

Promoters active in interphase are bookmarked during mitosis by ubiquitination

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ABSTRACT

We analyzed modification of chromatin by ubiquitination in human cells and whether this mark changes through the cell cycle. HeLa cells were synchronized at different stages and regions of the genome with ubiquitinated chromatin were identified by affinity purification coupled with next-generation sequencing. During interphase, ubiquitin marked the chromatin on the transcribed regions of ~70% of highly active genes and deposition of this mark was sensitive to transcriptional inhibition. Promoters of nearly half of the active genes were highly ubiquitinated specifically during mitosis. The ubiquitination at the coding regions in interphase but not at promoters during mitosis was enriched for ubH2B and dependent on the presence of RNF20. Ubiquitin labeling of both promoters during mitosis and transcribed regions during interphase, correlated with active histone marks H3K4me3 and H3K36me3 but not a repressive histone modification, H3K27me3. The high level of ubiquitination at the promoter chromatin during mitosis was transient and was removed within 2h after the cells exited mitosis and entered the next cell cycle. These results reveal that the ubiquitination of promoter chromatin during mitosis is a bookmark identifying active genes during chromosomal condensation in mitosis, and we suggest that this process facilitates transcriptional reactivation post-mitosis.

INTRODUCTION

During the eukaryotic cell cycle, the chromosomes undergo large structural changes, including reversible

post-translational modifications of the histone proteins and other chromatin associated proteins. One of the major post-translational modifications of histones is ubiquitination, primarily on H2A and H2B, although ubiquitinated H3 and H1 have also been reported in different cellular processes (1–3). Apart from the core histones, ubiquitination of some histone variants has also been reported (4,5). Monoubiquitinated H2B (ubH2BK120) is associated with transcribed regions of active genes where it is ubiquitinated by the E3 ubiquitin ligase RNF20 associated with the RNA Polymerase II Associated Factor PAF complex (6–9). Unlike ubH2B, monoubiquitinated H2A (ubH2A) is associated with transcriptionally repressed regions of the genome. UbH2A has been shown to be concentrated on the inactive X chromosome and other heterochromatic regions (10,11). UbH2A is deposited on the chromatin of silenced genes by the action of the polycomb group repressive complex (PRC-1) containing the Ring-finger protein Ring 1b (12). However, there are some known instances of ubH2A being associated with transcriptionally active genes, for example, ubH2A is present at the 5'-end of the actively transcribed mouse dihydrofolate reductase gene and also associated with the poised genes, hsp70 and copia (13,14). Given the role of ubiquitinated histones in gene regulation, several ubiquitin-specific proteases that catalyze removal of ubiquitin moiety from these histones also regulate gene expression as both stimulators and repressors (15).

Variation in histone ubiquitination has been associated with cell cycle progression. Both ubH2A and ubH2B are present in S and G2 phase but are deubiquitinated at prophase and then reubiquitinated in anaphase (16). H2A deubiquitination by Ubp-M precedes phosphorylation of histone H3, chromosome condensation and progression into mitosis (17). However, there are instances of ubiquitinated histones being present on the chromatin

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during mitosis. For example, ubH2A enriched at the inactive X chromosome persists through mitosis (18). Similarly, ubiquitinated histone H3 is present on elongating spermatids in rat testes (1).

Additional evidence for an important role of ubiquitination of chromatin during the cell cycle comes from the mouse G2 phase mutant cell line ts85, which has a temperature sensitive mutation in the ubiquitin-activating enzyme E1 (19,20). These cells when cultured at non-permissive temperatures are blocked in G2 and have reduced ubH2A levels (20,21). Deubiquitination of histones H2A and H2B by USP3 is required for progression through the S phase and for genomic stability (22). Although it is evident that changes in histone ubiquitination are crucial for cell cycle progression, how the global distribution of these ubiquitin marks at genomic loci changes during cell cycle progression has not been studied.

In this work, we studied the global pattern of ubiquitin conjugates on human chromatin and how it changes with the progression of cell cycle. We find that during interphase, ubiquitination marks the transcribed regions of the genome. We had anticipated that ubiquitin would be removed from chromatin during mitosis, but contrary to our expectation, we found that at the promoters of active genes chromatin ubiquitination levels actually increase.

MATERIALS AND METHODS

Cell culture, cell cycle synchronization, transfection and reagents

HeLa cells were grown in DMEM supplemented with 10% BS, glutamax, penicillin/streptomycin and sodium pyruvate (Invitrogen). HeLa cells expressing the HBT-tagged Ubiquitin (HeLa-Ub) (23) were grown in DMEM containing biotin (0.5 μ M, Sigma Aldrich) and puromycin (1.5 μ g/ml, Invitrogen). Cells were either arrested by a thymidine–nocodazole block and released for 0 (mitosis), 4 or 7 h (G1) or with a double thymidine block and released for 0, 2, 4, 6, 8, 10 or 12 h. For transcription inhibition, HeLa-Ub cells were treated with flavopiridol (1 μ M) or α -amanitin (50 μ g/ml) for 3 and 5 h, respectively, before crosslinking for Chromatin Affinity Purification (ChAP). For western blots, antibodies used in this study were anti-Ubiquitin (24), Streptavidin–horse radish peroxidase (HRP) (GE healthcare), anti-Rabbit IgG (GE healthcare), anti-phospho H3 (Ser28) (Millipore -07-145), alexafluor647 tagged anti-Rabbit (Invitrogen—A21244), anti-RNF20 (Novus Biologicals—NB100-2242), anti-Lamin-B (Abcam—ab16048), anti-ubH2B (cell signaling technology) and anti-TFIIH p89 subunit (25).

HeLa cells were transfected twice with 100 pmol of siRNA against RNF20 (sense strand: 5'-AAGAAG GCA GCU GUU GAA GAU-3') or to luciferase (GL2) using Oligofectamine (Invitrogen) using manufacturer's protocol at 48-h interval. Cells were blocked using thymidine or nocodazole 24 h after the second round of transfection and collected for ChAP the next day.

Chromatin fractionation

Chromatin fractionation was done as previously described (26). Briefly, nuclei were prepared by lysing HeLa cells in buffer containing 0.3% NP-40. Nuclei were collected by centrifugation at 2000g for 5 min and were then lysed in PIPES buffer containing EDTA and protease inhibitors. The chromatin fraction was pelleted by centrifugation at 6000g for 20 min at 4°C. The chromatin fraction was washed with PIPES buffer three times before adding SDS-loading buffer to the samples. For Figure 1D, chromatin was prepared as described below for ChAP using uncrosslinked HeLa cells.

FACS analysis

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 were analyzed using the FlowJo software. For phospho-H3 and propidium iodide stained cells, cells were first incubated with anti-phospho-H3 for 2 h, then with Alexafluor 687-labeled goat anti-rabbit for 1 h and last with propidium iodide.

ChAP and immuno precipitation

ChAP samples for sequencing by Illumina GA II were prepared as follows. ChAP was based on a standard Chromatin Immunoprecipitation (ChIP) method (27) with modification of a two-step affinity purification. HeLa-Ub cells were cross-linked with 1% formaldehyde (Sigma) and the reaction stopped by adding 1/20 volume of 2.5 M glycine. The cross-linked material was then washed with PBS, lysed as for the ChIP protocol and sonicated to an average DNA fragment size of 200 bp. All buffers were freshly supplemented with 10 mM *N*-ethylmaleimide (Sigma), 1 mM PMSF (Sigma), 1 \times Protease inhibitor cocktail (Sigma). The sheared chromatin was incubated with 375 μ l Ni-NTA beads (Qiagen) for 16 h at 4°C. An aliquot of the input DNA was saved prior to immunoprecipitation as reference sample. After washing in 6 ml of wash buffer I (50 mM Tris pH 8; 0.01% SDS; 1.1% Triton X-100; 150 mM NaCl), chromatin fragments were eluted in three cycles of 2 ml elution buffer I (50 mM Tris pH 8; 0.01% SDS; 1.1% Triton X-100; 150 mM NaCl; 300 mM Imidazole). The nickel eluate was incubated with 375 μ l of avidin beads (Thermo Scientific) for 6 h at 4°C. After washing in 1 ml of wash buffer II (50 mM Tris pH 8; 1% SDS; 1.1% Triton X-100; 1 M NaCl) followed by two washes in low salt buffer (50 mM Tris pH 8; 1% SDS; 1.1% Triton X-100; 0.5 M NaCl), then two washes with 1 ml Tris EDTA (TE) buffer (100 mM Tris pH 8; 10 mM EDTA; 50 mM NaCl). Crosslinks were reversed by adding 2 ml of elution buffer (50 mM Tris pH 8; 10 mM EDTA; 1% SDS; 200 mM NaCl) to the beads and incubating at 65°C for 15 h. The supernatant was collected and diluted 1:2 with TE buffer. The eluate was treated with RNase (0.2 mg/ml final concentration; Sigma) for 2 h at 37°C, followed by adding Proteinase K (0.2 mg/ml final concentration;

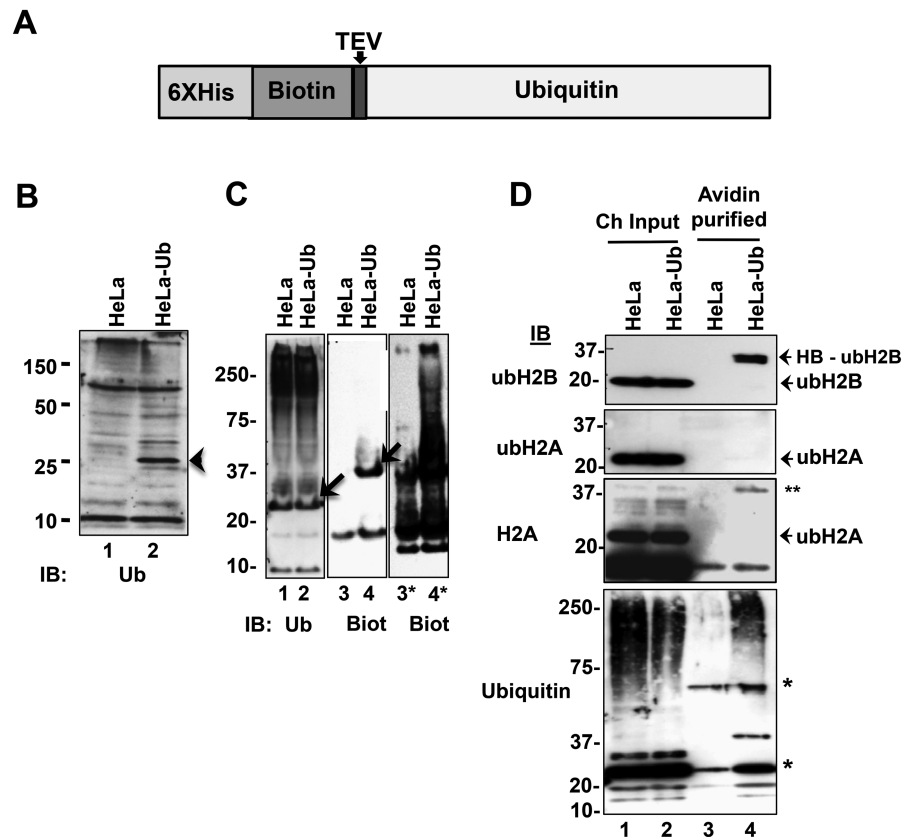


Figure 1. Ubiquitin fusion protein forms high molecular weight conjugates similar to the endogenous ubiquitin. (A) Schematic diagram of the expressed tagged ubiquitin protein with the hexahistidine peptide, the biotinylation domain, and the cleavage site for the TEV protease at its amino terminus. (B) Immunoblot showing ubiquitinated proteins in whole cell lysates from HeLa (lane 1) and HeLa-Ub (lane 2) cells. The recombinant ubiquitin protein in the HeLa-Ub cells is indicated by a black arrowhead. (C) Immunoblot analysis of ubiquitinated substrates in chromatin fractions from HeLa (lanes 1, 3 and 3*) and HeLa-Ub cells (lanes 2, 4 and 4*). Lanes 1 and 2 show ubiquitin conjugates on the chromatin using an ubiquitin-specific antibody. Lanes 3 and 4 show biotinylated conjugates using streptavidin linked with HRP. Lanes 3* and 4* are a longer exposure of the samples in lanes 3 and 4 to show higher molecular weight substrates. Red arrows indicate migration of mono-ubiquitinated H2A/H2B in HeLa cells at ~23kDa and in HeLa-Ub cells at ~37kDa. (D) Immunoblot analysis of input (chromatin) and streptavidin affinity-purified chromatin samples from HeLa and HeLa-Ub cells using antibodies indicated on the left side of the blots. Chromatin was prepared as for the ChAP-Seq samples with the exception that non crosslinked cells were used. Input lanes show 2% of the total chromatin sample used for affinity purification. The identity of bands when known is indicated on the right side of the blots with black arrows. * indicates non-specific bands. ** indicates a band detected with the H2A antibody in the affinity-purified sample that has a migration consistent with monoubiquitinated H2A.

Sigma) for 2 h at 55°C, and DNA was extracted in phenol/chloroform/isomyl alcohol, and the DNA was precipitated in 200 mM NaCl (final concentration), 30 µg of glycogen (Ambion), 2× of the volume of ice cold 100% ethanol and incubation at -20°C for 1 h followed by centrifugation. The pellet was washed in 500 µl of 70% ethanol, then the DNA was finally recovered, and its concentration was quantified by Picogreen assay (Invitrogen).

For ChIP, chromatin was prepared as above for ChAP. The chromatin was pre-cleared using protein-A sepharose beads for 1 h, then applied to protein-A beads that were incubated with either 10 µl of anti-ubH2B or Rabbit-IgG (mock). Protein-A-bound immune complexes were washed once with IP wash buffer 1 (20 mM Tris pH 8, 2 mM EDTA, 50 mM NaCl, 1% Triton X-100, 0.01% SDS), twice with IP high salt wash buffer (20 mM Tris pH 8, 2 mM EDTA, 500 mM NaCl, 1% Triton X-100, 0.01% SDS), once with IP wash buffer 2 (10 mM Tris pH 8, 1 mM EDTA, 250 mM LiCl, 1% NP-40 and 1% deoxycholic acid) and twice with TE buffer (100 mM

Tris pH 8; 10 mM EDTA; 50 mM NaCl). Immune complexes were eluted from protein-A beads by incubating at 65°C for 30 min with rotation. Cross-link reversal and downstream steps were carried out as described above for ChAP. Re-ChIP was done by first performing affinity purification of ubiquitinated chromatin using avidin beads as described above. After washing, the bound ubiquitinated substrates were eluted from the avidin beads by cleavage using TEV protease. Eluted chromatin was diluted with Lysis buffer III and was then used for ChIP using anti-ubH2B, anti-H2B or rabbit IgG antibody.

ChAP DNA preparation for Illumina GAI1 sequencing

ChAP DNA samples were then prepared for ChIP-sequencing library construction following the Illumina ChIP-Seq Sample Prep protocol. Briefly, the DNA samples were blunt-ended by using End-it DNA End-Repair Kit (Epicentre) according to the manufacturer's instructions. dA overhangs were then added and

Illumina adapters ligated. Adapter-ligated DNA was subject to 15 cycles of PCR before size selection of 200–300 bp by agarose gel electrophoresis. Amplified DNA was recovered using the MinElute PCR purification kit (Qiagen). The purified DNA was quantified with an Agilent Bioanalyzer and diluted to a working concentration of 10 nM prior to sequencing. Sequencing on an Illumina GAII instrument was carried out at the Nucleic Acid Shared Resource of The Ohio State University Comprehensive Cancer Center. Primary analysis of ChAP-Seq datasets: the image analysis and base calling were performed using Illumina Genome Analysis pipeline. The sequencing reads were aligned to the human genome UCSC build hg18. Only uniquely aligned reads were used for further analysis and multiple reads were eliminated to reduce PCR-generated artifacts. The aligned reads were further used for peak finding algorithm.

Data analysis

Ingenuity pathway analysis

Ingenuity pathway analysis was done using standard methods. A gene list containing the top 1000 genes that were ubiquitinated in mitosis were analyzed.

ChAP-Seq peak finding

FindPeaks 4.0.10 was used to generate peaks for all the ChAP-Seq and ChIP-Seq data with options of subpeaks 0.5, trim 0.2. A minimum height threshold for each dataset was established so that FDR is <0.1% based on the Monte-Carlo simulation of that dataset.

Principal component analysis of ChAP-Seq datasets

For each sample, chr1 tag counts histogram (without the centromeric region to avoid biased due to the high tag counts) was used for principal component analysis (PCA) analysis using Matlab, with a bin-size 1 kb. The first three principle components were plotted in Matlab program.

Histogram of genome-wide tag counts

Raw tags were counted in a 1-kb bin-size for every chromosome for each sample using a Matlab code.

Sorting peaks into different annotated regions

RefSeq database was used to obtain promoter [5-kb upstream of a transcription start site (TSS)], exon, intron and transcribed region DNA sequences. Gene desert (Intergenic gaps >1 Mb) data were obtained from a published report (28). CpG island region coordinates were obtained from UCSC genome browser website. The small intergenic region (<1 Mb) refers to the genome region that excludes all above annotations. A peak was sorted to a specific region if there is at least 1-bp overlap with that region. A peak can be sorted into different annotated region if there are overlaps between the two regions. Active and inactive promoters were classified based on GEO database asynchronous HeLa cell gene expression microarray dataset GDS885. Gene expression was grouped based on their expression levels, and high-activity promoters were defined from the top 20 percentile gene groups, while low-activity promoters were defined from the bottom 20 percentile groups. Each contains about 2400 genes.

Extended TSS region tag density profiling

The RefSeq database was used to obtain start and end coordinates of 10-kb up- and downstream of TSS for each gene that is included in the GDS885 dataset. The extended TSS regions of 12013 genes were used. Raw tags were extended according to the average length of each ChAP sample. A Matlab code was used to compute the average tag density of 5-bp bin along the extended TSS region. To generate the average TSS tag density profile, the tag density data from three biological replicates of the same cell stage sample were first normalized by their respective total tag counts then averaged. In the TSS heatmap, each row corresponds to an extended TSS region of a gene, which is 10-kb up- and downstream of a TSS. The normalized and averaged densities (from the three replicates) were used. In the sorted TSS heatmap, the rows (genes) were arranged according to either to the asynchronous HeLa cell microarray dataset GDS885 or the synchronized HeLa cell microarray dataset (GSE26922) gene expression level from low to high of S12 stage (equivalent of G1 stage) (29,30). For the former, 12013 probes of GDS885 were used; for the latter, the 11 660 overlapping genes between GDS885 and GSE26922 were used.

Ubiquitin ChAP-Seq and histone methylation data comparison

Publicly available HeLa cell ChIP-Seq datasets, including H3K4me3 (GSM566169), H3K36me3 (GSM766169) and H3K27me3 (GSM566170) were downloaded from the GEO database. For all ChIP-Seq datasets, the raw reads were extended to 200 bp. Peaks were generated using FindPeaks 4.0.10 with subpeaks option on. RefSeq gene promoter (5-kb upstream of a TSS) and transcribed region were used to search for peaks that have at least 90% of its range overlapping with specific annotated regions of a specific gene. Genes with ChAP-seq/ChIP-seq peaks overlapping a specific annotated region of that gene were obtained using BEDTools (31) from each datasets, and the gene lists from different datasets were crosschecked to generate Venn diagram.

Quantitative polymerase chain reaction analysis

For quantitative polymerase chain reaction (qPCR) analysis, ChAP or ChIP chromatin was prepared as described above. For ChAP, affinity purification was performed using only avidin beads to purify the ubiquitinated chromatin instead of sequential purification on nickel and avidin beads. Input sample was saved before purification and was treated similar to the affinity-purified DNA. Affinity-purified DNA or immunoprecipitated DNA and input DNA were used as a template for qPCR.

RESULTS

Conjugation of ubiquitin to chromatin changes during the cell cycle

In this study, we hypothesized that the ubiquitination of chromatin components was dynamic through the cell cycle. From published results (7,9), we anticipated

finding the chromatin on the transcribed portion of active genes to be transiently ubiquitinated. We were particularly interested in comparing late S phase chromatin when the heterochromatin of the newly replicated DNA would be established. To our surprise and as will be discussed later, we found ubiquitin to be dynamically associated with the chromatin on regulatory portions of active genes.

We established, using a published vector (23), a HeLa derived cell line that expresses the ubiquitin fusion protein with an amino-terminal tag encoding hexahistidine and a biotin-bound peptide (referred to as HeLa-Ub) (Figure 1A). This 19-kDa fusion protein is expressed at levels similar to the endogenous 8-kDa ubiquitin as detected as monomeric proteins in crude whole cell extracts (Figure 1B). We detected minor changes in the overall pattern of ubiquitination in the HeLa-Ub cells presumably due to the increased mass of the tagged ubiquitin molecule (Figure 1B). To determine if this tagged ubiquitin can be conjugated to chromatin substrates at efficiency similar to the endogenous ubiquitin protein, we fractionated chromatin from HeLa and HeLa-Ub cells and detected ubiquitinated substrates using anti-ubiquitin antibody and a Streptavidin–HRP antibody that detects the biotinylated fusion protein. Immunoblot analysis shows that the tagged (biotinylated) ubiquitin forms higher molecular weight conjugates similar to the endogenous protein on the chromatin (Figure 1C). In both cell lines, there is a prominent ubiquitin containing band with migration consistent with a molecular mass of ~23 kDa, and in the HeLa-Ub cells a protein of ~35 kDa is detected via the biotin tag. This band migrates at a position consistent with monoubiquitinated H2A or H2B, and in the HeLa-Ub cells the corresponding protein with the fusion tag would be predicted to migrate at this molecular mass (as described later and Figure 1D). There is a biotinylated protein band in HeLa cell chromatin with an estimated mass of 12–15 kDa (Figure 1C, lane 3). This may be an endogenously biotinylated protein which is likely to be removed from the affinity-purified ubiquitin containing samples by the metal ion affinity purification step (described later). Direct biotinylation of histones or any other proteins can only contribute minor background to the subsequent results since similar analysis using untagged HeLa cell chromatin revealed only background level signal. (Figure 2A, top tracing). We fractionated chromatin from HeLa and HeLa-Ub cells and affinity-purified biotinylated chromatin proteins by streptavidin beads. Immunoblot analysis using an anti-ubiquitin antibody showed the presence of high-molecular-weight ubiquitinated proteins that were purified by this approach (Figure 1D, bottom panel). We also detected monoubiquitinated H2B (at ~35 kDa) that was modified using the tagged ubiquitin (HB-ubH2B) in the streptavidin purified chromatin from the HeLa-Ub cells but not from HeLa cells (lanes 3 and 4, Figure 1D, top panel). The tagged ubiquitin molecule was a substrate for some ubiquitin ligases but not others. We were unable to detect ubiquitinated H2A in the affinity-purified chromatin sample using an ubH2A-specific antibody that recognizes H2A ubiquitinated at lysine 119. Another study has reported detection of ubH2A using the same HB-tagged

ubiquitin fusion protein by mass spectrometry analysis (32), but this tagged ubH2A had low abundance in our samples. Thus, the tagged ubiquitin could be ligated to H2B, but not to H2A, suggesting that this reagent is not suitable for scoring silencing by the Polycomb complex, but it is useful for other chromatin modifiers. As another test of the tagged ubiquitin we asked if RNA Polymerase II is polyubiquitinated (33,34) by the tagged ubiquitin molecule and we found that the tagged ubiquitin was incorporated into the polyubiquitin chain on the largest subunit of RNAPII (Supplementary Figure S1).

To identify ubiquitinated regions of the chromatin, we used metal ion affinity purification via the hexahistidine tag, followed by purification on avidin agarose via the biotin tag on the ubiquitin fusion molecule. In particular, the Streptavidin–biotin affinity purification permitted us to apply stringent washes of the bound chromatin. This method is similar to the standard ChIP method, but since the purification steps did not use an antibody, we called the procedure ChAP. Following this sequential affinity purification, the bound DNA was sequenced on the Illumina Genome Analyzer II. To determine cell-cycle-specific ubiquitinated regions of the genome, ChAP-Seq was performed using HeLa-Ub cells synchronized at several distinct cell cycle stages by a double thymidine or thymidine–nocodazole block and release strategy (see ‘Materials and Methods’ section for details; Supplementary Figure S2). As a control to determine non-specific background, affinity-purified DNA from asynchronously growing HeLa cells that did not express the tagged ubiquitin (untagged HeLa control) was sequenced similarly as the above samples. Sequencing of the affinity-purified DNA from the synchronized cells resulted in 10–30 million unique reads that were mapped to the human genome (Supplementary Table S1).

Mapping of the ubiquitin mark on interphase chromatin indicated that it was distributed unevenly through the length of the chromosome with ubiquitination being more enriched in some regions as compared to the others. As an example, the distribution of ubiquitin on chromosome 3 at the indicated cell cycle stages is shown in Figure 2A. A very low background signal was obtained using control HeLa cells not expressing the tagged protein, indicating the specificity of the affinity purification method (brown tracing in Figure 2A). Comparison of ubiquitination in the different interphase samples showed that at a chromosomal scale, no evident large scale changes occurred in ubiquitination through these points in the cell cycle. In contrast to the interphase samples, distribution of ubiquitin changed considerably on the mitotic chromatin indicating a global redistribution of this post-translational mark during cell division (orange tracing in Figure 2A).

To determine if these ubiquitination patterns on the genome were reproducible, ChAP-Seq for each cell cycle stage was performed in triplicate. The percentage of peak overlaps ranged from 60% to 85% when comparing all of the peaks in the G1 samples, the mid-S samples (S2) and the M phase samples (Supplementary Table S2). We did observe a somewhat lower level of overlap in the peaks of genome locations with ubiquitination during the beginning of S phase (S0) and late S phase (S4). As will be

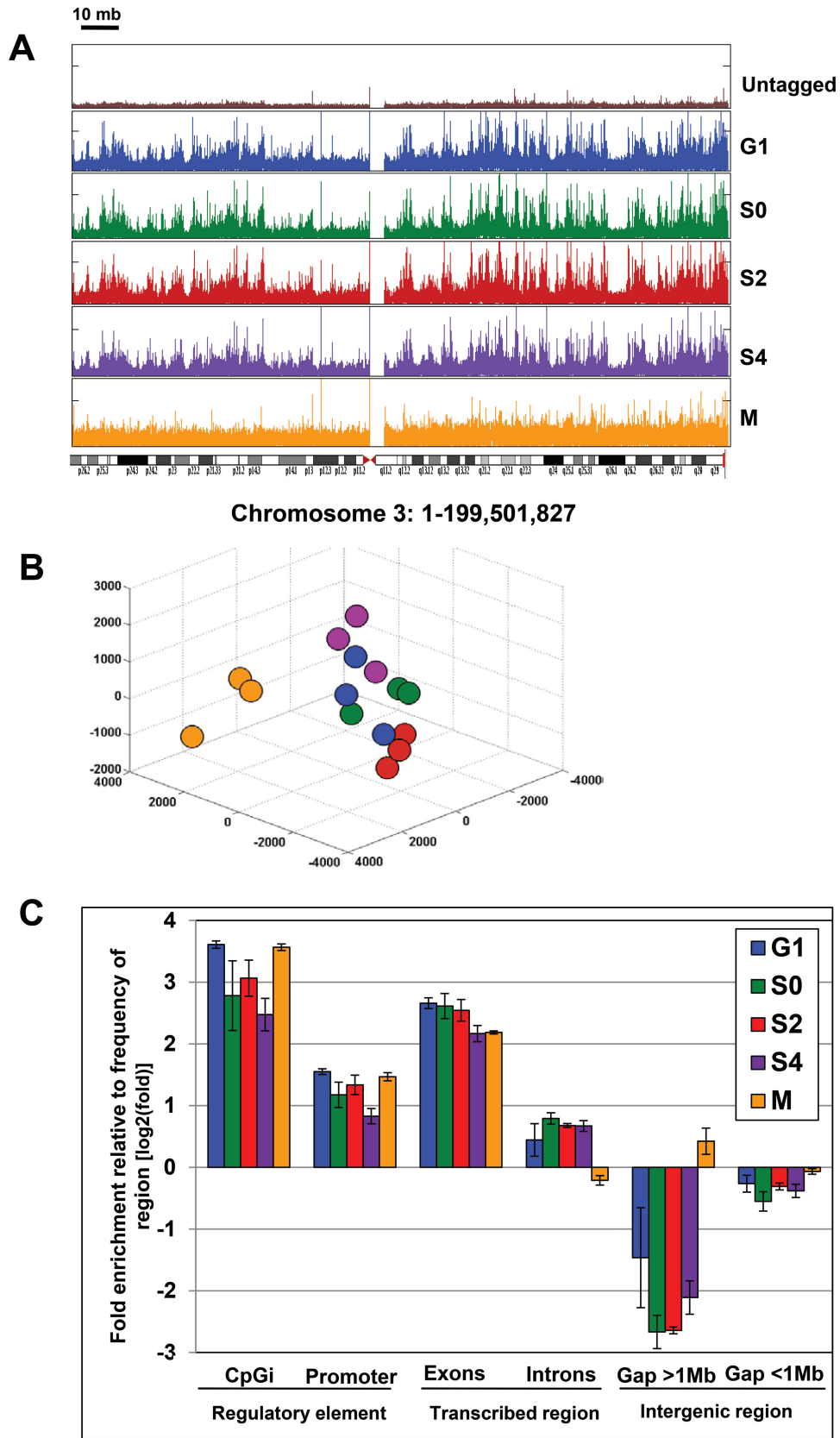


Figure 2. Distribution of the ubiquitin mark on chromatin through the cell cycle. (A) Each track displays raw ChAP-Seq data for each cell cycle stage (G1-blue, S0- green, S2- red, S4- purple, M- orange) across the length of chromosome 3. The ‘untagged’ track (brown, top) shows the background signal obtained by nickel and avidin affinity purification of chromatin using the HeLa cells that do not express the fusion protein. (B) PCA of the ChAP-Seq data reveals differences among stages of the cell cycle. PCA of each ChAP-Seq dataset is represented as a 3D graph. The

(continued)

discussed below, we observed some unanticipated features in the samples derived from cells in mitosis. Data obtained from all the replicates were also compared for consistency using PCA. For this analysis, the sequencing data from each replicate were plotted on a 3D graph with each point representing a single ChAP-Seq sample and each color representing a particular cell cycle stage (Figure 2B). As seen from the graph, biological repeats of the same point in the cell cycle plotted closer to each other than to the other stages suggesting that there are real differences in chromatin ubiquitination as cells traverse the cell cycle. Of note, the mitotic samples were distinct from the interphase samples supporting the observation that the ubiquitination pattern on interphase chromatin is very different from the pattern in the mitotic samples.

Ubiquitination mark redistributes in the genome during mitosis

Visualizing ubiquitination on the chromosomal scale suggested that ubiquitin mapped unevenly across the genome, being more enriched in certain regions of the genome than the others. To identify regions of the genome that were preferentially marked by this modification, sequence data obtained from ChAP-Seq were translated into peaks of ubiquitin enriched genomic regions using a peak calling software, FindPeaks. For each sample, the number of peaks mapping to a specific type of genomic element was calculated and expressed as percentage of total peaks in that sample (Supplementary Figure S3). To determine if there is an enrichment of ubiquitination on a particular type of element, the percentages of peaks in each region were expressed as fold change over the percentages of genome represented by that region (Figure 2C). We found that the majority of ubiquitination in interphase was mapped to the gene and gene regulatory regions of the genome. During mitosis ubiquitination in the introns was sharply reduced while inactive regions like gene deserts (gaps >1 Mb) were modestly enriched for this modification. Surprisingly, we did not see a reduction in the number of peaks mapped to CpG islands, promoters or exons during this stage. Further classification of the ubiquitination signal mapping to exon 1 and all other exons showed that while the number of peaks mapping to exon 1 during mitosis is similar to that during G1, this number is considerably reduced in all other exons (Supplementary Figure S4) suggesting that during mitosis some ubiquitination is observed just downstream of the TSS, near the promoters. This observation is consistent with the profiling of ubiquitination around the TSS in mitosis in Figure 3A.

Ubiquitination is enriched at active genes throughout the cell cycle

Stable promoter ubiquitination during mitosis led us to probe the distribution of ubiquitination around this region. To gain further insight into the relationship between ubiquitination and transcription levels of genes, we compared the expression levels of ubiquitination target genes in interphase and mitosis using publically available gene expression data from HeLa cells. Genes were classified into deciles based on their expression level (mRNA abundance) and for each group of genes average ubiquitination intensity was calculated in a 20-kb region (10 kb on each side) flanking the TSS (TSS indicated by the bent arrow; Figure 3A) (5). The most active genes (90–100%) were represented by the red tracing; the second decile of active genes (80–90%) was represented by the blue tracing and down to inactive genes (0–10%) in black. The results from the three biological replicates were averaged and normalized to the other samples (see ‘Materials and Methods’ section), and the relative sequence tag density of ubiquitinated chromatin is on the y-axis of the tracings.

During interphase, ubiquitination marked the transcribed region of genes and this mark showed a strong direct correlation with the level of gene expression. This signal possibly represents the histone H2B ubiquitinated at lysine 120 (ubH2BK120) as ubiquitination of this histone has been previously linked to transcribed regions of active genes (9) although presence of other ubiquitinated substrates, such as ubiquitinated large subunit of RNA polymerase II, cannot be ruled out. A smaller ubiquitination signal was observed in the promoter regions, which also correlated with the expression level. This pattern of ubiquitination around the TSS was maintained in G1 and through S phase (Figure 3A). During mitosis, ubiquitination associated with the transcribed region was dramatically reduced downstream of the TSS. Surprisingly, contrary to the ubiquitination levels in the transcribed region, the average ubiquitination levels of chromatin at promoters increased during mitosis. This ubiquitination level also correlated well to the mRNA levels as seen during interphase—genes that are active during interphase have ubiquitination over their promoter during mitosis (Figure 3A, upper left). This observation was surprising since most epigenetic marks are erased, not increased, during mitosis (35). In most instances of reported promoter ubiquitination have been associated with silenced promoters (36,37), but in this study, since the ubH2A was not abundantly labeled with the tagged ubiquitin, we detected examples of gene activation correlated with promoter ubiquitination. Consistent with

Figure 2. Continued

three principal components are denoted by the three axes in the graph. Each sample is denoted by a point of a specific color based on its cell cycle stage (as specified in panel A). (C) Enrichment of ubiquitination peaks in the specified region of the genome is shown as fold enrichment on a log₂ scale relative to the frequency of that element in the human genome. Each color histogram represents data from a specific cell cycle stage [as in (B)]. Peak enrichment for a region was calculated as described in the ‘Materials and Methods’ section. Fold enrichment of peaks for each element was calculated from the ratio of percentage of peaks in that sample mapping to the element to the percentage of genome represented by that element and then converting it to log scale. Error bars denote standard error of mean (SEM) based on three biological replicates. Genomic element types are indicated on the x-axis.

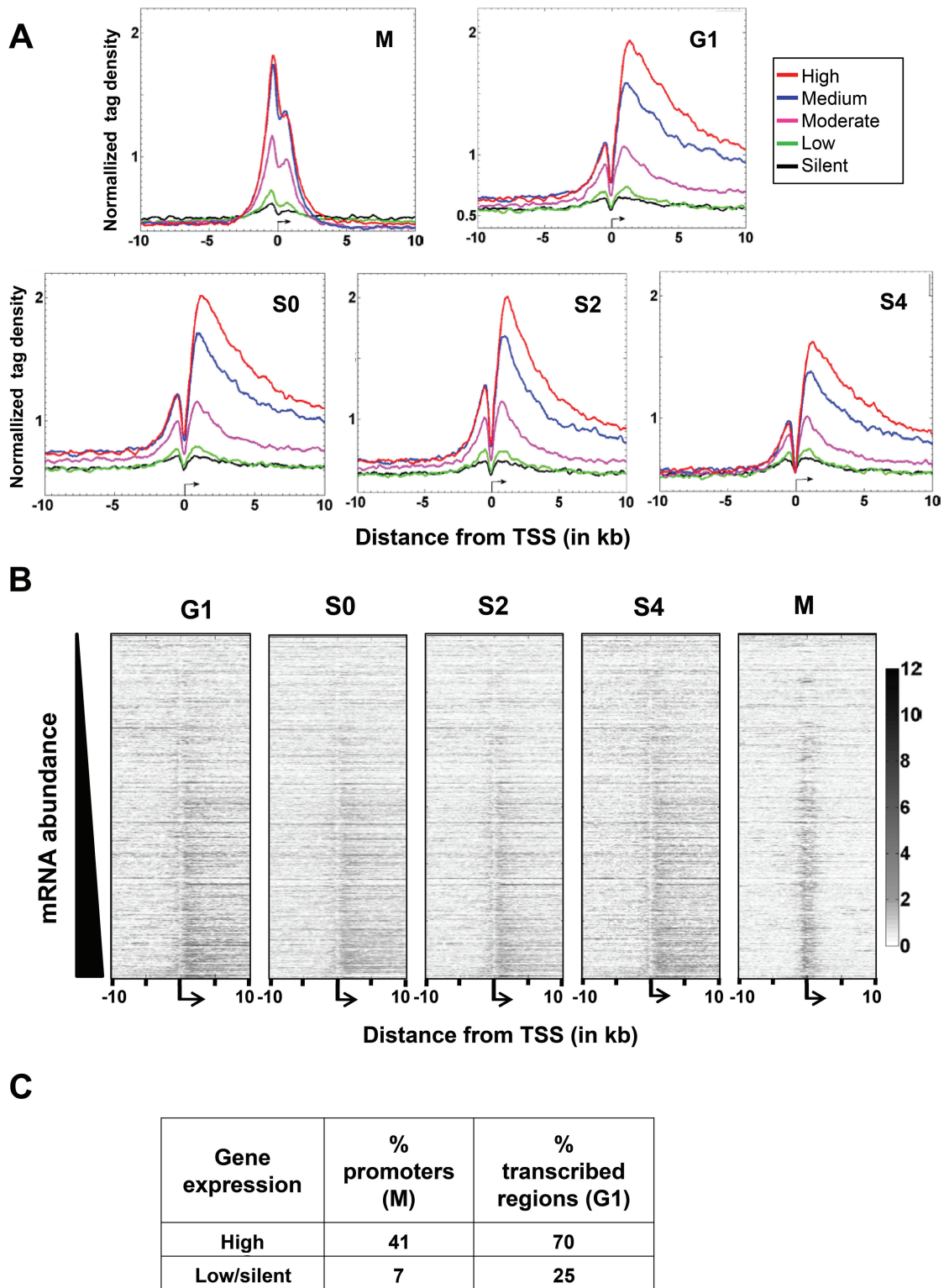


Figure 3. Ubiquitination marks active genes throughout the cell cycle. **(A)** Normalized ubiquitin tag density on a 20-kb region across the TSS (bent arrow) for highly active, medium and silent genes for the indicated cell cycle stage. Genes were classified in groups based on their mRNA abundance as obtained from HeLa microarray data: highest abundance (red, 90–100 percentiles), medium high abundance (blue, 80–90 percentiles), medium (pink, 50–60 percentiles), low (green, 10–20 percentiles) and very low (black, 0–10 percentiles). Each curve represents the average ubiquitin tag density around the TSS of 1200 genes from three biological replicates. **(B)** Heatmaps of ubiquitin tag density around the TSS for 12013 genes. The TSS for all genes is at the center column of the heatmap and genes are arranged in rows based on their mRNA abundance level from lowest (top) to highest (bottom). The normalized tag density of ubiquitin along the length of the gene (x-axis) is indicated in black. **(C)** Percentage of promoters/transcribed regions of highly active (90–100 percentiles) or low activity genes (0–10 percentiles) marked by ubiquitination during mitosis (M) or G1.

our observation, there are reports of histone modifications associated with active genes that mark previously active promoters in mitosis (38,39).

The above analysis of the density of ubiquitin tags mapped relative to the TSS was based on averaging the gene promoters and on the transcriptional activity of each group. In order to evaluate each gene, rather than an average for a decile of genes, we generated a heat map of the raw ubiquitination signal at the TSS of 12000 genes arranged from top to bottom of the heat map according to the abundance of their mRNA with highest abundance genes in the lower part of the graph and the low abundance ones at the top of the heat map. The TSS of all genes is at the center column (Figure 3B). As seen from the traces for high mRNA abundance genes (lower half of the heat map), during interphase ubiquitination was mostly present downstream of the TSS (right half of the heat map). However, we did not observe this downstream ubiquitination for all the high expression genes but only 70% of the high expression genes (defined as the top two deciles in mRNA abundance) had ubiquitin mark associated with their transcribed regions (Figure 3B–C). This indicates that not all, but rather a subset of actively transcribed genes have ubiquitinated chromatin over transcribed DNA. It remains a formal possibility that the ubiquitination on the transcribed regions of the remaining 30% of the genes may have been missed due to technical reasons. During mitosis, 41% of the promoters of the high expression genes were marked with ubiquitin while the signal from the transcribed regions was lost (Figure 3B and C, *M*). This observation was consistent with the reduction in total peaks in the transcribed region (introns) during mitosis (Supplementary Figure S3).

Ubiquitination at transcribed regions is sensitive to transcriptional inhibition

To test whether the loss of downstream ubiquitination during mitosis was associated with lack of transcription, we inhibited RNAPII dependent transcription using α -amanitin during S phase and analyzed the effect on chromatin ubiquitination by ChAP-Seq. We observed that treatment of S phase cells with α -amanitin resulted in a significant, though not complete, reduction in the ubiquitination at the transcribed regions for most high expression genes when compared to the untreated sample (Figure 4A). We also observed a reduction in the promoter ubiquitination during S phase after treatment with α -amanitin. We also tested by ChAP-qPCR the effect of flavopiridol, a CDK inhibitor, on chromatin ubiquitination. Consistent with the results obtained using α -amanitin, treatment of asynchronously growing cells with flavopiridol resulted in reduction of ubiquitination at both promoters and transcribed region (Figure 4B).

RNF20 is required for ubiquitination of H2B associated with active transcription

The PAF complex associated with transcription elongation is known to carry an E3 ubiquitin ligase activity that ubiquitinates histone H2B (7,8,40). We reasoned

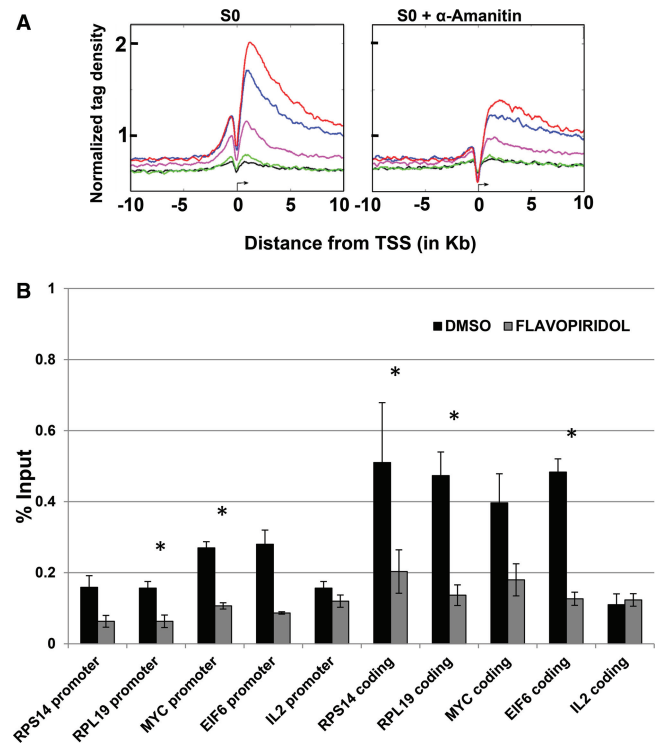


Figure 4. Chromatin ubiquitination on the transcribed regions is reduced upon transcriptional inhibition. (A) Normalized ubiquitin tag densities are shown as an average of two experiments from cells blocked in early S phase with (S0+amanitin; *right*) or without (S0; *left*) treatment with α -amanitin. Different color tracings show ubiquitination levels in different groups of genes as described in Figure 3. (B) Chromatin ubiquitination at select promoters and transcribed regions of high expression genes from cells treated with either flavopiridol or DMSO (control). Chromatin ubiquitination is reduced at both transcribed and promoters in flavopiridol treated cells as compared to control cells ($*P < 0.05$). Ubiquitination at a transcriptionally silent gene – *IL2*, serves as a negative control.

that this region of enriched ubiquitination over the transcribed portion of the genes is present during interphase due to transcriptional elongation activity and may be due to the ubiquitination of the histone H2B but is lost during mitosis as there is a general inhibition of transcription during this period. To verify if the ubiquitination on coding regions of the active genes detected by our ChAP approach is indeed due to ubH2B, we first confirmed detection of ubH2B at these regions by ChIP using anti-ubH2B antibody (Supplementary Figure S5A). We then performed sequential affinity purification for the ubiquitin tag followed by immunoprecipitation using antibody specific to ubH2b, antibody specific to H2B or a control rabbit IgG antibody (41). Re-ChIP results showed that the ubH2B signal was enriched in the affinity tag purified chromatin sample (Supplementary Figure S5B). To further confirm that the ChAP-Seq results on the coding regions during interphase represent ubiquitinated H2B, we compared our ChAP-Seq data with two publically available ubH2B ChIP-Seq data (GSM818830 and GSM264618) (9,42). 53% and 62% of the genes with ubH2B peaks from these datasets were also found to be ubiquitinated in our ChAP-Seq data (Supplementary Figure S5C).

Since most promoter ubiquitination was associated with highly active genes, we reasoned that the E3 ubiquitin ligase responsible for this modification must also be one that associates with active genes. Since RNF20 is the E3 ubiquitin ligase that associates with active transcription elongation complexes, we tested its role in promoter and transcribed region ubiquitination during interphase and during mitosis. RNF20 was depleted by a specific siRNA, resulting in a decrease in ubH2B levels (Figure 5A). Depletion of RNF20 by siRNA in HeLa-Ub cells reduced both the ubiquitination and ubH2B levels associated with the transcribed regions of the tested genes during interphase (Figure 5B and D). As expected, depletion of RNF20 significantly reduced the level of ubiquitination over the coding sequence. By contrast, depletion of RNF20 had no effect on ubiquitination of promoters during mitosis (Figure 5C), indicating that the E3 ubiquitin ligase responsible for promoter modification is different from the one required for ubiquitination on transcribed regions of active genes.

Ubiquitination bookmarks promoters of a subset of active genes

Consistent with the fact that transcriptional activity in the cell is reduced to a minimum during mitosis, ubiquitination at transcribed regions was reduced to minimum during this stage. However, there was an increase in the ubiquitination at the promoters of a set of genes that are expressed at high levels during interphase. There is a report of ubiquitination of H2B at promoters of active genes (43). The transient boost observed in promoter chromatin ubiquitination during mitosis may be a generalization of this earlier observation. To test if ubH2B is enriched at promoters during mitosis, we determined levels of ubH2B at promoters and coding regions during interphase and mitosis by ChIP-qPCR. As has been previously reported (9), ubH2B was enriched at transcribed regions and slightly at the promoters during interphase, but we failed to detect any enrichment of ubH2B at the promoters or the coding regions during mitosis (Figure 5E and Supplementary Figure S5A). This observation suggests that H2B (H2BK120) is not the ubiquitinated substrate at the promoters during mitosis and corroborates the previously published observation that H2B is deubiquitinated before onset of mitosis (16).

To validate the above observations made from ChAP-Seq data, the ubiquitination level at promoters and coding regions of selected high expression genes was determined in cells blocked in mitosis and released for 0, 4 and 7 h by ChAP-qPCR. FACS analysis of propidium iodide stained cells showed that >90% of the cell population was in mitosis after the thymidine-nocodazole block with no release (0 h), ~60–70% cells were in G1 after 4 h and 80% of the cells were in G1 phase 7 h post-release from the block (Supplementary Figure S6). Consistent with the ChAP-Seq data, promoter ubiquitination was highest at 0 h while the level of ubiquitination decreased as cells exited mitosis and entered the G1 phase with lowest at 7 h post-release for promoters of genes that are active during interphase (Figure 6A, left panel). At the

promoter of a gene that is not expressed in these cells (IL2) the promoter ubiquitination was low and did not change as cells traversed the cell cycle. These results indicated that promoter ubiquitination was abundant during mitosis and was removed once the cells entered G1. In contrast to ubiquitination at the promoters, ubiquitination at the transcribed regions was at the lowest at 0 h and steadily increased as the cells exited mitosis (at 4 and 7 h) representing ubiquitination due to transcription of these genes (Figure 6A, right panel). To determine at what point in the cell cycle the promoter chromatin becomes ubiquitinated, we performed a time course after release from a double thymidine block for 6, 8, 10 and 12 h. At time points correlating with G2, 6 and 8 h post-release, ubiquitination at these promoters was low. Promoter chromatin ubiquitination reached the highest level at 10 h and was again reduced at 12 h (Figure 6B, left panel). These results were most consistent with the marking of chromatin at promoters by ubiquitination specifically during mitosis and removed once the cells enter G1. Ubiquitination at the transcribed regions was high immediately before mitosis and immediately after (Figure 6B, right panel). However, the ubiquitin was detected associated with the transcribed regions in the sample taken 10 h post-release (S10), probably due to synchrony of the cells being not as tight as when they were blocked in mitosis using nocodazole. Flow cytometry analysis of phospho-H3 and propidium iodide-labeled cells revealed that at 10 h post-release ~30% of the cells were in mitosis (phospho-H3 positive) and by 12 h post-thymidine release, most cells had completed mitosis and entered G1 phase (Supplementary Figure S6B). Based on these results, we suggest that the ubiquitination peak at the promoters during mitosis functions as a bookmark to facilitate the resumption of transcription of these genes when the cells re-enter interphase.

Gene ontology analysis of the top 1000 genes whose promoters are ubiquitinated during mitosis revealed that ubiquitination tended to occur at genes encoding cell cycle regulators and those involved in protein synthesis, gene expression and DNA replication and repair (Supplementary Table S3). The cell cycle genes included many that are required in G1 or at G1/S transition (P -value = $6.13E-08$). The concentrations of the ubiquitin mark at promoter and transcribed regions of select genes known to be expressed in G1 are shown in Figure 6C. As an example, the GAPDH gene had a high concentration of promoter ubiquitination during mitosis, and during G1 the promoter ubiquitination was not detected but ubiquitination over the transcribed portion of the gene was abundantly detected (Figure 6A, bottom). These data indicated that ubiquitination occurs during mitosis at promoters of a subset of genes that are highly expressed during interphase. To understand if there is a correlation between mitotic promoter bookmarking and gene expression in G1, we obtained another publicly available gene expression dataset (GSE26922) (30) and sorted the genes according to their mRNA abundance in G1 and mapped ubiquitination at these genes during both mitosis and G1. From these heatmaps, we observed that genes highly expressed in G1 were also the most

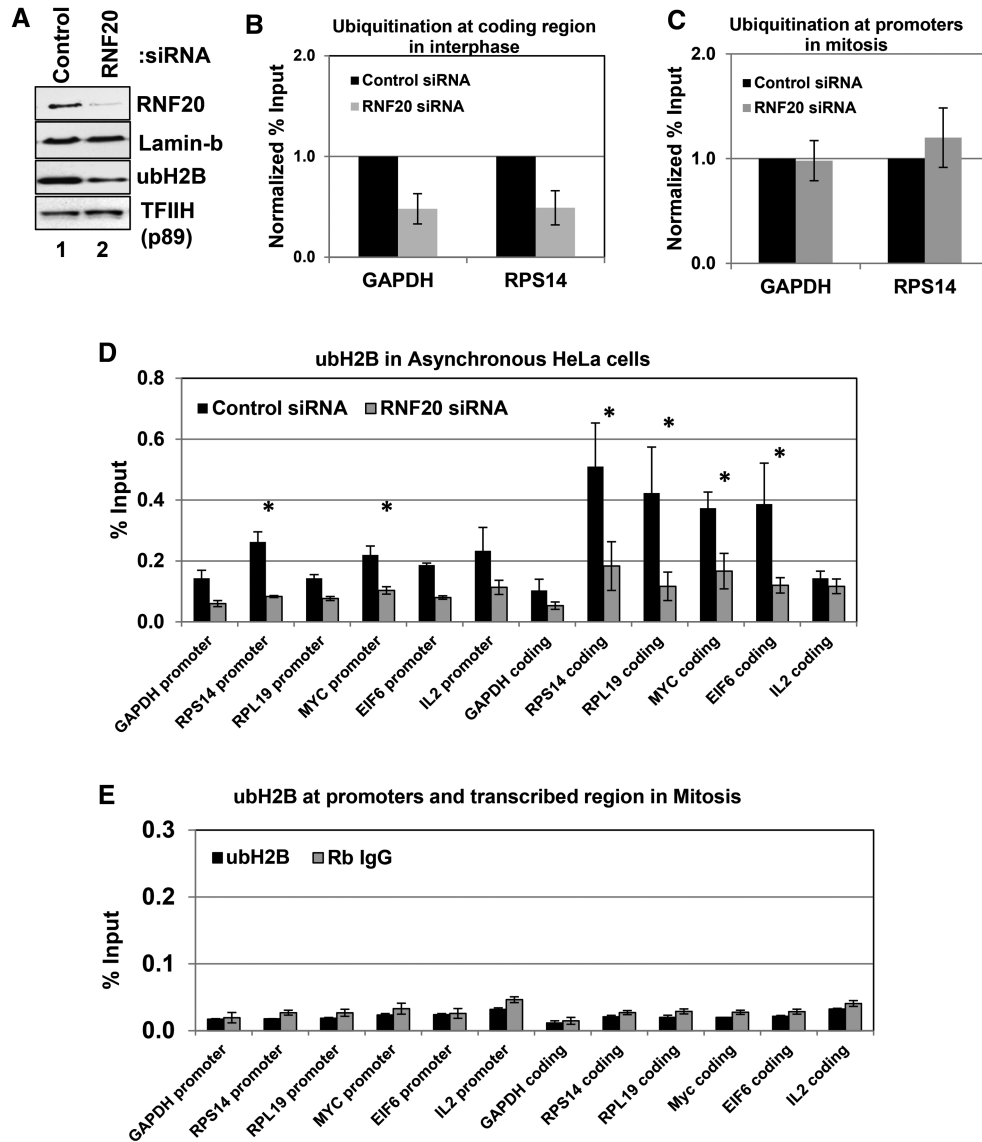


Figure 5. RNF20 depletion reduces ubiquitination of regions downstream of TSS but not at promoters. (A) Western blot analysis shows levels of RNF20 protein and ubH2B in HeLa cells after transfection with either a control siRNA (GL2; lane 1) or RNF20-specific siRNA (lane 2). Lamin-B and TFIIH (89-kDa subunit) levels serve as loading controls. (B and C) ChIP results showing ubiquitination at the promoters regions of indicated genes in mitosis (C) or at the transcribed regions during interphase (B) after transfection with control (black) or RNF20 (gray) siRNA. C_t values obtained in each sample were normalized to the input DNA value and the percentage input values thus obtained were further normalized to the percentage input values obtained in the control sample. (D) ChIP analysis of ubH2B levels at promoters and transcribed regions in asynchronously growing HeLa cells transfected with control (black) or RNF20 (gray) siRNA. UbH2B-specific antibody was used to immunoprecipitate ubH2B enriched DNA. The immunoprecipitated DNA and input DNA were amplified using primers specific to either promoters or transcribed region of the genes labeled on the X axis and enrichment is denoted as percentage of input sample on the Y axis (**P* < 0.05). (E) ChIP analysis of ubH2B using ubH2B-specific antibody or a mock (rabbit IgG) antibody at promoters and transcribed regions of indicated genes in mitosis.

ubiquitinated on their chromatin and at their TSS during mitosis (Figure 7A). Furthermore, 75% of genes with bookmarked promoters during mitosis were ubiquitinated during G1 (data not shown), corroborating that genes bookmarked by ubiquitination at promoters during mitosis are expressed at high levels during G1.

Ubiquitination during mitosis correlates with genes carrying active histone marks during interphase

Monoubiquitination of histone H2B precedes histone H3K4 trimethylation (44,45)—a post-translational

modification linked with active genes (46). Our results indicated that ubiquitination during mitosis mapped preferentially to chromatin at the promoters of highly expressed genes (Figures 3A and 7A). To determine if these genes were marked by other active or repressive epigenetic marks, we compared our ubiquitin localization data with three major histone modifications linked to gene expression—H3K4me3 (active gene mark at promoters) (46–48), H3K36me3 (active gene mark on gene bodies) (49–51) and H3K27me3 (repressed gene mark) (52). We obtained publically available ChIP-Seq datasets

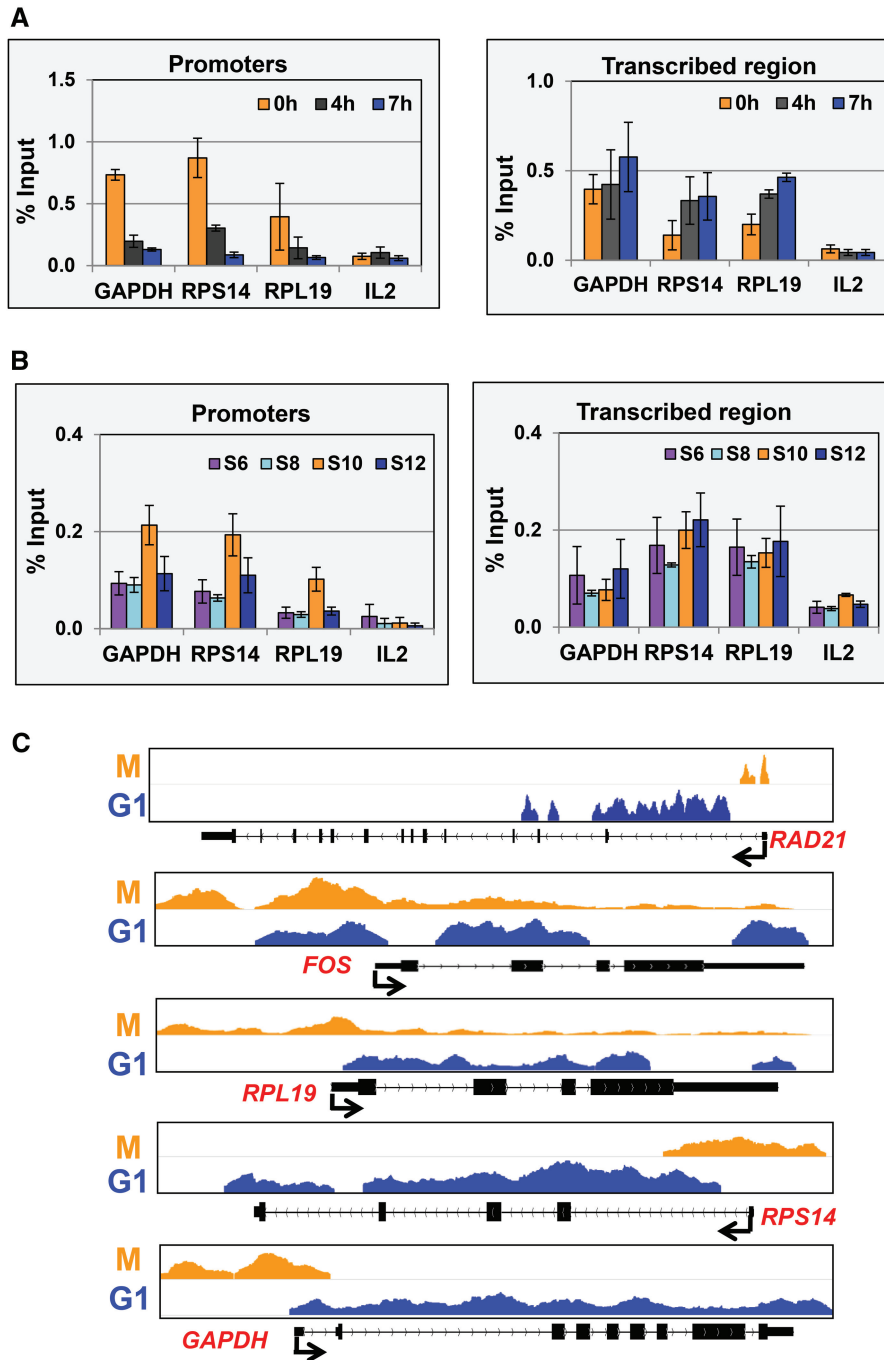


Figure 6. Chromatin ubiquitination at promoters is increased during mitosis. (A) Ubiquitination determined using ChAP-PCR at promoters (*left*) and transcribed regions (*right*) of the indicated genes at 0, 4 and 7 h post-release from a thymidine-nocodazole block. (B) Ubiquitination, measured using ChAP-PCR, at indicated promoters and transcribed regions at 6, 8, 10 and 12 h post-release from a double thymidine block. Ct values obtained by qPCR for each sample were normalized to the Ct value for input DNA and represented in the graph as ‘percentage input values’ on the y axes. (C) Ubiquitination at promoters and transcribed regions of select genes known to be expressed in G1, *RPS14*, *RPL19*, *FOS*, *RAD21* and a housekeeping gene expressed throughout cell cycle, *GAPDH*. Histograms of ubiquitin marks for each gene are labeled during mitosis (M, top) and during G1 (G1, bottom). The TSS is denoted by the bent arrow.

for the three modifications and identified the genes that are marked by each and compared them to the ubiquitination data from mitosis and G1. Genes ubiquitinated in mitosis and G1 correlated with the genes with the highest level of H3K4me3 labeling near the promoter ($R = 0.6932$) (Figure 7A). By contrast, there was no

apparent correlation between promoter ubiquitination and H3K27me3 ($R = 0.1306$) (Figure 7). During G1 phase, 75% of genes with ubiquitinated chromatin on their transcribed regions were also marked by H3K4me3 and 60% were marked by H3K36me3, whereas only 11% of these genes were marked by the repressive mark

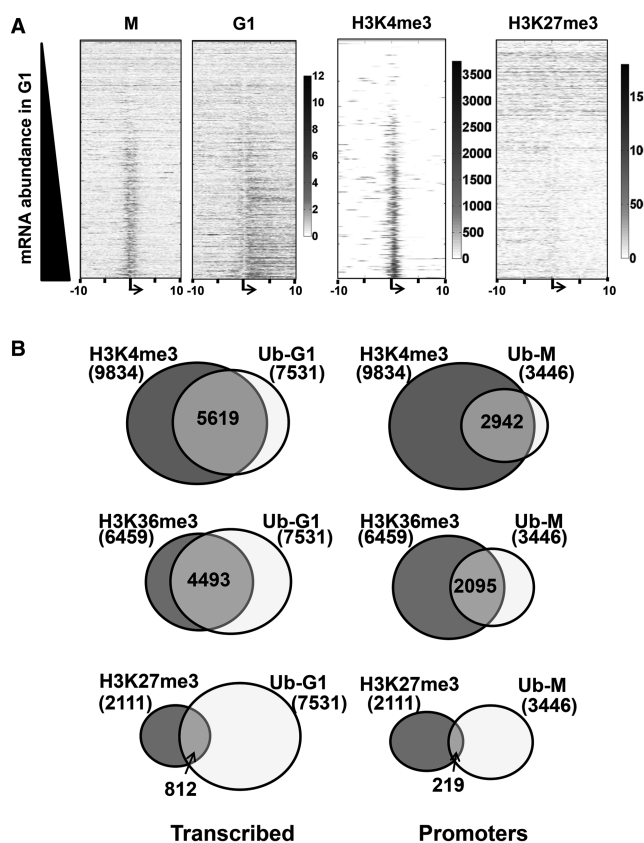


Figure 7. Ubiquitin marks on the chromatin correlate with active histone marks during all stages of the cell cycle. (A) Heatmaps show ubiquitination in M and G1 phases of the cell cycle (as in Figure 3B), H3K4me3 and H3K27me3 tag density around the TSS of 12013 genes expressed in HeLa cells. Rows in the heatmap represent genes that are arranged from top to bottom based on mRNA abundance during G1 phase of cell cycle. mRNA abundance in G1 was obtained from expression microarray data synchronized in G1 (S12, GSE26922). (B) Venn diagrams showing overlap in the genes that are marked by either H3K4me3, H3K36me3 or H3K27me3 and ubiquitination in the transcribed region in G1 (left) or in the promoter regions (right). Total number of genes marked by the modification is shown in parenthesis and the number of genes marked by both modifications is indicated in on the Venn diagram overlap.

H3K27me3 (Figure 7B). Active genes are ubiquitinated in the chromatin on the transcribed sequences by the PAF-elongation complex via the RNF20 subunit (6). Many active genes were not ubiquitinated over the coding DNA, and we suggest that either the ubiquitination of chromatin by the elongation complex was not essential for transcription of every gene or that the ubiquitination tag on some genes was more labile than on other genes.

Eighty-five percent of genes whose promoter chromatin was ubiquitinated during mitosis also carried the H3K4me3 mark and 61% carried H3K36me3 mark during interphase (Figure 7B). Since the histone methylation data are obtained from asynchronously growing cells, this high overlap suggested that genes with promoter ubiquitination during mitosis were active during interphase. A recent study showed that MLL1 (Mixed Lineage Leukemia-1), an H3K4 methyltransferase activity, remains bound to promoters of some of its

target genes during mitosis (53). However, the relevant physical interactions causing MLL to bind specifically to these promoters were not determined. To determine if ubiquitination also occurs at these MLL occupied genes, we compared MLL occupancy to ubiquitination during mitosis. We compared the ubiquitination status of 70 promoters that were bound by MLL1 and ChIP-PCR validated during this stage revealed that 88% of these promoters were also ubiquitinated during mitosis (data not shown). This correlation between MLL occupancy, H3K4me3 and ubiquitination suggests that apart from interphase, ubiquitination may also be required as a mark recognized by MLL1 to bind to its target promoters during mitosis.

DISCUSSION

In this study we investigated changes in the ubiquitin mark on the chromatin throughout the human cell cycle. We observed that during interphase, ubiquitination primarily marked the transcribed regions of the genome with a preference for genes with high mRNA levels. Apart from the coding regions, promoters and CpG islands were also labeled by this modification. Deposition of the ubiquitin mark on the transcribed regions was dependent on active transcription since cells in mitosis or inhibition of RNAPII elongation by α -amanitin or by flavopiridol reduced the levels of ubiquitination in the transcribed regions. Depletion of the ubiquitin ligase associated with transcription elongation, RNF20, reduced the abundance of the ubH2B over the downstream sequences of active genes and at promoters. H2B is known to be cotranscriptionally ubiquitinated at high expression genes by RNF20, an E3 ubiquitin ligase associated with the transcription elongation complex PAF (7). Contrary to our expectation, we noticed that not all high expression genes were ubiquitinated in their transcribed regions with only ~70% of the genes possessing this mark. This observation indicated that ubiquitination of H2B may not be essential for all transcribed genes. Consistent with this idea, studies done in fission yeast and human cells reveal that loss of ubiquitinated H2B or of RNF20 affects the transcription of only a subset of genes (54).

Histone H2A is ubiquitinated by the polycomb repressive complex (PRC1) at promoters of silenced and imprinted genes (12,36) and heterochromatic regions in mammalian cells (11). Our detection method did not detect polycomb-mediated monoubiquitination of H2A, since the tagged-ubiquitin was not coupled to H2A. It is important to note that the ubH2A was present in the sample but not detected. We thus focus on the presence of the tagged-ubiquitin in the genome-wide analysis, and we make no conclusions about where it was not detected.

A surprising and interesting finding of this work was that ubiquitination on the chromatin surrounding the promoters increased dramatically at mitosis—a time when most transcription factors are removed from the condensed chromosomes. This modification was not a common feature of all promoters but was specifically

seen at promoters of genes that were transcribed during G1 phase. The deposition of ubiquitin on chromatin at the promoters during mitosis was a separate phenomenon than the marking of transcribed regions with ubiquitin. The latter process is a consequence of active transcription, but the marking of promoters was not affected by treatment with α -amanitin (data not shown).

We also compared the genes ubiquitinated in mitosis with those carrying another post-translational chromatin modification—SUMOylation (Liu H.W. *et al.*, submitted for publication). We find that ~50% of the genes carrying the ubiquitin bookmark during mitosis have SUMO-1 associated with their promoters during interphase (P -value $< 2.2E-16$). Thus our data support the concept that SUMOylation is a mark for active genes.

A dramatic reduction in the levels of histone H2A and H2B ubiquitination occurs before the start of mitosis (55) and deubiquitination of H2A precedes chromatin condensation during mitosis (17). Our results from the ChIP analysis of ubH2B at promoters during mitosis are consistent with this previous observation and show that ubH2B, although modestly enriched at promoters during interphase, shows no enrichment at these sites during mitosis. Thus, H2B is ruled out as the substrate ubiquitinated and acting as the bookmark during mitosis. Since we were unable to detect ubH2A in our affinity-purified samples, we can also rule out involvement of ubH2A as the bookmarked substrate in this stage.

Other histone modifications and transcription factors have been shown to remain associated with the mitotic chromatin (56). For example, MLL binds to mitotic chromatin and occupies a specific set of promoters differing from the genes it occupies during interphase (53). Interestingly, ~90% of the promoters bound by MLL were also ubiquitinated. It is plausible then that ubiquitination of the chromatin at promoters may act as a recognition mark for MLL or other transcription factors to bind to specific sets of promoters in mitosis. Conversely, it can also be envisioned that some of these transcription factors may act to recruit an E3 ubiquitin ligase, which may then modify the chromatin leading to downstream effects.

Consistent with our observation of ubiquitination on promoters during mitosis, the E1 ubiquitin-activating enzyme has been shown to associate with mitotic chromatin in HeLa cells (57). Mammalian cells express hundreds of E3 ubiquitin ligases. Which enzyme is responsible for this specific post-translational modification of the chromatin at promoters during mitosis? Based on the loss of function phenotype and association with active genes, we tested some plausible candidates for this function. Although depletion of RNF20 affected ubiquitination of the transcribed regions in interphase, its depletion did not affect the ubiquitination of promoters during mitosis. We inferred from this result that ubiquitination of the promoters during mitosis and of the transcribed regions during interphase are two separate phenomena requiring the actions of different E3 ubiquitin ligases. We tested several other reasonable candidates, including TAF1, which is identical to the gene CCG1 implicated in regulation of cell cycle progression through G1 (58,59). The

TAF1-containing TFIID complex is also known to bind to the mitotic chromosomes (60) and TAF1 monoubiquitinates histone H1 in drosophila (2). Considering its homology to E1 or E2 ubiquitin ligases, it has been proposed to be a histone-specific ubiquitin-activating/conjugating enzyme. Although depletion of TAF1 caused a G1 block in the HeLa cells as expected, its absence did not affect the ubiquitination at promoters during mitosis (data not shown). Other candidate proteins are in the process of being tested.

In summary, we show that ubiquitination on the human chromatin is dynamic through the cell cycle with global pattern changing with cell cycle progression. Our data also suggest that ubiquitination of specific promoters may be a mode of cellular transcriptional memory to mark active genes while the silenced chromatin transits through mitosis.

SUPPLEMENTARY DATA

Supplementary Data are available at NAR Online: Supplementary Tables 1–4, Supplementary Figures 1–6 and Supplementary Reference [61].

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