Polycomb Group Protein-associated Chromatin Is Reproduced in Post-mitotic G1 Phase and Is Required for S Phase Progression

Received for publication, November 13, 2007, and in revised form, April 8, 2008 Published, JBC Papers in Press, May 2, 2008, DOI 10.1074/jbc.M709322200

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Polycomb group (PcG) proteins form two distinct complexes, PRC1 and PRC2, to regulate developmental target genes by maintaining the epigenetic state in cells. PRC2 methylates histone H3 at lysine 27 (H3K27), and PRC1 then recognizes methyl-H3K27 to form repressive chromatin. However, it remains unknown how PcG proteins maintain stable and plastic chromatin during cell division. Here we report that PcG-associated chromatin is reproduced in the G1 phase in post-mitotic cells and is required for subsequent S phase progression. In dividing cells, H3K27 trimethylation (H3K27Me3) marked mitotic chromosome arms where PRC2 (Su(z)12 and Ezh2) co-existed, whereas PRC1 (Bmi1 and Pc2) appeared in distinct foci in the pericentromeric regions. As each PRC complex was increasingly assembled from mitosis to G1 phase, PRC1 formed H3K27Me3-based chromatin intensively during middle and late G1 phase; this chromatin was highly resistant to in situ nuclease treatment. Thus, the transition from mitosis to G1 phase is crucial for PcG-mediated chromatin inheritance. Knockdown of Su(z)12 markedly reduced the amount of H3K27Me3 on mitotic chromosomes, and as a consequence, PRC1 foci were not fully transmitted to post-mitotic daughter cells. S phase progression was markedly delayed in these Su(z)12-knockdown cells. The fact that PcG-associated chromatin is reproduced during post-mitotic G1 phase suggests the possibility that PcG proteins enable their target chromatin to be remodeled in response to stimuli in the G1 phase.

Development of multicellular organisms depends on the generation and propagation of diverse cell types. Differentiating cells begin to express lineage-specific genes during an early stage of development in response to external signals and then maintain patterns of gene expression even after the initial signals are lost (1). Polycomb group (PcG) genes have been shown to maintain such lineage-specific gene expression via a chromatin-mediated repressive mechanism (2). PcG proteins form two distinct multisubunit complexes, called polycomb-repressive complex-1 (PRC1) and PRC2. PRC2 contains the SET (Su(var)3-9, Enhancer-of-zeste, Trithorax) domain containing protein Ezh2, which trimethylates histone H3 at lysine 27 (H3K27), together with the essential noncatalytic subunit Suz12 (3, 4). PRC1 includes the chromodomain protein Pc2, which recognizes H3K27 trimethylation (H3K27Me3) (5). Other PRC1 subunits, like Ring1 or Bmi1, are also involved in the ubiquitination of H2A at lysine 119, a prerequisite for full repression by PcG proteins (6). Thus, PcG-mediated gene repression is achieved through two processes, PRC2-mediated H3K27 trimethylation and recruitment of PRC1 onto the H3K27Me3 (2, 3). Although a transient interaction between the two complexes has been reported (7), it remains unknown how the two PRC complexes coordinately function during the cell cycle, and especially whether the means by which PRC2 associates with chromatin is equivalent to that used by PRC1.

One of the most remarkable features of the PcG-repressive system is inheriting patterns of gene expression by daughter cells, but the mechanism of how PcG proteins maintain repressive chromatin through H3K27Me3 during cell division is poorly understood (1, 8). For proper inheritance, chromatin must endure the passage of S phase-coupled DNA replication and survive mitosis, during which chromosomes are condensed, segregated, and decondensed (9–11). By contrast, a mechanism of inheritance for constitutive heterochromatin has been well studied (12, 13). DNA methyltransferase 1 is involved in maintaining patterns of genomic CpG methylation, which is closely coupled to the DNA replication process in S phase and may play an additional role in keeping them during G2/M phase (14–16). Methylated DNA-binding proteins immediately recognize methyl-CpG dinucleotides and maintain constitutively condensed heterochromatin by recruiting histone deacetylases and H3K9 methyltransferases (17–19). However, PcG-associated chromatin is likely to be distinct from the conventional heterochromatin, because it possesses...
significant plasticity for developmental reprogramming (1, 2, 13). For example, mouse ES cells sustain the silenced status of multiple lineage-specific genes by PcG proteins during self-renewal, whereas differentiation stimuli relieve this repression by inducing the loss of PcG proteins and, subsequently, of H3K27Me3 from target genes (20, 21). On the other hand, the pluripotent cell marker Oct3/4 is initially expressed in undifferentiated ES cells and then repressed in terminally differentiated neurons because of the marking of its core promoter region with H3K27Me3 (22). These observations emphasize that PcG-regulated chromatin is reversibly erased or established by the removal or addition of H3K27Me3, respectively, during the process of cell differentiation. It should be noted that a large number of transcription factors were found in promoter regions of the PRC1 target genes in Drosophila cells (23), implying that PcG-associated chromatin, to some degree, allows other regulatory molecules to access target motifs unlike constitutive heterochromatin (2, 13). Thus, PcG proteins produce facultative chromatin that can be either stably maintained or flexibly reorganized. Therefore, it is of great importance to understand the fundamental properties of metastably inheritable PcG-associated chromatin marked with H3K27Me3, which is applicable to other epigenetic mechanisms (1, 8).

In this study, we investigated the associations of endogenous PRC2 and PRC1 with chromatin using cytological and biochemical approaches. The mean by which PRC2 associates with chromatin is distinct from that used by PRC1, and localization of both PRC complexes was distinguished from either HP1-enriched condensed heterochromatin or transcriptionally active sites during interphase. Although the association of PRC1 with H3K27Me3-marked chromatin was largely lost in dividing cells, most PRC2 co-existed with H3K27Me3-marked mitotic chromosome arms. After cell division, PRC2-dependent H3K27Me3-marked regions were targeted by PRC1 to form a higher order chromatin structure, intensively, during middle and late G1 phase. Furthermore, knockdown of Su(z)2 abrogated H3K27Me3 marking of mitotic chromosomes and then PRC1 foci formation during G1 phase, resulting in defects in S phase progression. These findings provide insights into the molecular basis of PcG-mediated epigenetic inheritance during the cell cycle.

**EXPERIMENTAL PROCEDURES**

**Cell Culture, Cell Cycle Synchronization, and Transfection—**
HeLa S3 and NIH3T3 cells were maintained in Dulbecco's modified Eagle's medium/F-12 medium (Sigma) supplemented with 10% (v/v) fetal bovine serum. U2OS and HCT116 cells were grown in McCoy's 5A (Sigma) supplemented with 10% fetal bovine serum and 2 mM Glutamax (Invitrogen). ES cells were cultured as described previously (22). These cells were grown under 5% CO2 at 37 °C. Cells were synchronized with 100 ng/ml nocodazole (Sigma) at prometaphase or with 2 μg/ml thymidine at G1/S phase (Sigma). Each cell stage was confirmed by the expression of Nek2 (S/G2-specific marker) or cyclin B1 (G2/M-specific marker). Single and double plasmid transfections were performed using FuGENE 6 and FuGENE HD (Roche Applied Science), respectively. The double transfection condition was optimized to yield about 90% co-expression. For siRNA transfection, Lipofectamine RNAi Max (Invitrogen) was used. siRNA was transfected at 10 nM to minimize off-target effects. Hygromycin (Invitrogen) and puromycin (Sigma) were used for selection at 300 ng/ml and 2 μg/ml, respectively. Colony staining was performed using methylene blue (Sigma) in 50% methanol.

**Chromatin and Nuclear Fractionations—**
Nuclear fractions were prepared as described previously (24). Briefly, cells were lysed in NB buffer (15 mM Tris-HCl (pH 7.4), 0.3% Nonidet P-40, 5 mM MgCl2, 60 mM KCl, 15 mM NaCl, 1 mM dithiothreitol, 250 mM sucrose and protease inhibitors, including 4-(2-aminoethyl)benzenesulfonyl fluoride hydrochloride, chymostatin, leupeptin, pepstatin A, antipain, aprotinin, and trichostatin A). Micrococcal nuclease (MNase) digestion was performed as described previously (25). Briefly, isolated nuclei equivalent to 100 μg of DNA was treated with 0.2 units of MNase (Sigma) at room temperature in MNase buffer (15 mM Tris-HCl (pH 7.4), 5 mM MgCl2, 60 mM KCl, 15 mM NaCl, 2 mM CaCl2, and the protease inhibitors). After a 20-min digestion, samples were cooled and centrifuged. After removing the supernatants (S1), pellets were incubated in 2 mM EDTA (pH 8.0) on ice for 30 min, and supernatant (S2) was prepared. The subnuclear fractionation procedure was performed as described by Kreitz et al. (26) with some modifications. Briefly, cells were incubated in CSK100, 0.5% TX buffer (10 mM PIPES (pH 6.8), 300 mM sucrose, 3 mM MgCl2, 100 mM NaCl, 0.5% Triton X-100 with the protease inhibitors and ribonucleoside vanadyl complex (New England Biolabs)) to remove cytosol. Isolated nuclei were incubated in CSK buffer with 250 mM (NH4)2SO4 to extract salt-sensitive chromatin-binding proteins (fraction “A”). Remaining chromatin was digested with 100 units/ml RNase-free DNase I (Roche Applied Science) in CSK100/Ca2⁺ buffer (10 mM PIPES (pH 6.8), 300 mM sucrose, 3 mM MgCl2, 100 mM NaCl, 2 mM CaCl2, 20 units/ml rRNasin (Promega) with the protease inhibitors) for 60 min at 37 °C, and 250 mM (NH4)2SO4 was then added to extract proteins (fraction “B”). 2 mM NaCl was added to completely extract histones. Insoluble pellets were lysed in SDS sample buffer (57.5 mM Tris-HCl (pH 6.8), 2% SDS, 20% glycerol) with sonication (fraction “C”).

**Immunoprecipitation—**
HeLa cells were suspended in NB buffer as described above. After centrifugation to remove soluble proteins, the chromatin-bound fraction was further extracted with TTEM-N250 buffer (20 mM Tris-HCl (pH 7.4), 1% Triton X-100, 2 mM EDTA, 10 mM MgCl2, 250 mM NaCl, 10% glycerol, and the protease inhibitors) on ice for 30 min. After centrifugation at 10,000 × g for 20 min, supernatants were precleared using protein A and G beads (Amersham Biosciences) for 2 h at 4 °C with rotation. Precleared samples were then subjected to immunoprecipitation overnight at 4 °C, using specific antibodies or control IgG that was pre-bound to protein A and G beads. The beads were washed with TTEM-N250 buffer five times. The proteins that were bound to beads were finally eluted with SDS sample buffer containing 10 mM dithiothreitol and subjected to Western blot analysis, as described previously (22). The antibodies used are shown in supplemental Table 1. Band intensity was quantified using Scion image α 4.302 (Scion Corp., Frederick, MD).
Immunoﬂuorescence Analysis and in Situ Nuclease Sensitivity Assay—Cells grown on gelatin-coated coverslips were ﬁxed in 4% paraformaldehyde (pH 7.4) at room temperature for 10 min, washed twice with PBS, permeabilized with 0.5% Triton X-100 in PBS for 5 min on ice, and subjected to immunoﬂuorescence analysis as described previously (22). To analyze mitotic cells, nocodazole-treated cells were collected by shake-off, washed twice with pre-warmed Dulbecco’s modiﬁed Eagle’s medium/F-12 medium, and gently placed onto poly-L-ornithine-coated coverslips for immunoﬂuorescence analysis. For detection of proteins on metaphase chromosomes, cells were swollen with 60 mM KCl for 30 min, ﬁxed with 4% paraformaldehyde (pH 7.4) at room temperature for 10 min, and then post-ﬁxed with 100% methanol at −20 °C for 10 min. For pre-extraction experiments, cells were treated with CSK100 buffer containing 0.2% Triton X-100 on ice for 5 min. For in situ nuclease assays, pre-extracted cells were further incubated in DNase buffer (500 units/ml DNase I (Roche Applied Science), 10 mM PIPES (pH 6.8), 300 mM sucrose, 2 mM CaCl2, 3 mM MgCl2, 50 mM NaCl, RNasin (Promega), and the protease inhibitors) or RNase buffer (1/25 diluted RNase mixture (Ambion) containing a mixture of 20 units/ml RNase A and 800 units/ml RNase T1 with the protease inhibitors, in PBS) for 60 min at 37 °C, prior to ﬁxation.

Plasmids and RNA Interference—To generate expression vectors for H2B-EGFP, EGFP-PSDL, and DsRed monomer-PSDL, the coding sequences for mouse H2B and the subcellular localization domain (PSLD) of human DNA helicase B were PCR-ampliﬁed from mouse ES cell and HeLa cell cDNA libraries using LA-Taq (Takara). The primers used are as follows (restriction enzyme sites are underlined): for mouse H2B, 5’–CGGGGAATTCCGACCAATGCTAGCGGCTGCAAGTCC–3’ and 5’–CGGCTGAGGCCGCGCTTGGGCTAGTATACCTTG–3’; for human PSDL, 5’–GCGGAAATTCTCTCTCTCAGGCGGCACCTCGACGAGCTGAGTTTTCTTGATTTACGCTGGGGC–3’. The PCR products were digested with EcoRI and Xhol, and cloned into ppyCAGIP-EF GFP-N, pPyCAGIP-EF GFP-C or pPyCAGIP-DsRed monomer-C vectors. A 21-mer siRNA duplex was designed to target hSuz12 (GenBankTM accession number NM015355).

RESULTS

PcG Proteins Are Uniquely Distributed to Repressive Chromatin—To identify the fundamental properties of PcG-associated chromatin, we visualized constituent proteins of the PRC complexes in HeLa and NIH3T3 cells (Fig. 1A). Immunoﬂuorescent images combined with line scan plot analyses showed that endogenous Suz12 (green) and Bmi1 (red) were localized at multiple foci in the nuclei of both cell types. Although a few overlapping foci were seen between Suz12 and Bmi1, most of them were excluded from each other or from DNA-condensed heterochromatin stained with 4’,6-diamidino-2-phenylindole (DAPI) (blue). The immunolocalization pattern was speciﬁc to Suz12 and Bmi1, as we conﬁrmed the speciﬁcity of antibodies used in this study (supplemental Fig. 1). We then checked the locations of PcG-associated foci relative to those of histone-condensed chromatin (Fig. 1B and supplemental Fig. 2A), using the signals of histone H2B fused to EGFP (H2B-EGFP), which were reported to be highly concentrated at the nuclear periphery and in the perinuclear region (29). Suz12 and Bmi1 (red) were accumulated in discrete foci, which did not always overlap with the H2B-EGFP signals. Next we compared their locations with the sites of transcription, using the antibodies recognizing both phosphorylated and nonphosphorylated forms of RNA polymerase II (N-20) (Fig. 1C and supplemental Fig. 2B). Most of Ezh2 and Bmi1 foci had no or limited association to the sites where bulk RNA polymerase II is enriched. The use of antibodies raised against phosphorylated Ser-5 of the carboxyl-terminal region of RNA polymerase II (CTD4H8), indicative of transcription initiation (30, 31), also showed little co-localization, suggesting that Bmi1 and Suz12 are unlikely to be at the sites of ongoing transcription (supplemental Fig. 3). These results suggest that PRC1-associated chromatin and PRC2-associated chromatin are distinct and that neither form represents typical heterochromatin or actively transcribed sites.

Because the biochemical chromatin fractionation method has been well established in HeLa cells (26), we then investigated whether PRC1 and PRC2 are uniquely distributed on chromatin (Fig. 1D). By treating the nuclei of HeLa cells with MNase, we prepared two soluble fractions and the remaining pellet fraction; the S1 fraction contained histone H1-depleted mononucleosomes; the S2 fraction included H1-containing oligonucleosomes and the insoluble P fraction (MNase+). It had been originally described that the S1, S2, and P fractions approximately corresponded to open chromatin, heterochromatin, and nuclear matrix-associated chromatin, respectively (25). Suz12 and Ezh2 appeared in all three fractions, whereas Pc2 was found only in the nuclease-resistant P fraction. This suggests that PRC2- and PRC1-associated chromatin are biochemically nonequivalent. In addition, the heterochromatin

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FIGURE 1. Unique chromatin distribution of PRC1 and PRC2. A, localization of Suz12 and Bmi1 relative to heterochromatin. HeLa (left) and NIH3T3 (right) cells were immunostained with anti-Suz12 (green) and anti-Bmi1 (red) antibodies. Heterochromatic DNA was detected with DAPI (blue) in NIH3T3 cells. Bar, 5 μm. Line scan plots along the track of white arrows in the images are shown below. Relative fluorescence intensity (R.F.I.) and the distance from the start of arrow are also indicated. B, localization of Suz12 and Bmi1 relative to histone-enriched chromatin. Suz12 and Bmi1 (red) were detected in NIH3T3 cells expressing histone H2B fused to GFP (H2B-GFP). The condensed chromatin and Bmi1 foci are indicated with arrows and the arrowhead, respectively. Enlarged views of the white-boxed area in merged images are shown in red boxes. Bar, 5 μm. C, localization of Ezh2 and Bmi1 relative to RNA polymerase II-transcribed sites. Ezh2 (green) and Bmi1 (green) were stained together with RNA polymerase II (Pol II) (red) and DAPI (blue). Rabbit polyclonal antibodies (N-20) detected both phosphorylated and nonphosphorylated forms of RNA polymerase II. Bar, 5 μm. PcG proteins did not overlap with DAPI-stained heterochromatin (A), H2B-GFP-enriched chromatin (B), or Pol II-transcribed sites (C) during interphase. D, distinct chromatin association of PRC2 and PRC1. HeLa nuclei were partially digested with MNase for 20 min to prepare fractions S1, S2, and P as described under “Experimental Procedures.” Western blot analysis was performed together with ethidium bromide staining of nucleosomal DNA. Both H1 and core histones are shown with Coomassie Blue staining. E, different sensitivities of PRC1 and PRC2 to DNase I treatment. An in situ nuclease assay was performed in U2OS cells. Fibrillarin, DAPI, and the promyelocytic leukemia protein (PML) were used as controls to test the effects of RNase A/T1 and DNase I. Nuclear localization of Pc2 and Bmi1 was resistant to DNase I. Bars, 10 μm.

protein HP1 was present in the S2 and P fractions, and the transcription factor Sp1 was found in the S1 and P fractions. On the other hand, without the use of MNase, all of these proteins were detected in the P fraction (MNase−).

To further elucidate the differences between the PRC2 and PRC1 complexes, we performed in situ nuclease digestion analysis. Because polycomb foci were evidently detected, U2OS cells were used for this experiment (Fig. 1E). The cells were
of silenced chromatin marked with H3K27Me3 from mother to daughter cells during the cell cycle. Especially during the transition from mitosis to the G1 phase, the overall chromatin architecture changes dynamically because of chromosome condensation, segregation, and then decondensation. Therefore, we examined the association of H3K27Me3 with chromatin during cell division (Fig. 2A and supplemental Fig. 4A). HeLa cells were synchronized with nocodazole treatment and released for re-growth. Each stage from prometaphase to early G1 phase was determined by the combinations of DAPI, lamin B1, and β-tubulin staining. H3K27Me3 marking was stably maintained on mitotic chromosomes. By contrast, acetyl-H3K9/14 (H3K9/14Ac) disappeared between prometaphase and anaphase and appeared again during telophase. It was shown that the absence of H3K9/14Ac from mitotic cells was because of a loss of acetylation of H3 during mitosis but not because of phosphorylation of H3S10 (11).

We then examined the localization of PRC complexes relative to the DAPI-stained region, centromeres, and H3K27Me3-marked regions in mitotic HeLa cells (Fig. 2, B–D). Suz12 and Ezh2 existed on mitotic chromosome arms and were frequently co-localized with H3K27Me3 (Fig. 2, B and D). Two different monoclonal antibodies against Ezh2 (AC22 and C11) showed very similar results (supplemental Fig. 4B). In addition, two independent shRNAs against Suz12 mRNA eliminated the signal for Suz12 on mitotic chromosomes (Fig. 4B and data not shown), emphasizing that Suz12 and Ezh2 maintain their association with mitotic chromatin. Localization of Suz12 and Ezh2 in mitotic chromosomes was also observed in asynchronously growing embryonic stem cells, E14tg2a, which were originally derived from a mouse male blastocyst (32) (supplemental Fig. 4C), suggesting that the association is neither a drug-induced artifact nor related to an inactive X chromosome. We further examined the localization of Bmi1 on mitotic chromosomes (Fig. 2, C and D). A previous immunofluorescence study showed that Bmi1 was highly accumulated in the large block of pericentromeric heterochromatin (1p12) of mitotic chromosomes when the cells were pretreated with a hypotonic buffer (33, 34). Under similar conditions, we also observed 1–3 Bmi1 foci adjacent to the centromeric region where CREST antigen existed (Fig. 2C), and they did not overlap with H3K27Me3 marking (Fig. 2D). Consistently, GFP-
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A

Bmi1 H3K27Me3 merge

Early G1

Middle G1

Late G1

B

| G2 | M | G1 | S |
|----|---|----|---|
| A | B | C | A | B | C | A | B | C |

Pc2

Suz12

LaminB1

C

| G2 | M | G1 | S |
|----|---|----|---|
| 6  | 8  | 10 | 12 |

H3K27Me3

H3K9Me2

β-tubulin

FIGURE 3. PRC1 forms H3K27Me3-marked chromatin during middle and late G1 phase. A, formation of Bmi1-containing PRC1 foci during G1 phase. Nocodazole-arrested U2OS cells were released for 2 h (early to middle G1) and 8 h (late G1) for immunostaining. The cells were further subjected to in situ DNase I extraction prior to immunofluorescent analysis (right panel). Bars, 5 μm. B, increase in the amount of DNase I-resistant Pc2 during the transition from mitosis to G1 phase. Double thymidine-blocked HeLa cells were released to each phase during the cell cycle. The cells were lysed and divided into fractions A (250 mM (NH4)2SO4 extract), B (DNase I-digested extract), and C (2 M NaCl/DNase I-resistant) for Western blot analysis. Lamin B1 represents fraction C. The band intensity of Pc2 was normalized to the sum of band intensities of Pc2 in all fractions. The percentage of each fraction is shown in the graph. C, up-regulation of H3K27Me3 from mitosis to G1 phase. Western blot analysis of double thymidine-treated HeLa cells was performed. The signal intensities for modified histones were normalized to β-tubulin, and values relative to those in G2 phase (6 h after the release) are shown. M indicates mitosis.

fused Bmi1 was barely detected in chromosome arms between prometaphase and anaphase in living cells (data not shown). These data suggest that most Bmi1 dissociates from mitotic chromatin and then re-associates with interphase chromatin, as shown in Fig. 1. Additional analysis of other chromatin proteins showed that the chromatin remodeling factors Brg1 and HP1β were not concentrated on mitotic chromosomes (supplemental Fig. 4D), as also found for DNMT1, MeCP2, SirT1, and HDAC1 (data not shown). These data suggest that both PRC2 and H3K27Me3 markages co-exist on mitotic chromosomes, whereas PRC1 is dissociated from them.

To clarify whether PRC complexes are functionally formed on chromatin, we examined PRC complex formation during mitosis and G1 phases. HeLa cells were arrested at metaphase by nocodazole and released to subsequent G1. After removing the cytosol/nuclear soluble fraction, the chromatin-bound fraction was prepared by 250 mM NaCl extraction and subjected to immunoprecipitation analysis (Fig. 2E). Both Suz12-Ezh2 complexes (PRC2) and Pc2-Bmi1 complex (PRC1) were increasingly formed from mitosis to G1 phase. A quantitative analysis was performed after normalizing each immunoprecipitated signal against the input. Approximately 31% of the Suz12-Ezh2 complex in G1 phase was present during mitosis, whereas only about 5% of the Pc2-Bmi1 complex in G1 phase was detectable during mitosis. Reciprocal immunoprecipitations showed that Ezh2 was present in the Suz12 immunoprecipitates during both mitosis and G1 phase (data not shown). These findings may agree with a previous report showing that Bmi1 and Pc2 are both expressed and mainly localized in the cytoplasm or soluble nuclear fractions during mitosis (33, 34). Thus, our results suggest that PRC2 is associated with mitotic chromosome arms and that this association contributes to the inheritance of H3K27Me3 to post-mitotic cells, whereas most of PRC1 is targeted to chromatin in the subsequent G1 phase.

PRC1 Foci and H3K27Me3 Are Reproduced during the Post-mitotic G1 Phase—To further determine the dynamics of PRC1, we investigated how Bmi1 is localized to chromatin during G1 phase, relative to H3K27Me3 marking (Fig. 3A). U2OS cells were synchronized with nocodazole and released to G1 phase. During early G1, Bmi1 foci are small, DNase I-sensitive and distinct from H3K27Me3 distribution. As cells progress through late G1, the foci became larger, resistant to DNase I, and co-localized with H3K27Me3, implying that Bmi1 is targeted and incorporated to highly organized chromatin with K27 trimethylated histone gradually during middle and late G1 phase. We could not detect co-localization of Bmi1 with H3K27Me3, implying that Bmi1 is specifically associated with H3K27Me3-enriched chromatin (data not shown). It was of interest that DNase I treatment led to the loss of H3K27Me3 signal, whereas Bmi1 foci remained intact during the middle and late G1 phase. This observation emphasizes that the Bmi1 foci represent the chromatin-bound form of PRC1, which is targeted to H3K27Me3 and further formed higher ordered structure that no longer requires DNA/chromatin content for its maintenance, once it is formed.
Chromatin sub-fractionation experiment also implied that biochemical property of Pc2 changes during the cell cycle (Fig. 3B). The cells were synchronized with double thymidine blocks and then released to a cycling state. The cells were lysed and divided into three fractions for Western blot analysis, termed fractions A (250 mM (NH₄)₂SO₄ extract), B (DNase I-sensitive extract), and C (2 M NaCl/DNase I-resistant pellet). Fraction C included higher ordered chromatin proteins as well as lamin B1. In G₂ phase, approximately half the amount of Pc2 protein was in fraction C, which dropped to ~30% at mitosis and recovered to 50% in the following G₁ phase. To further examine the amounts of H3K27Me³ and H3K9Me² during the cell cycle, Western blot analysis was performed using double thymidine-treated HeLa cells (Fig. 3C). The amount of H3K27Me³ decreased 0.55-fold during mitosis, and then increased more than 2-fold during late G₁ and S phase. By contrast, the level of H3K9Me² was constant throughout the cell cycle. These data suggest that patterns of H3K27Me³ marking and PRC1 foci are gradually regenerated during G₁ and S phase progression, unlike the methylation of H3K9.

Suz12 Is Essential for Both Maintenance of H3K27Me³ during Mitosis and Post-mitotic Assembly of PRC1 Foci—To test whether PRC2 is required for maintaining H3K27Me³-marked mitotic chromatin, we knocked down Suz12 using two independent siRNAs or shRNA vectors (Fig. 4A). Suz12 knockdown decreased the amount of this protein to 30–50%, resulting in a reduction in the level of H3K27Me³, and somewhat increased the amount of Bmi1. We then examined whether depletion of Suz12 affects H3K27Me³ marking of mitotic chromosomes during the first cell division after knockdown (Fig. 4B). HeLa cells were transfected with shRNA and GFP-PSLD plasmids for 24 h and treated with nocodazole for 16 h for immunostaining analysis. Co-transfection of cells with the GFP-PSLD plasmid enabled us to identify Suz12-depleted cells. Interestingly, Bmi1 foci as well as Suz12 were lost in the daughter cells. Bars, 10 μm.
down cells (Suz12 knockdown numbers 1 and 2). Thus, PRC2 constantly acts on mitotic chromatin, and Suz12 is essential for maintaining H3K27Me3 markings on mitotic chromosomes. Because PRC1 recognizes and binds methyl-H3K27, we tested whether PRC1 foci formation is post-mitotically affected when daughter cells do not receive the modified H3.

To address this, we visualized the formation of Bmi1 foci during the subsequent G1 phase in Suz12-depleted cells (Fig. 4C). For this experiment, cells were pre-extracted with CSK/Triton X-100 buffer to efficiently visualize chromatin-bound proteins. In control cells, Bmi1 foci were increasingly observed in cells from early G1 (2 h after the nocodazole release) to late G1 phase (8 h after the release). However, the Suz12-depleted cells largely lost such Bmi1 foci during G1 phase. Thus, PRC2 is essential for not only the initial targeting of Bmi1 to chromatin during early G1, but also the formation of PRC1 foci during late G1 phase. Because the total amount of Bmi1 was constant even after Suz12 knockdown (Fig. 4A), PRC1 foci formation itself was disturbed by Suz12 depletion. Taken together, our results indicate that Suz12 is required for inheritance of H3K27Me3 marks during mitosis and for PRC1 foci re-formation during G1 phase.

Autonomous Growth Defect and Delayed S Phase Progression in Suz12-depleted Cells—To finally demonstrate whether PcG-mediated chromatin formation is functionally linked to cell cycle regulation, we performed a colony formation analysis of Suz12-knockdown HCT116 cells. Compared with control cells, Suz12-depleted cells significantly suppressed colony formation (supplemental Fig. 5A). This result may be due to an autonomous cell cycle defect induced by the loss of PcG-associated chromatin or due to derepression of PRC target genes that encode certain nonautonomous factors, such as hormones or cytokines (20, 21). To address this, we performed a heterocellular co-culturing assay, using isogenic control cells to supply a growth-supportive environment, and examined whether the control cells could alleviate growth inhibition of co-cultured Suz12-knockdown cells in a dose-dependent manner (Fig. 5A). We transfected cells with either control shRNA and DsRed-PSLD (as indicated with red) or Suz12 shRNA and EGFP-PSLD (green), mixed these cells at different ratios, and plated a total of 1 × 10^5 cells under appropriate antibiotic selection for 10 days. The use of Suz12 shRNA induced effective depletion of this protein (data not shown). In both HeLa and HCT116 cells, we found that the number of formed colonies was in proportion to the number of control cells but not to the number of Suz12-depleted cells. In addition, a 50-fold excess of co-cultured control cells did not rescue the growth defect of Suz12-knockdown cells (data not shown). DsRed-PSLD-expressing control cells formed large colonies, whereas small colonies expressed EGFP-PSLD, suggesting that the propagation of Suz12-knockdown cells was autonomously prohibited. A PSLD reporter was also used to serve as an indicator of cell cycle stage, because fluorescent protein-fused PSLD distributes in the nucleus during G1 phase, in both the nucleus and cytoplasm in the G1/S transition, and mostly in the cytoplasm during S/G2 phase (35). EGFP-PSLD in Suz12-knockdown cells was concentrated in the nuclei, suggesting that impaired G1/S transition occurs in Suz12-depleted cells.

To determine whether Suz12 depletion affects S phase progression within a single cell cycle, HeLa cells were synchronized with thymidine and then released, transfected with shRNA and EGFP-PSLD, and blocked in G1/S phase by the second thymidine treatment (Fig. 5B). We examined S phase progression in these cells by monitoring the localization of EGFP-PSLD protein. At 2–4 h after the second release, ~40–60% of the control cells proceeded to S phase, whereas S phase progression was observed in only 15–30% of the Suz12-knockdown cells. Even at 8 h after the release, about 30% of the Suz12-knockdown cells showed S phase progression, suggesting that Suz12 depletion causes a delay in S phase progression. Requirement of Suz12 for S phase progression was further confirmed by similar experiments using different S phase marker proliferating cell nuclear antigen (supplemental Fig. 5B). The Suz12-knockdown cells progressed to middle/late S phase less efficiently (supplemental Fig. 5B).

We then tested the involvement of the G1/S checkpoint via p16 Ink4a/pRb and p14 ARF/p53 pathways in cultured human cells (supplemental Fig. 6). Irrespective of the cell type, Suz12-knockdown cells were arrested at the G1/S phase, even in Saos-2 cells that did not express the retinoblastoma protein and p53. Following Suz12 knockdown, the G1/S checkpoint controlled by p16 Ink4a, p14 ARF, and p53 was not activated in HCT116 cells that originally expressed wild-type retinoblastoma protein and p53, suggesting that the failure of S phase progression may be directly induced by a defect in PcG-associated chromatin. Taken together, our data suggest that PcG-mediated chromatin assembly occurs during the post-mitotic G1 phase and is a prerequisite for S phase progression.

DISCUSSION

For inheritance of cellular phenotypes to descendant cells, the defined transcriptional state is maintained over many cell divisions, which is called “cellular Memory” (1, 8). PcG-mediated gene repression is thought to play an essential role in cellular memory by forming heterochromatin-like structures (1, 2, 8). In this study, we identified fundamental properties of PRC2- and PRC1-associated chromatin and the mechanism underlying PcG-associated chromatin formation during the cell cycle. First, most of PRC1 and PRC2 exist distinctly in interphase chromatin that does not correspond to conventional heterochromatin or transcriptionally active sites. Second, PRC2 is responsible for the inheritance of genome-wide H3K27Me3 markings to post-mitotic cells, leading to the reproduction of PRC1 foci. Third, PRC1-associated chromatin is reconstructed intensively during middle and late G1 phase in post-mitotic cells. Fourth, depletion of Suz12 results in the loss of not only H3K27Me3 but also PRC1 foci, leading to delayed S phase progression. Collectively, our findings suggest that PcG-mediated chromatin remodeling is established during the post-mitotic G1 phase and is required for S phase progression.

There have been conflicting reports about PcG-associated chromatin and the precise distribution of PRC1 and PRC2 pro-
Our cytological and biochemical analyses of endogenous PcG proteins showed that PRC2 and PRC1 are mostly present in distinct chromosomal sites during both interphase and mitosis. During mitosis, PRC2 containing Suz12 and Ezh2 were associated with mitotic chromosome arms that were widely marked with H3K27Me3, whereas PRC1 was concentrated in some pericentromeric regions. During interphase, PRC1 increasingly formed chromatin foci containing H3K27Me3 from early to late G1 phase. The resulting PRC1 foci became resistant to nuclease treatment and did not require H3K27Me3 marks any longer, suggesting that PcG-mediated chromatin remodeling occurs from early to late G1 phase. Our RNA interference analysis revealed that H3K27Me3 was lost in mitotic chromosomes at the first cell division of Suz12-depleted cells. Subsequently, post-mitotic cells derived from Suz12-depleted mother cells showed disturbed formation of PRC1 foci containing Bmi1. These observations suggest that PRC complexes are differently but coordinately regulated in cycling cells to form H3K27Me3-marked inactive chromatin. Both the marking of H3K27Me3 by PRC2 and the targeting of PRC1 to these marks is properly controlled during every cell cycle. It is interesting that PRC2-like complexes are evolutionarily conserved among most multicellular organisms, whereas PRC1 is absent from plants, nematodes, and funguses (41–43), suggesting that PRC2 is a prototype of the PcG system. The observation that plants generally have higher regeneration capacity than animals may be correlated with cellular memory produced only by PRC2 (42). Thus, PRC1 is not always required for epigenetic regulation by PcG proteins and might be essential only for long term repression (41, 42).

During the G1 phase, H3K27Me3-marked chromatin gradually gained PRC1 and assembled into several large foci. Consequentially, PRC1 K.D. #1 and K.D. #2 formed in control (red) and Suz12-depleted cells (green). These observations suggested that post-mitotic reproduction of PcG-associated chromatin occurs from early to late G1 phase and is properly controlled during every cell cycle.
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gesting that higher ordered chromatin structures are formed for stable repression (44). PcG-associated chromatin is actively reproduced during G1 phase, and it may provide a chance to remodel chromatin and gene expression in response to external stimuli, including hormones or growth factors (45, 46). Thus, PcG-mediated chromatin regulation is involved not only in stable maintenance of the repressive state but also in the flexible changes in gene expression required during the developmental process.

Because DNA and chromatin status is replicated in S phase (1, 8, 16), an entire re-organization of PcG-associated chromatin must be completed before the onset of S phase. Our data revealed that abrogation of H3K27Me3 marking by Suz12 knockdown impaired S phase progression. It remains unknown whether or not this defect in S phase progression is because of de-repression of a critical cell cycle inhibitory factor. Previously, it was shown that defects of PcG-mediated repression of p16 Ink4a/ARF induced activation of the pRb/p53 pathway (47). However, we did not find any changes shown to be involved in the activation of G1/S or G2/M arrests cell cycle progression, analogous to DNA damage checkpoints (48). Therefore, there may be other early responsible PcG target genes or pathways that are critical for regulating the cell cycle. Alternatively, there may be some unknown surveillance mechanism that senses epigenetic lesions and arrests cell cycle progression, analogous to DNA damage checkpoints (48). Indeed, multiple PcG proteins have been shown to be involved in the activation of G1/S or G1/M checkpoints (49). In this context, it is interesting that, compared with normal diploid fibroblast cells, undifferentiated stem cells and various cancer cells express PcG proteins at high levels (supplemental Fig. 7), possibly leading to high proliferative rates via repression of such an epigenetic checkpoint gene. In summary, our study sheds light on the molecular basis of PcG-mediated chromatin formation and its inheritance to post-mitotic cells. It is possible that the functional combination of PRC1 and PRC2 is responsible for the metastable inheritance of H3K27Me3 modifications and chromatin reproduction during every cell cycle to maintain and alter cellular memory.

Acknowledgments—We are grateful to all of the members of our laboratory and Dr. Kenji Watanabe for helpful discussions.

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