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Supplemental material

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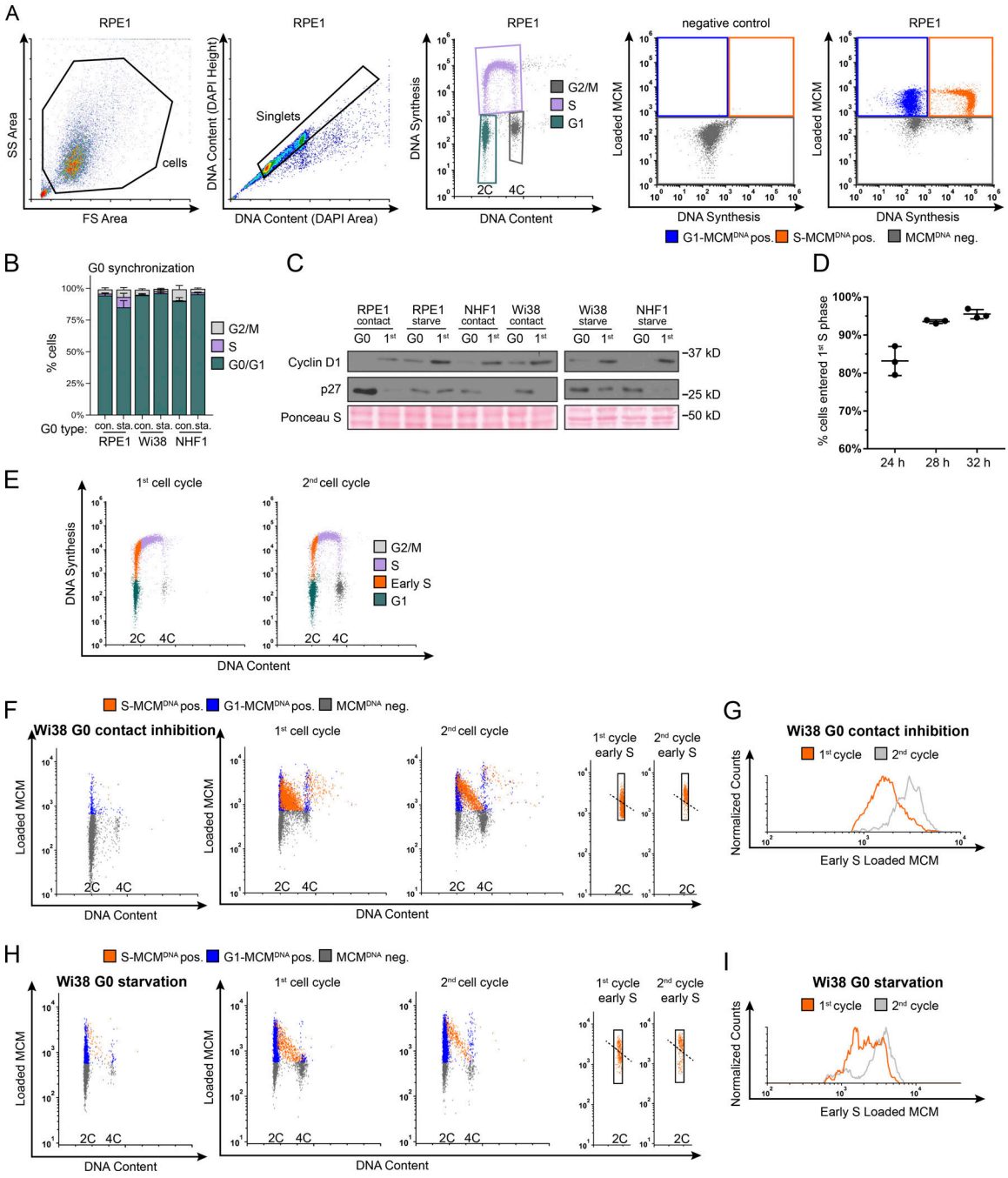

Figure S1. **Flow cytometry gating and alternate cell lines. (A)** Flow cytometry gating to isolate cells for analysis. Proliferating RPE1 cells (Fig. 1 A) were processed for analytical flow cytometry for chromatin-bound proteins, labeling DNA content (DAPI), loaded MCM (anti-Mcm2), and DNA synthesis (EdU). Cells were labeled with 10 µM EdU for 30 min before harvesting. Gating to isolate cells from debris is forward scatter (FS) area versus side scatter (SS) area, cells gate. Gating to isolate single cells from doublets is DAPI area versus DAPI height, singlets gate. Gating to determine cell cycle phase distributions is DNA content versus DNA synthesis. Color gating for S-MCM^{DNA}–positive (orange), G1-MCM^{DNA}–positive (blue), and MCM^{DNA}-negative (gray) is on DNA synthesis versus loaded MCM using a negative control sample without Mcm2 primary antibody or EdU, but with donkey-anti-mouse-488 secondary antibody and 647-azide as a measure of background signals. **(B)** Cell cycle phases of RPE1, Wi38, and NHF1 cells synchronized in G0 by contact inhibition or mitogen starvation from F–I; Fig. 1 E; and Fig. S2, A–F. *n* = 3, mean with SD. **(C)** Immunoblot for cyclin D1 or p27 of total protein lysate from RPE1 cells synchronized in G0 or released into the first cell cycle (24 h) as in Fig. 1 C. **(D)** Percentage of S phase cells (EdU-positive) defined by flow cytometry. RPE1 cells were synchronized in G0 by contact inhibition and released into the cell cycle with continuous 1 µM EdU at the time of release, harvesting cells 24, 28, and 32 h after release from G0. *n* = 3, mean with SD. **(E)** Flow cytometry of DNA synthesis versus DNA content of cells from Fig. 1 E, marking early S phase in orange. **(F)** Flow cytometry of chromatin-bound protein as in Fig. 1 A for Wi38 cells synchronized in G0 by contact inhibition in 0.1% FBS for 72 h and released from G0 into the cell cycle, harvesting 24 h after release (first cell cycle) and 48 h after release (second cell cycle). Early S gates applied as in Fig. 1 E. **(G)** Loaded MCM in early S phase determined by flow cytometry as in Fig. 1 E from Wi38 in F. **(H)** Flow cytometry of chromatin-bound protein as in Fig. 1 A for Wi38 cells synchronized in G0 by mitogen starvation in 0.1% FBS for 72 h and released from G0 into the cell cycle, harvesting 24 h after release (first cell cycle) and 48 h after release (second cell cycle). Early S gates applied as in Fig. 1 E. **(I)** Loaded MCM in early S phase determined by flow cytometry as in Fig. 1 E from Wi38 in H.

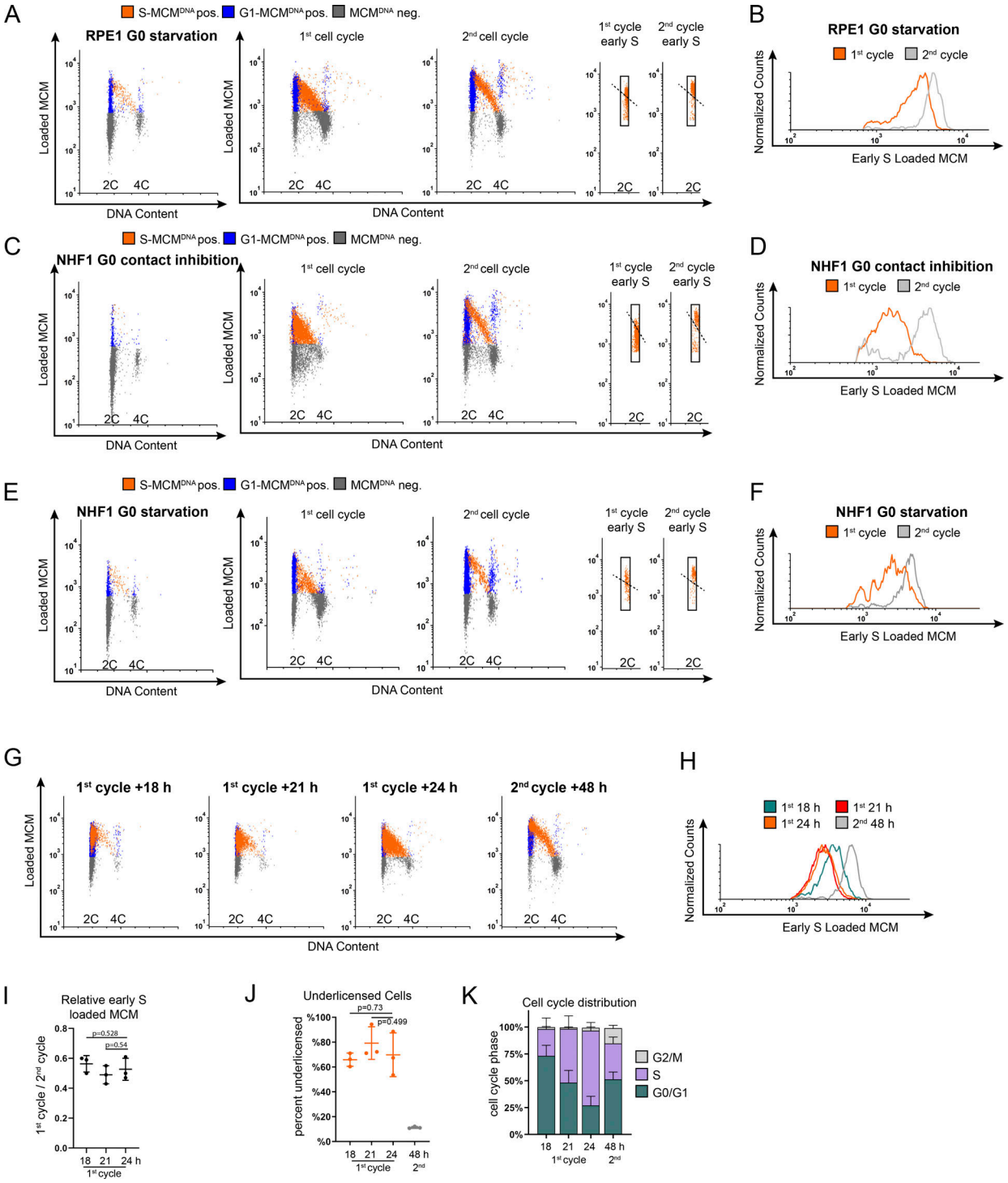

Figure S2. **Additional cell lines are underlicensed in the first cell cycle. (A)** Flow cytometry of chromatin-bound protein as in Fig. 1 A for RPE1 cells synchronized in G0 by mitogen starvation in 0% FBS for 72 h and released from G0 into the cell cycle, harvesting 24 h after release (first cell cycle) and 48 h after release (second cell cycle). Early S gates applied as in Fig. 1 E. **(B)** Loaded MCM in early S phase determined by flow cytometry as in Fig. 1 E from A. **(C)** Flow cytometry of chromatin-bound protein as in Fig. 1 A for NHF1 cells synchronized in G0 by contact inhibition in 0.1% FBS for 72 h and released from G0 into the cell cycle, harvesting 24 h after release (first cell cycle) and 48 h after release (second cell cycle). Early S gates applied as in Fig. 1 E. **(D)** Loaded MCM in early S phase determined by flow cytometry as in Fig. 1 E from NHF1 in C. **(E)** Flow cytometry of chromatin-bound protein as in Fig. 1 A for NHF1 cells synchronized in G0 by mitogen starvation in 0.1% FBS for 72 h and released from G0 into the cell cycle, harvesting 24 h after release (first cell cycle) and 48 h after release (second cell cycle). Early S gates applied as in Fig. 1 E. **(F)** Loaded MCM in early S phase determined by flow cytometry as in Fig. 1 E from NHF1 in E. **(G)** Flow cytometry of chromatin-bound protein as in Fig. 1 A for RPE1 cells synchronized in G0 by contact inhibition in 10% FBS and released, collecting 18, 21, 24 h (first cell cycle) or 48 h (second cell cycle) after release. **(H)** Loaded MCM in early S phase determined by flow cytometry as in Fig. 1 E from NHF1 in G. **(I)** Relative early S phase DNA-loaded MCM plotted as mean loaded MCM in the first cycle divided by mean loaded MCM in second cycle from cells in H. $n = 3$, mean with SD. **(J)** Percentage of underlicensed cells from G. $n = 3$, mean with SD. **(K)** Cell cycle phase percentages of cells from G. $n = 3$, mean with SD.

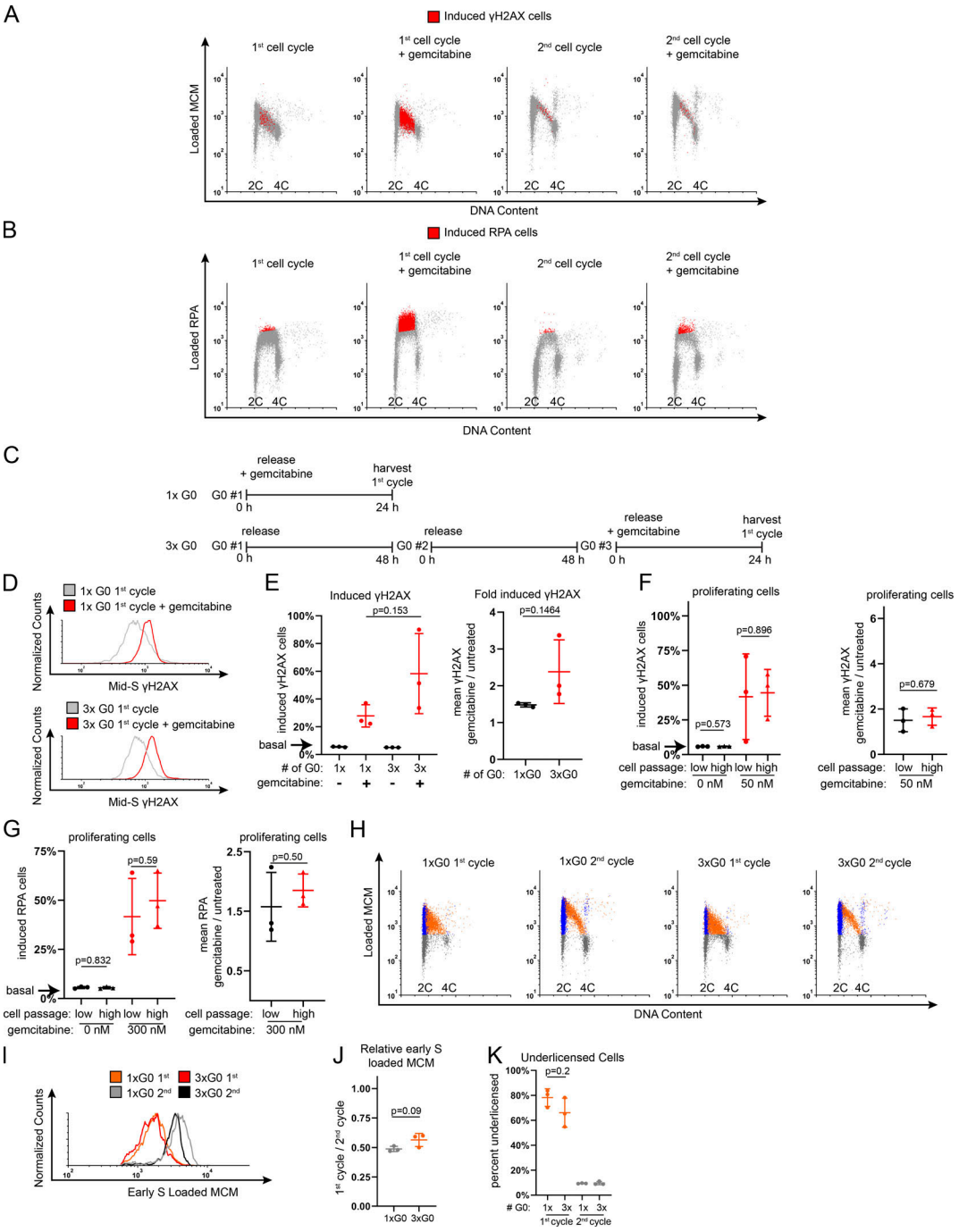

Figure S3. **Replication stress sensitivity after single or repeated transitions between G0 and proliferation. (A)** Flow cytometry of chromatin-bound protein from cells in Fig. 2 B. Red cells are gemcitabine (replication stress)-induced γH2AX-positive, as indicated in Fig. 2 C. **(B)** Flow cytometry of cells treated as Fig. 2 F i. Red cells are gemcitabine (replication stress)-induced RPA-positive. **(C)** Plan. For 1× G0: RPE1 cells were synchronized in G0 by contact inhibition for 48 h, then released into the cell cycle with 5 nM gemcitabine, harvesting cells 24 h after release. For 3× G0: RPE1 cells were synchronized in G0 by contact inhibition for 48 h, released into the cell cycle without gemcitabine, and grown to confluency to repeat the G0 by contact inhibition a second time, then repeating once again for a third G0. Then cells were released into the cell cycle with 5 nM gemcitabine, harvesting cells 24 h after release. **(D)** Flow cytometry of chromatin-bound proteins from cells treated as in C, measuring DNA content (DAPI) and γH2AX (anti-H2AX phospho S139). Histograms plot γH2AX of mid-S phase cells as described in Fig. 2 C. **(E)** Left: Percentage of replication stress–induced γH2AX in 1× G0 and 3× G0 released into the first cycle from C. Right: Comparison of γH2AX intensity per cell presented as fold-change between gemcitabine-treated and untreated cells from C. *n* = 3, mean with SD. **(F)** Proliferating cells passage 1 from storage (low) or passage 9 (high, ∼24 more cell cycles) were treated with 50 nM gemcitabine for 2 h, and then analyzed by flow cytometry as in Fig. 2 C. Left: Percentage of replication stress–induced γH2AX in low and high passage. Right: Comparison of γH2AX intensity per cell presented as fold-change between gemcitabine-treated and untreated cells. *n* = 3, mean with SD. **(G)** Cells treated and analyzed as in F stained for DNA-bound RPA. *n* = 3, mean with SD. **(H)** Cells synchronized in 1× G0 or 3× G0 as in C, collected without drug treatment for flow cytometry as in Fig. 1 A. **(I)** Loaded MCM in early S phase determined by flow cytometry as in Fig. 1 E from H. **(J)** Relative mean MCM loaded in early S phase in the first cycle divided by the second cycle as indicated. *n* = 3, mean with SD. **(K)** Percentage of underlicensed cells from H. *n* = 3, mean with SD.

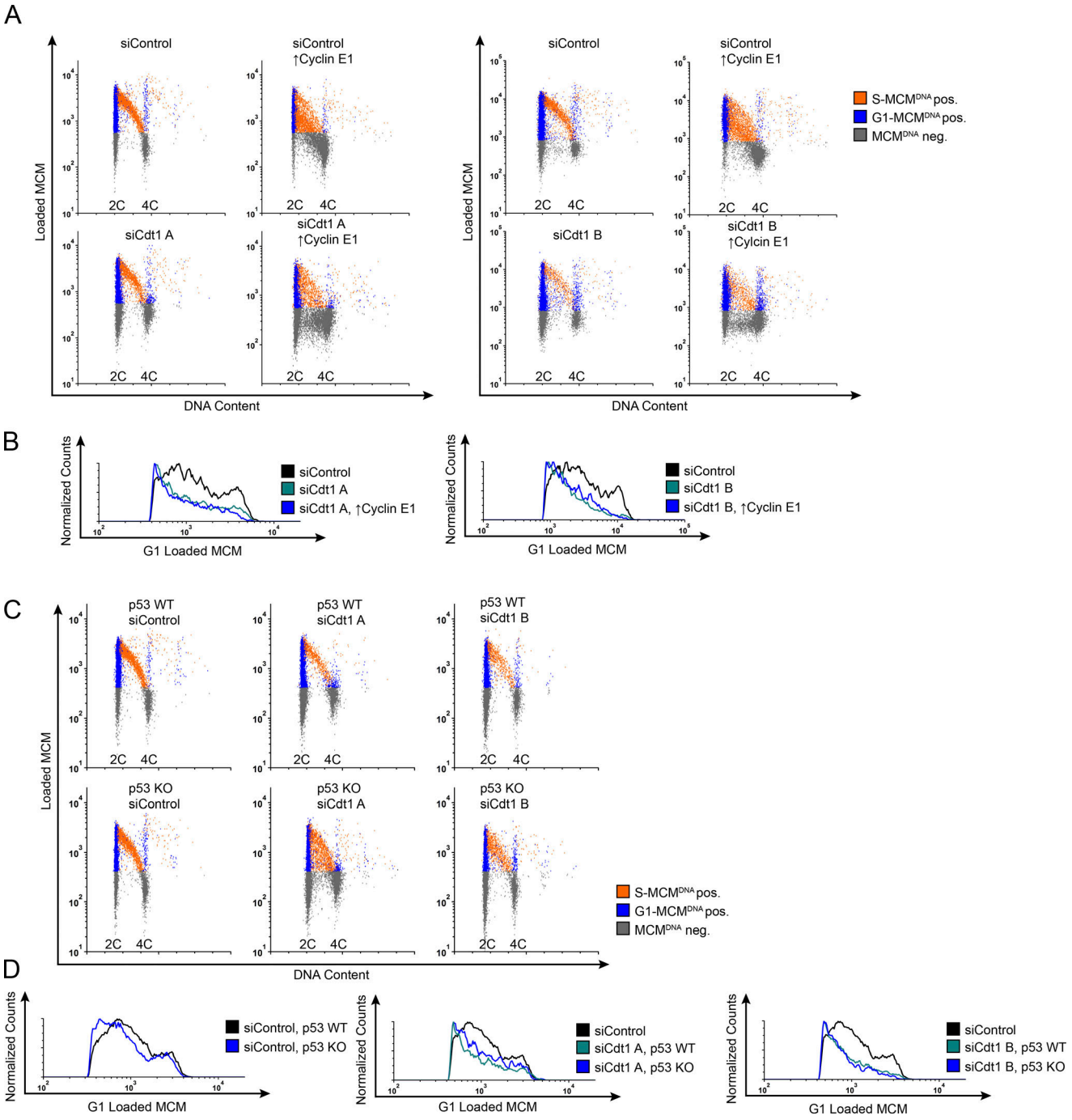

Figure S4. **Flow cytometry plots of siRNA in proliferating cells. (A)** Flow cytometry of chromatin-bound proteins for cells shown in Fig. 3 C, measuring DNA content (DAPI), loaded MCM (anti-MCM2), and DNA synthesis (EdU). **(B)** Loaded MCM in G1 cells from Fig. 3 C. **(C)** Flow cytometry of chromatin-bound protein for cells shown in Fig. 4 C, measuring DNA content (DAPI), loaded MCM (anti-Mcm2), and DNA synthesis (EdU). **(D)** Loaded MCM in G1 cells from Fig. 4 C. Note the black siControl p53 WT are the same sample on all three histograms.

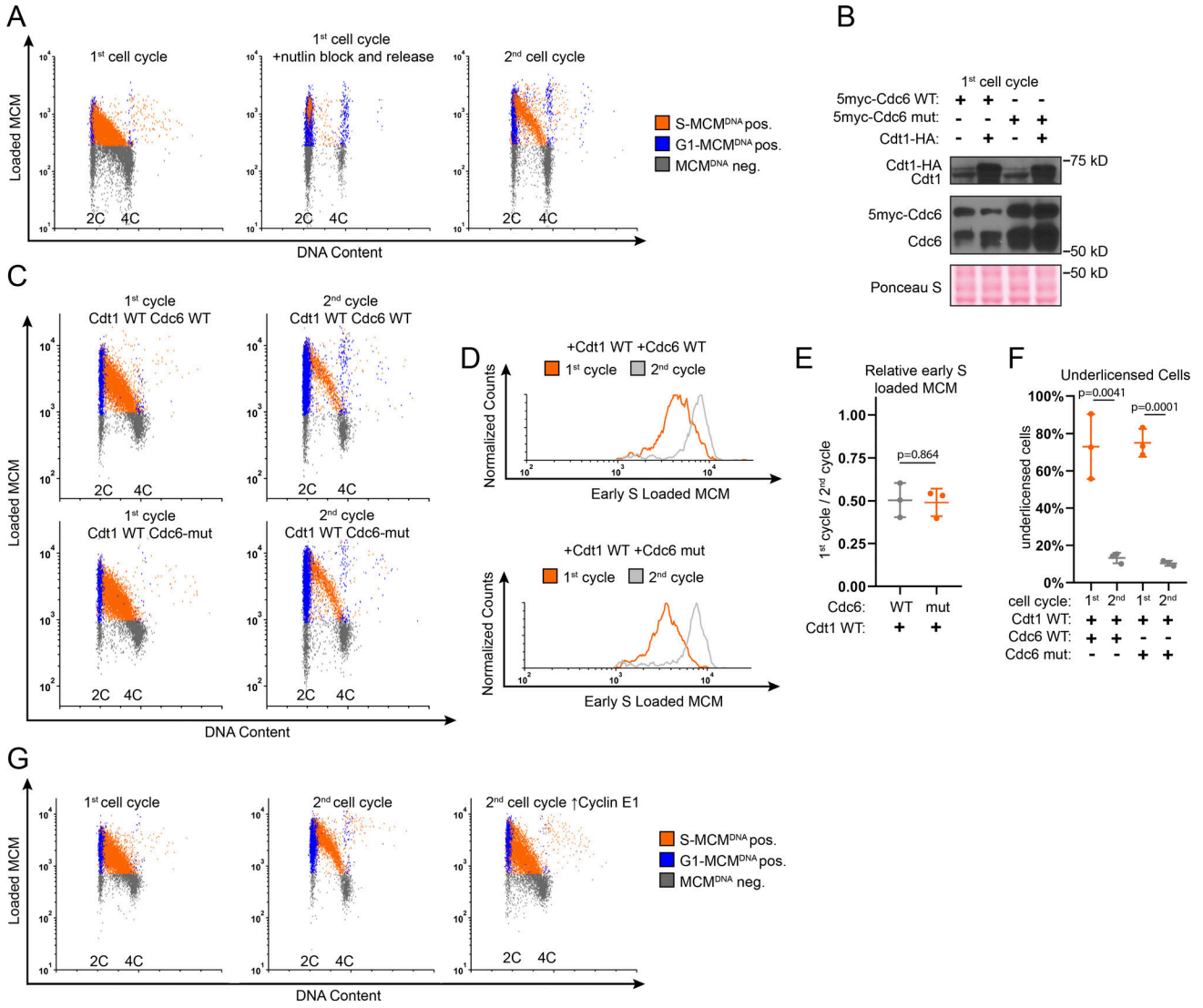

Figure S5. **Overproduction of Cdt1 and a stable Cdc6 mutant do not rescue underlicensing in the first cell cycle after G0. (A)** Flow cytometry of chromatin-bound protein from cells shown in Fig. 7 C, measuring DNA content (DAPI), loaded MCM (anti-Mcm2), and DNA synthesis (EdU). The first cell cycle population is 24 h from G0. The first cycle with nutlin block and release is cells treated with 10 µM nutlin-3a from 10–18 h, then released and harvested 26 h after G0. The second cell cycle is 48 h from G0. **(B)** Immunoblot for Cdc6 and Cdt1 of total protein lysates from RPE1 cells constitutively producing either 5myc-Cdc6 WT or 5myc-Cdc6-mut (a mutant of Cdc6 that is not targeted for degradation by APC^{Cdh1}) and a doxycycline-inducible Cdt1-HA. RPE1 cells were synchronized in G0 by contact inhibition, treated with 100 ng/ml doxycycline for 4 h before replating cells to release into the first cell cycle, and harvested 24 h after release. **(C)** Flow cytometry of chromatin-bound protein for cells treated as in B, harvested 24 h (first cell cycle) and 48 h (second cell cycle) after G0 release, measuring DNA content (DAPI), loaded MCM (anti-Mcm2), and DNA synthesis (EdU). **(D)** Loaded MCM of early S phase from C. Top: RPE1 cells producing Cdt1-HA WT and 5myc-Cdc6 WT. Bottom: RPE1 cells producing Cdt1-HA WT and 5myc-Cdc6-mut. **(E)** Comparison of early S phase DNA-loaded MCM per cell from D. Values plotted are the ratio of mean loaded MCM of the first cell cycle divided by mean loaded MCM of the second cell cycle. *n* = 3, mean with SD. **(F)** Percentage of underlicensed cells from early S phase cells in D. *n* = 3, mean with SD. **(G)** Flow cytometry of chromatin-bound protein from cells shown in Fig. 7 H, measuring DNA content (DAPI), loaded MCM (anti-Mcm2), and DNA synthesis (EdU).