Supporting Information

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SI Materials and Methods

Cell Lines and Plasmids. Hct116 (J. Simon, Fred Hutchinson Cancer Research Center) and U2OS cells (American Type Culture Collection) were maintained in DMEM with 10% (vol/vol) FBS and penicillin/streptomycin. The cyclin-dependent kinase (Cdk) 2 plasmid was described (1) and the Cdk2AF expression plasmid was obtained from J. Roberts (Fred Hutchinson Cancer Research Center).

Antibodies and immunocytochemistry. The antibodies used were as follows: cyclin E (HE12 for Western Blotting, HE111 for immunoprecipitation, Santa Cruz Biotechnology), Cdk2 (Western blotting: M2, immunoprecipitation D-12; Santa Cruz Biotechnology), anti-Cdk1 phospho-Tyr15 (Calbiochem), phosphohistone H2A.X (JBW301; Millipore), histone H2A.X (07-627; Millipore), protein phosphatase 2A (PP2A) (610555; BD Biosciences), Mus81 (MTA30 2G10/3, Abcam), and anti-HA (12CA5). The cyclin A antibody was a gift from J. Roberts (Fred Hutchinson Cancer Research Center). Immunocytochemistry using anti-phosphohistone H2A.X antibody was performed according to manufacturer's instructions (Millipore).

Flow Cytometry. For γH2AX staining, cells were harvested and fixed in 70% (vol/vol) ethanol overnight at 4 °C. Cells were washed twice in BSA-T-PBS [1% (vol/vol) BSA and 0.2% (vol/vol) Triton X-100 in PBS] and incubated with mouse γH2AX antibody (1:500) for 1 h at room temperature (RT). Cells were washed twice in BSA-PBS and incubated with goat anti-mouse Alexa Fluor 488 conjugated secondary antibody (Invitrogen) for 30 min at RT. After washing twice in BSA-PBS, samples were stained with propidium iodide. For annexin V Apoptosis assay, cells were harvested by trypsinization, washed twice with PBS, and resuspended in 1× binding buffer (10 mM Hepes at pH 7.4, 140 mM NaCl, and 2.5 mM $CaCl_2$) at a concentration of 1×10^6 cells per mL, 100 μL of cells were incubated with 5 μL of FITC-annexin V antibody (BD Biosciences) and 5 μg/mL propidium iodide for 15 min at RT. Four hundred microliters of 1× binding buffer was added, and cells were analyzed by flow cytometry.

Immunoprecipitations, Immunoblotting, and Kinase Assays. Cells were lysed in Nonidet P-40 lysis buffer [50 mM Tris·HCl at pH 8.0, 150 mM NaCl, and 1% (vol/vol) Nonidet P-40] supplemented with protease and phosphatase inhibitors. Lysates were normalized by Bradford assay and analyzed by immunoblotting as described (1). Histones were extracted by lysing cells in Triton extraction buffer [TEB: PBS containing 0.5% (vol/vol) Triton X-100, 2 mM PMSF, and 0.02% (wt/vol) NaN_3]. After centrifugation of lysate at 6,500 × *g* for 10 min, nuclei were washed in TEB and resuspended in 0.2 M HCL at a density of 4×10^7 nuclei per mL and incubated overnight at 4 °C. Samples were centrifuged at 6,500 × *g* for 10 min, and supernatant was neutralized with NaOH. Immunoprecipitation of Cdk2 was performed from whole-cell lysates for greater than 2 h at 4 °C by using Protein G beads. We noted that Cdk2AF did not immunoprecipitate as well as wt-Cdk2. For cyclin E and cyclin A kinase assays, histone H1 phosphorylation was measured. One hundred fifty micrograms of lysate (cyclin E) or 10 μg of lysate (cyclin A) was immunoprecipitated at 4 °C for no more than 2 h. IPs were washed twice in Nonidet P-40 lysis buffer and once in kinase buffer (50 mM Hepes at pH 7.4, 10 mM $MgCl_2$, and 1 μM DTT). Beads were resuspended in 25 μL of reaction buffer [50 mM Hepes at pH 7.4, 10 mM $MgCl_2$, 1 μM DTT, 1 μg/mL histone H1, 30 μM ATP, and 0.2 μCi/μL ATP (γ^{32P}; Perkin-Elmer)] and incubated at 37 °C for 30 min with intermittent mixing. Laemmli sample buffer was added, samples were boiled at 95 °C, and run on a 12% (wt/vol) polyacrylamide gel. Gels were dried and imaged by using film or a Typhoon Trio imager.

Cyclin E Pulse–Chase Analysis. Cells were starved in media without methionine for 20 min, labeled for 20 min in 0.3 mCi/mL TranS^{35}-label (MP Biomedicals), and chased in normal media with 0.8 mg/mL methionine for the indicated times. Cells were harvested by trypsinization and lysed in Nonidet P-40 Lysis buffer. Extracts were precleared with protein A beads before immunoprecipitation with cyclin E antibody. Beads were washed three times with RIPA buffer, resuspended in Laemmli sample buffer, boiled at 95 °C, and run on an 8% (wt/vol) polyacrylamide gel. Gels were dried and imaged by using a Typhoon Trio imager.

Quantitative PCR Assays. Total RNA was isolated from cells by using TRIzol Reagent according to the manufacturer's instructions (Life Technologies). RNA was treated with DNase and cDNA was synthesized with the High Capacity Reverse Transcription Kit (Applied Biosystems). Quantitative PCR was performed by using Taqman Universal PCR Master Mix and cyclin E specific Taqman gene expression assay on a ABI Prism 7700 (Applied Biosystems).

Microfluidics-Assisted Replication Track Analysis. Microchannel fabrication, DNA fiber stretching, and replication track analysis was done as described (2, 3). Labeling of cells was done as described in figure legends. Percent origin firing was calculated as (number of tracks with three consecutive segments IdU-EdU-IdU/total number of tracks counted) × 100. Mean EdU segment lengths of EdU-IdU tracks were used to derive fork progression rate (kilobases per minute), with the conversion factor 1 μm = 3.9 kb and labeling interval, 30 min, as a denominator. The presented values are averages of fork rates across three experiments. Percent of ongoing forks was calculated as [number of EdU-IdU tracks/(number of EdU-only tracks + number of EdU-IdU tracks)] × 100. Statistical significance of the differences in origin firing frequencies and percent ongoing forks was determined by Student's *t* tests. For track measurements in normal replication (Fig. 1*D*), lengths of EdU and IdU segments in EdU-IdU tracks were quantified and plotted as frequency distributions. For track length measurements in the presence of HU, EdU segments in EdU-IdU tracks of untreated cells or HU-treated cells were quantified and plotted as frequency distributions (Fig. 5*F*). To determine statistical significance, the difference in length distributions of EdU segments in untreated vs. treated cells was evaluated by the Kolmogorov–Smirnov (KS) test (2). Values from three experiments were pooled. In both $Cdk2^{+/+}$ and $Cdk2^{AF/AF}$ cells, the difference between untreated and HU-treated EdU tracks was significant ($P < 0.001$) in both cell types. However, the D-statistic (maximal difference) was higher in the $Cdk2^{AF/AF}$ cells compared with the $Cdk2^{+/+}$ cells (0.4509 vs. 0.2102), indicating that the $Cdk2^{AF/AF}$ cells replicate DNA to a greater extent than $Cdk2^{+/+}$ cells in HU. Representative images of labeled tracks are shown in Fig. S8.

1. Clurman BE, Sheaff RJ, Thress K, Groudine M, Roberts JM (1996) Turnover of cyclin E by the ubiquitin-proteasome pathway is regulated by cdk2 binding and cyclin phosphorylation. *Genes Dev* 10(16):1979–1990.
2. Sidorova JM, Kehrli K, Mao F, Monnat R, Jr. (2013) Distinct functions of human RECQ helicases WRN and BLM in replication fork recovery and progression after hydroxyurea-induced stalling. *DNA Repair (Amst)* 12(2):128–139.
3. Sidorova JM, Li N, Schwartz DC, Folch A, Monnat RJ, Jr. (2009) Microfluidic-assisted analysis of replicating DNA molecules. *Nat Protoc* 4(6):849–861.

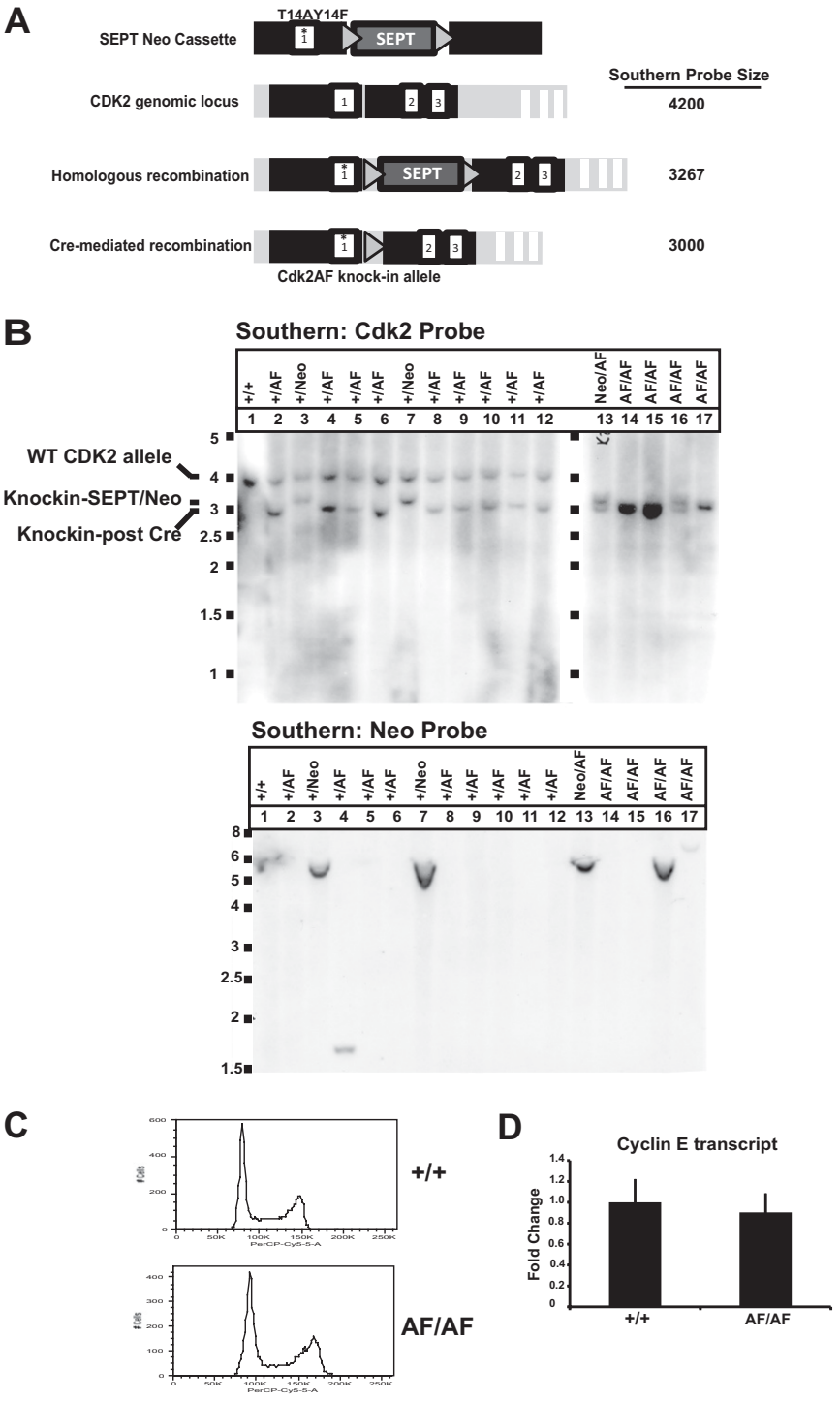

Fig. S1. Adeno-associated virus (AAV)-gene targeting and characterization of Cdk2$^{AF/AF}$ cells. (*A*) AAV targeting strategy and indicated Cdk2 Southern fragment size for various Cdk2 alleles. Cdk2 homology arms are shown in black. (*B*) Southern blots of Cdk2$^{+/+}$, Cdk2$^{+/AF}$, Cdk2$^{+/-}$ (Cdk2$^{+/Neo}$), Cdk2 $^{Neo/AF}$, and Cdk2$^{AF/AF}$ clones probed with Cdk2 probe (*Upper*) or Neo probe (*Lower*). Note: Clones 4 and 16 were incorrect and were not used. (*C*) Asynchronous cell cycle profiles of Cdk2$^{+/+}$ and Cdk2$^{AF/AF}$ cell lines. (*D*) Cyclin E transcript levels were assayed by quantitative PCR 13 h after serum/leucine release. Data represents average with SD from three independent data points.

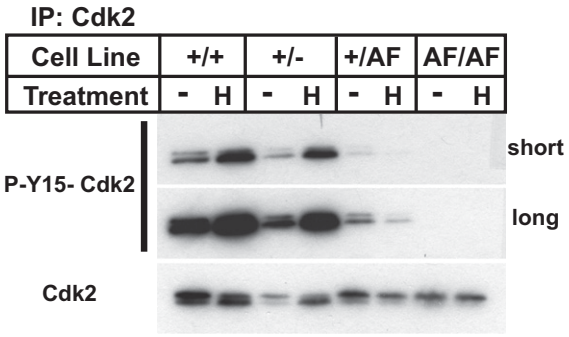

Fig. S2. Cdk2 inhibitory phosphorylation is increased after HU treatment. Cdk2$^{+/+}$, Cdk2$^{+/-}$, Cdk2$^{+/AF}$, and Cdk2$^{AF/AF}$ cells were untreated or treated with HU. Cdk2 Y15 phosphorylation and Cdk2 abundance are shown.

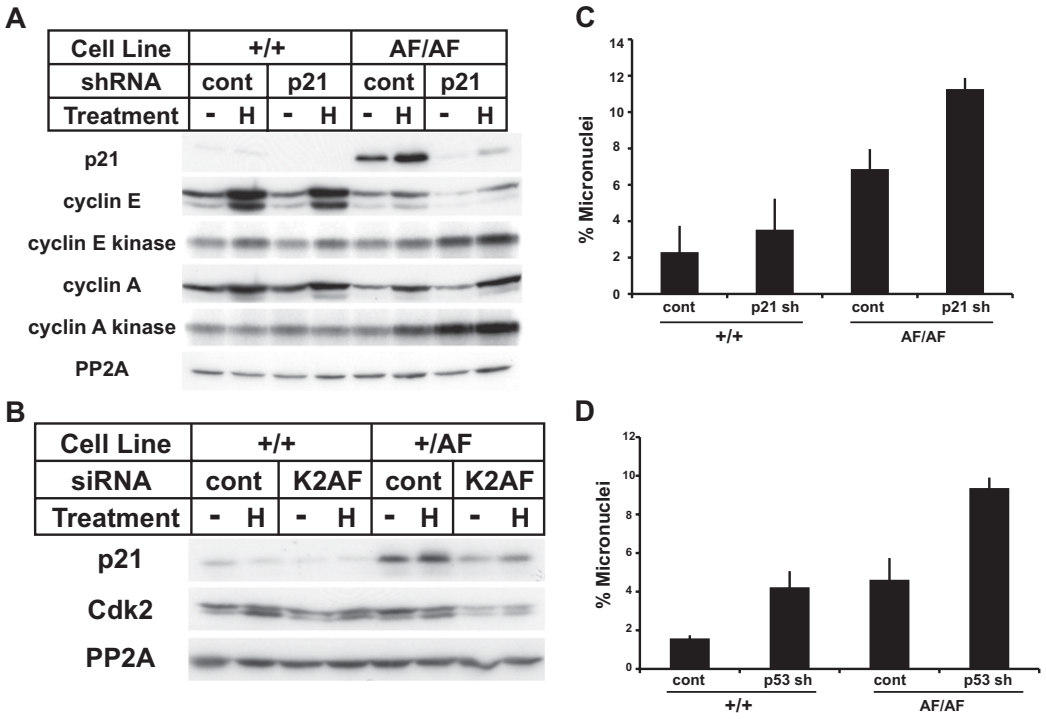

Fig. S3. Loss of Cdk2 inhibitory phosphorylation induces p21/Cip1 expression and causes genomic instability. (*A*) Cdk2$^{+/+}$ and Cdk2$^{AF/AF}$ cells were transduced with control or p21 shRNA lentiviruses and treated with HU as indicated. p21, cyclin E, and cyclin A abundance is shown, as well as cyclin E and cyclin A kinase activity. (*B*) Cdk2$^{+/+}$ and Cdk2$^{AF/AF}$ cells were treated with either control or Cdk2AF-specific siRNA and grown asynchronously or treated with HU. (*A* and *B*) PP2A-loading control. (*C* and *D*) Cdk2$^{+/+}$ and Cdk2$^{AF/AF}$ cells were transduced with control, p21 shRNA lentivirus (*C*), or p53 shRNA retrovirus (*D*), and the percentages of cells with micronuclei are shown.

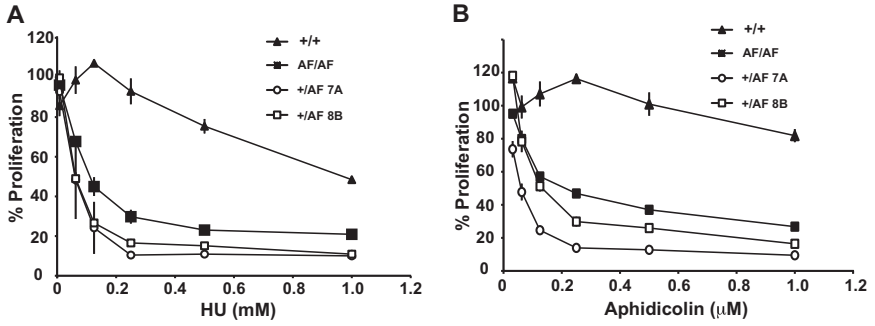

Fig. S4. Cdk2$^{AF/AF}$ cells are sensitive to replication inhibitors. (*A*) Cdk2$^{+/+}$, Cdk2$^{AF/AF}$, Cdk2$^{+/AF}$ clone 7A, and Cdk2$^{+/AF}$ clone 8B cells were seeded on day 0. On day 1, HU was added at the indicated concentrations and was removed after 24 h. Proliferation was assayed 3 d later (day 5) by using Crystal Violet. (*B*) Same as in *A*, but with Aphidicolin. Percent proliferation is calculated as the percentage of proliferation relative to untreated cells.

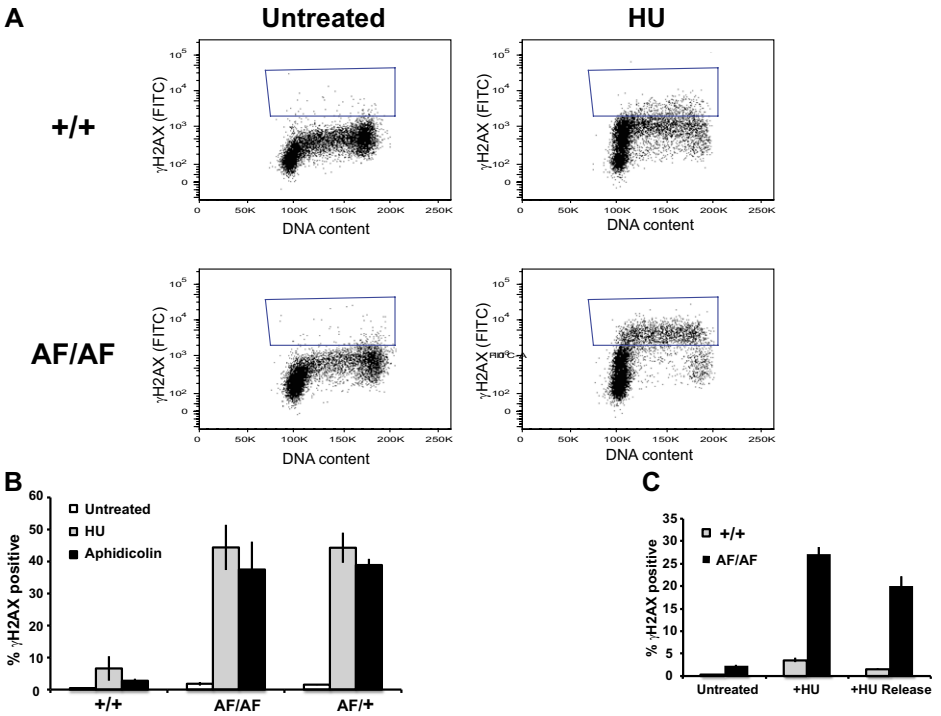

Fig. S5. Loss of inhibitory phosphorylation leads to accumulation of irreparable DNA damage during replication stress. (*A*) Flow cytometry-based assay to measure γH2AX abundance. Highly positive γH2AX cells were gated as shown. The $Cdk2^{+/+}$ HU sample was used to position the γH2AX-positive gate to include only those cells that equaled or exceeded the most highly positive cells. (*B*) $Cdk2^{+/+}$, $Cdk2^{AF/AF}$, or $Cdk2^{+/AF}$ cells were untreated or treated for 7 h with either HU or aphidicolin and γH2AX abundance was assayed by flow cytometry. (*C*) $Cdk2^{+/+}$ and $Cdk2^{AF/AF}$ cells were treated with HU for 7 h or treated with HU and released into media without HU for 17 h. γH2AX-positive cells were identified by flow cytometry.

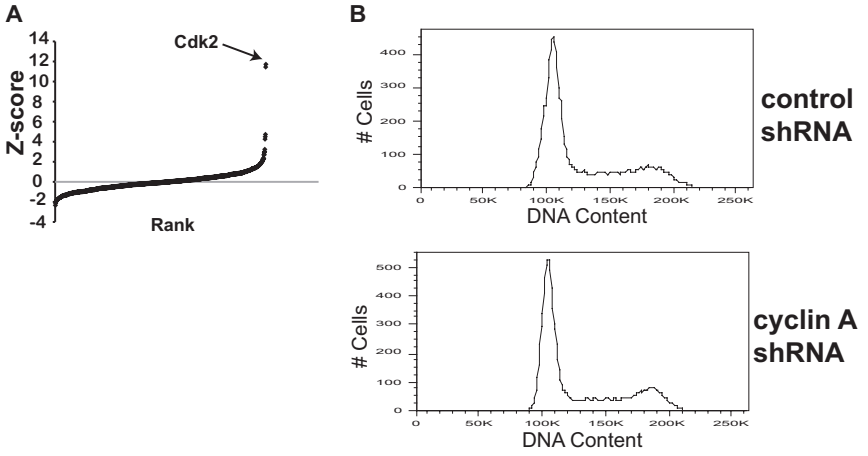

Fig. S6. Characterization of cyclin and kinase requirements in the Cdk2AF replication stress phenotype. (*A*) Identification of hits of kinome siRNA screen by Z-score and rank. Z-scores were calculated for each siRNA set (*y* axis) and numerically ranked (*x* axis). Cdk2 was the top hit. For more details about the siRNA screen, see *Materials and Methods*. (*B*) Cell cycle profile of $Cdk2^{AF/AF}$ cells expressing either a control or cyclin A shRNA after 7 h of HU treatment.

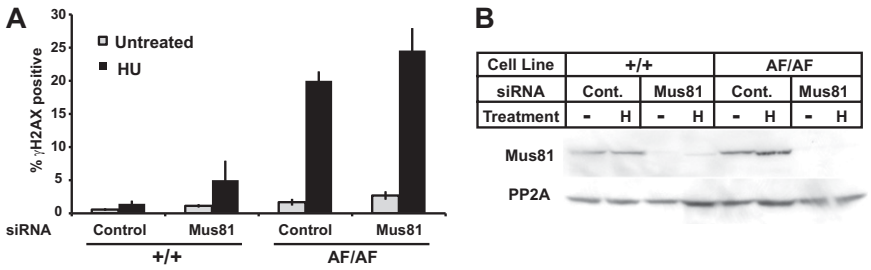

Fig. S7. Mus81 is not required for DNA damage during replication stress in Cdk2$^{AF/AF}$ cells. (*A*) Cdk2$^{+/+}$ or Cdk2$^{AF/AF}$ cells were transfected with control or Mus81 siRNA. Forty hours later, cells were left untreated or treated with HU for 7 h. Cells were harvested and analyzed by flow cytometry for γH2AX abundance. (*B*) Western blot from lysates isolated from *A* to confirm Mus81 depletion.

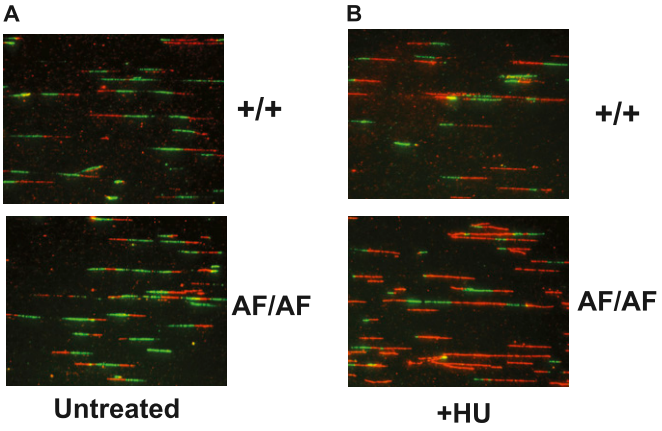

Fig. S8. Representative images of labeled forks from the mARTA analysis. Images from untreated (*A*) or HU treated (*B*, 3-h treatment) Cdk2$^{+/+}$ or Cdk2$^{AF/AF}$ cells.