Mitotic Accumulation of Dimethylated Lysine 79 of Histone H3 Is Important for Maintaining Genome Integrity During Mitosis in Human Cells

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ABSTRACT
The loss of genome stability is an early event that drives the development and progression of virtually all tumor types. Recent studies have revealed that certain histone post-translational modifications exhibit dynamic and global increases in abundance that coincide with mitosis and exhibit essential roles in maintaining genomic stability. Histone H2B ubiquitination at lysine 120 (H2Bub1) is regulated by RNF20, an E3 ubiquitin ligase that is altered in many tumor types. Through an evolutionarily conserved trans-histone pathway, H2Bub1 is an essential prerequisite for subsequent downstream dimethylation events at lysines 4 (H3K4me2) and 79 (H3K79me2) of histone H3. Although the role that RNF20 plays in tumorigenesis has garnered much attention, the downstream components of the trans-histone pathway, H3K4me2 and H3K79me2, and their potential contributions to genome stability remain largely overlooked. In this study, we employ single-cell imaging and biochemical approaches to investigate the spatial and temporal patterning of RNF20, H2Bub1, H3K4me2, and H3K79me2 throughout the cell cycle, with a particular focus on mitosis. We show that H2Bub1, H3K4me2, and H3K79me2 exhibit distinct temporal progression patterns throughout the cell cycle. Most notably, we demonstrate that H3K79me2 is a highly dynamic histone post-translational modification that reaches maximal abundance during mitosis in an H2Bub1-independent manner. Using RNAi and chemical genetic approaches, we identify DOT1L as a histone methyltransferase required for the mitotic-associated increases in H3K79me2. We also demonstrate that the loss of mitotic H3K79me2 levels correlates with increases in chromosome numbers and increases in mitotic defects. Collectively, these data suggest that H3K79me2 dynamics during mitosis are normally required to maintain genome stability and further implicate the loss of H3K79me2 during mitosis as a pathogenic event that contributes to the development and progression of tumors.

KEYWORDS chromosomal instability; H2Bub1; H3K79me2; mitotic increase; RNF20; DOT1L

Over the past few decades, a wealth of studies has correlated aberrant patterning of histone post-translational modifications (PTMs) with the development and progression of cancer. Histones are the primary compaction units of DNA and are the substrates for numerous PTMs including acetylation, phosphorylation, methylation, and ubiquitination. Histone PTMs have classically been examined in the context of gene expression and regulation and typically at the single-gene or single-nucleosome resolution. However, recent evidence has emerged that shows the global abundance of certain histone PTMs is highly dynamic and maximal during mitosis, when transcription rates are extremely low (Taylor 1960). Included among these dynamic PTMs are H3S10ph (histone H3 phosphorylation at serine 10) (Hendzel et al. 1997; Goto et al. 2002), H3K9me3 (histone H3 trimethylation at lysine 9) (Peters et al. 2001; McManus et al. 2006a), and H4K20me (histone H4 monomethylation at lysine 20) (Houston et al. 2008). The misregulation of these PTMs has
functional implications in chromosome biology and is known to affect mitotic fidelity through a variety of pathways, including chromosome condensation (Wei et al. 1999), chromosome segregation (McManus et al. 2006a), and kinetochore structure (Heit et al. 2009). Thus, disrupting the normal mitotic dynamics of certain histone PTMs may be a contributing factor in tumorigenesis. Indeed, many of the genes encoding the corresponding regulating enzymes (e.g., AURKB, SUV39H1, SETD8, etc.) are somatically altered in numerous tumor types, including lung, breast, and colon cancers (Forbes et al. 2011), suggesting that aberrant expression and/or function of these enzymes may be causally linked to cancer (Thompson et al. 2013). Thus, characterizing the cell cycle dynamics of mitotic-associated histone PTMs and identifying the enzymes that regulate these dynamic PTMs will provide insight into the etiological origins of genome instability and tumorigenesis.

RNF20 is an evolutionarily conserved E3 ubiquitin ligase that regulates the abundance of H2Bub1 (histone H2B mono-ubiquitinated at lysine 120) (Shema et al. 2008). RNF20 is somatically mutated in a number of tumor types (Supporting Information, Table S1), and aberrant H2Bub1 levels are associated with genome instability (Chernikova et al. 2012). However, the biological consequences of aberrant RNF20 expression are not limited to H2Bub1, as an evolutionarily conserved trans-histone pathway exists where H2Bub1 is an essential upstream prerequisite for subsequent dimethylation events that occur on histone H3 at lysines 4 (H3K4me2) and 79 (H3K79me2) (Dover et al. 2002; Feng et al. 2002; Ng et al. 2002; Sun and Allis 2002). Given the hierarchal position of RNF20 and H2Bub1 within the trans-histone pathway, the impact aberrant levels of H3K4me2 and H3K79me2 have on genomic instability is poorly understood. Accordingly, characterizing the global abundance of these histone PTMs throughout the cell cycle and evaluating their roles in genome stability will provide new insight into the mechanisms required to maintain genome stability.

In this study, we characterize the spatial and temporal dynamics of key components in a trans-histone pathway (RNF20, H2Bub1, H3K4me2, and H3K79me2) throughout the cell cycle. Using imaging microscopy and biochemical approaches, we show that RNF20 and H2Bub1 are highly dynamic in interphase, RNF20 is spatially confined within the nucleus and corresponding global levels of H2Bub1 are maximal. However, as cells enter mitosis, RNF20 relocates from the compacting chromosomes into the cytoplasm, and H2Bub1 levels are rapidly lost such that they are no longer detectable within metaphase chromosomes. We also characterize distinct cell cycle kinetics for H3K4me2 and H3K79me2 that are independent of H2Bub1 during mitosis. While the global abundance of H3K4me2 remains relatively constant throughout the cell cycle, H3K79me2 levels are highly dynamic but opposite to those of H2Bub1. Surprisingly, H3K79me2 attains maximal abundance during mitosis, suggesting it may have a functional role in mitotic chromosome biology and genome stability. In support of this possibility, we identified DOT1L as a mitotic-specific H3K79me2 histone methytransferase. Using RNAi and chemical-based approaches, we show that the loss of mitotic H3K79me2 is associated with genome instability that manifested as increases in aberrant chromosome numbers. Collectively, these data indicate there is a functional uncoupling of the trans-histone pathway specifically during mitosis. They further show that misregulation of mitotic increases in H3K79me2 is associated with genome instability and supports the possibility that aberrant regulation of mitotic H3K79me2 levels may be a pathogenic event that contributes to tumorigenesis.

**Materials and Methods**

**Cell culture**

HCT116 cells (human epithelioid colorectal carcinoma) were purchased from American Type Culture Collection (Rockville, MD) and the immortalized (telomerase) BJ normal skin fibroblasts (hTERT) were a generous gift from C. P. Case (University of Bristol, Bristol, UK). HCT116 cells were grown in modified McCoy’s SA (HyClone, Logan, UT) and hTERT cells were grown in Dulbecco’s modified Eagle’s medium (HyClone). Both media were supplemented with 10% fetal bovine serum. Cells were grown in a 37°C humidified incubator containing 5% CO2. Cell lines were authenticated on the basis of recovery, viability, growth, morphology, and spectral karyotyping (Sajesh et al. 2013).

**Peptide competition and dot blot assays**

Antibody epitope specificities were evaluated by immunofluorescent peptide competition assays and dot blot assays as detailed previously (McManus and Hendzel 2005a). Briefly, the H2Bub1, H3K4me2, and H3K79me2 antibodies were pre-incubated with a 10-fold molar excess with specific or nonspecific peptides at 4°C for 16 hr, and indirect immunofluorescent imaging was performed as described below. Dot blot assays were performed by dispensing 20 ng of each peptide (control or experimental) onto a 0.2-μm PVDF membrane. Peptide aliquots were allowed to dry and antibody specificity was determined using a standard Western blot approach (McManus and Hendzel 2005a). The H2Bub1 peptide was custom synthesized by Biomatik (Cambridge, ON, Canada), while the remaining peptides were purchased from Abcam: H2B (ab5489), H3 (ab7228), H3K4me1 (ab8895), H3K4me2 (ab7766), H3K4me3 (ab8580), H3K79me1 (ab4555), H3K79me2 (ab4556), and H3K79me3 (ab4557).

**Western blot**

Western blots were performed as detailed previously (McManus and Hendzel 2005a). Table S2 lists the primary and secondary antibodies and dilutions employed. Loading controls include α-tubulin; unmodified histone H3; or copper phthalocyanine tetrarsulfonic acid tetrasodium salt (CPTS), a protein stain. Western blot images were imported into ImageJ software (v1.48s) and background-corrected mean signal intensities were normalized to respective loading controls.
**Indirect immunofluorescence**

Asynchronous cells were seeded onto sterilized glass cover-slips 1 day prior to analysis, such that they were ~80% confluent the following day. Cells were fixed, permeabilized, immunofluorescently labeled, and counterstained with DAPI as detailed elsewhere (McManus and Hendzel 2003). Table S2 presents the antibodies and dilutions employed. An antigen retrieval step was employed prior to indirect immunofluorescence (IF) labeling with the H2Bub1 antibody. Briefly, cells were fixed in paraformaldehyde for 10 min, washed twice in nanopure water, permeabilized with ice-cold (−20°C) acetone for 2 min, and rinsed five times with nanopure water. Cells were incubated in 2 N HCl for 15 min at room temperature prior to neutralization with two washes of 0.1 M NaBorate (pH 8.5) for 2 min each. Cells were incubated in PBS (pH 7.4) for 5 min prior to IF labeling as above.

**Gene silencing (small inhibitory RNA)**

A lipid-based transfection method (RNAiMAX; Invitrogen, Carlsbad, CA) was employed to deliver small inhibitory RNA (siRNA) duplexes into HCT116 cells as described in Barber et al. (2008). Cells were seeded in six-well dishes at a cell density of 210,000 cells per well (HCT116) and permitted to grow for 24 hr prior to treatment. Cells were transfected according to the manufacturer’s instructions with 30 pmol of siRNA diluted in 6 μl of RNAiMAX. Cell culture media were replaced 24 hr post-transfection and every 48 hr thereafter.

**DOT1L inhibition**

Cells were seeded as above [Gene silencing (small inhibitory RNA)] and incubated with 5 μM SGC0946 (Selleck Chemicals), an established DOT1L inhibitor (Yu et al. 2012), or DMSO (vehicle control). Cells were incubated with chemicals for either 2 or 48 hr.

**Cell synchronization**

A standard nocodazole arrest was employed to generate a mitotic-enriched population of cells as detailed elsewhere (McManus and Hendzel 2005a). Briefly, asynchronous cells were treated with 15 nM nocodazole (Sigma-Aldrich, St. Louis) for 14 hr prior to harvesting. Flow cytometric cell cycle analyses were performed following propidium iodide incorporation as detailed elsewhere (McManus et al. 2006a).

**Image acquisition and deconvolution**

Imaging was performed using an AxioImager.Z1 (Carl Zeiss, Thornwood, NY) microscope equipped with a 12-bit AxioCam HRm cooled-coupled device camera and a Z-stage motor. All images were collected with a 40× oil-immersion plan-neofluar lens [numerical aperture (NA) = 1.3] or a 63× oil-immersion plan-apochromat lens (NA = 1.4). All three-dimensional (3D) image series were acquired at 200-nm intervals. Image deconvolution was performed using a maximum-likelihood expectation deconvolution in AutoQuant X3 (Media Cybernetics), using a constrained iterative algorithm and theoretical point spread functions for 460-nm (DAPI), 505-nm (Alexa Fluor 488), and 565-nm (Cy3) emission wavelengths. Deconvolved images were imported into ImageJ software (v1.48s) and analyzed as detailed below. Figures and panels were assembled in Photoshop Creative Suite 6 (Adobe).

**Semiquantitative IIF imaging**

Two-dimensional (2D) IIF imaging was employed to acquire a minimum of 20 interphase and 20 mitotic cells (prophase, prometaphase, and metaphase) for control and experimental conditions. All images were collected with identical exposure times and thus semiquantitative imaging analyses were used to reveal changes in the global abundance of a particular fluorescently labeled epitope (McManus and Hendzel 2005b). Images were imported into ImageJ software (v1.48s) and mean fluorescent signal intensities were determined for both the DAPI and H3K79me2 channels for each individual cell. To account for increases in histone contents associated with DNA replication, H3K79me2 signal intensities were normalized to the corresponding DAPI intensities. Normalized H3K79me2 signal intensities were exported into Prism v6 software (GraphPad) for statistical analyses (see below).

**Line scans**

A single representative line was placed through an entire nucleus for each condition evaluated (McManus et al. 2006b), and a fluorescence intensity profile was generated for each channel, using plot profile tools in ImageJ (v1.48s). Relative fluorescent intensity values were plotted in Prism v6 software (GraphPad) to allow for the comparison of fluorescent intensities between channels on a pixel-by-pixel basis. Graphs were exported as Tiff images and assembled in panels, using Photoshop CS6 (Adobe).

**Mitotic chromosome spreads**

Subconfluent cells were processed as described elsewhere (Barber et al. 2008), and 100 mitotic chromosome spreads for each control and experimental condition were manually evaluated.

**Mitotic defect analysis**

HCT116 cells were transfected or chemically treated as detailed above. Cells were fixed and immunofluorescently labeled as above, using an anti-H3S10ph antibody to identify mitotic cells (Hendzel et al. 1997). Images were acquired with a Cytoation3 Imager (BioTek) equipped with a 10× long working distance air lens (UPLFLN10×; NA = 0.3). Each experimental condition was repeated at least four times and a minimum of 275 mitotic cells were evaluated per condition. All data were normalized to the respective controls (siGAPDH or DMSO) and are presented as the fold increase in mitotic defects relative to controls.

**Statistical analyses**

All data were imported into Prism v6 (GraphPad). Where Student’s t-tests were performed to evaluate the difference in
mean values between control and experimental conditions, a P-value <0.05 was considered significant. For the semi-quantitative IIF analyses, the DAPI-normalized H3K79me2 total signal intensities were employed. All graphs were generated in Prism, exported as Tiff images, and assembled into panels using Photoshop CS6 (Adobe).

Results

**RNF20 is rapidly redistributed into the cytoplasm during mitosis**

We first wished to determine whether RNF20 exhibits a distinct localization pattern throughout the cell cycle that may affect the temporal progression patterns of H2Bub1, H3K4me2, and H3K79me2. Using an established single-cell immunofluorescent approach (McManus and Hendzel 2005b), a validated anti-RNF20 antibody (Figure S1) (Van Pel et al. 2013), and asynchronous HCT116 cells, we show that RNF20 exhibits a dynamic localization pattern. In interphase cells, RNF20 is spatially confined to the nucleus where it is present as hundreds of immunofluorescent foci that are visually depleted from nucleoli (Figure 1). Line scans of interphase nuclei reveal that RNF20 localization is nonrandom (Figure S2). RNF20 is enriched with euchromatin as visually defined by less intensely stained DAPI regions and is depleted from heterochromatin as defined by intensely stained DAPI regions. As cells enter mitosis (i.e., prophase and prometaphase), RNF20 is rapidly redistributed away from the compacting chromosomes and into the cytoplasm (Figure 1B). However, as cells reenter G1, RNF20 is reincorporated and retained within the nucleus. To confirm the above results were not restricted to HCT116 cells, analogous experiments were performed in hTERTs (an immortalized fibroblast cell line) with very similar findings (Figure S3). Finally, to determine whether changes in RNF20 expression levels accompany changes in localization Western blots were performed that show RNF20 levels are relatively consistent between mitotically enriched (Figure S4) and asynchronous (predominantly interphase) populations (Figure 1C).

**The global abundance of H2Bub1 decreases rapidly during mitosis**

Having established that RNF20 exhibits a dynamic localization pattern, we now sought to determine whether H2Bub1 would also exhibit dynamic temporal patterning. However, we first confirmed the specificity of the H2Bub1 antibody, using a peptide competition assay and a dot blot assay (Figure S1). Using the single-cell approach employed above, we show that the global abundance of H2Bub1 is highly dynamic (Figure 2). In interphase cells, H2Bub1 localizes within nuclei and forms hundreds of immunofluorescent foci that are depleted from nucleoli. Line scans revealed that the H2Bub1 foci are preferentially enriched within euchromatic regions rather than heterochromatic regions (Figure S2). As cells enter mitosis there is a dramatic decrease in the abundance of H2Bub1 such that levels are below the limits of detection by metaphase. Global levels remain low throughout anaphase and telophase and begin to rise rapidly as cells enter G1 (Figure 2B), which coincides with the reincorporation of RNF20 within interphase nuclei. To confirm the above results were not restricted to the HCT116 cells, similar experiments were performed in hTERT with very similar results (Figure S5).

Although the in situ data generated above suggest there is a dramatic loss of H2Bub1 during mitosis, epitope accessibility/occlusion may adversely affect these results, particularly in mitotic cells with extensive chromosome compaction. To address this possibility, standard biochemical approaches were employed, and denaturing Western blots were performed on histones isolated from asynchronous or mitotically enriched populations. In agreement with the imaging data, there was a striking decrease in the abundance of H2Bub1 within the mitotic population compared to the interphase (asynchronous) population (Figure 2C). Collectively, the above data show that the global abundance of H2Bub1 is highly...
dreds of immunofluorescent foci that are highly enriched within nuclei but absent from nucleoli. Line scans revealed that H3K4me2 foci are preferentially enriched within euchromatin compared to heterochromatin (Figure S2). In contrast to H2Bub1, however, the global abundance of H3K4me2 does not decrease rapidly during mitosis but rather is maintained along the length of the chromosome arms in a heterogeneous punctate pattern. hTERT cells were also evaluated and produced very similar results (Figure S7). Finally, denaturing Western blots confirmed the relatively consistent levels (i.e., minimal changes) observed via indirect immunofluorescence (Figure 3C). Collectively, the above data show that the global abundance of H3K4me2 is relatively static throughout the cell cycle.

**Global levels of H3K79me2 are dynamic and attain maximal abundance during mitosis**

Having established that the temporal dynamics of H3K4me2 are distinct from H2Bub1, we now sought to characterize the temporal and spatial patterning of H3K79me2, using a validated antibody (Figure S6, C and D) and similar approaches. Surprisingly, H3K79me2 levels are highly dynamic and in stark contrast to those of H2Bub1—minimal H3K79me2 levels occurred during interphase and maximal levels of H3K79me2 occurred during mitosis (Figure 4). The difference in H3K79me2 signal intensities is so pronounced that interphase intensities are not readily detected when the image exposure time is optimized for the mitotic signals (Figure 4A, left). Nevertheless, longer exposure times clearly show H3K79me2 signals within interphase nuclei (Figure 4A, right) that present hundreds of immunofluorescent foci, which are enriched within nuclei. Line scans show that H3K79me2 foci are preferentially associated with euchromatic regions rather than heterochromatic regions (Figure S2). Furthermore, as cells enter prophase there is an increase in H3K79me2 levels throughout the length of the chromosomes that continues until maximal abundance is achieved by metaphase (Figure 4B). As cells enter anaphase and telophase, H3K79me2 levels rapidly decrease and return to basal levels as cells enter G1. Semiquantitative imaging analyses of DAPI-normalized, H3K79me2 total signal intensities revealed a highly statistically significant, ~31-fold increase within mitotic cells relative to interphase cells (Figure 4C and Table S3), while Western blots confirmed the large increase in the global levels within mitotically enriched cells (Figure 4D). Analogous experiments were performed in hTERT cells with very similar findings (Figure S8). Thus, the above data show that the global abundance of H3K79me2 is dynamic, and unlike H2Bub1 it attains maximal levels during mitosis.

**DOT1L expression and function are required for the mitosis-specific increases in H3K79me2**

The dramatic increase in the abundance of H3K79me2 specifically during mitosis mimics that of other histone PTMs with established roles in chromosome biology and stability.
To determine whether DOT1L function is essential for the mitotic-associated increases in H3K79me2 levels, and to address potential off-target effects associated with siRNA, similar experiments were performed using SGC0946, an established DOT1L inhibitor (Yu et al. 2012), or DMSO. Semiquantitative microscopy was performed as above on asynchronously growing cells, and the analyses were restricted to mitotic cells. In agreement with the silencing data, DOT1L inhibition produced a statistically significant decrease (~24% of control levels) in mean DAPI-normalized H3K79me2 total signal intensity within the mitotic cells (Figure 5B and Table S4) that was confirmed by Western blots (Figure 5D). It should be noted that the mitotic decrease in H3K79me2 levels was more pronounced following SGC0946 treatment than following DOT1L silencing. Although the underlying mechanism accounting for this difference is beyond the scope of the present study, it likely stems from the heterogeneous nature of siRNA-based silencing or more likely the residual amounts of functional DOT1L remaining following silencing. In any case, the siRNA- and chemical-based data show that DOT1L expression and function are normally required to induce the dramatic increase in H3K79me2 levels specifically within mitotic cells.

**H2Bub1 is not a prerequisite for H3K79me2 during mitosis**

The observation that the global abundances of H2Bub1 and H3K79me2 are dynamic but opposite in mitosis suggests there is transient uncoupling of the trans-histone pathway during mitosis. Although our initial immunofluorescent and Western blot analyses did not detect H2Bub1 within mitotic cells, the possibility remained that extremely low levels of H2Bub1, below the limits of detection, persisted within mitotic cells that could affect H3K79me2 levels. Accordingly, we sought to decrease any residual H2Bub1 within mitotic cells by silencing RNF20 and examine H3K79me2 levels. However, we first confirmed the existence of an intact trans-histone pathway within the interphase cells of our experimental system. As expected, RNF20 silencing dramatically decreased the global abundance of H2Bub1 within interphase cells (Figure 5E, top), and in agreement with previous studies (Ng et al. 2002; Sun and Allis 2002) also decreased the global abundance of H3K79me2. Although RNF20 silencing reduced H3K79me2 to similar levels achieved following DOT1L silencing, SGC0946 treatments appeared to be the most efficient at depleting H3K79me2 within interphase cells (Figure 5E, bottom). However, within
mitotically enriched populations (Figure 5F), RNF20 silencing had very little, if any, impact on the global abundance of H3K79me2 and supports the transient uncoupling of the trans-histone pathway during mitosis.

Loss of mitotic-associated H3K79me2 is associated with chromosome instability

The global abundance of many histone PTMs is dynamic and exhibits mitosis-specific increases required for mitotic fidelity and genome stability (Hendzel et al. 1997; Goto et al. 2002; McManus et al. 2006a; Houston et al. 2008). Thus, we wished to determine whether the large increases in H3K79me2 that occur during mitosis have a similar role. HCT116 cells were purposefully selected for this analysis as they are a karyotypically stable line that has been employed extensively in chromosome instability assays (Barber et al. 2008; McManus et al. 2009; Sajesh et al. 2013; Van Pel et al. 2013). Accordingly, we employed DOT1L silencing and inhibition (SGC0946) to decrease H3K79me2 levels during mitosis and evaluated the impact on chromosome stability within mitotic chromosome spreads (Figure 6A). As predicted, there was an overall increase in the number of aberrant spreads within the silenced and inhibited populations relative to controls (Figure 6B). More specifically, the frequency of spreads harboring >45 chromosomes increased from 18% in GAPDH-silenced controls to 30% in DOT1L-silenced cells and from 11% in DMSO-treated controls to 49% in SGC0946-treated cells, which Student’s t-tests revealed to be statistically significant (Table S6). Flow cytometry was also performed and confirmed that DOT1L silencing and inhibition do not adversely affect cell cycle progression (Figure S9 and Table S5). Thus, the loss of H3K79me2 in mitotic cells following DOT1L silencing or inhibition is associated with increases in chromosome numbers and the loss of genome stability.

To gain insight into the mechanism(s) underlying the increases in chromosome numbers observed above, mitotic cells were evaluated for the presence of aberrant mitotic events, including chromosome congression errors, anaphase bridges, and segregation defects (Figure 6C). Mitotic levels of H3K79me2 were depleted as above, and mitotic defects were manually scored. Overall, the loss of H3K79me2 during mitosis was associated with statistically significant increases (P-value <0.05) in aberrant mitotic events (Figure 6D). More specifically there was a 1.7-fold increase in the DOT1L-silenced cells (9.7%) relative to a siGAPDH-silenced control (5.6%) and a 2.5-fold increase within the
SGC0946-treated cells (9.3%) relative to vehicle control (3.7%) (Table S7). Thus, the loss of H3K79me2 levels within mitotic cells is associated with chromosome instability and an increase in aberrant mitotic events, which suggests that the mitotic increases in H3K79me2 levels are required for mitotic fidelity.

**Discussion**

In this study, we employ asynchronous and untreated human cell lines along with complementary single-cell microscopy and biochemical approaches to provide the first in situ spatial and temporal progression patterns of four key components of an established trans-histone pathway, RNF20, H2Bub1, H3K4me2, and H3K79me2. We specifically show that the global abundance of H2Bub1 is highly dynamic and reflects the spatial distribution of RNF20. H2Bub1 levels are highest in interphase, when RNF20 is spatially constrained within the nucleus, and are rapidly lost upon entry into mitosis in a manner coincident with RNF20 becoming spatially redistributed away from the chromosomes and into the cytoplasm. To our surprise, and in opposition to the established trans-histone pathway (Dover et al. 2002; Feng et al. 2002; Sun and Allis 2002), the temporal progression patterns characterized for H3K4me2 and H3K79me2 were in contrast to that of H2Bub1. While H3K4me2 levels remain relatively constant throughout the cell cycle, H3K79me2 levels are highly dynamic and are minimal during interphase and maximal during mitosis. To identify the mechanism responsible for the mitotic-associated increases in H3K79me2, we employed a combination of reverse and chemical genetics and show that DOT1L expression and function are essential requirements. Although our results are most consistent with DOT1L being the mitosis-specific methyltransferase, they do not preclude the possibility that DOT1L indirectly regulates the large mitotic increases in H3K79me2 abundance through an unknown mechanism. Finally, based on similar dynamics observed for other histone PTMs (e.g., H3S10ph, H3K9me3, and H4K20me2) with established roles in chromosome biology and genome stability, we predicted that H3K79me2 would exhibit a similar role during mitosis. Using siRNA and chemical treatments, we show that the loss of H3K79me2 levels during mitosis is associated with increases in chromosome instability and aberrant mitotic events. Collectively, our data
suggest there is a transient uncoupling of the trans-histone pathway during mitosis, which is normally required to ensure mitotic fidelity and chromosome stability in human cells.

The dynamic temporal progression pattern we observe for H2Bub1 extends the observations of Wu et al. (1981), who showed that ubiquitinated H2B, but not specifically H2Bub1, is reduced within mitotically enriched histones isolated from mouse and Chinese hamster cells. Although it is unclear why there is such a dramatic loss of H2Bub1 during mitosis, there are a number of possibilities. First, because H2Bub1 is associated with transcription (Davie and Murphy 1990; Shema et al. 2008), the low levels may simply reflect the transcriptionally silent status of mitotic cells (Egli et al. 2008). Second, replication-independent nucleosome assembly may occur (Ahmad and Henikoff 2002), which is a mechanism that affects the global abundance of other histone PTMs through histone replacement. However, replication-independent nucleosome assembly is typically associated with transcription and/or DNA repair (Janicki et al. 2004), and neither process occurs to a large extent within mitotic cells. It is also unlikely that this mechanism accounts for the dramatic loss of H2Bub1 along the entire length of the chromosome arms, particularly as they are compacting during mitosis and histone replacement would not be easily achieved. Finally, it is possible that the removal of ubiquitin is a normal prerequisite for proper chromosome condensation to occur. Ubiquitin is a relatively large ∼8.5-kDa covalent modification that is added onto the carboxy-terminal tail (K120) of histone H2B (∼13.8 kDa). It is plausible that H2Bub1 represents a structural barrier to the compacting mitotic chromosomes that must be removed for proper compaction and segregation to occur. In support of this possibility, Fierz et al. (2011) recently showed that H2Bub1 disrupts local and higher-order chromatin compaction in chemically defined nucleosomal arrays. Moreover, studies from a variety of model organisms have shown that higher-order chromosome compaction is necessary to ensure mitotic fidelity (Wei et al. 1999; Bui et al. 2004). In any case, the functional significance and underlying mechanism accounting for the loss of mitotic H2Bub1 warrant further investigation.

Numerous studies have characterized the interrelationship of H2Bub1 and H3K79me2 (Chandrasekharan et al. 2010). However, since these studies focused on transcription, they are biased toward describing relationships within interphase populations. Unlike these previous studies, the present study focuses on the relationship and changes that occur within mitotic populations, when transcription rates are low (Taylor 1960). Given the existence of the well-characterized trans-histone pathway, the identification of distinct temporal progression patterns for H2Bub1, H3K4me2, and H3K79me2 during mitosis is surprising. Here we show that the global abundance of H3K4me2 remains relatively consistent throughout the cell cycle and is in agreement with previous work demonstrating that histone methylation was classically considered a static epigenetic mark (Byvoet et al. 1972; Duerre and Lee 1974). Perhaps most interesting is the observation that H3K79me2 levels are highly dynamic and
maximal during mitosis, which are opposite to those of H2Bub1. Our findings support those of Feng et al. (2002), who reported an overall increase in the abundance of H3K79 methylation within mitotically enriched HeLa cells. However, they did not distinguish between the methylation subtypes (i.e., mono-, di-, and trimethylation). More recently, Kim et al. (2012) employed Western blots to show that H3K79me2 levels are only marginally dynamic within in a lung cancer cell line. Using synchronized populations of cells, they show that H3K79me2 levels peak in S phase, but not during mitosis as shown in the present study. Although the underlying reason for this discrepancy is unclear, it may be due to the different cell models employed or to the fundamental differences of population-based, biochemical approaches they employed, relative to the single-cell, semiquantitative imaging microscopy employed in the present study. Furthermore, the observations and conclusions of the present study are based on experiments conducted in two distinct cellular contexts (HCT116, transformed epithelial cells; and hTERT, immortalized fibroblast cells) and are in agreement with those gleaned from studies in model organisms including Trypanosoma brucei (Janzen et al. 2006) and Saccharomyces cerevisiae (Schulze et al. 2009). Thus, our data show that H3K79me2 is highly dynamic during mitosis and further suggest that the mitotic-associated dynamics are evolutionarily conserved in eukaryotes.

A number of studies exist that show certain histone PTMs exhibit mitosis-specific increases that are essential for mitotic fidelity (reviewed in Thompson et al. 2013). Our data show that H3K79me2 levels are dynamic and suggest that these dynamics are essential requirements for genome stability. In support of this possibility, depleting the mitotic-associated levels of H3K79me2 by DOT1L silencing or inhibition was associated with increases in chromosome numbers and aberrant mitotic events (Figure 6). Interestingly, DOT1L is somatically altered in a number of tumor types (Table S1), and murine models have shown that Dot1l deficiencies correlate with increases in DNA content (Jones et al. 2008; Barry et al. 2009). Accordingly, DOT1L expression and function and concomitant mitotic increases in H3K79me2 are implicated as essential components required to maintain genome stability in human cells.

In summary, our findings show that H2Bub1, H3K4me2, and H3K79me2 exhibit unique temporal progression patterns throughout the cell cycle. Of particular interest, we show that H3K79me2 is highly dynamic during mitosis and identify DOT1L as a mitosis-specific histone methyltransferase. We further show that H3K79me2 dynamics are normally required to preserve genome stability, which adds H3K79me2 to the emerging list of histone PTMs with similar dynamics and roles in chromosome biology and genome stability. Thus, our data coupled with DNA sequencing data from various tumor types and recent Dot1l animal models strongly suggest that altered DOT1L function, particularly during mitosis, may be a pathogenic event that drives genome instability and contributes to tumorigenesis.

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Mitotic Accumulation of Dimethylated Lysine 79 of Histone H3 Is Important for Maintaining Genome Integrity During Mitosis in Human Cells

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Table S1  Frequency of Somatic Gene Alterations in Cancer (FORBES et al. 2011; CERAMI et al. 2012).

| Gene | Tumor Type | nA | Mutation | DelB | AmpC | Total |
|------|------------|----|----------|------|------|-------|
| RNF20 | Colorectal | 72 | 5.6% (4) | 0.0% (0) | 0.0% (0) | 5.6% (4) |
| RNF20 | Uterine | 240 | 5.0% (12) | 0.8% (2) | 0.0% (0) | 5.8% (14) |
| RNF20 | Melanoma | 121 | 5.0% (6) | 0.0% (0) | 0.0% (0) | 5.0% (6) |
| RNF20 | Lung | 129 | 4.7% (6) | 0.0% (0) | 0.0% (0) | 4.7% (6) |
| RNF20 | Ovarian | 316 | 0.9% (3) | 0.6% (2) | 0.3% (1) | 1.8% (6) |
| DOT1L | Colorectal | 72 | 11.1% (8) | 0.0% (0) | 0.0% (0) | 11.1% (8) |
| DOT1L | Bladder | 127 | 4.7% (6) | 0.0% (0) | 1.6% (2) | 6.3% (8) |
| DOT1L | Melanoma | 121 | 5.8% (7) | 0.0% (0) | 0.0% (0) | 5.8% (7) |
| DOT1L | Lung | 129 | 4.7% (6) | 0.8% (1) | 0.0% (0) | 5.5% (7) |
| DOT1L | Ovarian | 311 | 0.3% (1) | 3.5% (11) | 0.3% (1) | 4.1% (13) |

A Number of tumors sequenced (n)
B Homozygous Deletion (Del)
C Amplification (Amp)
Table S2  Antibodies and Dilutions Employed.

| Antibody/Epitope | Source     | Catalog No. | Species | Working Dilution^A | IIF^B | WB^C |
|------------------|------------|-------------|---------|--------------------|-------|------|
| RNF20            | Abcam      | ab32629     | Rabbit  | 1:400              | 1:6,000 |
| H2Bub1           | Millipore  | 05-1312     | Mouse   | 1:200              | 1:10,000 |
| H3K4me2          | Abcam      | ab32356     | Rabbit  | 1:200              | 1:5,000 |
| H3K79me2         | Abcam      | ab3594      | Rabbit  | 1:400              | 1:15,000 |
| H3S10ph          | Abcam      | ab14955     | Rabbit  | 1:10,000           | 1:10,000 |
| DOT1L            | Abcam      | ab180483    | Mouse   | n/a                | 1:2,000 |
| α-Tubulin        | Abcam      | ab7291      | Mouse   | n/a                | 1:20,000 |
| Rabbit IgG-Alexa Fluor 488 | Molecular Probes | A-11034 | Goat    | 1:200              | n/a    |
| Mouse IgG-Cy3    | Molecular Probes | A-10521 | Goat    | 1:200              | n/a    |
| Rabbit IgG-HRP   | Jackson    | 111-035-006 | Goat    | n/a                | 1:15,000 |
| Mouse IgG-HRP    | Jackson    | 115-035-146 | Goat    | n/a                | 1:10,000 |

^Anot applicable (n/a)
^BIndirect immunofluorescence (IIF)
^CWestern blot (WB)
Table S3  Student t-test for the Mean DAPI-Normalized H3K79me2 Total Signal Intensities as Determined by Semi-Quantitative Imaging Microscopy.

| Cell Cycle Stage | n^A | Mean ± Standard Deviation | p-value^B | Fold Increase |
|------------------|-----|---------------------------|-----------|---------------|
| Interphase       | 23  | 1.00 ± 0.02               | n/a       | n/a           |
| Mitosis^C        | 23  | 31.04 ± 1.63              | <0.0001   | 31            |

^A Number of cells analysed (n)
^B Based on Student’s t-tests comparing the mean DAPI-normalized H3K79me2 total signal intensity of interphase and mitotic cells. Not applicable (n/a)
^C Limited to only those cells in prophase, prometaphase and metaphase as identified by cytological features
| Condition                          | n<sup>А</sup> | Mean ± Standard Deviation | p-value<sup>А</sup> |
|-----------------------------------|---------------|---------------------------|---------------------|
| **48 Hours Post Silencing**       |               |                           |                     |
| Mitotic Cells + siGAPDH           | 23            | 100.00 ± 4.97             | n/a                 |
| Mitotic Cells + siDOT1L           | 23            | 54.72 ± 3.78              | <0.0001             |
| **2 Hours Post Treatments**       |               |                           |                     |
| Mitotic Cells + DMSO              | 23            | 100.00 ± 4.13             | n/a                 |
| Mitotic Cells + SGC0946           | 23            | 24.36 ± 1.60              | <0.0001             |

<sup>А</sup>Number of cells analysed (n)  
<sup>B</sup>Based on Student’s *t*-tests comparing the mean DAPI-normalized H3K79me2 total signal intensity of mitotic cells for either DOT1L silenced or inhibited conditions with their respective controls. Not applicable (n/a)
Table S5  Cell Cycle Analyses Following Protein Silencing or Drug Treatment.

| Condition/Treatment | G0/G1   | S-phase | G2/M   |
|---------------------|---------|---------|--------|
| Non-treated         | 41.9%   | 35.9%   | 20.6%  |
| siGAPDH             | 41.8%   | 35.9%   | 20.6%  |
| siRNF20             | 36.3%   | 35.2%   | 26.5%  |
| siDOT1L             | 39.9%   | 40.0%   | 18.8%  |
| DMSO                | 35.6%   | 36.4%   | 26.3%  |
| SGC0946             | 38.2%   | 34.4%   | 25.5%  |
Table S6  Student $t$-tests Comparing Mean Chromosome Numbers Following DOT1L Silencing or Inhibition.

| Condition   | n$^A$ | Mean ± Standard Deviation | p-value$^B$ |
|-------------|-------|----------------------------|-------------|
| siGAPDH     | 100   | 44.80 ± 0.07               | n/a         |
| siDOT1L     | 100   | 48.38 ± 1.38               | 0.0102      |
| DMSO        | 100   | 44.97 ± 0.05               | n/a         |
| SGC0946     | 100   | 46.30 ± 0.49               | 0.0075      |

$^A$Number of cells analysed (n)

$^B$Not applicable (n/a)
Table S7  The Loss of Mitotic H3K79me2 Levels is Associated with Increases in Aberrant Mitotic Events

| Condition  | Total Mitotic Cells Analyzed | Mean Frequency of Defects (%) (± Standard Deviation) | Fold Increase<sup>A</sup> |
|------------|-----------------------------|-------------------------------------------------------|---------------------------|
| siGAPDH    | 315                         | 5.570 ± 0.8907                                        | n/a                       |
| siDOT1L    | 337                         | 9.696 ± 2.163                                        | 1.74-fold                 |
| DMSO       | 306                         | 3.742 ± 1.007                                        | n/a                       |
| SGC0946    | 318                         | 9.303 ± 1.918                                        | 2.49-fold                 |

<sup>A</sup>Fold increase is calculated relative to the corresponding control. n/a; not applicable
Figure S1  Validation of the RNF20 and H2Bub1 Antibodies.
Representative low-resolution images collected from the IIF peptide competition assays using a non-specific peptide (NS peptide) or the RNF20-specific, immunizing peptide (A), or the H2Bub1-specific peptide (B). The epitope of interest and DAPI are pseudo-colored green and red, respectively, within the merged images. Images were acquired using identical exposure times and thus qualitative comparisons can be made between the non-specific and the corresponding specific peptide for each epitope. Arrowheads identify mitotic cells and the scale bar represents 5μm. (C) Dot blot assays demonstrating the specificity of the H2Bub1 antibody for the H2Bub1 epitope and not the unmodified control (H2B).
Figure S2  Defining the Spatial Relationships of RNF20, H2Bub1, H3K4me2 and H3K79me2 within Interphase Chromatin.

Representative high-resolution (63×) deconvolved images of interphase HCT116 cells immunofluorescently labeled for RNF20 (A), H2Bub1 (B), H3K4me2 (C), and H3K79me2 (D), and counterstained with DAPI. The epitope of interest and DAPI are pseudo-colored green and red, respectively, within the merged images. Scale bar represents 2 μm. Line scans (right panels) show the spatial juxtaposition of the four specific epitopes evaluated (green) relative to DAPI (red). Lines and arrows within the Merge indicate the position and direction presented in the graph. Each epitope appears to be enriched within or adjacent to euchromatic regions (less intense DAPI stained regions), and reduced within heterochromatic regions (intense DAPI stained regions).
Figure S3  RNF20 is Spatially Relocalized in Mitotic hTERT Cells.
(A) Representative image of asynchronous hTERT cells immunofluorescently labeled with an anti-RNF20 antibody (green) and counterstained with DAPI (red). Arrowhead identifies a mitotic (prometaphase) cell, while the scale bar represents 5 μm. (B) High-resolution images depicting the temporal and spatial progression pattern of RNF20 (green) throughout the various stages of the cell cycle (indicated). Note that variable exposure times were employed for each cell cycle stage so as to best capture the localization of RNF20 and thus qualitative comparisons cannot be made between stages. Scale bar represents 2 μm.
Figure S4  Flow Cytometric Analyses of Asynchronous and Mitotically-enriched Populations. 
Cell cycle analyses of asynchronous (left) or a mitotic-enriched (nocodazole treated [right]) populations of HCT116 cells labeled with PI. Markers are included to identify the various cell cycle stages with the percentage of cells within each stage provided.
Figure S5  H2Bub1 Levels Decrease Dramatically During Mitosis in hTERT Cells.
(A) Representative image of asynchronous hTERT cells immunofluorescently labeled with an anti-H2Bub1 antibody (green) and counterstained with DAPI (red). Arrowhead identifies a mitotic (metaphase) cell, while the scale bar represents 5μm. (B) High-resolution images depicting the temporal and spatial progression pattern of H2Bub1 (green) throughout various stages of the cell cycle (indicated). Note that the exposure times were maintained constant for each cell cycle stage so as to show qualitative changes in abundance during mitosis relative to interphase or early G1. Scale bar represents 2μm.
Figure S6  Validation of the H3K4me2 and H3K79me2 Antibodies.
Representative low-resolution images collected from the IIF peptide competition assays using a non-specific peptide (NS peptide) or the H3K4me2-specific peptide (A), or the H3K79me2-specific peptide (C). The epitope of interest and DAPI are pseudo-colored green and red, respectively, within the merged images. Images were acquired using identical exposure times and thus qualitative comparisons can be made between the non-specific and the corresponding specific peptide for each epitope. Arrowheads identify mitotic cells and the scale bars represent 5 μm. Dot blot assays demonstrating the high specificity of the H3K4me2 (B) or H3K79me2 (D) antibodies for their cognate epitope, but not closely related mono- or tri-methylated species.
Figure S7  H3K4me2 Levels in hTERT Cells are Relatively Constant Throughout the Cell Cycle.
(A) Representative image of asynchronous hTERT cells immunofluorescently labeled with an anti-H3K4me2 antibody (green) and counterstained with DAPI (red). Arrowhead identifies a mitotic (prophase) cell, while the scale bar represents 5μm. (B) High-resolution images depicting the spatial and temporal progression pattern of H3K4me2 (green) throughout the cell cycle (indicated). Note that variable exposure times were employed for each cell cycle stage so as to best capture the localization of H3K4me2 and thus qualitative comparisons cannot be made between stages. Scale bar represents 2μm.
Figure S8   The Global Abundance of H3K79me2 Increases Dramatically during Mitosis in hTERT Cells. (A) Representative image of asynchronous hTERT cells immunofluorescently labeled with an anti-H3K79me2 antibody (green) and counterstained with DAPI (red). Arrowhead identifies a mitotic (prometaphase) cell and the scale bar represents 5 μm. (B) High-resolution images depicting the spatial and temporal progression pattern of H3K79me2 (green) throughout the cell cycle (indicated). Note that variable exposure times were employed for each cell cycle stage so as to best capture the localization of H3K79me2 and thus qualitative comparisons cannot be made between stages. Scale bar represents 2 μm. (C) Western blot analysis of H3K79me2 from asynchronous (A) and mitotic-enriched (M; nocodazole treated) hTERT populations. Phosphorylation of serine 10 of histone H3 was included as a mitosis-specific marker, while CPTS serves as the loading control.
Figure S9  Cell Cycle Analyses of Silenced and Drug Treated Cellular Populations.
Flow cytometric analyses of DNA content profiles from asynchronously growing cells harvested from controls (Non-treated [NT] and DMSO-treated [DMSO]), protein silenced populations (siGAPDH, siRNF20 or siDOT1L) or drug treated populations (SGC0946). Percentage of cells within each stage (i.e. G0/G1, S-phase or G2/M) are presented in Table S7.
SUPPORTING INFORMATION REFERENCES

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