Ki-67 Contributes to Normal Cell Cycle Progression and Inactive X Heterochromatin in p21 Checkpoint-Proficient Human Cells

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ABSTRACT The Ki-67 protein is widely used as a tumor proliferation marker. However, whether Ki-67 affects cell cycle progression has been controversial. Here we demonstrate that depletion of Ki-67 in human hTERT-RPE1, WI-38, IMR90, and hTERT-BJ cell lines and primary fibroblast cells slowed entry into S phase and coordinately downregulated genes related to DNA replication. Some gene expression changes were partially relieved in Ki-67-depleted hTERT-RPE1 cells by codepletion of the Rb checkpoint protein, but more thorough suppression of the transcriptional and cell cycle defects was observed upon depletion of the cell cycle inhibitor p21. Notably, induction of p21 upon depletion of Ki-67 was a consistent hallmark of cell types in which transcription and cell cycle distribution were sensitive to Ki-67; these responses were absent in cells that did not induce p21. Furthermore, upon Ki-67 depletion, a subset of inactive X (Xi) chromosomes in female hTERT-RPE1 cells displayed several features of compromised heterochromatin maintenance, including decreased H3K27me3 and H4K20me1 labeling. These chromatin alterations were limited to Xi chromosomes localized away from the nuclear lamina and were not observed in checkpoint-deficient 293T cells. Altogether, our results indicate that Ki-67 integrates normal S-phase progression and Xi heterochromatin maintenance in p21 checkpoint-proficient human cells.

KEYWORDS cell cycle, heterochromatin

Ki-67 was first identified via an antibody raised against Hodgkin's lymphoma cell nuclei (1). Because Ki-67 is generally expressed strongly in proliferating cells and poorly in quiescent cells (2), anti-Ki-67 antibodies are frequently used to detect proliferative cells in clinical studies (3, 4). In interphase cells, Ki-67 primarily localizes to the nucleolus (5–7), whereas during mitosis, it coats the chromosomes (8–10). In the past few years, several studies have greatly increased our understanding of Ki-67 function. This is particularly true for its mitotic roles. Specifically, Ki-67 is required for formation of the mitotic perichromosomal layer (11, 12), a proteinaceous sheath that coats mitotic chromosomes (13, 14). In this layer, Ki-67's large size and highly positively charged amino acid composition keep individual mitotic chromosomes dispersed rather than aggregated upon nuclear envelope disassembly, thereby ensuring normal kinetics of anaphase progression (15). At anaphase onset, Ki-67 binds protein phosphatase 1γ (PP1γ) to form a holoenzyme (16) important for targeting substrates that must be dephosphorylated during mitotic exit (10). In contrast to its structural role on the

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mitotic chromosomal surface, Ki-67 does not appear to affect nucleosomal spacing (15) or condensation of individual mitotic chromosomes (11, 15).

In addition to its expression in proliferating cells, other experiments suggested that Ki-67 has a positive role in regulating cell proliferation. In early studies, antisense oligonucleotides targeting Ki-67 expression in human IM-9 multiple myeloma cells blocked [³H]thymidine incorporation, indicating an inhibition of proliferation (17). Likewise, Ki-67-targeted phosphorothioate antisense oligonucleotides that resulted in partial depletion of Ki-67 protein inhibited proliferation of human RT-4 bladder carcinoma and other tumor cell lines (18). More recently, small interfering RNA (siRNA)-mediated depletion of Ki-67 resulted in reduced proliferation in human 786-0 renal carcinoma cells (19).

However, despite its utility as a proliferation marker, the contribution of Ki-67 to cell proliferation has recently been questioned. For example, in one recent study, genetic disruption of Ki-67 in human MCF-10A epithelial breast and DLD-1 colon cancer cells did not affect cell proliferation rates in bulk culture, although the clonogenic growth of highly diluted cell populations was reduced (20). In another recent study, depletion of Ki-67 in human HeLa or U2OS cells did not alter cell cycle distribution (12). These data raise the possibility that Ki-67 function may have different consequences in different cell types.

In our previous studies, we demonstrated that the interphase and mitotic localization of Ki-67 is partially dispersed in cells lacking the N-terminal domain of the p150 subunit of chromatin assembly factor 1 (21, 22). We therefore began to explore the functions of Ki-67 in several human cell types. Here we show that the contribution of Ki-67 to cell proliferation depends on the cell type. In hTERT-RPE1, WI-38, IMR90, and hTERT-BJ cells and primary foreskin fibroblasts, depletion of Ki-67 resulted in reduced frequencies of S-phase cells and concomitant reductions of S-phase-related transcript levels. We show that in female hTERT-RPE1 cells, these phenotypes required a p21 checkpoint-mediated delay in S-phase entry and were accompanied by altered nucleolar association and chromatin characteristics of the inactive X (Xi) chromosome. Notably, none of these phenotypes were observed in human cells unable to induce p21 in response to Ki-67 depletion. Therefore, Ki-67 is important for normal S-phase progression in p21 checkpoint-proficient human cells, in a manner correlated with its contribution to Xi heterochromatin composition.

RESULTS

Ki-67 affects S-phase gene expression and progression. To explore how Ki-67 affects gene expression, we performed transcriptome sequencing (RNA-seq) analyses of control and Ki-67-depleted hTERT-RPE1 cells, a diploid retinal pigment epithelial cell line immortalized by an hTERT transgene (23). Duplicate analyses were highly reproducible (Fig. 1A and B). Ki-67 depletion resulted in approximately equal numbers of reduced and increased RNA levels across the transcriptome (Fig. 1C). However, reactome pathway analysis of RNA abundance changes showed that the most altered functional sets of genes included those involved in DNA replication and cell cycle progression (Fig. 1D). For example, levels of RNAs encoding all subunits of several protein complexes involved in S-phase progression were concertedly reduced, including those for the DNA replication clamp loader RFC (RFC1-5 genes), the single-stranded DNA (ssDNA)-binding complex RPA (RPA1-3), the replicative helicase (MCM1-6), the GINS replication initiation complex (GINS1-4), and the DNA polymerase alpha/primase complex, as well as the DNA replication clamp PCNA and the flap endonuclease FEN1, involved in Okazaki fragment maturation (see Tables S1 and S2 in the supplemental material). We confirmed the reduced levels of a subset of replication-related RNA targets by real-time quantitative PCR (RT-qPCR) analyses (Fig. 1E and F). Notably, the RT-qPCR data obtained with two distinct Ki-67 depletion reagents were very similar; one reagent was a synthetic siRNA and the other a cocktail of *in vitro*-diced double-stranded RNAs (dsRNAs) not overlapping the siRNA target, both of which efficiently depleted steady-state Ki67 levels (Fig. 1M and N).

FIG 1 Ki-67 depletion in hTERT-RPE1 cells reduced S-phase-related mRNA abundance and the proportion of cells in S phase. (A) Scatterplot analysis of RNA levels, comparing two replicate si-scramble RNA-seq analyses of hTERT-RPE1 cells. R^2 , Pearson's correlation coefficient. (B) Scatterplot analysis comparing two replicate si-Ki-67 RNA-seq analyses. (C) Distribution of RNA fold changes (FC) measured by RNA-seq, comparing si-scramble- and si-Ki-67-treated hTERT-RPE1 cells. The x axis shows the mean $\log_2$ value for normalized counts of abundance levels for each RNA species. The y axis shows the $\log_2$ fold change upon Ki-67 depletion. The symmetry of the plot above and below the zero point on the y axis indicates that similar numbers of genes were up- and downregulated upon Ki-67 depletion. (D) Reactome evaluation of RNA-seq

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We also observed that fluorescence-activated cell sorting (FACS) analysis of bromodeoxyuridine (BrdU)-labeled cells showed that the proportion of S-phase cells decreased upon depletion of Ki-67 in hTERT-RPE1 cells with either depletion reagent (Fig. 1G and H; quantified in Fig. 1I and K). In contrast, proportions of G₁ and G₂/M populations were not significantly altered (Fig. 1J and L). Therefore, Ki-67 is important for normal S-phase distribution and gene expression in hTERT-RPE1 cells.

As an additional control for the direct effect of the siRNA treatment, we used clustered regularly interspaced short palindromic repeats (CRISPR)/Cas9 to mutate the siRNA target site in hTERT-RPE1 cells (Fig. 2A and B). In the resulting homozygous mutant cell line (Fig. 2C), si-Ki-67 no longer depleted Ki-67 protein levels, but as expected, the esi-Ki-67 reagent was still effective (Fig. 2D). We also observed that si-Ki-67 no longer altered candidate S-phase RNA levels in the resistant cell line, but esi-Ki-67 did (Fig. 2E). We concluded that the transcriptional response to acute depletion of Ki-67 mRNA was due to a loss of Ki-67 protein.

However, recent studies challenged the view that Ki-67 is important for human cell proliferation; for example, depletion of Ki-67 had minimal effects on the cell cycle distribution of tumor-derived HeLa or U2OS cells (12). Because our data indicated that Ki-67 contributes to normal cell cycle progression in hTERT-RPE1 cells, we hypothesized that the contribution of Ki-67 to cell cycle progression would be cell type dependent and may be related to checkpoint function. To explore this idea, we depleted Ki-67 in several additional cell lines. We compared diploid, nonimmortal WI-38 and IMR90 fibroblasts, hTERT-immortalized BJ fibroblasts, and primary human foreskin fibroblasts (HFFs) with tumor-derived cell lines, namely, virally transformed kidney (293T) or cervical carcinoma (HeLa) cells and U2OS osteosarcoma cells. Importantly, we confirmed that all these cells could be efficiently depleted of Ki-67 protein by use of either of our depletion reagents (Fig. 2F to L).

In all of the experiments with the non-tumor-derived cells, we observed reduced replication factor RNA levels and fewer cells in S phase, similar to our results for hTERT-RPE1 cells, and these results were independent of which Ki-67 depletion reagent was used (Fig. 3 and 4). In contrast, in the tumor-derived cell lines, Ki-67 depletion did not result in uniform downregulation of the S-phase genes tested, nor were there changes in cell cycle distribution (Fig. 5 and 6). These data are consistent with RNA-seq data sets for HeLa and U2OS cells (12) that did not display a concerted downregulation of DNA replication genes. We concluded that the effects of Ki-67 depletion on RNA levels and cell cycle distribution are cell type dependent.

FACS analyses of asynchronous cell populations indicated that Ki-67 depletion most significantly affected S phase in the sensitive cell types. To examine this in more detail, we analyzed the kinetics of DNA synthesis in synchronized hTERT-RPE1 cells. Cells were blocked near the G₁-S transition of the cell cycle by use of hydroxyurea (HU) for 15 h (Fig. 7A) (24), which provided efficient arrest (Fig. 7D). Cells were then released into drug-free medium and pulse labeled with the deoxynucleotide analog 5-ethynyl-2-deoxyuridine (EdU) at 2-h time points across an 8- to 10-h time course. For control cells

FIG 1 Legend (Continued)

analysis of si-Ki-67-treated cells. The PATH terms with P values of $<5 \times 10^{-5}$ are graphed. (E) RNA levels of DNA replication genes are coordinately downregulated in si-Ki-67-treated cells. RT-qPCR measurements are presented as fold changes relative to the scramble siRNA control measurements after normalization. *MKI67* mRNA levels indicate the effectiveness of the siRNA treatment. Data are means and standard deviations (SD) for 3 biological replicates. (F) Analysis of RNA levels as described for panel E, except that cells were treated with *in vitro*-diced esiRNAs as depletion reagents. (G) FACS analysis of siRNA-treated cells. Cells were pulsed with BrdU for 20 min and then analyzed via two-dimensional flow cytometry to monitor BrdU incorporation (y axis) and DNA content (x axis). G₁ (lower left)-, G₂ (lower right)-, and S (upper)-phase populations are boxed for each sample, with percentages of the total population shown. Data shown are from one representative experiment of three biological replicates. (H) FACS analysis as described for panel G, except that cells were treated with esiRNAs. (I) Percentage of cells in S phase in siRNA-treated hTERT-RPE1 populations from three biological replicates of the BrdU labeling experiment. The P value for comparison of the si-scramble and si-Ki-67 treatments is indicated and was calculated via an unpaired, two-tailed parametric t test. (J) Percentage of cells in G₁ or G₂/M phase from the same three experiments as those analyzed for panel I. (K) Percentage of S-phase cells as described for panel I, except that cells were treated with *in vitro*-diced esiRNAs. (L) Percentage of cells in G₁ or G₂/M phase from the same three experiments as those analyzed for panel K. (M) Immunoblot analysis of Ki-67 depletion in siRNA-treated hTERT-RPE1 cells from panel E. Marker molecular weights are indicated on the left. (N) Immunoblot analysis of Ki-67 depletion in esiRNA-treated hTERT-RPE1 cells from panel F.

FIG 2 Validation of specificity and effectiveness of Ki-67 depletion reagents. (A) CRISPR/Cas9-based strategy for generating siRNA-resistant mutations in the endogenous Ki-67 gene. The si-Ki-67 target (green letters) and sgRNA-directed cleavage site (triangle) are indicated in the upper diagram of the endogenous locus. Altered nucleotides (red) and the novel EcoO109I restriction site are shown in the lower diagram of the repair template. (B) DNA sequence analysis of PCR products from wild-type hTERT-RPE1 cells and si-Ki-67-resistant clone 7. (C) EcoO109I digestion of the same PCR products sequenced for panel B. (D) Immunoblot analysis of clone 7 treated with the indicated reagents. (E) RT-qPCR analysis of clone 7. (F to L) Immunoblot analyses of the indicated cell lines treated with the indicated RNA depletion reagents.

treated with the scrambled siRNA, EdU labeling was first detected at the 2-h time point and displayed a typical early-S-phase pattern consisting of many small foci (25). At later time points, the pulse of EdU labeled larger foci, indicative of mid-to-late-S-phase patterns. Upon Ki-67 depletion, we observed a delay in the initial detection of EdU incorporation of approximately 2 h (Fig. 7B). Notably, the Ki-67-depleted population also displayed a larger percentage of cells that did not incorporate EdU during the time course (Fig. 7C). Together, these data are consistent with our transcriptomic and FACS data indicating that Ki-67 depletion affects S phase in hTERT-RPE-1 cells (Fig. 1).

FIG 3 siRNA-mediated Ki-67 depletion affected S-phase gene expression and cell cycle distribution in diploid human cells. The WI-38 (A to D), IMR90 (E to H), and hTERT-BJ (I to L) cell lines and primary human foreskin fibroblasts (HFFs) (M to P) were analyzed. (A, E, I, and M) RT-qPCR analyses as described in the legend to Fig. 1E. (B, F, J, and N) FACS analyses as described in the legend to Fig. 1G. (C, G, K, and O) Percentages of S-phase cells as described in the legend to Fig. 1I. (D, H, L, and P) Percentages of G₁- and G₂/M-phase cells as described in the legend to Fig. 1J.

Checkpoint responses to Ki-67 depletion. Because Ki-67 depletion did not affect S-phase transcription or cell cycle progression in tumor-derived cell lines, our data suggested that functional checkpoints are required for sensitivity to Ki-67 depletion. Consistent with this idea were comparisons of our RNA-seq data with metadata analyses of genes regulated by cell cycle status or by E2F transcription factors (26) that are important for G₁/S cell cycle phase transcription (26–28). These meta-analyses aggregated multiple data sets and found that similar results in multiple data sets strongly predicted regulatory network connections that could be missed in single experiments. Of the cell cycle-regulated genes identified in that study, we found that those that peak during G₁/S phase were more frequently downregulated than upregulated upon Ki-67 depletion (Fig. 8A; Table S3). Consistent with this observation, E2F target RNA levels (Fig. 8B) were much more frequently downregulated than upregu-

FIG 4 esiRNA-mediated depletion of Ki-67 in diploid cells resulted in the same phenotypes as those observed with siRNA treatments. Cells and assays were the same as those described in the legend to Fig. 3.

lated upon Ki-67 depletion. These comparisons were consistent with the idea that checkpoint activation contributed to the observed delay of S-phase entry and transcriptional phenotypes of Ki-67-depleted cells.

To test this, we performed experiments to codeplete checkpoint proteins. First, we took advantage of derivative hTERT-RPE1 cells that have an integrated, doxycycline-inducible short hairpin RNA (shRNA) that targets the Rb mRNA (29). Rb levels remained unchanged in these cells in the absence of doxycycline (Fig. 8C), and siRNA-mediated depletion of Ki-67 resulted in reduced S-phase-related RNA levels as observed previously (Fig. 8D). Addition of doxycycline to deplete Rb, together with a control scrambled siRNA leaving Ki-67 levels unchanged, did not significantly alter S-phase-related target RNA levels (Fig. 8D). In contrast, simultaneous depletion of Rb and Ki-67 resulted in RNA levels at two of the four loci tested that were significantly elevated compared to those in cells depleted of Ki-67 alone (Fig. 8D). FACS analysis indicated that Rb depletion was insufficient to significantly change the cell cycle profile of Ki-67-depleted cells (Fig. 8E and F). We concluded that depletion of Rb only partially relieves the cellular response to Ki-67 depletion in hTERT-RPE1 cells.

Therefore, we reasoned that other factors must contribute to this phenomenon. One clue was provided by comparison of the si-Ki-67 RNA-seq data to a metadata analysis of binding by subunits of a transcription repressor complex termed DREAM (26). We

FIG 5 Ki-67-insensitive cells. Ki-67 depletion did not affect S-phase gene expression and cell cycle distribution in the HeLa (A to D), U2OS (E to H), and 293T (I to L) cell lines. (A, E, and I) RT-qPCR analyses. (B, F, and J) FACS analyses. (C, G, and K) Percentages of S-phase cells. (D, H, and L) Percentages of G₁- and G₂/M-phase cells.

observed that genes with the highest predicted probability of DREAM binding were very frequently downregulated upon Ki-67 depletion (Fig. 8G). In mammalian cells, DREAM is a master regulator of cell cycle-dependent gene expression, repressing both G₁/S and G₂/M targets, and gene repression by DREAM requires the p21 checkpoint protein (27, 30, 31). p21 is a potent universal cyclin-dependent kinase (CDK) inhibitor (CKI). During G₁ and S phases, p21 directly binds to and inhibits the kinase activity of cyclin E-CDK2, cyclin B1-CDK1, and cyclin D-CDK4/6 complexes (32–34). Furthermore, p21 also directly inhibits DNA synthesis by binding to PCNA, the sliding clamp required for processive DNA polymerase activity (35). Therefore, we hypothesized that p21 may be important for the response to Ki-67 depletion (Fig. 8H).

Consequently, we next tested the effects of Ki-67 depletion on the *CDKN1A* gene, which encodes p21. Our RNA-seq data indicated increased *CDKN1A* RNA levels in Ki-67-depleted hTERT-RPE1 cells ($\log_2$ fold change = +0.48; $P = 0.016$), although multiple-hypothesis testing indicated that these values did not achieve the stringent statistical significance cutoff of a false discovery rate (q value) of <0.05 (Table S2). RT-PCR measurements of *CDKN1A* RNA levels demonstrated significant increases in four diploid cell types but not in 293T cells (Fig. 9A). We note that induction of *CDKN1A* RNA was dependent on having an intact siRNA target site in the Ki-67 gene, indicating that this was a direct effect of Ki-67 depletion (Fig. 2E). Consistent with the increased RNA levels, we detected elevated p21 protein levels in hTERT-RPE1 but not 293T cells (Fig. 9B).

FIG 6 esiRNA-mediated depletion of Ki-67 in insensitive cells resulted in the same phenotypes as those observed with siRNA treatments. Cells and assays were the same as those described in the legend to Fig. 5.

p21 is a direct target of transcriptional induction by the tumor suppressor p53, and the cell lines examined thus far therefore implicated active p53 in the sensitivity of cells to Ki-67 (36, 37). To examine this relationship further, we compared the effects of Ki-67 depletion in additional cancer cell lines, including two expressing wild-type p53, i.e., MCF7 and HCT116 cells, and MDA-MB-231 cells, which express a mutant p53 protein defective for p21 induction (38). In MCF7 breast adenocarcinoma cells, we observed that Ki-67 depletion elevated p21 RNA and protein levels (Fig. 9G and H) and down-regulated replication-related RNAs (Fig. 9H). In contrast, in HCT116 colorectal carcinoma and MDA-MB-231 breast adenocarcinoma cells, Ki-67 depletion did not increase p21 expression or cause a concerted downregulation of S-phase genes (Fig. 9G, I, and J). Because HCT116 and MDA-MB-231 cells differ in their p53 status, we concluded that p53 status cannot always predict the response to Ki-67 depletion. Instead, we find that induction of p21 upon Ki-67 depletion is a consistent hallmark of this form of checkpoint activation.

To assess the functional consequence of p21 induction, we performed codepletion experiments in hTERT-RPE1 cells (Fig. 9D). We observed that cells simultaneously depleted of both Ki-67 and p21 no longer displayed reduced levels of any of the four S-phase-related mRNAs analyzed (Fig. 9D). Furthermore, FACS analysis of the codepleted cells showed that there was significant restoration of the percentage of cells in S phase upon codepletion of p21 and Ki-67 (Fig. 9E and F). We concluded that induction of p21 is functionally important for the effects of Ki-67 depletion on cell cycle distribution in hTERT-RPE1 cells.

FIG 7 Depletion of Ki-67 delayed S-phase entry in hTERT-RPE1 cells. (A) Schematic of short-pulse assay. hTERT-RPE1 cells were released from HU arrest for the indicated periods, pulsed for 20 min with EdU, and analyzed by click chemistry and fluorescence microscopy for EdU incorporation. (B) Representative cells from the indicated time points, showing EdU staining (green) to detect the progression of S phase. Total DNA was visualized with DAPI staining (blue). S-phase cells were categorized into 3 substages based on the number, size, shape, and distribution of fluorescence-labeled replication foci. During early S phase, small and numerous replication foci are scattered in the nuclear interior but excluded from the nucleolus, the nuclear periphery, and other heterochromatic regions. At mid-S phase, replication takes place at the nuclear periphery and at perinuclear regions. Late in S phase, there are several large foci throughout the nucleus (25). Bars, 5 μ m. (C) Distributions of EdU morphologies during the time courses. Percentages of early-S-phase, middle-S-phase, and late-S-phase EdU staining morphologies were determined for >300 total cells per time point. (D) FACS histograms showing cell cycle profiles of propidium iodide-stained hTERT-RPE1 cells. asy, asynchronous cells were incubated with the dimethyl sulfoxide (DMSO) vehicle control; HU block, cells were incubated with hydroxyurea. (Left) si-scramble-treated cells were incubated with or without 2 mM hydroxyurea for 15 h. (Right) si-Ki-67-treated cells were incubated with or without 2 mM hydroxyurea for 15 h. Histograms were generated using FlowJo v9.9.4. (E) A longer (3 h) EdU pulse prevented detection of S-phase alterations in Ki-67-depleted hTERT-RPE1 cells. Asynchronous cells were treated with either si-scramble or si-Ki-67 for 72 h, incubated with EdU for the final 3 h, and analyzed via click chemistry. The total numbers of cells assayed were 218 for si-scramble and 265 for si-Ki-67, in three independent experiments. Bar, 20 μ m. (F) Ratios of EdU-positive cells to the total cell number. The difference between groups was not significant ($P = 0.77$). (G) Cell cycle distributions of si-scramble- and si-Ki-67-treated hTERT-RPE1 cells as analyzed by one-dimensional FACS profiling of propidium iodide-stained cells.

Ki-67 affects heterochromatic characteristics of the inactive X chromosome.

Ki-67 is required for the normal cellular localization of heterochromatin-associated histone modifications (12) and for the interphase nucleolar association of heterochromatic loci (22). Because the inactive X (Xi) chromosome is a well-studied example of facultative heterochromatin that associates with the nucleoli of female mouse (24) and human (39–41) cells, we tested whether Ki-67 affected characteristics of Xi heterochromatin. Indeed, Ki-67 depletion in hTERT-RPE1 cells resulted in a subset of cells that displayed reduced staining intensities for antibodies recognizing H3K27me3 and H4K20me1, histone modifications that are enriched on the Xi chromosome (42, 43) (Fig. 10A, C, E, and G). H3K27me3 is generated by the Polycomb PRC2 complex and is a keystone of facultative heterochromatic silencing (44–46). H4K20me1 is generated by the PR-Set7/Set8/KMT5a enzyme (47) and, together with H3K27me3, is an early mark on Xi chromosomes during the process of XIST-mediated inactivation (43, 47). Notably,

FIG 8 Rb contributes to transcriptional downregulation caused by Ki67 depletion. (A) Summary of transcriptional changes of cell cycle target genes (based on Table S10 in reference 26). The adjusted cell cycle scores on the x axis are values based on a meta-analysis of 5 different cell cycle expression data sets plus information regarding binding by the Rb/E2F and MMB/FOXO1 transcription factors. Negative values indicate frequent detection of G₁/S expression and binding by Rb/E2F, and positive values indicate frequent detection of G₂/M expression and binding by MMB/FOXO1.

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FIG 9 Cell-type-specific induction of a p21-dependent checkpoint upon depletion of Ki-67. (A) RT-qPCR analysis demonstrates cell-type-specific induction of *CDKN1A* (p21) RNA upon Ki-67 depletion. (Left) The indicated cells were treated with either si-scramble or si-Ki-67 for 72 h. (Right) Same as the left panel, except that cells were treated with esiRNAs. (B) Cell-type-specific induction of p21 protein levels upon Ki-67 depletion. hTERT-RPE1 and 293T cells were treated with the indicated siRNA or *in vitro*-diced esiRNA depletion reagents. p21 protein levels were quantified as ratios to β -tubulin levels and normalized to the levels in control-treated cells. Quantification was done in ImageJ 10.2. (C) Immunoblot analysis of hTERT-RPE1 cells depleted of the indicated proteins via siRNA treatments. Marker molecular weights are indicated on the right. (D) RT-qPCR analysis of DNA replication genes in hTERT-RPE1 cells from panel C. Asterisks indicate values for the si-p21 + si-Ki-67 samples that were significantly different ($P < 0.05$) from those for the si-Ki-67 samples. P values were calculated via unpaired, two-tailed parametric t tests and corrected for multiple comparisons by using the Holm-Sidak method. (E) Percentages of S-phase cells calculated from three BrdU labeling experiments. P values were calculated via unpaired, two-tailed parametric t tests. (F) FACS analysis of a representative BrdU labeling experiment. (G) Immunoblot analysis of additional siRNA-treated cell lines tested for p21 induction. (H) RT-qPCR analysis of the indicated genes in MCF7 cells. P values calculated as described for panel D were < 0.05 for all genes. (I) RT-qPCR analysis of the indicated genes in MDA-MB-231 cells. (J) RT-qPCR analysis of the indicated genes in HCT116 cells.

changes to either of these histone modifications were observed only in cells in which the Xi chromosome was localized away from the nuclear periphery (Fig. 10B, D, F, and H). Furthermore, these changes were not observed in 293T cells that also lacked the cell cycle response to Ki-67 depletion (Fig. 11). Therefore, the response of hTERT-RPE1 cells

FIG 8 Legend (Continued)

by MMB-FOXM1. (B) P values for transcription changes of E2F target genes (based on Table S9 in reference 26), with greater scores on the x axis representing higher frequencies of detection as an E2F target. As expected from panel A, E2F targets were commonly downregulated upon Ki-67 depletion. (C) Immunoblot analysis of hTERT-RPE1 Tet-sh-Rb cells. Cells were treated with either vehicle (Rb +) or 2 μ g/ml doxycycline (Rb -) for 72 h, as indicated, to induce sh-Rb expression and were also incubated in the presence of either si-scramble (Ki-67 +) or si-Ki-67 (Ki-67 -). (D) RT-qPCR analysis of DNA replication genes in cells treated as described for panel C. Measurements are presented as fold changes relative to the scramble siRNA control levels without doxycycline induction. Data are means and SD for 3 biological replicates. P values were calculated via unpaired, two-tailed parametric t tests and corrected for multiple comparisons by using the Holm-Sidak method. (E) Percentages of S-phase cells calculated from three BrdU labeling experiments. P values were calculated via unpaired, two-tailed parametric t tests. (F) FACS analysis of a representative BrdU labeling experiment. (G) P values for transcription changes of DREAM target genes (based on Table S7 in reference 26), with greater scores on the x axis representing more frequent detection as a DREAM target. (H) Proposed model. In "Ki-67-sensitive" cells, depletion of Ki-67 leads to p21 induction. The elevated p21 levels are predicted to downregulate the Rb/E2F and DREAM target G_1/S cell cycle genes and to inhibit DNA synthesis by binding to PCNA. Together, these effects delay S-phase entry.

FIG 10 H3K27me3 and H4K20me1 staining of the inactive X chromosome was altered upon Ki-67 depletion in a subset of hTERT-RPE1 cells. Bars, 5 μ m. (A) Immunofluorescence analysis of H3K27me3 overlapping XIST in siRNA-treated hTERT-RPE1 cells. Note that in the si-Ki-67-treated cell, the H3K27me3 signal overlapping XIST displays a reduced intensity and is localized away from the nuclear lamina. (B) Percentages of cells that displayed reduced H3K27me3 enrichment on the Xi chromosome in the experiments for panel A. Enrichment was calculated as the ratio of the mean H3K27me3 signal overlapping XIST divided by the mean H3K27me3 signal from the remainder of the entire nucleus. Cells with ratios of <1.5 were defined as having reduced enrichment, as described previously (48). Percentages were calculated for the total cell population as well as for the nuclear lamina-associated XIST foci and the non-lamina-associated foci, as indicated. The total numbers of cells assayed were 250 for si-scrabble and 239 for si-Ki-67. Means and SDs for three biological replicate experiments were graphed. *P* values were determined by unpaired Student's *t* tests. (C) Analysis of H3K27me3 enrichment on the Xi chromosome, as described for panel A, for hTERT-RPE1 cells treated with *in vitro*-diced esiRNAs. (D) Quantitation as described for panel B. The total numbers of cells assayed were 236 for esi-luciferase and 220 for esi-Ki-67. (E) Immunofluorescence analysis of H4K20me1 overlapping XIST in siRNA-treated hTERT-RPE1 cells. Note that the H4K20me1 signal colocalizing with XIST is reduced in the Ki-67-depleted cell. (F) Quantitation of the experiments for panel E as described for panel B. The total numbers of cells assayed were 204 for si-scrabble and 216 for si-Ki-67. (G) Analysis of H4K20me1 in cells treated with esiRNAs. (H) Quantitation of the panel G experiments. The total numbers of cells assayed were 164 for esi-luciferase and 182 for esi-Ki-67. (I) Example of H3K27me3 signal intensity quantification. (J) RT-qPCR analysis of XIST RNA levels in hTERT-RPE1 cells.

FIG 11 H3K27me3 and H4K20me1 staining of the inactive X chromosome was unaltered upon Ki-67 depletion in 293T cells. Bars, 5 μ m. (A) Immuno-FISH analysis of H3K27me3 overlapping XIST in siRNA-treated 293T cells. Note that 293T cells have two Xi chromosomes. In these cells, the H3K27me3 foci overlapping XIST remained unchanged upon Ki-67 depletion. (B) Quantitation of the data from panel A. The total numbers of alleles assayed were 136 for si-scrabble and 146 for si-Ki-67. (C and D) Analysis of H3K27me3 in esiRNA-treated 293T cells. The total numbers of alleles assayed were 196 for esi-luciferase and 198 for esi-Ki-67. (E) Immuno-FISH analysis of H4K20me1 overlapping XIST in 293T cells. In these cells, the H4K20me1 foci overlapping XIST remained unchanged upon Ki-67 depletion. (F) Quantitation of the data from panel E. The total numbers of alleles assayed were 180 for si-scrabble and 162 for si-Ki-67. (G and H) Analysis of H4K20me1 in esiRNA-treated cells. The total numbers of alleles assayed were 180 for esi-luciferase and 162 for esi-Ki-67.

to Ki-67 depletion involves two classes of correlated events that are both absent in 293T cells: checkpoint-mediated perturbation of S phase and altered Xi heterochromatin.

Increased levels of repetitive element-rich Cot-1-hybridizing transcripts and RNA polymerase II (Pol II) have previously been observed in breast cancer cell lines that display perturbations in Xi chromatin (48). We tested for changes in these properties as well, and we detected an increase in the frequency of cells that displayed Cot-1-

hybridizing transcripts or RNA polymerase II on the Xi chromosome upon Ki-67 depletion in hTERT-RPE1 cells (Fig. 12 and 13). As observed above for the histone modifications (Fig. 10), increased levels of Cot-1 RNA and Pol II within the XIST domain were observed only for cells in which the Xi chromosome was localized away from the nuclear periphery. Also, as for all other phenotypes detected, these changes were similar with either Ki-67 depletion reagent (Fig. 12 and 13).

However, not all aspects of Xi heterochromatin were sensitive to Ki-67 depletion. For example, Ki-67 depletion did not alter the overall appearance of the XIST “cloud” that covers the Xi chromosome (Fig. 14A). In addition, RT-PCR showed no significant downregulation of XIST transcript expression (Fig. 10J). Also, there was no evidence of Xi chromosome-wide derepression of transcription, as shown by an analysis of X-linked gene expression (Fig. 14B) and an analysis of allele-specific transcription of X-linked genes detected via analysis of known single-nucleotide polymorphisms (SNPs) (Table S4). Furthermore, an additional mark associated with the Xi chromosome, the histone variant macroH2A, did not change in appearance upon depletion of Ki-67 (Fig. 14C to H). Together, the Xi data indicate that acute depletion of Ki-67 alters several, but not all, characteristics of Xi heterochromatin in hTERT-RPE1 cells. Importantly, changes in H3K27me3 and H4K20me1 staining were not observed in 293T cells, indicating a correlation between checkpoint activation and effects on the Xi chromosome upon Ki-67 depletion.

Ki-67 affects the S-phase nucleolar association of the inactive X chromosome.

The perinucleolar space is a subset of the heterochromatic compartment; another frequent location for heterochromatin is at the nuclear periphery, adjacent to the nuclear lamina (49). Accordingly, the Xi chromosome is usually localized to one of these two preferred locations (24). However, heterochromatic sequences can dynamically relocate from nucleoli to the periphery, either during cell division or upon perturbation of the nucleolus by use of actinomycin (49–52). Because Ki-67 depletion affected heterochromatic marks only on Xi chromosomes away from the nuclear periphery (Fig. 10 to 13), we hypothesized that it might also affect the interphase localization of the Xi chromosome. We first examined Xi chromosome localization in asynchronous hTERT-RPE1 cells, using immuno-RNA-fluorescence *in situ* hybridization (immuno-RNA-FISH) to detect the Xi chromosome-associated long noncoding RNA (lncRNA) XIST and the nucleolar protein fibrillarin. Indeed, Ki-67 depletion resulted in a partial but statistically significant reduction in Xi chromosome-nucleolus associations (Fig. 15A to D). This loss of nucleolar association was accompanied by an increase of similar magnitude in Xi chromosome-lamina associations, and similar results were observed with our two distinct Ki-67 depletion reagents (Fig. 15B and D). However, in 293T cells, we observed no significant alteration in Xi chromosome-nucleolus associations (Fig. 15E to H). Thus, the distribution of the Xi chromosome within the interphase nucleus is sensitive to Ki-67 depletion in a cell type that induces p21.

Previous studies with mouse cells showed that the Xi chromosome-nucleolus association is cell cycle regulated, occurring most prevalently in S-phase cells (24). Therefore, we examined Xi chromosome localization in hTERT-RPE1 cells prepared in the same manner as that in the cell synchronization experiments reported in Fig. 7A and B. Consistent with published data from mouse cells (24), the frequency of Xi chromosome-nucleolus associations peaked in the transition from middle S to late S phase; this was true for the frequency of Xi chromosome associations that were exclusively at the nucleolus (Fig. 15I) and the frequency of Xi chromosomes simultaneously associated with both the nucleolus and the lamina (Fig. 15J). These peaks occurred in both the control and Ki-67-depleted populations, with the Xi chromosome-nucleolus interaction peaks being delayed 2 h in the latter case. This 2-h shift correlates with the delay in S-phase entry in Ki-67-depleted cells (Fig. 7).

As suggested by the Xi chromosome localization data from asynchronous cells (Fig. 15A to D), Xi chromosome associations with the nucleoli and lamina were inversely related, so the peaks of nucleolar associations (Fig. 15I and J) coincided with the lowest frequencies of laminar associations (Fig. 15K). We note that when the total Xi

FIG 12 Analysis of Cot-1 and Pol II enrichment on the Xi chromosome in siRNA-treated hTERT-RPE1 cells. Bars, 5 μ m. (A and B) Localization of Cot-1-hybridizing transcripts relative to that of XIST domains when XIST is localized away from (A) or at (B) the nuclear lamina. A line scan (arrows) across the XIST signal (green) was used to analyze the Cot-1 hybridization level (red); fluorescence densities across the line scan are plotted in the panels to the right of the images. Cot-1 RNA was considered to be reduced across the XIST domain when the average Cot-1 signal overlapping XIST was lower than the average Cot-1 signal across the nucleus. The average nuclear Cot-1 signal is depicted by a dotted line. (A) In the examples shown where the Xi chromosome was within the cell interior, Cot-1 RNA was excluded from XIST in the si-scramble-treated cell but not the si-Ki-67-treated cell. (B) In contrast, si-Ki-67 treatment did not affect Cot-1 enrichment on the Xi chromosome in cells where the Xi chromosome was at the lamina. (C and D) Analysis of RNA Pol II localization (red) relative to that of XIST (green) when XIST is localized away from (C) or at (D) the nuclear lamina. Exclusion was analyzed as

(Continued on next page)

chromosome-lamina association frequencies were counted by summing the exclusively lamina associations (Fig. 15K) and those also associated with nucleoli simultaneously (Fig. 15J), we observed little change during the experiment (Fig. 15L). Thus, the biggest change during S phase was that lamina-associated Xi chromosomes became transiently also associated with nucleoli (e.g., compare Fig. 15J and K). Together, these data indicate that cell cycle-regulated Xi chromosome-nucleolus associations are delayed in concert with DNA synthesis upon depletion of Ki-67 in hTERT-RPE1 cells. Thus, checkpoint activation upon Ki-67 depletion affects cell cycle progression and gene expression, and these effects are correlated with altered Xi heterochromatin in female hTERT-RPE1 cells.

DISCUSSION

Cell-type-specific responses to Ki-67 depletion. Our studies show that Ki-67 expression is important for normal S-phase progression in primary human foreskin fibroblasts, nontransformed fibroblast lines (WI-38 and IMR90), hTERT-immortalized BJ fibroblasts, and hTERT-immortalized RPE1 cells (23), a human female retinal pigment epithelial cell line with a diploid karyotype (53). In contrast, in cancer-derived 293T, U2OS, and HeLa cells, depletion of Ki-67 did not cause defects in cell proliferation. Therefore, we concluded that Ki-67 is required for normal cell cycle progression in some but not all cell lines. Our data distinguish two types of responses to depletion of Ki-67 in human cells, depending on whether cells are able to mount a p21-dependent checkpoint.

hTERT-BJ fibroblasts were sensitive to Ki-67 depletion in our assays; however, a previous study indicated that shRNA-mediated Ki-67 depletion did not affect hTERT-BJ cell cycle reentry after starvation (12). Therefore, not all assays can detect the effects of Ki-67 depletion. For example, two assays used in the previous study are insensitive to the cell cycle progression delays that we observe upon Ki-67 depletion. First, one-dimensional flow cytometry of propidium iodide-stained asynchronous populations cannot detect the S-phase delays we observe in BrdU labeling experiments; second, a 3-h EdU pulse is too long to capture the 2-hour S-phase entry delay. Together, our data indicate that the effects of Ki-67 on S-phase progression are transient in sensitive cell types and therefore most easily observed using short pulses of labeled deoxynucleotides.

Characteristics of checkpoint activation caused by Ki-67 depletion. Meta-analyses of our RNA-seq data showed that Ki-67 depletion in hTERT-RPE1 cells resulted in frequent repression of Rb/E2F-regulated, G₁/S-expressed genes. However, depletion of the Rb checkpoint protein only partially relieved transcriptional repression and did not restore normal percentages of S-phase cells, indicating that additional factors were responsible for the altered cell cycle profile in Ki-67-depleted cells.

A strong candidate for such a factor is the DREAM complex, which represses G₁/S-expressed cell cycle genes in a p21-dependent manner (26, 54). We noticed frequent downregulation of DREAM complex targets in Ki-67-depleted cells, which led us to test whether sensitivity to Ki-67 depletion was also p21 dependent. Notably, both the transcriptional alterations and cell cycle perturbations caused by Ki-67 depletion were partially relieved by simultaneous depletion of p21. Importantly, cell lines that induce p21 upon Ki-67 depletion are those that inhibit transcription of DNA replication genes and delay entry into S phase. Thus, this study implicates a p21-dependent checkpoint in cells sensitive to Ki-67 depletion.

A recent study showed that PD0332991, a small-molecule CDK4/CDK6 inhibitor (CDKi), depletes Ki-67 protein levels in some but not all cell lines (55). In “CDKi-sensitive”

FIG 12 Legend (Continued)

described for panels A and B. (E) Quantitation of Cot-1 RNA overlapping XIST RNA domains. Mean (and SD) percentages of cells displaying Cot-1 RNA overlapping XIST foci were plotted for three biological replicate experiments. The total numbers of cells assayed were 404 for si-scramble and 465 for si-Ki-67. *P* values were determined by unpaired Student's *t* tests. (F) Percentages of cells showing the presence of RNA Pol II at XIST RNA domains. The total numbers of cells assayed were 362 for si-scramble and 367 for si-Ki-67. (G) Immunoblot analysis of Ki-67 depletion in siRNA-treated hTERT-RPE1 cells.

FIG 13 Analysis of Cot-1 and Pol II enrichment on the Xi chromosome in esiRNA-treated hTERT-RPE1 cells. Analyses were performed as described in the legend to Fig. 12. Bars, 5 μ m. (A and B) Cot-1 enrichment. The total numbers of cells assayed were 178 for esi-luciferase and 180 for esi-Ki-67. (C and D) Pol II enrichment. The total numbers of cells assayed were 160 for esi-luciferase and 176 for esi-Ki-67. (E) Quantitation of Cot-1 RNA overlapping XIST RNA domains in the images from panels A and B. (F) Quantitation of RNA Pol II at XIST RNA domains in the images from panels C and D. (G) Immunoblot analysis of Ki-67 depletion in esiRNA-treated hTERT-RPE1 cells.

FIG 14 Some aspects of Xi chromosome structure and function were resistant to Ki-67 depletion. (A) The XIST clouds in hTERT-RPE1 cells have similar appearances regardless of Ki-67 depletion. Cells were treated with the indicated siRNAs for 72 h and analyzed by RNA-FISH to localize XIST (green) and DAPI (blue) staining. Bar, 10 μ m. (B) Average RNA levels of X-linked genes did not change upon Ki-67 depletion in hTERT-RPE1 cells. Analyses of log₂ fragments per kilobase per million (FPKM) for the RNA-seq data from two biological replicates for the two indicated siRNA treatments are shown. (C to H) MacroH2A enrichment at the XIST domain was not altered upon Ki-67 depletion. hTERT-RPE1 cells were treated with siRNAs (C to E) or *in vitro*-diced esi-Ki-67 (F to H) for 72 h. (C and F) Cells were analyzed by immuno-RNA-FISH to localize XIST (green) and macroH2A (red). Bar, 10 μ m. (D and G) Quantitation of cells that displayed reduced macroH2A staining. The total numbers of cells assayed were 248 for si-scramble, 291 for si-Ki-67, 218 for esi-luciferase, and 236 for esi-Ki-67. (E and H) Immunoblot analyses of Ki-67 depletions.

cells, this compound causes G₁ cell cycle arrest via an Rb-mediated checkpoint, inhibiting Ki-67 and cyclin A gene transcription, while proteasome-mediated degradation destroys existing Ki-67 protein molecules. CDKi sensitivity appears to be similar to many of the responses we observed upon Ki-67 depletion, and CDKi-sensitive cells include IMR90 and primary fibroblasts that we found to be “Ki-67 sensitive.” MCF7 breast adenocarcinoma cells are also CDKi sensitive (55), and we found that, upon Ki-67 depletion, MCF7 cells induce p21 and downregulate DNA replication genes. In contrast, HeLa and U2OS cells are not sensitive to either CDKi or Ki-67 depletion. Thus, depletions of Ki-67 via CDKi treatment (55) or via siRNA (this work) often lead to similar outcomes.

However, there is a counterexample to these correlations. CDKi treatment blocks S-phase entry and depletes Ki-67 in MDA-MB-231 breast adenocarcinoma cells (55). In contrast, upon Ki-67 depletion, MDA-MB-231 cells do not display either p21 induction or transcriptional downregulation of S-phase genes. Therefore, proteasome-mediated degradation of Ki-67 via CDK4/6 inhibition is not equivalent to siRNA-mediated Ki-67 depletion in all cell types. We hypothesize that a key difference is related to induction of p21 in “Ki-67-sensitive” cell lines. p21 contributes to G₁/S arrest via multiple mechanisms. As a CDK inhibitor (32, 33), p21 blocks CDK-mediated Rb phosphorylation, thereby inhibiting E2F-driven transcription (56). Likewise, it maintains the activity of the transcriptionally repressive DREAM complex, which contains Rb-related p107/p130

FIG 15 Depletion of Ki-67 redistributed the Xi chromosome within hTERT-RPE1 but not 293T cell nuclei. Bars, 10 μ m. (A) Fluorescence microscopy images of representative hTERT-RPE1 cells treated with either scramble control or Ki-67-targeted siRNA, as indicated. Cells were analyzed by RNA-FISH to detect XIST RNA (green) marking the Xi chromosome and by immunofluorescence with antifibrillarin antibodies (red) to label nucleoli. DNA was stained with DAPI (blue). (B) Quantification of XIST association frequencies from the experiments for panel A. XIST associations with the indicated locations were counted; “total nucleolus” indicates the sum of XIST signals that were exclusively nucleolar plus those that were also on the nuclear periphery. Three biological replicate experiments were performed, and mean percentages of association and SD were graphed. The total numbers of cells assayed were 363 for si-scramble and 376 for si-Ki-67. Holm-Sidak-corrected *P* values comparing the si-scramble and si-Ki-67 treatments are indicated, with *P* values of <0.05 shown in red. (C) Fluorescence microscopy images of representative hTERT-RPE1 cells treated with either luciferase- or Ki-67-targeted esiRNA, as indicated. Cells were stained as described for panel A. (D) Quantification of association frequencies from panel C experiments. The total numbers of cells assayed were 348 for esi-luciferase and 391 for esi-Ki-67. (E) Fluorescence microscopy images of representative 293T cells treated with either scramble control or Ki-67-targeted siRNA. (F) Quantification of XIST association frequencies from panel E experiments. The total numbers of alleles assayed were 272 for si-scramble and 298 for si-Ki-67. Holm-Sidak-adjusted *P* values for comparisons of association frequencies were >0.97 for all comparisons. (G) Fluorescence microscopy images of representative 293T cells as described for panel E, except that the cells were treated with *in vitro*-diced esiRNAs as depletion reagents. (H) Quantification of XIST association frequencies from panel G experiments. The total numbers of alleles assayed were 250 for esi-luciferase and 270 for esi-Ki-67. Holm-Sidak-adjusted *P* values for comparisons of association frequencies were >0.98 for all comparisons. (I to L) Frequencies of Xi chromosome associations versus time. All associations were measured in HU-synchronized cells as described in the legend to Fig. 7A and B, and the averages and SD for 3 independent experiments are shown. For each time point, >300 cells were counted. (I) Xi chromosome-nucleolus-only associations (Xi chromosomes associated with nucleoli but not with the lamina). (J) Xi chromosomes simultaneously associated with both the lamina and nucleoli. (K) Xi chromosome-lamina-only associations (Xi chromosomes associated with the lamina but not with nucleoli). Note that Xi chromosome-nucleolus associations (I and J) peak and Xi chromosome-lamina-only associations (K) reach a minimum when the majority of cells are in mid- to late S phase, which is delayed by 2 h in Ki-67-depleted cells. (L) Total Xi chromosome-laminar associations (Xi chromosomes associated either with the lamina or with both the lamina and nucleoli simultaneously).

“pocket protein” subunits (26, 27, 54). p21 also directly interacts with PCNA and directly inhibits DNA synthesis (35). Therefore, the lack of p21 induction in MDA-MD-231 cells may be the key factor explaining the lack of “Ki-67 sensitivity” in this cell line. Future experiments will be required to determine whether the activation of the DREAM complex or direct inhibition of the DNA synthesis machinery is more important for the “Ki-67-sensitive” phenotype associated with p21 induction.

Regarding the defect in p21 induction in MDA-MB-231 cells, we note that they express a gain-of-function R280K allele, which dominantly blocks p21 induction (38). Thus, p53 status is likely a critical aspect of the different cell cycle responses to Ki-67 depletion in many cell lines. However, sensitivity to Ki-67 depletion cannot always be predicted strictly by p53 status. For example, HCT116 cells express wild-type p53 (38) but are not sensitive to the CDKi PD0332991 (55) or to Ki-67 depletion (Fig. 9J). Because

Ki-67 expression predicts the differential response of different cell lines to CDKi treatment during xenograft tumor formation (55), understanding how different checkpoint mutations alter Ki-67 expression and sensitivity to its depletion is an important goal for developing stratified approaches to cancer therapies.

Ki-67 contributes to the interphase localization of the Xi chromosome. Nucleoli are non-membrane-bound organelles within the nucleus. Not only are these sites of synthesis and assembly of ribosome components, but the periphery of these organelles plays an important role in higher-order chromosome localization (49, 57). Specifically, the nucleolar periphery houses a subset of the cellular heterochromatin, which exchanges dynamically with lamina-associated heterochromatin (50–52). As in other heterochromatin regions, high-resolution analysis of nucleolus-associated domains (NADs) in human cells reveals enrichment of satellite repetitive DNAs and repressive histone marks (51, 58, 59). Major questions in chromosome biology include how heterochromatin regions are partitioned into different intranuclear locations and how these interactions are governed by cell cycle progression.

As a region of facultative heterochromatin, the Xi chromosome in female cells is enriched in NAD loci, usually localized to either the nucleolar periphery or the nuclear lamina (24, 40, 58). In mouse cells, the Xi chromosome-nucleolus association is cell cycle dependent, with frequencies peaking during mid- to late S phase. A genetic deletion was used to show that the long noncoding RNA XIST, which is expressed from the Xi chromosome, is required for normal Xi chromosome-perinucleolus localization. Deletion of XIST results in diminished H3K27me3 enrichment and increased synthesis of Cot-1-hybridizing, repeat-derived RNAs on the Xi chromosome (24). These data suggest that perinucleolar localization of the Xi chromosome contributes to the maintenance of heterochromatin structure. More recently, depletion of the long noncoding RNA Firre was shown to reduce association of the Xi chromosome with the nucleolus in mouse cells, and it also reduced H3K27me3 density on the Xi chromosome (60). However, Firre depletion had minimal effects on Xi gene silencing, consistent with the idea that multiple functionally overlapping factors affect Xi heterochromatin localization and gene silencing.

In the present study, we discovered that Ki-67 affects the Xi chromosome-nucleolus interaction. Analysis of synchronized RPE-1 cells showed that the association appears more slowly in Ki-67-depleted cells, coincident with the delay in S-phase entry. In addition to this delay, Ki-67 depletion alters some of the heterochromatin characteristics of the Xi chromosome, causing significantly increased levels of Cot-1-hybridizing RNAs and Pol II and decreased enrichment of H3K27me3 and H4K20me1. This loss of heterochromatic properties is partial in the cell population, and we did not detect uniform reactivation of Pol II genes on the Xi chromosome. These data are consistent with previous studies showing that multiple overlapping mechanisms maintain the inactive status of the Xi chromosome (60, 61). We note that XIST levels are not reduced upon Ki-67 depletion (Fig. 10; see Table S2 in the supplemental material), suggesting that altered XIST levels are unlikely to explain the effect of Ki-67 on the Xi chromosome. Instead, our data are consistent with the view that Ki-67 is one of the factors that contributes to the maintenance of heterochromatic structures of the Xi chromosome (12), in this case in a manner coupled to S-phase progression.

Recent data show that the nuclear lamina localization mediated by the interaction between XIST and the lamina B receptor facilitates the spreading of XIST on the Xi chromosome, which in turn contributes to transcriptional silencing (62). This raises the question of whether there are specific protein factors that contribute to the association of the Xi chromosome with the nucleolus in addition to the lncRNAs XIST and Firre (24, 60). Could Ki-67 be such a factor? In support of this idea, Ki-67 is also required for association of other heterochromatic regions with nucleoli in interphase cells (11, 12, 22).

It appears that the erosion of heterochromatic features on the Xi chromosome occurs in a significant fraction of Ki-67-depleted cells away from the nuclear lamina but

that lamina-associated Xi chromosomes are not altered. There are two possibilities to explain these data. First, it may be that lamina association confers protection from heterochromatin changes. Alternatively, Xi chromosomes that are most severely affected by Ki-67 depletion may preferentially relocate away from the lamina. Because our shRNA-based experiments necessitate a 72-h period to achieve strong Ki-67 depletion, there is likely passage through multiple mitoses for each cell during this period. Because Ki-67 is a key component of the perichromosomal layer that envelopes each mitotic chromosome (11, 15), it is tempting to speculate that the loss of Ki-67 affects the reassociation of heterochromatic sequences with the nuclear lamina or nucleoli after mitotic exit.

MATERIALS AND METHODS

Antibodies and immunoblotting. The following antibodies were used in this work: rabbit anti-Ki-67 (Ab15580; Abcam), mouse anti-beta-tubulin (Y1060; Ubpbio), rabbit antifibrillarin (ab5821-100; Abcam), mouse anti-BrdU antibody (MoBu-1) (ab8039; Abcam), mouse anti-p21 antibody (ab109520; Abcam), rabbit anti-microH2A.1 (ab37264; Abcam), mouse anti-Rb antibody (4H1) (9309; Cell Signaling), mouse anti-H4K20me1 (39727; Active Motif), mouse anti-RNA polymerase II, clone CTD4H8 (05-623; Millipore), mouse antinucleophosmin (sc-32256; Santa Cruz Biotechnology), rabbit anti-WSTF (2152; Cell Signaling), rabbit anti-H3K27me3 (39535; Active Motif), Amersham ECL horseradish peroxidase (HRP)-linked rabbit IgG whole antibody (NA934; GE Life Science), Alexa Fluor 594-conjugated donkey anti-rabbit IgG(H+L) secondary antibody (A-21207; Life Technologies), Alexa Fluor 488-conjugated donkey anti-rabbit IgG(H+L) secondary antibody (A-21206; Life Technologies), Alexa Fluor 488-conjugated streptavidin (S-32354; Invitrogen), and DyLight 594-labeled anti-digoxigenin-digoxin (DI-7594; Vector Laboratories).

For immunoblotting, cells were collected 3 days after RNA interference (RNAi) transfection. Whole-cell lysates were extracted in 20 mM Tris-HCl, pH 7.5, 1% SDS, and 10% glycerol supplemented with protease inhibitor cocktail (Sigma). The lysates were sonicated in a Bioruptor set on high power for one 5-min cycle, with 30 s on and 30 s off. Fifteen micrograms of each lysate was separated by SDS-PAGE, transferred to a polyvinylidene difluoride (PVDF) membrane, and probed as described in the figure legends.

Cell cultures. hTERT-RPE1 cells (a kind gift from Judith Sharp) and hTERT-BJ cells were cultured in Dulbecco's modified Eagle's medium (DMEM)-F-12 medium (VWR) with 10% fetal bovine serum (FBS; HyClone), 1% penicillin-streptomycin, 5% L-glutamine, and 7.5% sodium bicarbonate solution.

HeLa and U2OS cells were propagated in DMEM supplemented with 10% fetal bovine serum. Cells were maintained at >25% confluence and passaged every 3 days.

Human foreskin fibroblasts (HFFs; a kind gift from Jennifer Benanti, University of Massachusetts Medical School [63]) were maintained in DMEM containing 10% FBS and antibiotic-antimycotic solution. HFF cells were grown at >25% confluence and were split 1:4 every 2 days. All cells were maintained in a 37°C incubator with 5% CO₂.

WI-38 cells were maintained in DMEM supplemented with 10% FBS, 2 mM L-glutamine, and antibiotic-antimycotic solution (Life Technologies, Carlsbad, CA). WI-38 cells were grown at >25% confluence and were split 1:4 every 2 days.

IMR-90 and MDA-MB-231 cells were maintained in DMEM with 10% FBS, 2 mM L-glutamine, and antibiotic-antimycotic solution. The cells were cultured at >25% confluence and were split every 2 days.

HCT116 (a kind gift from Anastasiia Vertii, University of Massachusetts Medical School) and 293T cells were cultured in DMEM with 10% FBS. The cells were split 1:4 every 3 days.

MC7 cells were grown in RPMI medium with 10% FBS. The cells were split 1:3 every 3 days.

For hydroxyurea treatment, hTERT-RPE1 cells were cultured in the presence of 2 mM hydroxyurea for 15 h, washed three times with phosphate-buffered saline (PBS), released into hydroxyurea-free medium, and harvested at the indicated time points.

sgRNA design. For editing of the siRNA target site in the endogenous Ki-67 locus, the four highest-scoring single guide RNAs (sgRNAs) targeting nucleotides 9661 to 9722 of the genomic DNA (accession no. [NG_047061](https://www.ncbi.nlm.nih.gov/nuccore/NG_047061)) were selected by using the CRISPR Design tool at <http://crispr.mit.edu/> (64). The sgRNA sequences were as follows: 5'-ACGTGCTGGCTCCTGTAAGT-3' (antisense), 5'-TCTAGCTTCTCTCTGACCC-3' (sense), 5'-GATCTTGAGACACGACGTGC-3' (antisense), and 5'-CTTCTGACCCTGGTGAGTAG-3' (sense).

These were cloned into a variant of the pX330 plasmid (64) with a puromycin resistance cassette (a kind gift from Kurtis McCannell and Thomas Fazzio, University of Massachusetts Medical School) as previously described (65). To determine the most efficient sgRNAs, 293T cells were transfected by use of FuGENE HD (Promega) according to the manufacturer's instructions. Five hundred nanograms of sequence-verified CRISPR plasmid (pSpCas9-sgRNA) was transfected into 200 × 10³ cells in each well of a 24-well dish. At 48 h posttransfection, DNA was extracted using QuickExtract DNA extraction solution (Epicentre) according to the manufacturer's instructions. Genomic DNA was PCR amplified using *Taq* DNA polymerase (New England BioLabs). The following primers were used for PCR amplification: F2 primer, GGGTTCACGAAATTCCTCTG; and R primer, TCACCAAGGAAAGTTAGGC. PCR products (514 bp) were run in an agarose gel, purified using a Zymoclean gel DNA recovery kit (Zymo Research), and sent for Sanger sequencing at the Genewiz sequencing facility. The following primer was used for sequencing: F primer, GCCAGGCTGTCTCAAACCTC.

To assess gene editing in 293T cells by use of the four sgRNA plasmids, the TIDE tool (<https://tide-calculator.nki.nl/>) was used (66). Trace data for PCR fragments from green fluorescent protein (GFP)-transfected 293T cells were used as a control sample chromatogram, and trace data for PCR fragments from CRISPR plasmid-transfected cells were used as test sample chromatograms. The following sgRNA was selected due to its efficiency in cutting and proximity to the siRNA site: 5'-GATCTTGAGACACGAC GTGC-3' (referred to as sgKi-67 from now on).

Cotransfection of CRISPR plasmid and HDR template into hTERT-RPE1 cells. A homology-directed repair (HDR) template carrying the siRNA resistance-conferring mutations, the EcoO109I site, and 700-bp homology flanks on each side was purchased as a gBlock from Integrated DNA Technologies. The template was cloned into pCR2.1 (Thermo Fisher Scientific) according to the manufacturer's instructions.

sgKi-67 was cloned into a variant of the pX330 plasmid (64) with a neomycin resistance cassette (a kind gift from Kurtis McCannell and Thomas Fazio, University of Massachusetts Medical School) to facilitate gene editing in hTERT-RPE1 cells. A total of 3.3 μ g of a 1:1 (vol/vol) mix of repair template and CRISPR plasmid was transfected by use of FuGENE HD into 75×10^3 cells in a 6-well dish. Starting at 48 h posttransfection, cells were cultured in selection medium with 800 μ g/ml of G418 (Sigma-Aldrich) for 7 days, with the selection medium being changed every other day. Cells were recovered in G418-free medium for 4 days, after which cells were trypsinized and diluted to 0.5 cells per 200 μ l and seeded into 96-well plates. A week later, the plates were inspected for wells with single colonies, and 4 days after that, the samples were replica plated into two 96-well plates. One plate was frozen, and the second was used to maintain and passage the cells. Once cells on the third plate were at least ~70% confluent, DNAs were extracted using QuickExtract DNA extraction solution and PCR amplified using the following primers: F3 primer, TGGCCCATTTATGAGAAACTGA; and R2 primer, GGGAACAGACTTCAATTCTCCA. PCR products (1,523 bp) were further digested with the EcoO109I restriction enzyme (New England BioLabs). PCR products from successfully integrated clones were expected to be digested into products of 751 and 772 bp. PCR products for clones positive for the EcoO109I-digested bands were run in an agarose gel, purified using a Zymoclean gel DNA recovery kit, and sent to Genewiz for Sanger sequencing. Primers F2 and R2, shown above, were used for Sanger sequencing.

Immunofluorescence (IF) assay. Cells grown on glass coverslips were fixed in 4% paraformaldehyde for 10 min and then permeabilized with 0.5% Triton X-100 for 10 min at room temperature. The fixed cells were blocked in 5% goat serum for 30 min and then incubated with a primary antibody at 37°C in a humidified chamber for 1 h. The cells were washed with PBS three times for 5 min each, incubated with a secondary antibody for 1 h at 37°C in a humidified chamber, and then washed with PBS three times for 5 min each. Slides were then incubated with 130 ng/ml 4',6-diamidino-2-phenylindole (DAPI) for 5 min and mounted in Vectashield mounting medium (Vector Lab).

Images were taken on a Zeiss Axioplan2 microscope with a 63 $\times$ objective. Entire cells were imaged via z stacks taken at 200-nm intervals. Approximately 25 stacks were taken per cell and were displayed as two-dimensional maximum-intensity projections generated using AxioVision, version 4.6.

The Xi chromosome-nucleolus association frequencies on individual coverslips were scored in a blinded manner. The criterion for Xi chromosome-nucleolus or -lamina association was that there were no pixels between the fluorescence signals from the XIST FISH probe and fibrillarin immunostaining (for nucleolar association) or DAPI staining of the nuclear edge (for lamina association). Densitometry of individual immunostained cells was performed in ImageJ 10.2 (48), using the macro script of the RGB Profiles tool for all experiments. The quantifications of H3K27me3, macroH2A, H4K20me1, Cot-1, and Pol II enrichment were also performed in ImageJ 10.2 (48).

Visualization of EdU-labeled nascent DNA. hTERT-RPE1 cells were grown on glass coverslips in DMEM-F-12 medium as described above. 5-Ethynyl-2-deoxyuridine (EdU) was added to the culture medium at 10 μ M for 20 min. After labeling, cells were washed three times with PBS. Cells were permeabilized in 0.5% Triton X-100 for 30 s and then fixed in 10% formaldehyde for 10 min. Cells were then rinsed twice with PBS and incubated for 30 min in 100 mM Tris-HCl, pH 8.5, 1 mM CuSO₄, 100 mM ascorbic acid, and 50 mM carboxyrhodamine 110-azide for click-chemistry labeling. After staining, the cells on coverslips were washed three times with PBS plus 0.5% Triton X-100 for 5 min each. Cells were then counterstained with DAPI, mounted in Vectashield, and imaged by fluorescence microscopy as described above.

Immuno-RNA-FISH and RNA-FISH. The plasmid pGIA, which contains human XIST exons 4, 5, and 6, was a gift from Judith Sharp. The Cot-1 probe was obtained from Invitrogen. The probes were labeled with either biotin-14-dCTP or digoxigenin-11-dUTP by using the BioPrime DNA labeling system (Invitrogen).

For IF assay combined with RNA-FISH (immuno-RNA-FISH), the IF assay was first performed as described above. Cells were then refixed in 4% paraformaldehyde for 10 min at room temperature. The cells were then dehydrated in 75%, 85%, 95%, and 100% ethanol for 2 min each.

Approximately 150 ng of each probe was mixed with 20 μ g single-stranded salmon sperm DNA (Sigma-Aldrich) and 12 μ g *Escherichia coli* tRNA and then air dried in a speed vacuum, resuspended in 20 μ l 50% formamide-50% hybridization buffer (20% dextran sulfate in 4 $\times$ SSC [1 $\times$ SSC is 0.15 M NaCl plus 0.015 M sodium citrate]), denatured at 80°C for 10 min, and preannealed at 37°C for 30 min prior to hybridization. Hybridizations were performed overnight in a humidified chamber at 37°C. The next day, cells were washed for 20 min in 50% formamide in 2 $\times$ SSC at 37°C and then for 20 min in 2 $\times$ SSC at 37°C and 20 min in 1 $\times$ SSC at 37°C. The hybridized probes were detected by incubation with either Alexa Fluor 488 conjugated to streptavidin (Invitrogen) or DyLight 594-labeled anti-digoxigenin-digoxin (Vector Laboratories) at a 1:500 dilution for 60 min in a 37°C humidified chamber. After incubation, slides

were washed twice in 50% formamide, 2× SSC for 5 min and once in 1× SSC for 5 min in a 37°C humidified chamber before DAPI staining as described above.

RNAi experiments. The siRNA targeting human Ki-67 is from the collection of Silencer Select predesigned siRNAs (Thermo Fisher Scientific) and targets nucleotides 559 to 577 of the cDNA (accession no. [NM_002417.4](#)). Its sequences are as follows (lowercase letters indicate 5′ overhangs after annealing): sense, CGUCGUGUCUACAAGAUUCUAtt; and antisense, UAGAUCUUGAGACACGACGtg. For human TP53 (hTP53) (accession no. [NM_00546.5](#)), the forward primer GAAUUUGCGUGUGGAGUAtt and the reverse primer UACUCCACACGCAAAUUUCct were used. For hp21 (accession no. [NM_078467.2](#)), the forward primer CAAGGAGUCAGACAUUUUAtt and the reverse primer UAAAAUGUCUGACUCCUUGtt were used.

The esiRNA targeting human Ki-67 was generated by *in vitro* RNase III cleavage of T7 RNA polymerase-synthesized transcripts, as previously described (21, 67), and targets internal repeat regions at nucleotides 3611 to 4047, 3979 to 4357, 4705 to 5098, and 6913 to 7347 of the cDNA (accession no. [NM_002417.4](#)). For hKi-67, the forward primer gcgtaatacgtactactataggGTGCTGCCGGTTAAGTTCTCT and the reverse primer gcgtaatacgtactactataggGCTCCAACAAGCACAAGCAA were used. For luciferase, the forward primer gcgtaatacgtactactataggAACAAATTGCTTTTACAGATGC and the reverse primer gcgtaatacgtactactataggAGGCAGACAGTAGATCC were used.

Cells were transfected by use of Lipofectamine RNAi Max (Invitrogen) following the manufacturer's instructions. For esiRNA transfection, 500 ng of esiRNA targeting either the luciferase control or Ki-67 was transfected into 40 × 10³ cells in a 6-well dish. For siRNA transfection, either a scrambled siRNA or si-Ki-67 (40 nM) was transfected into 40 × 10³ cells in a 6-well dish. The cells were harvested 72 h after transfection for immunoblotting, RT-qPCR, RNA-seq, FACS, or FISH analysis.

Flow cytometry. BrdU incorporation was analyzed based on published protocols (68). Cells were pulse labeled with 50 μM BrdU for the indicated periods. Cells were then washed twice with PBS and fixed in 70% ethanol at 4°C for 1 h. Postfixed cells were denatured in 2 N HCl–0.5% Triton X-100 for 30 min. After denaturation, cells were washed once in 0.1 M sodium tetraborate for 2 min and once in PBS–1% bovine serum albumin (BSA). After that, the cells were resuspended in 1 μg/ml anti-BrdU antibody, PBS, and 1% BSA for 1 h, followed by three washes with 0.5% Tween 20, 1% BSA, and PBS. The cells were incubated with 0.5 μg/ml secondary antibody, PBS, and 1% BSA for 30 min, counterstained with 50 μg/ml propidium iodide in PBS, and analyzed on an LSR II flow cytometer (BD Biosciences). The data were analyzed with FlowJo v9.9.4 software (TreeStar, Ashland, OR).

RNA isolation and real-time quantitative PCR. Total RNA from cells at 72 h posttransfection was isolated using TRIzol (Invitrogen) following the manufacturer's instructions and purified using an RNeasy kit (Qiagen).

One microgram of RNA was subjected to reverse transcription with SuperScript II reverse transcriptase (Invitrogen). qPCRs were performed on an Applied Biosystems StepOnePlus machine (Life Technologies), using Fast Sybr mix (Kapa Biosystems). The program used was as follows: hold at 98°C for 30 s followed by 40 cycles of 95°C for 10 s and 60°C for 30 s. All the signals were normalized to that of beta-actin as indicated in the figure legends, and the 2^{−ΔΔCT} method was used for quantification (Life Technologies). Primer sequences were designed by use of Primer3Plus software (69). All oligonucleotides for qPCR are listed in Table S5 in the supplemental material.

RNA-seq sample preparation and analysis. RNAs were isolated as described above. Libraries from two replicates for each condition were constructed in a strand-specific manner via the dUTP method by BGI and then sequenced as single-end 50-base reads, using the Illumina HiSeq 2000/2500 platform (BGI). Twenty-nine million and 31 million mapped reads were obtained from the two si-scramble-treated controls, and 28 million and 29 million mapped reads were obtained from the two si-Ki-67 knockdown replicates.

Reads were aligned to the human reference genome (hg19) by use of Tophat 2.0.14 (70, 71). Differential expression analysis was determined by use of Cufflinks 2.2.1 (72). In addition, reactome analyses were performed using the Bioconductor package ChIPpeakAnno (version 3.2.0) (73, 74). Genes that showed differential expression between control and Ki-67 depletion samples, with a Benjamini-Hochberg adjusted *q* value of <0.05 (75), were selected for the reactome analysis.

For the SNP analysis, the genotype (SNP) information from the RPE cell line (76) from GEO sample [GSM1848919](#) (<https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSM1848919>) was annotated based on the R bioconductor package SNPlocs.Hsapiens.dbSNP144.GRCh38 (SNP locations for *Homo sapiens* [dbSNP build 144; R package, version 0.99.20]) The SNP locations were further annotated by using the ChipPeakAnno package (73).

Accession number(s). The data discussed in this publication have been deposited in NCBI's Sequence Read Archive (SRA) (<http://www.ncbi.nlm.nih.gov/sra/>) and are accessible through SRA series accession numbers SRR4252548, SRR5353471, SRR5353778, and SRR5353934.

SUPPLEMENTAL MATERIAL

Supplemental material for this article may be found at <https://doi.org/10.1128/MCB.00569-16>.

SUPPLEMENTAL FILE 1, XLSX file, 0.1 MB.

SUPPLEMENTAL FILE 2, XLSX file, 3.5 MB.

SUPPLEMENTAL FILE 3, XLSX file, 0.2 MB.

SUPPLEMENTAL FILE 4, XLSX file, 0.1 MB.

SUPPLEMENTAL FILE 5, XLSX file, 0.1 MB.

SUPPLEMENTAL FILE 6, PDF file, 0.1 MB.

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