treated with 0.125 M glycine for 15 min at room temperature. Cells were scraped in cold phosphate-buffered saline (PBS). Cytoplasmic proteins were removed by incubation in hypotonic buffer (25 mM HEPES, pH 7.8, 1.5 mM $MgCl_2$, 10 mM KCl, 0.1% NP-40, 1 mM dithiothreitol, 0.25 mM PMSF, and protease inhibitors) for 10 min on ice. Then nuclei were pelleted and resuspended in sonication buffer (50 mM HEPES, pH 7.9, 140 mM NaCl, 1 mM EDTA, 1% Triton X-100, 0.1% SDS, 0.1% Na-deoxycholate, 0.5 mM PMSF, and protease inhibitors) and incubated for 10 min on ice. After sonication, the supernatant containing crude soluble chromatin was incubated with rat anti-BrdU antibody overnight at 4°C and then with protein A/G beads for 4 hr. The immunocomplex was eluted in loading buffer by boiling at 95°C for 5 min.

Comet Assay

DSB repair was analyzed by neutral comet assay using the Trevigen comet assay kit (4250-050-K) according to the manufacturer's instructions. Seventy-two hours after siRNA transfection, U2OS cells were subjected to comet analysis at the indicated time

points. After staining with SYBR green, comet images were captured by fluorescence microscopy.

HR Repair Analysis

DR-GFP, pCAGGS, and pCBASce plasmids were kindly provided by Dr. Maria Jasin (Memorial Sloan-Kettering Cancer Center, New York, NY). U2OS cells containing a single copy of the HR repair reporter substrate DR-GFP in a random locus were generated as previously described (19). GFP expressing plasmid (pEGFP-C1) was used for transfection efficiency control. Twenty-four hours after DNA2 siRNA transfection, cells were re-seeded; the next day, cells were transfected with pCBASce plasmids. Forty-eight hours later, flow cytometry analysis was performed to detect GFP-positive cells using a FACScalibur apparatus with CellQuest software (Becton Dickinson, San Jose, CA).

Flow Cytometry Analysis of BrdU Incorporation

After treatments, cells were labeled with BrdU (Sigma) for 30 min prior to fixation with 70% cold ethanol (-20°C). In the samples for CIdU (Sigma) and IdU (Sigma) staining, cells were labeled with CIdU (25 μM) for 30 min and then treated with CPT (1 μM) for 1 hr and released in culture medium containing IdU (250 μM) for 1 hr before fixation. The next day, cells were washed with cold PBS and incubated with 2 M HCl for 5 min at room temperature. Then cells were neutralized with 0.1 M sodium borate (pH 8.5). Cells were then incubated in 1% bovine serum albumin with 0.1% Triton X-100 for 30 min and mouse anti-BrdU antibody (1:400) for 1 hr to detect BrdU labeling or IdU labeling. Rat

anti-BrdU antibody (1:200) was used to detect CIdU labeling. After washing, cells were incubated in secondary antibodies (fluorescein isothiocyanate or rhodamine 1:400) for 30 min. Cells were resuspended in staining solution (10 μg/mL propidium iodide, 20 μg/mL RNAase A, and 0.05% Triton X-100). Cell cycle analysis was performed at the MD Anderson Cancer Center Flow Cytometry and Cellular Imaging Facility. For fluorescence-activated cell sorter (FACS) analysis, the percentage of cells with positive BrdU staining in each phase of the cell cycle was quantitated with CellQuest software and ModFit software (Verity Software House Inc.)

Immunofluorescent Staining

For detection of DNA-damage-induced foci of p-RPA34, RPA34, and RAD51, immunofluorescent staining was carried out essentially as described previously (19). To detect BrdU incorporation in the denatured conditions, U2OS cells were labeled with BrdU (10 μM) for 30 min before fixation. Then slides were incubated with 2 M HCl for 10 min and neutralized with 0.1 M sodium borate (pH 8.5). Then slides were incubated with mouse anti-BrdU antibody and fluorescein isothiocyanate-conjugated secondary antibody. Detection of BrdU incorporation in the native conditions was performed as previously described; cells were incubated with BrdU (10 μM) for 24 hr before fixation. Immunostaining was performed with anti-BrdU antibody without DNA denaturing. Slides were mounted in medium containing DAPI (Vector Laboratories, Burlingame, CA) and analyzed under a fluorescence microscope. The number of foci per cell was scored for at least 50 cells per sample.

Cell Proliferation Assay

Cell proliferation was measured by MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; Sigma) reduction. To test the cell proliferation rate, $1x10^4$ cells/well were seeded in a 96-well plate in a total volume of 100 μl in triplicate in each experiment. In the following days, 20 μl of MTT substrate (2 mg/mL) was added to each well and incubated with cells for 3 hr. Then the culture medium was removed and 100 μl of dimethyl sulfoxide was added. Plates were read at 490 nm and 650 nm (background) in a microplate reader (Molecular Devices). After subtraction of background, the cell viability was calculated as fold change relative to control cells. In MCF-7 cells transfected with NT siRNA or with DNA2 siRNA, we used trypan blue exclusion assay to determine cell viability at indicated time points and thereby monitor cell proliferation. In this assay, 0.1 mL of trypan blue stock solution (0.4% in PBS pH7.2) was added to 1 mL of cell suspension and the number of cells without taking up trypan blue (considered viable cells) were counted with a hemacytometer immediately in triplicates.

Colony-forming Assay

Transfection and colony-forming assay were performed as previously described. Briefly, U2OS cells were transfected with DNA2 siRNA or DNA2 constructs. The next day, cells were seeded at low density; cells were then left for 2 weeks to allow colonies to form. Colonies were stained with 2% methylene blue/50% ethanol, and colonies containing 50 or more cells were counted. To test the sensitivity of DNA2-depleted cells to replication-associated DSB-inducing agents, cells were treated with etoposide (Sigma) or CPT (Sigma) at the indicated concentrations. 1 hour later, the drug was removed and cells

were left to grow for 10-14 days to allow colony formation.

Soft Agar Assay

MCF-10A cells were transfected with indicated plasmids. Twenty-four hours later, cells were resuspended in culture medium containing 0.5% agarose (Sigma) and seeded onto a coating of 1% agarose. Colonies were scored 2 weeks after preparation. Colonies containing more than 50 cells were counted as positive.

Chromosomal Aberration Analysis

U2OS cells were transfected with control or DNA2 siRNAs. Seventy-two hours later, cells were subjected to cytological analysis at the MD Anderson Cancer Center Molecular Cytogenetics Core Facility. Briefly, cytological preparations were made following standard protocol. Cells were exposed to colcemid (0.04 μg/ml) for 1 hr, subjected to hypotonic treatment (0.075 M KCl for 20-25 min at room temperature), and fixed in a mixture of methanol and acetic acid (3:1). Slides were stained in Giemsa and examined blindly for structural and numerical abnormalities. These slides were decoded after the entire scoring of aberrations was completed. From each sample, a minimum of 35 metaphase spreads were analyzed, and representative spreads were captured using a Genetiscan imaging system.

Immunohistochemical Staining

Six cases of formalin-fixed, paraffin-embedded pancreatic tumors with their corresponding nonneoplastic tissues were obtained from US Biomax. After slides were deparaffinized with xylene and rehydrated through ethanol series, antigen retrieval was

carried out by placing the slides in 0.1 M sodium citrate buffer (pH 6.0) in a water bath for 30 min at 95°C. Endogenous peroxidase activity was quenched with 3% hydrogen peroxide in PBS for 10 min. Slides were washed three times with PBS and incubated for 1 hr in blocking buffer containing 10% goat serum for 30 min. Immunohistochemical staining was done by using the Vectastain ABC avidin biotin-peroxidase enzyme complex kit (Vector Laboratories), and DNA2 antibody (1:200) was incubated overnight at 4°C in a wet box. Slides were counterstained with hematoxylin and mounted with Permount aqueous medium.

ROS Measurements

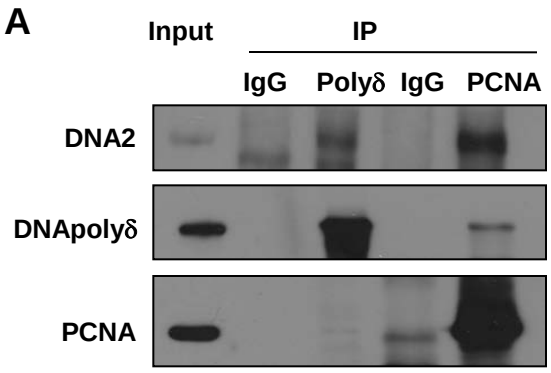
Cells were incubated with DCF-DA (Molecular Probes, 10 μM) for 1 hr. Then cells were collected in PBS and subjected to FACS using CellQuest 3.2 software for acquisition and analysis. Green fluorescence of DCF from oxidation of DCF-DA was measured through a 530-nm filter.

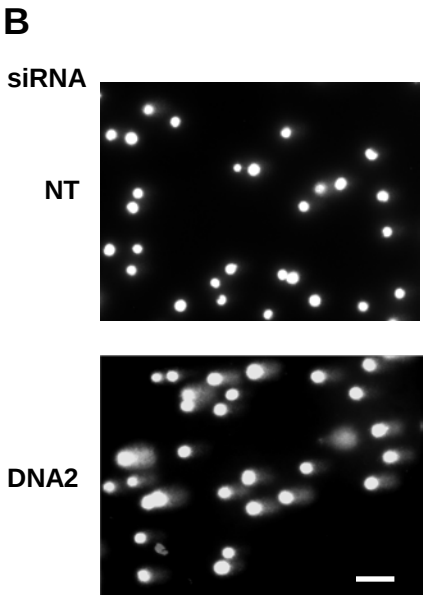
Senescence Assay

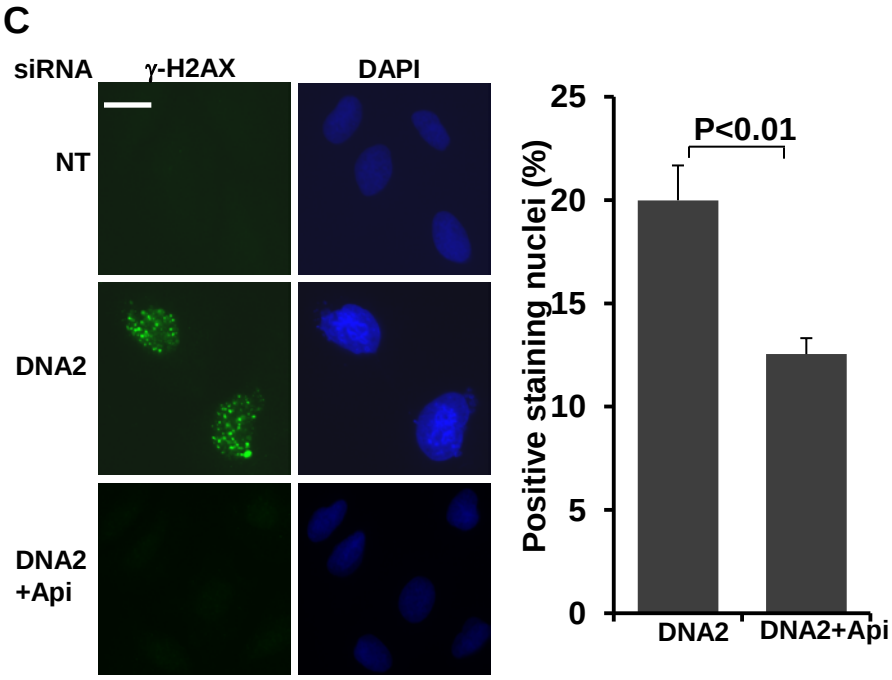
Senescence assay was performed with a senescence β-galactosidase staining kit (#9860; Cell Signaling) according to the manufacturer's protocol. Briefly, cells were seeded in six-well plates. After washing with PBS, cells were incubated with 1x fixation solution containing 2% formaldehyde and 0.2% glutaraldehyde solution in PBS. The cells were washed again and stained for 4 hr in solution containing 1 mg/ml X-gal, 40 mM citric acid/sodium phosphate (pH 6), 150 mM NaCl, and 2 mM MgCl2. The stain was removed, cells were rinsed with PBS, and positive staining was viewed and scored.

Gene expression data of breast cancer patients. Gene expression data from breast cancer patient cohort was used for analysis. Normalized gene expression data from NKI was obtained from pubic Merck website (http://www.rii.com/publications/2002/nejm.html).

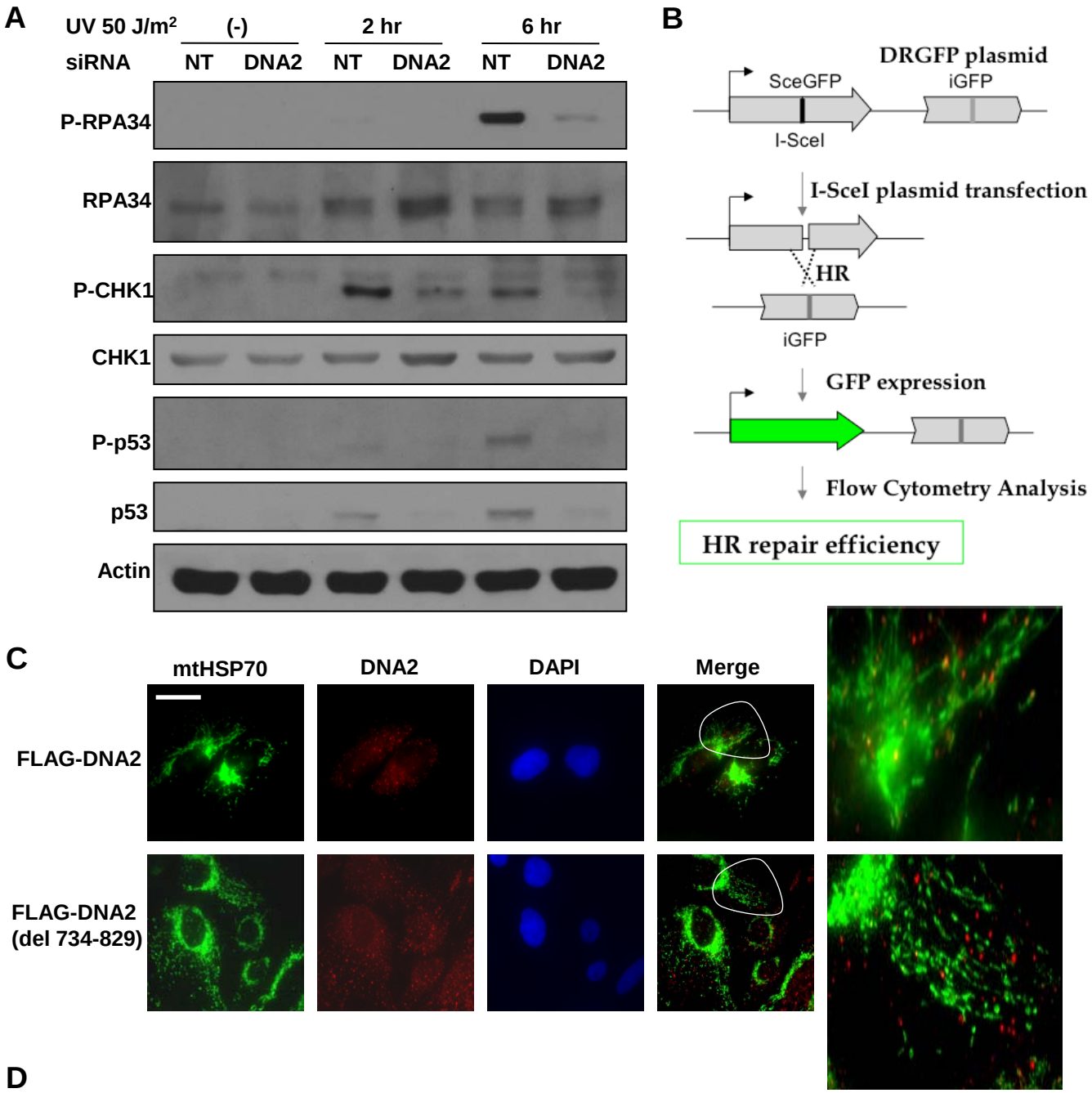
Supplemental Figure 1







Supplemental Figure 2



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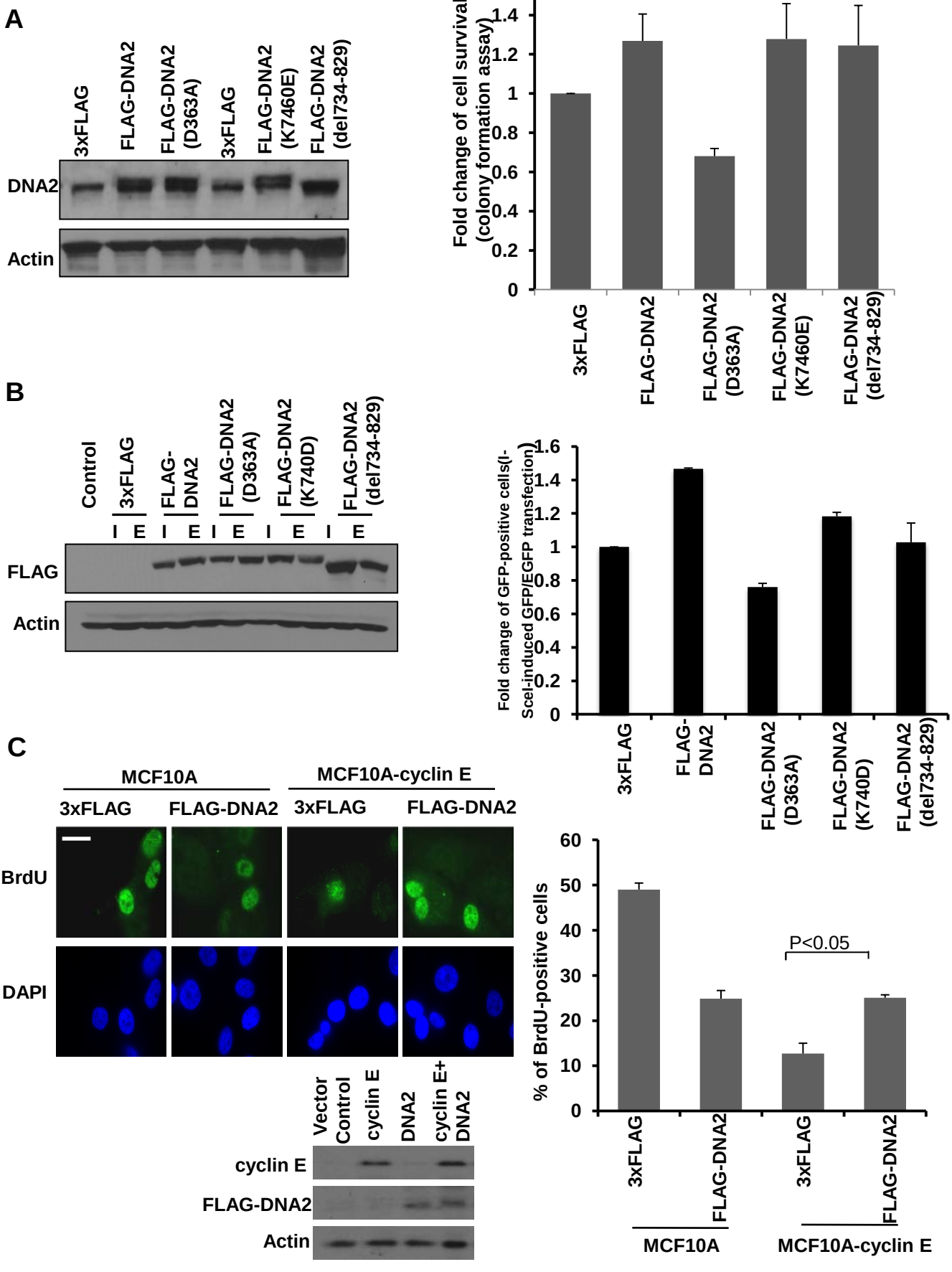

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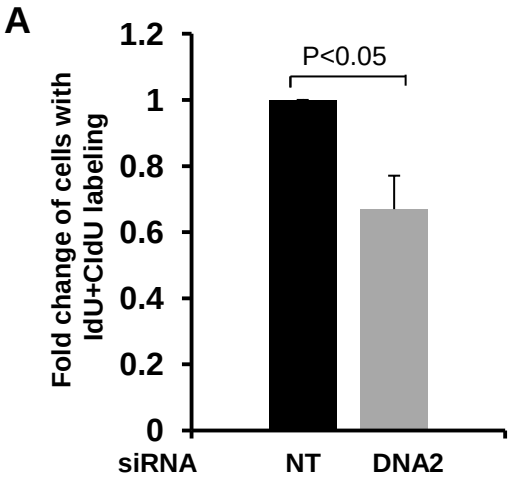

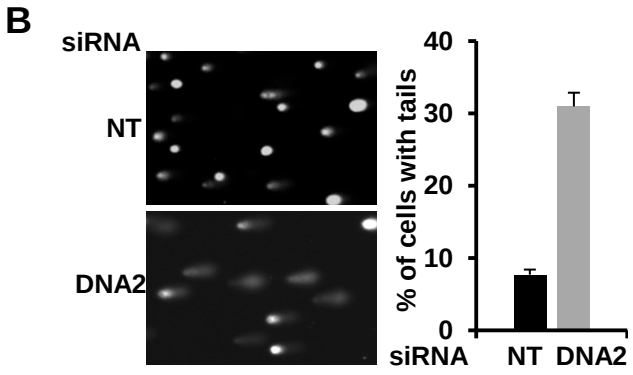

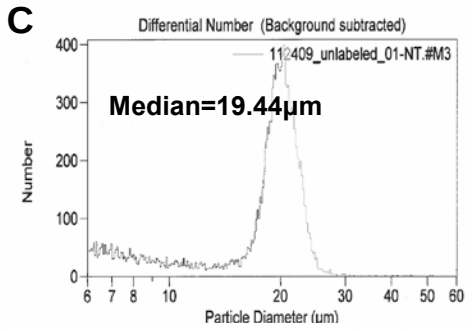

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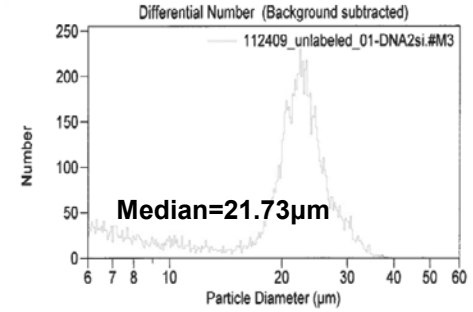

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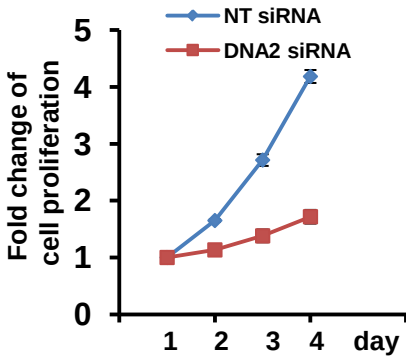

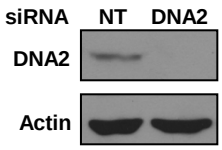

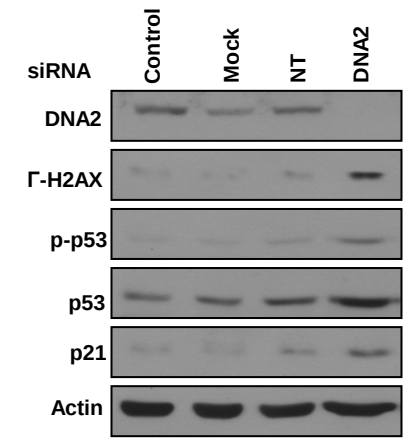

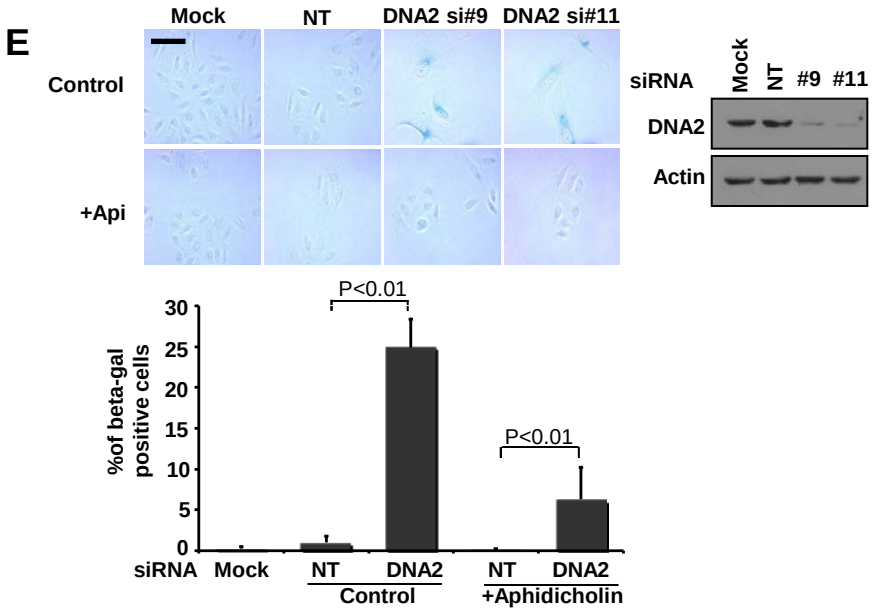

Supplemental Figure 5