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RING1A and BMI1 bookmark active genes via ubiquitination of chromatin-associated proteins

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ABSTRACT

During mitosis the chromatin undergoes dramatic architectural changes with the halting of the transcriptional processes and evacuation of nearly all transcription associated machinery from genes and promoters. Molecular bookmarking of genes during mitosis is a mechanism of faithfully transmitting cell-specific transcription patterns through cell division. We previously discovered chromatin ubiquitination at active promoters as a potential mitotic bookmark. In this study, we identify the enzymes involved in the deposition of ubiquitin before mitosis. We find that the polycomb complex proteins BMI1 and RING1A regulate the ubiquitination of chromatin associated proteins bound to promoters, and this modification is necessary for the expression of marked genes once the cells enter G1. Depletion of RING1A, and thus inactivation of mitotic bookmarking by ubiquitination, is deleterious to progression through G1, cell survival and proliferation. Though the polycomb complex proteins are thought to primarily regulate gene expression by transcriptional repression, in this study, we discover that these two polycomb proteins regulate the transcription of active genes during the mitosis to G1 transition.

INTRODUCTION

During mitosis, when the chromatin is condensed, global silencing of transcription occurs along with the displacement of the majority of general and tissue-/gene-specific transcriptional factors and other associated machinery from the chromatin (1). This provides a window of opportunity for the cells to undergo major reprogramming of their transcriptional states, but in most cases, cellular identity needs to be maintained and gene expression patterns are accurately restored upon exit from mitosis. Understanding how cells remember which genes to express after cell division is an important problem in biology, and though epigenetic regulation controls gene expression in development, it cannot explain how active genes remain active after the cells pass through mitosis.

Not all information is lost during this stage and a subset of factors remain bound to mitotic chromosomes, providing a molecular bookmark to direct proper chromatin reassembly (2–6). This process of transcriptional memory by molecularly marking these genes during mitosis is referred to as 'mitotic bookmarking', and it involves retention of histone modifications and histone variants and distortions in the chromatin such as nuclease accessibility (2,7,8). In addition, several transcription factors are also retained at a subset of their target genes during this stage. The factors that regulate the retention of these transcription factors at these mitosis specific sites versus their interphase binding sites are not well understood (9,10).

We recently discovered that an additional mechanism of gene bookmarking in HeLa cells occurs by ubiquitination of the proteins associated with the regulatory regions of active genes during cell division (2). This bookmark occurs specifically during mitosis and primarily on those genes that are highly expressed in these cells soon after finishing mitosis.

Polycomb group (PcG) proteins form two major types of the polycomb complexes—PRC1, typically consisting of core proteins BMI1, RING1B/RING1A, CBX4 and PHC1, and PRC2 consisting of core proteins EZH2, SUZ12, EED and RbAp46/48 (reviewed in (11,12)). Together, these complexes drive cell differentiation by silencing genes that are required for the undifferentiated/pluripotent state. Other complexes containing some of these PcG proteins have also been reported (13,14), raising the possibility that some of the PcG proteins have other functions not associated with the PRC1 complex. The primary function of the PRC1 complex is to execute the RING1B-dependent monoubiquitination of histone H2A, and BMI1 stimulates the E3 activity of RING1B (15,16). RING1A is a less efficient H2A ubiquitin ligase and is not the main H2A ubiquitin ligase for the PRC1 complex. H2A is the major but not the sole, ubiquitination substrate of these RING1 proteins. BMI1 and RING1A (but not RING1B) ubiquitinate

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TOP2A in cells treated with the topoisomerase inhibitor etoposide and lead to proteasomal degradation of TOP2A (17). Thus, the RING1A protein could have hitherto undiscovered ubiquitination targets other than H2A.

In this study, we screened likely chromatin associated ubiquitin ligases for the enzyme that ubiquitinates chromatin associated proteins at the promoters of active genes during mitosis. We found that the PcG proteins BMI1 and RING1A stimulate the ubiquitination of the chromatin at bookmarked promoters. Though several chromatin associated marks have been characterized as bookmarks (18–20) the discovery that BMI1 and RING1A specifically regulate the placement of one of these marks enables the investigation of how these bookmarks impact cell function. Bookmarking the promoters via ubiquitination during mitosis is necessary for the expression of these genes once the cells exit mitosis and enter G1. In addition, our data reveal that ubiquitination at the bookmarked promoters during mitosis is required for another mitotic bookmark, H3K4me3, to tag these genes. We also show that bookmarking of genes by ubiquitination is a crucial process and its perturbation is deleterious to the proliferation of cells.

MATERIALS AND METHODS

Cell culture, cell cycle synchronization and transfections

HeLa cells expressing the tagged ubiquitin (HeLa-Ub) (21) were grown under standard conditions and supplemented with biotin (0.5 μM, Sigma Aldrich) and puromycin (1.5 ug/ml, Life technologies). Cells were transfected with siRNA using oligofectamine or plasmids using lipofectamine 2000 using the manufacturer's protocol (Life technologies). Sequence information for the siRNAs used in this study is provided in Supplementary Table S1.

Cells were synchronized in mitosis using a thymidine-nocodazole block by treating them with thymidine for 18 h, releasing in thymidine free media for 3 h followed by a 12-h nocodazole block. To yield cells synchronized in G1, HeLa cells were first blocked in mitosis followed by release into a nocodazole free media for 7 h.

Cell proliferation assay

Forty-eight hours post-transfection of siRNAs into HeLa or U2OS cells, 2000 cells (for HeLa) or 20 000 cells (for U2OS) were seeded onto 6-well dishes. The cells were harvested at intervals of 2 days and counted using a hemocytometer.

Antibodies

The antibodies used in this study were specific for USP22 (Abcam), RING1B (MBL international), RING1A (Santa Cruz), BMI1 (Cell signaling technology), α-tubulin (Sigma), GAPDH (Advanced immunochemical inc.), histone H4 (Millipore), RHA (RNA Helicase A) (22), H3K4me3 (Cell signaling technology), H3K79me2 (Abcam), RNAPII 8WG16 (23), phospho-H3 (Santa Cruz), Rabbit IgG (GE healthcare) and mouse IgG (GE healthcare).

Flow cytometry

FACS analysis was done on at least 10 000 cells stained with propidium iodide from each stage of the cell cycle using a BD FACScalibur machine in the OSUCCC Analytic Cytometry shared resource. Data was analyzed using the FlowJo software.

ChIP, ChAP and qPCR

Chromatin immunoprecipitation and affinity purification were performed as described in (2) ChAP is similar to ChIP except affinity purification was performed using avidin beads to purify the ubiquitinated chromatin. Input sample was saved before purification and was treated similar to the affinity purified DNA.

For qPCR analysis, affinity purified DNA or immunoprecipitated DNA and input DNA was used as a template for qPCR. The enrichment of the affinity purified DNA was expressed as percent of input and of immunoprecipitated DNA was expressed as fold enrichment over mock ChIP using IgG antibody. The sequences of primers used for qPCR in this study are provided in Supplementary Table S2. Statistical significance was calculated by using student's paired *t*-test. *P*-values < 0.05 are indicated by a single asterisk (*), <0.001 by a double asterisk (**) and <0.0001 by three asterisks (***).

RESULTS

Polycomb repressive complex 1-associated proteins positively regulate promoter ubiquitination in mitosis

We previously used a cell line expressing biotin-tagged ubiquitin to identify genomic locations at which ubiquitin conjugated proteins are bound, and, importantly, we asked how the locations of the ubiquitin mark changed as cells traversed the cell cycle (2). We found that during interphase, the gene bodies of actively transcribed genes were ubiquitinated on H2B, consistent with the function of the Polymerase Associated Factor (PAF) complex (24) on templates undergoing transcription elongation. During mitosis, the gene bodies were no longer ubiquitinated, indicating the highly dynamic nature of that mark, but we found that the promoters of these genes were ubiquitinated specifically in mitosis. Once cells exited mitosis, the promoters were deubiquitinated and the transcribed regions of the genes were once again ubiquitinated, suggesting that the mitotic ubiquitination was a bookmark for active genes. In this study, we tested known chromatin associated ubiquitin ligases as candidates for the enzyme that places the ubiquitin on a chromatin associated protein at promoters during mitosis.

BMI1 is a homolog of the *Drosophila* Psc protein and is part of the polycomb repressive complex-1 (PRC1) along with two other E3 ubiquitin ligases, RING1A and RING1B, and its main function is to enhance the H2A ubiquitination activity of RING1B (and probably also RING1A) (15). To determine if BMI1 and the RING1 proteins play a role in ubiquitination of the chromatin at the promoters of genes that are active during interphase, we depleted these proteins via siRNA transfection and examined the effect on the level of ubiquitination at promoters.

HeLa-Ub cells express ubiquitin as a fusion protein with a domain that is naturally biotinylated (2,21). These cells were transfected and synchronized in mitosis using a sequential thymidine-nocodazole block, and loosely adherent mitotic cells were collected for analysis. Ubiquitination at the promoters was analyzed by ubiquitin-specific Chromatin Affinity Purification and quantitative PCR (ChAP-qPCR). This method is similar to ChIP except no antibody is used, but instead the affinity tag is used to specifically purify the target. In ChAP-qPCR, the ubiquitinated proteins and bound DNA were purified using an avidin agarose matrix that binds to the biotin moiety on the ubiquitin fusion protein. Immunoblot analysis showed a clear depletion of the specified proteins following siRNA treatment. Interestingly, while RING1B, the major effector E3 ubiquitin ligase of the PRC1 complex, had no effect on ubiquitination of chromatin proteins bound to the promoter (Figure 1A and B), BMI1 or RING1A depletion caused significant reductions in the ubiquitination at the promoters of the transcriptionally active ribosomal protein, GAPDH, EIF6 and MYC genes but not the transcriptionally inactive IL2 gene (Figure 1C–G). Non-overlapping siRNAs targeting RING1A showed a similar reduction in the ubiquitination levels of these genes (Figure 1E and F). In mitotic cells that were depleted of RING1A, the ubiquitination of proteins bound to the promoters of highly active genes were reduced to approximately the levels of ubiquitination observed in silent gene promoters. Ubiquitination of proteins at the promoters of silent genes including IL2, GAGE5, HIST1H2BA and the satellite Sat2 were low in control cells and not significantly affected by depletion of RING1A (Figure 1F). These results suggest that the promoter chromatin of active genes bookmarked during mitosis is modified by ubiquitination that is regulated by the BMI1-RING1A proteins but not by the RING1B enzyme. Since both siRNAs tested against RING1A showed similar reduction in mitotic promoter ubiquitination, the following experiments were performed using siRNA1.

Interestingly, when testing candidate deubiquitinases for removing the ubiquitin mark as cells enter G1 phase, we found that the SAGA complex associated deubiquitinase, USP22 (25) was also required for mitotic bookmarking and not the removal of the ubiquitin mark as cells enter G1 (Supplementary Figure S1). We interpret this result to indicate that USP22 indirectly regulates the mitotic bookmarking.

Chromatin ubiquitination at promoters during mitosis is essential for transcription of the bookmarked genes in G1

Since loss of RING1A led to the biggest decrease in the mitotic bookmark ubiquitination, we focused on RING1A for the remaining experiments. We hypothesized that RING1A mediated promoter ubiquitination during mitosis may play a role in the transcription of the bookmarked genes during G1. To test this hypothesis, we depleted RING1A and measured the abundance of RNAPII at these genes during G1. Cells were subjected to the thymidine-nocodazole block, released from nocodazole for 7 h and adherent and floating cells were collected and combined. During G1 of the cell cycle, active genes have RNAPII bound to the pro-

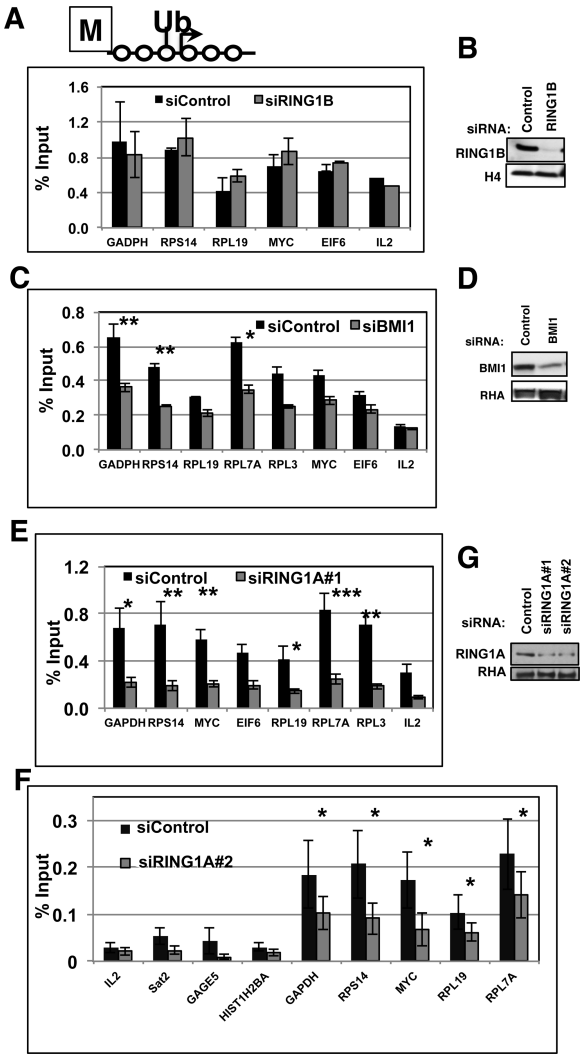


Figure 1. Polycomb proteins BMI1 and RING1A but not RING1B are essential for mitotic promoter ubiquitination. (**A**) A diagram of ubiquitination of chromatin-associated proteins during mitosis is depicted; the circles indicate nucleosomes and chromatin associated proteins (top). HeLa-Ub cells, which express biotinylated ubiquitin, were transfected with siRNA specific for RING1B (gray) or a control siRNA (black). Cells were arrested in mitosis, cross-linked and ChAP was performed using avidin agarose that purifies ubiquitinated proteins via the biotin tag. Promoter ubiquitination was determined by qPCR and normalized relative to the DNA content of the input for the ChAP reaction. Results were taken from three independent transfection experiments. Error bars indicate SEM. (**B**) The extent of depletion of RING1B was determined by immunoblot; Histone H4 was a loading control. (**C**) Ubiquitination of promoter-bound proteins during mitosis in cells depleted of BMI1 (gray) or a control siRNA (black) was determined in three replicate transfections, as in panel A. Error bars indicate SEM, and *($P < 0.05$) and **($P < 0.001$) indicate significant P-values using the student's paired t-test. (**D**) The extent of depletion of BMI1 was determined by immunoblot; RNA helicase A (RHA) was a loading control. (**E**) Ubiquitination of promoter-bound proteins during mitosis in cells transfected with a control siRNA (black) and with siRING1A #1 (gray) was determined in three replicate transfections, as in panel A. Error bars indicate SEM, and *($P < 0.05$), **($P < 0.001$) and ***($P < 0.0001$) indicate significant P-values using the student's paired t-test. (**F**) Ubiquitination of promoter-bound proteins during mitosis in cells transfected with a control siRNA (black) and with siRING1A #2 (gray) was determined in four replicate transfections, determined by the student's paired t-test using normalized values for the percent input. Error bars indicate SEM and *($P < 0.05$). (**G**) The extent of depletion of RING1A was determined by immunoblot; RHA was a loading control.

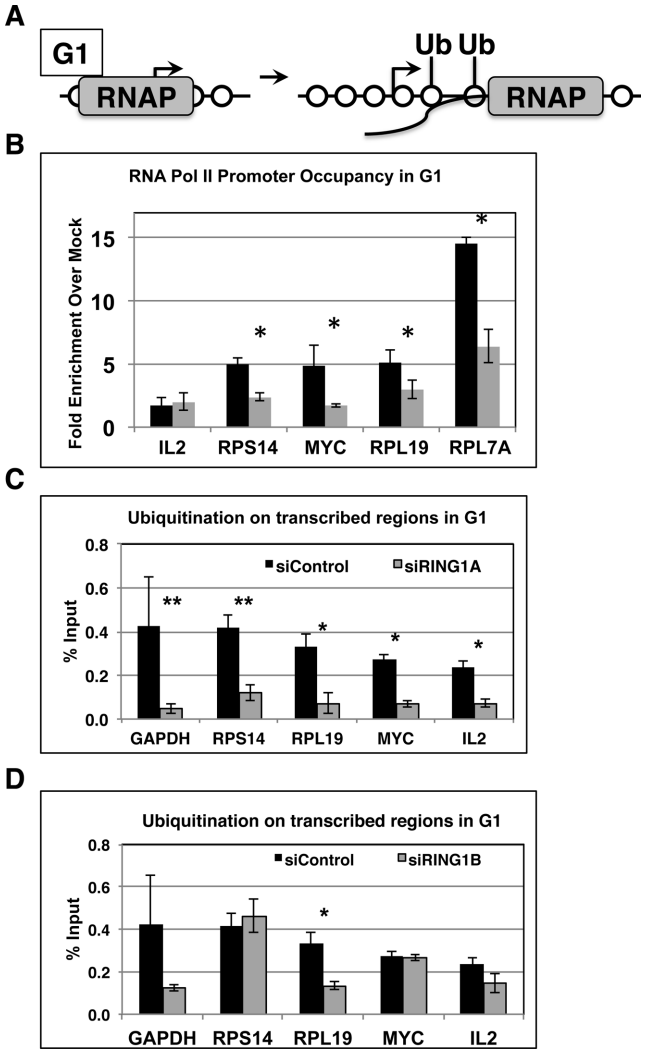


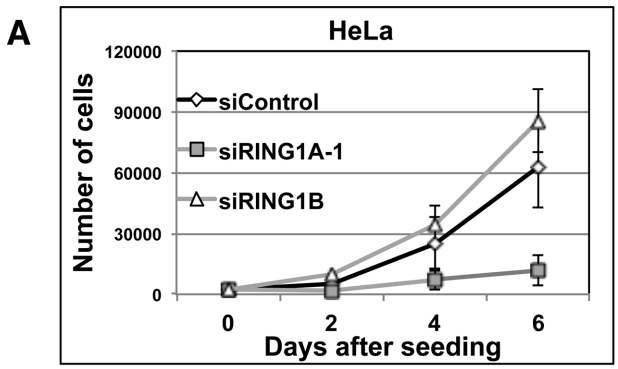
Figure 2. Depletion of RING1A in HeLa cells leads to reduction in transcription during G1 phase of the cell cycle. (**A**) The experimental setting is diagrammed with active transcription by the RNA polymerase II (RNAP) in complex with Paf1 causing ubiquitination of histone H2B downstream of the transcription start site. (**B**) HeLa cells were transfected with siRNA and synchronized in G1 using a thymidine-nocodazole block and a 7 h release. Association of RNAPII with promoters of the indicated genes was assessed by ChIP using the RNAPII-specific 8WG16 antibody followed by qPCR in cells transfected with the control siRNA (black) or the RING1A-specific siRNA (gray). Fold enrichment of RNAPII was determined by the ratio of DNA content in the 8WG16 ChIP relative to the control IgG ChIP. Results were taken from four independent transfection experiments. Error bars indicate SEM, and *($P < 0.05$), **($P < 0.001$) and ***($P < 0.0001$) indicate significant P-values. (**C**) HeLa-Ub cells were transfected with siRNA specific for RING1A and synchronized in G1 using a thymidine-nocodazole block and a 7 h release. To measure active transcription, ubiquitin bound to the chromatin at the coding regions of the indicated genes was determined by ChAP-qPCR, as in Figure 1A, in cells transfected (three replicate transfections) with the control siRNA (black) or the RING1A specific siRNA (gray). (**D**) Ubiquitination of coding regions was assayed in three replicate transfections as in panel B with the exception that RING1B (gray) was depleted from cells.

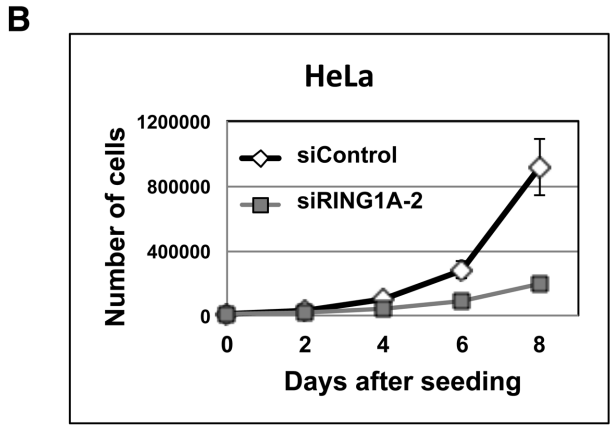
moter, and RNAPII binds to the transcribed region along with ubiquitinated H2B due to the action of the PAF complex regulation of transcriptional elongation (Figure 2A). Depletion of RING1A caused a decrease in the association of RNAPII with the promoters of the RPS14, MYC, RPL19 and RPL7A genes (Figure 2B). IL2 is not expressed in HeLa cells and its promoter is not ubiquitinated during mitosis (Figure 1C–F); consistent with this, only baseline levels of RNAPII were detected at the IL2 promoter and these were unaffected by depletion of RING1A (Figure 2B).

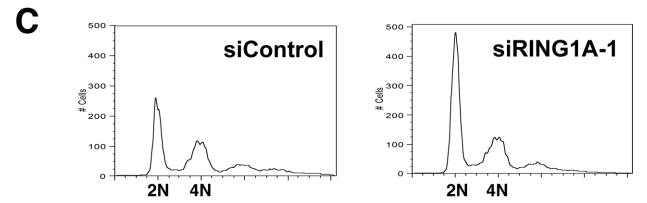
We have previously observed that a dynamic measure of active gene expression is the ubiquitination of chromatin bound to transcribed sequences due to the transcription elongation process (2,24,26). This ubiquitination of histone H2B bound to transcribed sequences was dependent on the RNF20 subunit of PAF and sensitive to inhibitors of transcription elongation, such as α-amanitin and flavopiridol (2). To corroborate the above results with RNAPII binding to promoters in G1, we tested whether ubiquitination of chromatin associated with the transcribed regions of these genes was affected by RING1A. We used the HeLa-Ub cells, which express the biotinylated ubiquitin and ubiquitin conjugates were purified by avidin affinity matrix. We depleted RING1A from HeLa-Ub cells that were synchronized in G1 as in Figure 2B, followed by qPCR to determine chromatin ubiquitination on the transcribed regions as indicators of active transcription (Figure 2A). As anticipated, the loss of RING1A caused a marked reduction in the ubiquitination at the transcribed regions of highly active genes suggesting a sharp decline in the transcription of these genes (Figure 2C). By contrast, RING1B depletion only had a modest and inconsistent effect on the transcription levels of these genes (Figure 2D). While it was anticipated that RING1A depletion would reduce expression of GAPDH, MYC, RPS14 and RPL19, as indicated by the reduced detection of ubiquitin on the transcribed region of each gene, we were surprised to note that the ubiquitination of the IL2 gene body was also reduced. RNA polymerase II (RNAPII) did not localize to the IL2 gene (Figure 2B). The ubiquitination of chromatin associated proteins bound to the genome is a gradient and the chromatin on the IL2 gene has very low amounts of associated ubiquitinated proteins. We interpret these results to reflect the sensitivity of the ChAP-qPCR assay detecting small changes on IL2. The combination of effects of depleting RING1A on RNAPII binding to promoters of active genes and diminished ubiquitination of H2B by PAF on these genes strongly suggests that mitotic bookmarking via RING1A mediated ubiquitination is important, and loss of this process has severe consequences on the transcription of the bookmarked genes during G1.

Proliferation block in RING1A depleted cells

We hypothesized that if bookmarking by ubiquitination is an important process for the cells, abrogation of this process by depletion of RING1A will affect their survival and/or proliferation. To test this, we depleted RING1A or RING1B using siRNA. Forty-eight hours post-transfection 2000 HeLa cells (or 20 000 U2OS cells) were seeded onto dishes and harvested at intervals of every 2 days for a total of 6 days for cell counting. While RING1B depleted cells grew at rates similar to the control cells, RING1A depleted cells failed to proliferate throughout the time course studied (Figure 3A and Supplementary Figure S2). Since proliferation can be a nonspecific readout, we tested whether the sec-







Control	G1	S	G2/M
% of cells	35.4	7.2	27.6

siRING1A-1	G1	S	G2/M
% of cells	50.8	8.2	23.9

Figure 3. Depletion of RING1A leads to proliferation block in HeLa cells. (**A**) Proliferation of HeLa cells after depletion of RING1A or RING1B. HeLa cells were transfected with the indicated siRNA and seeded onto plates 48 h post-transfection and were counted at 2-day intervals. Growth of cells transfected with the control siRNA (diamond), RING1B (triangle) and RING1A (square) is indicated. Results are from three replicate transfections. (**B**) Proliferation assay of HeLa cells, in three replicate transfections as in panel A, was repeated using siRING1A-2. (**C**) RING1A depletion leads to a cell cycle block in G1. FACS analysis evaluating cell cycle stage distribution in control (left) or RING1A depleted (right) cells 48 h after transfection of the siRNAs. The X-axis represents the DNA content per cell as gauged by propidium iodide staining and the Y-axis represents the number of cells. The table shows the percentage of cells at the indicated cell cycle stage for each sample.

ond non-overlapping siRNA targeting RING1A had a similar effect on HeLa proliferation, and it too resulted in very low levels of cell growth while control siRNA transfected cells proliferated as normal (Figure 3B). The failure to grow could be either due to a proliferation block or cell death or a combination of both. To test for proliferation block, we checked for any abnormalities in the cell cycle profiles of the RING1A depleted cells 48 h post-transfection with siRNA. The DNA content per cell was measured by staining with propidium iodide and analyzed by flow cytometry. RING1A depleted cells showed a higher accumulation of cells in the 2N peak as compared to the control cells, indicating an arrest or a delay in the progression through the G1 phase of the cell cycle (Figure 3C).

Since RING1A makes its mark during mitosis, we tested whether it affected passage through mitosis. Control siRNA or RING1A targeting siRNA transfected cells were subjected to a double thymidine block and released for times up to 12 h and then stained with propidium iodide to detect DNA content changes and phosphorylated histone H3 to detect cells in mitosis. In samples that were not released from the double thymidine block, most cells had unreplicated DNA content and no phospho-H3. The double thymidine block was imperfect as 21 to 31% of the cells had some replicated DNA content. Following a nocodazole block, 70–80% of the cells were phospho-H3 positive and had post-replication DNA content, indicating they were in mitosis. Following release from double thymidine block, in control and RING1A depleted cells at 8 h post-release about 75% of the cells had replicated DNA and some of these were phospho-H3 positive. From 8 to 11 h post-release the cells in both samples had cells in mitosis and an increase in cells with unreplicated DNA content and phospho-H3-negative, indicating passage into G1. The fraction of cells in each phase of the cell cycle was similar for control and RING1A depleted cells. As an example, at the 10 h time point, in control cells 35% of cells were in G2, 30% in M, and 35% in G1, and in RING1A depleted cells 25% in G2, 18% in M and 57% in G1 (Figure 4). These results suggest that RING1A depletion did not slow the passage of the cells through G2 and M phases. Thus, the proliferation block observed in Figure 3A and B, was most consistent with a delay in G1 phase of the cell cycle. These observations indicate that RING1A is required for ubiquitination at the promoters during mitosis, and this activity impacts normal progression through the cell cycle.

RING1A regulates mitotic bookmarking by H3K4 trimethylation

Some other factors including histone marks and transcription factors have been shown to persist at a subset of their target genes during mitosis and have been suggested to act as mitotic bookmarks. Since crosstalk has been observed with different histone modifications, we hypothesized that these different bookmarking mechanisms may interact. Since depletion of RING1A decreased the ubiquitin bookmark at the promoters during mitosis, we examined if the mitotic ubiquitination affected other mitotic bookmarks. We tested the effect of RING1A and RING1B depletion on the levels of two of the previously defined mitotic bookmarks, namely H3K4me3 and H3K79me2 at these promoters. Enrichment of H3K4me3 or H3K79me2 at select promoters was determined by performing ChIP-qPCR using antibodies specific to these modifications. We observed that depletion of RING1A (Figure 5A), but not RING1B (Supplementary Figure S2), led to a dramatic reduction in the H3K4me3 levels at the promoters of high

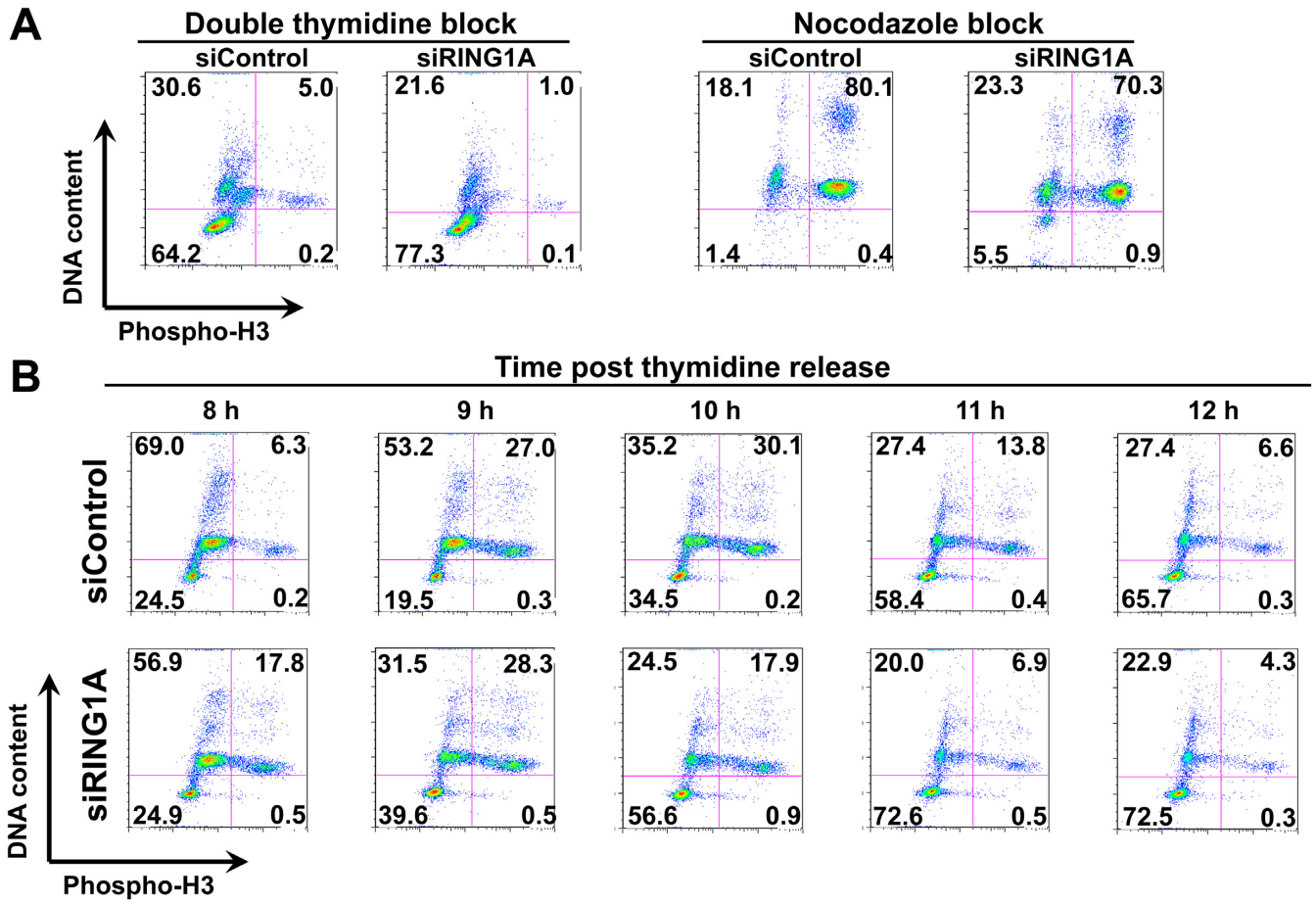


Figure 4. Depletion of RING1A does not slow passage through mitosis. (**A**) HeLa cells were transfected with a control siRNA or a RING1A specific siRNA as indicated and cell cycle arrested using a double-thymidine block or thymidine-nocodazole block as indicated. Cells were stained with propidium iodide to measure DNA content (vertical axis) and with antibody specific to phosphorylated histone H3 (horizontal axis) and analyzed by FACS. The percentage of cells in each quadrant is indicated. (**B**) Following transfection and cell cycle arrest as in panel A, double thymidine arrested cells were released into regular medium for the indicated times and DNA content and phospho-H3 were measured by FACS. Samples transfected with the control siRNA are on the top row and samples transfected with the RING1A siRNA are on the bottom row.

expression genes. However, these depletions had no effect on H3K79me2 levels at the same promoters (Figure 5B). We asked if this reduction in H3K4me3 levels is specific to mitosis or if deposition of this mark is affected throughout the cell cycle including interphase. Examining the effect of RING1A depletion on H3K4me3 levels during interphase showed that this reduction observed in the H3K4me3 levels was not a secondary effect caused by its reduction during interphase, as the H3K4me3 levels in asynchronously growing cells in interphase remained unchanged following RING1A depletion (Figure 5C). These results together suggest that there is crosstalk between H3K4me3 and ubiquitination occurring at these promoter sites specifically during mitosis and that ubiquitination at these promoters is essential for H3K4me3 maintenance during cell division.

DISCUSSION

Bookmark ubiquitination

In a previous study, we discovered that the chromatin-associated proteins bound to promoters of genes that were active during G1 phase of the cell cycle were ubiquitinated during mitosis (2). In this study, we show that the E3 ubiquitin ligases, BMI1 and RING1A, and deubiquitinase USP22 positively regulate the mitotic bookmarking by the ubiquitination of proteins bound to promoters of genes actively transcribed during interphase. Though depletion of RING1A causes mitotic bookmarking to be reduced to baseline levels, it is formally possible that it is not the ligase that mediates the bookmark ubiquitination but an upstream regulator of the actual factor. We also found that RING1A regulated modification of these promoters by another mitotic bookmark, H3K4me3.

This process of bookmarking via ubiquitination is important as inhibition of this process leads to transcriptional deregulation during G1 and a cell cycle and proliferation arrest. Both BMI1 and RING1A are subunits of the polycomb repressive complex 1. Since the PRC1 complex is typically associated with repression of transcription via epigenetic mechanisms, it was surprising to find components of this silencing complex associated with the activation of gene expression via bookmark ubiquitination. Of interest, the major PRC1 component that catalyzes the H2A ubiquitination is the RING1B subunit, but depletion of RING1B

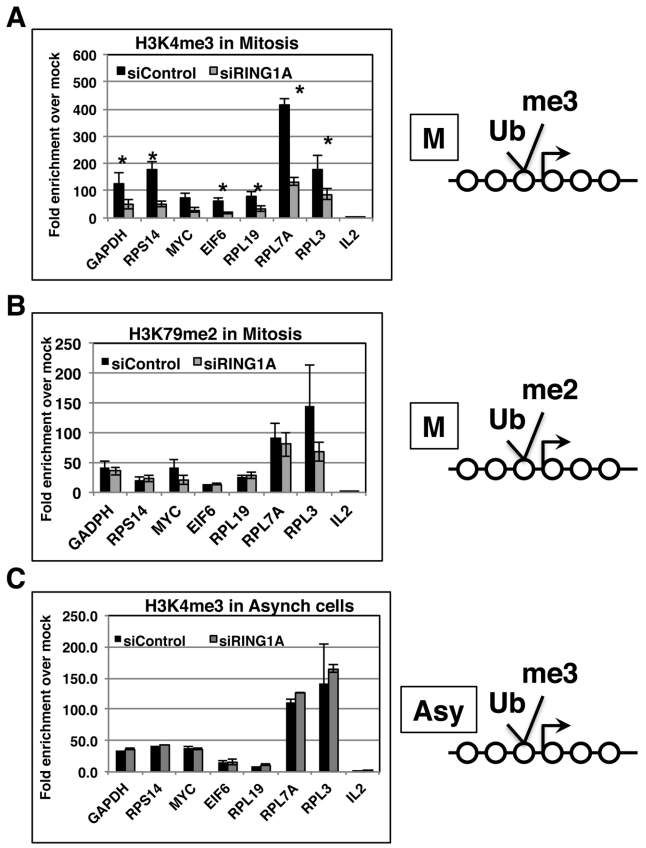


Figure 5. Effect of RING1A depletion on other mitotic bookmarks. (**A**) ChIP-qPCR analysis of H3K4me3 levels at promoters in HeLa cells arrested in mitosis for cells transfected with the control siRNA (black) or the RING1A siRNA (gray). H3K4me3 specific antibody was used to immunoprecipitate chromatin-bound DNA enriched for this modification. ChIP using rabbit IgG served as a control. The model for the ubiquitin and H3K4me3 bookmarks is diagrammed on the right. The circles indicate nucleosomes and chromatin associated proteins. The immunoprecipitated DNA and input DNA were amplified using primers specific to the promoters of the indicated genes labeled on the X axis and enrichment is denoted as fold enrichment over non-specific antibody on the Y axis. Results were from three independent transfection experiments. Error bars represent standard error of mean and significant *P*-values are indicated using asterisks. (**B**) ChIP-qPCR analysis of H3K79me2 levels at promoters in mitotic HeLa cells transfected with control (black) or RING1A (gray) siRNA. H3K79me2 specific antibody was used to immunoprecipitate chromatin-bound DNA in three independent transfection experiments as was done for panel A. (**C**) ChIP-qPCR analysis of H3K4me3 levels at promoters in HeLa cells grown without cell cycle synchronization (Asy), a mark associated with active transcription, was performed as in panel A.

had no effect on bookmark ubiquitination suggesting that the function of RING1A and BMI1 in bookmark ubiquitination may be independent of the polycomb complex.

Our results indicate that RING1A and BMI1 are both required for normal progression through the cell cycle since HeLa cells depleted of these proteins are blocked in G1. Though our results suggest a direct link between mitotic bookmark ubiquitination and cell cycle progression, it is possible that BMI1 and RING1A regulate the cell cycle via transcription silencing function of specific genes as components of PRC1, rather than via the bookmark ubiquitination. In addition, RING1A was a hit in an RNAi screen for proteins required for mitotic entry suggesting that RING1A may act as a cell cycle regulator (27).

Polycomb protein subunits that activate transcription

Polycomb proteins are involved in transcriptional repression. However, our results show that BMI1 and RING1A are positive regulators of transcription in G1 as depletion of the RING1A enzyme had a deleterious effect on the transcription levels during early G1. These effects may be solely due to absence of the mitotic bookmarks, though it cannot be ruled out that other interphase effects may be caused by the depletion of these proteins. Although conventionally linked to transcriptionally silenced genes, several recent reports link polycomb proteins to active transcription. MEL18 (a BMI1 homolog) and RING1A bind to actively transcribed cytokine genes in differentiated T cells and are required for their transcription (28). EZH1 and SUZ12, components of the PRC2 complex have been shown to co-occupy sites of active transcription in embryonic stem cells along with RNAPII (29). EZH2 is a co-activator of transcription factors such as androgen receptor (AR) and NF-κB (30,31). Although these above studies have shown polycomb proteins regulating active genes, the current study is the first to implicate some of the polycomb proteins in mitotic bookmarking of active genes.

Interestingly, although BMI1 and RING1A depletions had profound effects on the ubiquitination at the promoters during mitosis, RING1B, the principal effector ubiquitin ligase of the PRC1 complex did not regulate this process. These results suggest PRC1-independent roles of BMI1 and RING1A. It is plausible that BMI1 and RING1A may form a novel complex along with other polycomb or non-polycomb proteins. As previously shown in this experimental system (2), neither H2A ubiquitinated at lysine 119 nor H2B ubiquitinated at lysine 123 are the substrates that are present at the promoter chromatin during mitosis. The H2AK119ub modification is mediated by RING1B, and the H2BK123ub is catalyzed by RNF20, and the latter E3 ubiquitin ligase did not direct the bookmark ubiquitination (2,16,32). Thus, BMI1 and RING1A ubiquitinate a novel substrate that is yet to be identified. In this context, BMI1 and RING1A have been shown to ubiquitinate topoisomerase IIA independent of the RING1B enzyme and thus independent of the PRC1 complex (17). We tested TOP2A as a potential substrate of bookmark ubiquitination, but we could not detect its ubiquitination in the absence of added topoisomerase inhibitors (data not shown).

Cross talk with other mitotic bookmarks

Several other mitotic bookmarks including some histone modifications and some transcription factors have been identified (8,33). We show that one of these bookmarks, H3K4me3, is sensitive to RING1A depletion. Interestingly, MLL1, the enzyme responsible for catalyzing this modification of H3K4 is also retained at specific sites on the condensed chromosomes (9). It would be tempting to speculate that RING1A may affect the levels of H3K4me3 at these promoters during mitosis by regulating the binding of the MLL1 enzyme. However, Blobel *et al*. showed that

the levels of H3K4me3 during mitosis are actually independent of MLL1 binding thus suggesting an alternate mechanism by which RING1A would regulate this modification. H3K4me3 has been shown to be dependent on the ubiquitination of H2B (34,35). The methylation of H3K4 at these promoters is, however, independent of this modification, since H2B is deubiquitinated before the onset of mitosis (2,36).

The majority of the transcription factors involved in mitotic bookmarking have been shown to bind to only a subset of their target sites bound during interphase. Some even bind to novel sites that are not bound during interphase but only during mitosis (9). What determines transcription factor binding to this specific subset of their target sites during mitosis is not known. We speculate that modification of these target sites by ubiquitination during mitosis may act as a recognition motif and thus play a role in mitosis specific binding of these transcription factors.

An important question in biology is how daughter cells remember the set of genes that were expressed by the mother cell. Epigenetic regulation is a major component of generational memory, particularly during development, but it does not explain the retention of gene expression programs of cells in culture as they pass through mitosis. Bookmarking of genes during mitosis marks the genes active in the cell before its chromosomes condense, and our results indicate that this bookmarking process is essential for appropriate regulation of gene expression and for cell viability.

SUPPLEMENTARY DATA

Supplementary Data are available at NAR Online.

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