Role of Bmi1 in H2A Ubiquitylation and Hox Gene Silencing*

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Posttranslational histone modifications play a crucial role in the regulation of chromatin structure and gene activity. In previous studies, we identified the histone H2A ubiquitin ligase as Ring2, together in a complex with Ring1, Bmi1, and HPH2 (human polyhomeotic 2). We report here that the oncogene Bmi1 stimulates H2A ubiquitylation both in vitro and in vivo and that Bmi1-regulated H2A ubiquitylation is required for Hox gene silencing and normal cell growth. Our studies indicate that Bmi1 maintains the integrity of the complex through simultaneous interactions with the other subunits. We reconstituted the functional human H2A ubiquitin ligase complex and a panel of subcomplexes of different subunits. Comparisons of the H2A ubiquitin ligase activities of these different complexes revealed that Bmi1 stimulates the H2A ubiquitin ligase activity of Ring2 (and Ring1). Additionally, we demonstrated that the HoxC5 gene is regulated by ubiquitylated H2A in HeLa cells and that ubiquitylated H2A is localized on 5′ regulatory regions of the HoxC5 gene. The role of Bmi1 in H2A ubiquitylation and HoxC5 gene expression in vivo was analyzed by RNA interference experiments. Knockdown of Bmi1 causes a global and locusspecific loss of H2A ubiquitylation, up-regulation of the HoxC5 gene, and slower cell growth. Intriguingly, Ring2 binds to its target regions in Bmi1 knockdown cells. Therefore, our studies reveal that Bmi1 is required for H2A ubiquitylation and suggest that H2A ubiquitylation regulates Bmi1-mediated gene expression.

Covalent modifications of histones are emerging as key mechanisms to regulate the structure and function of chromatin (1). Of these modifications, histone ubiquitylation has recently been shown to regulate transcription and other chromatin-based processes (2, 3). Studies of H2B ubiquitylation in budding yeasts revealed a critical role of this modification in transcription activation, elongation, and gene silencing. During gene activation, a dynamic change of ubiquitylated H2B at the promoter region is required for optimal gene expression (4). Disturbance of ubiquitylated H2B by deletion of the H2B ubiquitin-conjugating enzyme Rad6 (4) or the H2B deubiquitinase Ubp8 (5) caused defects in the expression of specific genes. Moreover, through interaction with the Paf1-elongating complex (6, 7), Rad6 regulates transcription elongation, at least in part, by H2B ubiquitylation. Rad6 distributes across the coding region rapidly after gene activation, coinciding precisely with the appearance of RNA polymerase II (8). In addition, H2B ubiquitylation is also required for proper gene silencing. Deubiquitylation of H2B by Ubp10 maintains low levels of ubiquitylated H2B at telomere and rDNA regions, facilitates the binding of silencing protein Sir2, and leads to gene repression (9, 10). The most intriguing finding concerning H2B ubiquitylation is that ubiquitylated H2B is a prerequisite for subsequent histone H3 Lys4 and Lys79 methylation, a phenomenon termed “trans-histone” regulation (11–14). Although the underlying mechanism remains unclear, this observation does provide a molecular explanation for the function of H2B ubiquitylation in transcription and gene silencing.

H2A was the first protein shown to be ubiquitylated (15), and ubiquitylation occurs on 5–15% of total cellular H2A in mammalian cells (16). Although earlier studies implicated the involvement of H2A ubiquitylation in gene activation (16), recent studies established a role of H2A ubiquitylation in gene repression (3). Identification of the H2A ubiquitin ligase shed light on the functional significance of this modification in chromatin regulation. Employing a biochemical approach, we purified the histone H2A ubiquitin ligase shed light on the functional significance of this modification in chromatin regulation. Employing a biochemical approach, we purified the histone H2A ubiquitin ligase directly from HeLa cells. Further characterization of the H2A ubiquitin ligase complex revealed that H2A ubiquitylation plays a critical role in the polycomb group (PcG)3 protein-mediated gene silencing (17). Using a different approach, de Napoles et al. (18) found that ubiquitylated H2A (uH2A) and a subgroup of PcG proteins localized on the inactive X chromosome and that uH2A was abolished from the inactive X chromosome in a mouse line with Ring1A and Ring1B knockouts. These studies revealed a connection between H2A ubiquitylation and PcG protein-mediated gene silencing.

PcG proteins are a group of evolutionarily conserved proteins that play a key role in the maintenance of the repression state of homeobox (Hox) genes and other genes through multiple rounds of cell division during development (19, 20).

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3 The abbreviations used are: PcG, polycomb group; RT, reverse transcription; ChIP, chromatin immunoprecipitation; ChDIP, chromatin double immunoprecipitation; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; HA, hemagglutinin; RNAi, RNA interference; siRNA, small interfering RNA; uH2A, ubiquitylated H2A; Ft, flow-through.
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Moreover, PcG genes also regulate cell proliferation and differentiation, stem cell renewal, and certain types of cancer development (21, 22). Biochemical and genetic evidence indicates that PcG proteins exist in at least two distinct complexes, the PRC1 (polycomb-repressive complex 1) and PRC2 (polycomb-repressive complex 2) (19, 20). Although PRC2 was found to possess histone methyltransferase activity (23), no enzymatic activity has been attributed to PRC1. The finding that H2A ubiquitylation is required for PcG protein-regulated gene expression suggests that H2A ubiquitylation may be part of the mechanism underlying PcG protein-regulated gene silencing.

To gain insight into the relationship between H2A ubiquitylation and PcG protein-regulated cell function, we reconstituted the H2A ubiquitin ligase complex and analyzed the role of individual subunits in H2A ubiquitylation as well as in gene expression and cell proliferation. Our results indicate that the oncogene Bmi1 stimulates the H2A ubiquitin ligase activity of Ring2 both in vitro and in vivo. Moreover, knockdown of Bmi1 causes a decrease of H2A ubiquitylation, abnormal gene expression, and slow cell growth. Therefore, this study establishes a critical role for Bmi1 in H2A ubiquitylation and demonstrates the importance of H2A ubiquitylation in Bmi1-regulated cell function.

EXPERIMENTAL PROCEDURES

Construction of Recombinant Baculoviruses and Coimmunoprecipitation Assay—cDNAs encoding Ring1 (NM_002931), Ring2 (NM_007212), Bmi1 (NM_005180), and HPH2 (human polyhomeotic 2) (NM_004427) were cloned into pFASTBAC vector (Gibco) with or without a FLAG tag at its N terminus. Recombinant baculovirus expressing individual subunits of the H2A ubiquitin ligase complex was generated and amplified following the manufacturer’s protocol.

Recombinant baculoviruses expressing FLAG-tagged subunits and non-FLAG-tagged subunits were pairwise infected into Sf9 cells in 10-cm plates (as indicated in Fig. 1). After 2 days of infection, cells were lysed (50 mM Tris-HCl (pH 7.5), 150 mM NaCl, 4 mM MgCl2, 0.4 mM EDTA, 2 mM dithiothreitol, 20% glycerol, protease inhibitors) by homogenization with pestle B for three times (10 strokes each) in a period of 30 min. After being cleaned up by centrifugation at 11,000 rpm for 10 min, the supernatant was diluted to 300 mM NaCl by mixing with F dilution buffer (20 mM Tris (pH 7.9), 0.2% Nonidet P-40, 10% glycerol). The diluted supernatant was then incubated with M2 α-FLAG-agarose beads (Sigma) for 4 h at 4 °C. Then beads were washed with F washing buffer (20 mM Tris (pH 7.9), 150 mM NaCl, 2 mM MgCl2, 0.2 mM EDTA, 1 mM dithiothreitol, 15% glycerol, 0.01% Nonidet P-40), and bound proteins were eluted with FLAG peptide (0.4 mg/ml) in washing buffer. The eluted material was concentrated and subjected to a Superdex 200 column. Fractions of the Superdex 200 were analyzed by silver staining, Western blot, and histone ubiquitin ligase assay. Histone ubiquitin ligase assay and Western blot assay were carried out as previously described (17).

Reverse Transcription-PCR, Chromatin Immunoprecipitation (ChIP), and Chromatin Double Immunoprecipitation (ChDIP) Assays—For reverse transcription-PCR, total RNAs were first isolated from control, Ring2 knockdown, and Bmi1 knockdown cells with an RNAeasy kit (Qiagen) following the manufacturer’s protocol. Reverse transcription was then carried out using oligo(dT)18 as primer. The resulting cDNAs were used as templates for PCR amplification. GAPDH was used as a control. The primer sequences used for PCR amplification were as follows: HoxA9, 5′-AGTGTTTATGGCAATAGGCCG; HoxC5, 5′-TGGTGGGAGACTGATGCGG and 3′-ACGGTGAATCTGTGTGGCCG; HoxB1, 5′-TCAGGGTGTTAGACGATGTTG and 3′-ATGCTGCGGAGATATGGCC; HoxD10, 5′-GATTCCCTTGATCAGGTCCGTCG and 3′-GCCGAATGTGTTGTCGCG; GAPDH, 5′-TCTGGAAGACGTGTTGGCAG and 3′-TCTGGAAAGCTGTGGCGTG.

For ChIP and ChDIP assays, cells (control, Ring2 knockdown, Bmi1 knockdown, and FLAG-H2A and HA-ubiquitin double-tagged cell) from 15-cm plates at 70–80% confluence were cross-linked with 1% formaldehyde in Dulbecco’s modified Eagle’s medium for 10 min at room temperature. The reaction was quenched by adding glycine to a final concentration of 0.125 M. Cells were then washed twice with phosphate-buffered saline and collected by scraping. Cells were lysed in 300 μl of buffer B (10 mM HEPES (pH 7.9), 0.5% Nonidet P-40, 1.5 mM MgCl2, 10 mM KCl, 0.5 mM dithiothreitol, protease inhibitors) by incubation on ice for 10 min. The released nuclei were collected by centrifugation at 4000 rpm for 5 min and lysed in buffer C (20 mM HEPES (pH 7.9), 25% glycerol, 0.5% Nonidet P-40, 0.42 mM NaCl, 1.5 mM MgCl2, 0.2 mM EDTA plus protease inhibitors) by incubation at 4 °C for 20 min. The lysed nuclei were then sonicated to generate chromatin fragments with an average length of 0.4–0.7 kb. After centrifugation at 13,200 rpm for 10 min, the supernatant was diluted with an equal volume of buffer D (1% Triton X-100, 2 mM EDTA, 20 mM Tris-HCl (pH 7.9), 50 mM NaCl plus protease inhibitors). The

Purification of the Native and Reconstituted H2A Ubiquitin Ligase Complexes—The native H2A ubiquitin ligase complex was purified from HeLa cell nuclear pellets as described (17). To reconstitute and purify the H2A ubiquitin ligase complex and different subcomplexes, baculoviruses expressing FLAG-tagged Bmi1 and different combinations of other subunits (Fig. 2B) or FLAG-tagged HPH2 and other subunits (Fig. 2D) were used to coinfect Sf9 cells. After 2.5 days of infection, cells were collected and washed with phosphate-buffered saline. Cells were lysed in F lysis buffer (20 mM Tris-HCl (pH 7.9), 500 mM NaCl, 4 mM MgCl2, 0.4 mM EDTA, 2 mM dithiothreitol, 20% glycerol, protease inhibitors) by homogenization with pestle B for three times (10 strokes each) in a period of 30 min. After being cleaned up by centrifugation at 11,000 rpm for 10 min, the supernatant was diluted to 300 mM NaCl by mixing with F dilution buffer (20 mM Tris (pH 7.9), 0.2% Nonidet P-40, 10% glycerol). The diluted supernatant was then incubated with M2 α-FLAG-agarose beads (Sigma) for 4 h at 4 °C. Then beads were washed with F washing buffer (20 mM Tris (pH 7.9), 150 mM NaCl, 2 mM MgCl2, 0.2 mM EDTA, 1 mM dithiothreitol, 15% glycerol, 0.01% Nonidet P-40), and bound proteins were eluted with FLAG peptide (0.4 mg/ml) in washing buffer. The eluted material was concentrated and subjected to a Superdex 200 column. Fractions of the Superdex 200 were analyzed by silver staining, Western blot, and histone ubiquitin ligase assay. Histone ubiquitin ligase assay and Western blot assay were carried out as previously described (17).

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For ChIP and ChDIP assays, cells (control, Ring2 knockdown, Bmi1 knockdown, and FLAG-H2A and HA-ubiquitin double-tagged cell) from 15-cm plates at 70–80% confluence were cross-linked with 1% formaldehyde in Dulbecco’s modified Eagle’s medium for 10 min at room temperature. The reaction was quenched by adding glycine to a final concentration of 0.125 M. Cells were then washed twice with phosphate-buffered saline and collected by scraping. Cells were lysed in 300 μl of buffer B (10 mM HEPES (pH 7.9), 0.5% Nonidet P-40, 1.5 mM MgCl2, 10 mM KCl, 0.5 mM dithiothreitol, protease inhibitors) by incubation on ice for 10 min. The released nuclei were collected by centrifugation at 4000 rpm for 5 min and lysed in buffer C (20 mM HEPES (pH 7.9), 25% glycerol, 0.5% Nonidet P-40, 0.42 mM NaCl, 1.5 mM MgCl2, 0.2 mM EDTA plus protease inhibitors) by incubation at 4 °C for 20 min. The lysed nuclei were then sonicated to generate chromatin fragments with an average length of 0.4–0.7 kb. After centrifugation at 13,200 rpm for 10 min, the supernatant was diluted with an equal volume of buffer D (1% Triton X-100, 2 mM EDTA, 20 mM Tris-HCl (pH 7.9), 50 mM NaCl plus protease inhibitors). The
diluted chromatin solution was preclear with 60 µl of protein A/G-Sepharose beads pretreated with sheared salmon sperm DNA. Antibodies were then added to the cleared chromatin, and the mixture was incubated at 4 °C overnight. Protein A/G beads were then added to the antibody-chromatin mixture and subjected to incubation for an additional 1 h with rotating. Agarose beads were then collected by centrifugation. For ChIP assay, beads was sequentially washed with TSE1 (0.1% SDS, 1% Triton X-100, 2 mM EDTA, 10 mM Tris-Cl, pH 8.1), TSE2 (0.1% SDS, 1% Triton X-100, 20 mM Tris-Cl, pH 8.1), and TSE3 (0.25 M LiCl, 1% Nonidet P-40, 1% deoxycholate, 1 mM EDTA, 10 mM Tris-Cl, pH 8.1), each for 5–10 min. For ChDIP, beads were washed with buffer containing an equal volume of Buffer C and D twice. The bound material was eluted with 0.4 mg/ml FLAG peptide. The eluted material was then subjected to second round immunoprecipitation with anti-HA antibody. Beads from anti-HA immunoprecipitation were washed with TSE1, TSE2, and TSE3 as described. After further washing the beads with TE buffer (10 mM Tris-Cl, 1 mM EDTA, pH 8.0) twice, bound chromatin was eluted with 100 µl of buffer containing 1% SDS and 0.1 M NaHCO₃. After reversing the cross-linking by incubation at 65 °C overnight, immunoprecipitated DNA was extracted by phenol/chloroform, precipitated by ethanol, and analyzed by PCR using primer pairs as follows: A, 5'-GGCATCAACCAACCACTA and 3'-GGCTAGAGCTTGATAATAATTCA-3' and 3'-CTGAGCCGACCACCTTACC and 3'-GACATGAGCCAGCGATACCA and 3'-AATAAAGCACCCTCGATGCGC; B, 5'-GCCAAGGCACTATAACGG and 3'-TCAGGCCTTAAAGCCTCCTA; C, 5'-TGGGATACGCGCCACACTTACC and 3'-CTGAGGGCACCACCTAATA and 3'-GTCGAACCTGCACTTAA; D, 5'-GCCAATGAGCCAGCGATACCA and 3'-AATAAAGCACCCTCGATGCGC; E, 5'-CCATGAGCTCTACTAGGCC and 3'-TCAGAGGAGCGGCTCGT.

RNAi and Stable Cell Line Establishment—siRNA oligonucleotides were purchased from Invitrogen in a purified and annealed duplex form. Cells with 30–40% confluence in a 15-cm dish were used for transfection. First, we diluted 80 µl of siRNA (20 µM) in 3.2 ml of Opti-MEM I reduced serum medium (Invitrogen). At the same time, we mixed 32 µl of Lipofectamine 2000 (Invitrogen) with 3.2 ml of Opti-MEM I reduced serum medium (Invitrogen) and incubated at room temperature for 15 min. Then we mixed the diluted siRNA and Lipofectamine 2000 and incubated for 15 min at room temperature. Finally, we added the mixture to cells and mixed well. Cells were cultured for 36 h and processed for the ChIP assay. An aliquot of cells were used for a Western blot assay to confirm the RNAi efficiency.

The sequences targeting Ring2 are as follows: RNAi1, 5'-UUCUUCUAAAGCUAACCUCACGCGC; RNAi2, 5'-UUUAAUACAGUGAAACUGCCGCAUGGG. The sequences targeting Bmi1 are as follows: RNAi1, 5'-AAGUAUGUGAAACUCGUGAGUAAGG; RNAi2, 5'-AACCAAGAAGAAGUGUGAGUGAGAAG.

For stable knockdown of Ring2 and Bmi1, vectors expressing FLAG-H2A (G418-resistant) and HA-ubiquitin (hygromycin-resistant) were first linearized with MfeI and then cotransfected into HeLa cells with Effectene (Invitrogen) following the manufacturer’s instructions. For establishment of the flag-H2A and HA-ubiquitin cell line, vectors expressing FLAG-H2A (G418-resistant) and HA-ubiquitin (hygromycin-resistant) were first linearized with MfeI and then cotransfected into HeLa cells with Effectene (Invitrogen) following the manufacturer’s instructions. G418 at 0.5 mg/ml and hygromycin at 0.3 mg/ml were added to the medium until clones formed. Individual clones were amplified and analyzed by Western blot with anti-FLAG and HA antibodies.

RESULTS

Determination of the Architecture of the Human H2A Ubiquitin Ligase Complex—Previous studies identified Ring2 as the catalytic subunit of the human histone H2A ubiquitin ligase complex (17). Although Ring2 alone could ubiquitylate histone H2A, the activity was severely compromised when compared with an equal amount of Ring2 in the native complex (about 200-fold less active; data not shown). This indicates that other subunit(s) may regulate the H2A ubiquitin ligase activity of Ring2.

FIGURE 1. Determination of the architecture of the human H2A ubiquitin ligase complex.

The two siRNAs that target Bmi1 are as follows: 5'-TGATGCCACACCATAATTCAGAGATATTTGCTATTAGCTTCT-3' and 5'-GGCTAGAGCTTGGTATCTGCAGCCATTCGG-3'. The two siRNAs that target Ring2 are as follows: 5'-AACCAGCAAGTATTGTCCTATTC-3' and 5'-ACCAGAAGTATTGCTCCTATTTCAGAGATAGG-3'. For establishment of the Flag-H2A and HA-ubiquitin cell line, vectors expressing Flag-H2A (G418-resistant) and HA-ubiquitin (hygromycin-resistant) were first linearized with MfeI and then cotransfected into HeLa cells with Effectene (Invitrogen) following the manufacturer’s instructions. G418 at 0.5 mg/ml and hygromycin at 0.3 mg/ml were added to the medium until clones formed. Individual clones were amplified and analyzed by Western blot with anti-FLAG and HA antibodies.
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To identify such subunit(s), we set out to reconstitute the human H2A ubiquitin ligase complex with baculovirus expression system. As a prerequisite for reconstitution, we first determined the pairwise interaction between individual subunits of the complex by coimmunoprecipitation assays. When HPH2 (F-HPH2) was expressed together with Bmi1 in Sf9 cells, Bmi1 could be efficiently coimmunoprecipitated by HPH2 (Fig. 1, lanes 1–3). However, under the same conditions, HPH2 failed to coimmunoprecipitate Ring1 (Fig. 1, lanes 4–6) and Ring2 (Fig. 1, lanes 7–9). These data indicate that HPH2 interacts with Bmi1 but not with Ring1 and Ring2 in the complex. Moreover, Bmi1 can be coimmunoprecipitated with both Ring1 (Fig. 1, lanes 10–12) and Ring2 (Fig. 1, lanes 13–15), suggesting that there are direct interactions between Bmi1 and Ring1 and between Bmi1 and Ring2. Since Ring2 was not coimmunoprecipitated with Ring1, we conclude that Ring1 does not interact with Ring2 directly (Fig. 1, lanes 16–18). In summary, we suggest the architecture of the complex as follows. Bmi1 is located in the center of the complex and interacts with Ring1, Ring2, and HPH2, whereas there is no strong interaction between HPH2, Ring1, and Ring2 (Fig. 1B).

Reconstitution and Purification of the Human H2A Ubiquitin Ligase Complex—After establishing the role of Bmi1 in the integrity of the H2A ubiquitin ligase complex, we produced and purified the complex by coinfection of Sf9 cells with baculoviruses encoding FLAG-tagged Bmi1, Ring1, Ring2, and HPH2. As indicated in Fig. 2A, the reconstituted complex was first affinity-purified by M2 anti-FLAG-agarose beads and then further purified by gel filtration through a Superdex S200 column. The latter step helped remove excess F-Bmi1 that was not incorporated into the complex (Fig. 2B, top). Silvering staining of the fractions derived from the Superdex 200 column revealed that the complex was successfully reconstituted (Fig. 2B, top, individual subunits are marked by asterisks). The reconstituted complex eluted at 443–220 kDa, and this molecular mass is consistent with the size of the native H2A ubiquitin ligase complex.

Western blot analysis confirmed the identity of the subunits as Ring1, Ring2, and Bmi1 (anti-FLAG) (three middle panels). Since an antibody against HPH2 is not available, we attempted to confirm the presence of HPH2 in the reconstituted complex using an alternative approach.

The protein band that migrated at a size corresponding to FLAG-Bmi1 was more intensively stained than Ring1 and Rings even after the excess FLAG-Bmi1 was removed by gel filtration (Fig. 2B, top). We speculated that it may contain both HPH2 and FLAG-Bmi1. To explore this possibility, we performed immunoprecipitation assays under denaturing conditions (24) (Fig. 2C). After disrupting the interaction between the different subunits by detergent, the denatured complex was subjected to immunoprecipitation with anti-Ring1 antibody. Proteins in input (In), Ft, and bound (B) were analyzed by silver staining (bottom panel) and Western blot (top three panels).
complex by coinfection of S9 cells with baculoviruses expressing FLAG-HPH2, Ring1, Ring2, and Bmi1 (Fig. 2D). Western blot analysis of the anti-FLAG immunoprecipitates revealed that HPH2 could communoprecipitate Ring1, Ring2, and Bmi1 (Fig. 2D, lane 3). This result indicates that HPH2 is indeed a component of the reconstituted complex. However, the reconstituted complex in FLAG-HPH2 immunoprecipitates (Fig. 2D, lane 3) was low (as revealed by Western blot analysis with anti-Ring1, Ring2, and Bmi1 antibodies), and after further purification through a Superdex 200 column, only FLAG-HPH2 was revealed by silver staining (data not shown). Taken together, we concluded that we have reconstituted the human H2A ubiquitin ligase complex containing Ring1, Ring2, Bmi1, and HPH2.

It is possible that the reconstituted complex contains two subcomplexes: one contains Bmi1 and Ring1, and the other one contains Bmi1 and Ring2 (Fig. 2B). To test this possibility, we performed immunoprecipitation experiments with antibody against Ring1 (which has been previously linked to protein A-agarose beads) in the purified reconstituted complex (fraction 47) (Fig. 2B). As shown in Fig. 2E, silver staining and Western blot analysis indicate that both Ring2 and Bmi1 are communoprecipitated by anti-Ring1 immunoprecipitation. These results indicate that there is only a single Bmi1-containing complex that contains all of the four proteins.

The reconstituted complex robustly ubiquitylated histone H2A in a histone ubiquitin ligase assay with HeLa nucleosomes as substrates (Fig. 2B, bottom panel). To further determine whether the reconstituted complex is functionally similar to the native complex, we compared the histone ubiquitin ligase activity and substrate specificity of these two complexes. When equal amounts of Ring2 were used in the histone ubiquitin ligase assay (Fig. 3A, top), the H2A ubiquitin ligase activity of the reconstituted complex was equivalent to the native complex (Fig. 3A, bottom). Moreover, the reconstituted complex also preferred nucleosomes as substrates to core histones (Fig. 3B), similar to the native complex (17). Therefore, we conclude that the reconstituted complex is fully functional, and its properties are similar to the native H2A ubiquitin ligase complex.

**Purification of Different H2A Ubiquitin Ligase Subcomplexes and Comparison of Histone Ubiquitin Ligase Activities**

To determine the role of individual subunits in the regulation of H2A ubiquitin ligase activity, we reconstituted and purified a panel of subcomplexes produced by dropouts of one or two subunits. Silver staining revealed that these complexes were nearly homogeneous (Fig. 4A). Western blot analysis confirmed the identity of individual subunits as Ring1, Ring2, and Bmi1 (anti-FLAG) (Fig. 4C). When different subcomplexes were analyzed for histone ubiquitin ligase activity with nucleosomes (Fig. 4B, top) and histones (Fig. 4B, bottom) as substrates, we found that both Ring2 alone and Bmi1 alone failed to ubiquitylate histone H2A at the amount of protein used (Fig. 4B, lanes 6 and 7). However, a robust H2A ubiquitin ligase activity was observed when the two subunits formed a subcomplex (Fig. 4B, lane 5), suggesting a critical role of Bmi1 in the regulation of H2A ubiquitin ligase activity. Additional incorporation of either HPH2 (lane 4) or Ring1 (lane 2) into the complex did not further stimulate the H2A ubiquitin ligase activity (Fig. 4B, compare lanes 2 and 4 with lane 5). As predicted, when Ring2...
was dropped out of the complex, the H2A ubiquitin ligase activity decreased dramatically (Fig. 4B, compare lane 3 with lane 5). However, the activity was significantly higher than control, suggesting that Ring1 could also ubiquitylate histone H2A (compare lane c with lane 3). This observation is consistent with a previous report that cells derived from Ring1B (mouse homolog of Ring2) knock-out mice still contain H2A ubiquitylation on the inactive X chromosome (18). In addition, all of the subcomplexes preferred nucleosomes as substrates to core histones (Fig. 4B). Taken together, we conclude that Bmi1 is not only required for the integrity of H2A ubiquitin ligase complex but also plays a central role in the regulation of the H2A ubiquitin ligase activity of Ring2 and Ring1.

**Identification of the uH2A-regulated Genes in HeLa Cells**—Studies in Drosophila identified *ubx* as the uH2A-regulated gene (17). However, it is not known what genes uH2A regulates in mammalian cells. To identify the uH2A-regulated genes in mammalian cells, we generated two stable cell lines with knockdown of Ring2. Compared with previous reports (17), the efficiency of Ring2 knockdown in these cell lines is significantly higher (Fig. 5A, top, compare lanes 2 and 3 with lane 1). Importantly, uH2A was reduced substantially in these knockdown cells (Fig. 5A, middle, compare lanes 2 and 3 with lane 1). When the expression of selected Hox genes is compared in control and Ring2 knockdown cells, we found that *HoxC5* was significantly up-regulated in Ring2 knockdown cells, whereas the expression of *HoxA9*, -B1, and -D10 was not changed (Fig. 5B). These results indicate that uH2A may regulate the expression of *HoxC5* in HeLa cells.

To confirm the role of H2A ubiquitylation in HoxC5 expression, we investigated the distribution of uH2A across the entire *HoxC5* gene. Since the commercially available anti-uH2A antibody works poorly
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In ChIP assays, we pursued an alternative approach. We first established a cell line that stably expresses both FLAG-H2A and HA-ubiquitin (Fig. 5C, compare lanes 4 and 6 with lanes 3 and 5). After confirming that uH2A-containing nucleosomes can be efficiently isolated by anti-FLAG and anti-HA tandem immunoprecipitation (Fig. 5D, top two panels), we performed ChDIP in this double-tagged cell line. As indicated in Fig. 5E, uH2A was localized on 5’ upstream regulatory regions (around 1–1.5 kb upstream of the transcription start site) of HoxC5 (Fig. 5E, lanes 1–5, top). Consistent with the role of Ring2 and Bmi1 in H2A ubiquitylation, Ring2 and Bmi1 were also localized in the same region (lanes 1–5, second and third panels). Importantly, uH2A was abolished from this region (Fig. 5E, top panel, compare lanes 6–10 with lanes 1–5) when Ring2 was knocked down by siRNA (Fig. 5, E second panel and F). Interestingly, knockdown of Ring2 also abolished the binding of Bmi1 to these target regions (Fig. 5E, third panel, compare lanes 6–10 with lanes 1–5). Therefore, these studies identify HoxC5 as one of the uH2A-regulated genes in HeLa cells.

Role of Bmi1 in H2A Ubiquitylation, Gene Expression, and Cell Proliferation—Since Bmi1 stimulates the histone ubiquitin ligase activity of Ring2 (and Ring1) in vitro (Fig. 4), we investigated the role of Bmi1 in H2A ubiquitylation in vivo. For this purpose, two cell lines with stable knockdown of Bmi1 were established based on the vector-derived siRNA strategy (17). Western blot analysis revealed that Bmi1 levels were significantly reduced in these cells (Fig. 6A, top panel, compare lanes 2 and 3 with lane 1). Interestingly, the levels of H2A ubiquitylation in these cells were also substantially reduced (Fig. 6A, middle panel, compare lanes 2 and 3 with lane 1). Consistent with its role in promoting cell proliferation, knockdown of Bmi1 caused cells to grow slower than control cells (Fig. 6B). This indicates that Bmi1 is required for H2A ubiquitylation and normal cell growth in vivo.

To explore the role of Bmi1 in gene expression, we measured the expression of HoxA9, -C5, -B1, and -D10 in Bmi1 knockdown and control cells. Similar to the results from a knockdown of Ring2, the expression of HoxC5 was significantly up-regulated in Bmi1 knockdown cells, whereas the expression of HoxA9, -B1, and -D10 was not changed (Fig. 6C). To further determine the relationship between H2A ubiquitylation and Bmi1-regulated gene expression, we performed ChDIP assays in control and Bmi1 knockdown cells. Consistent with previous results (Fig. 5C), uH2A was localized to 5’ regulatory regions of HoxC5 in control cells. When the expression of Bmi1 was reduced by RNA interference (Fig. 6E), the levels of uH2A on 5’ regulatory regions of HoxC5 were greatly reduced (Fig. 6D, top panel, compare lanes 1–5 with lanes 6–10). Interestingly, we found that Ring2 was still recruited to its target sequences in Bmi1 knockdown cells (Fig. 6D, second panel, compare lanes 1–5 with lanes 6–10). These results indicate that there might be a sequential assembly of the polycomb complex during gene repression. Taken together with previous results (Fig. 4), we suggest that Bmi1-stimulated and Ring2-mediated H2A ubiquitylation may be important for PcG protein-regulated gene expression.

DISCUSSION

We report here the reconstitution and characterization of the human H2A ubiquitin ligase complex. Our studies call attention to Bmi1, an oncogene implicated in cell proliferation, stem cell renewal, and certain types of cancer development. We provide evidence that Bmi1 stimulates the ligase activity of Ring2 (and Ring1) both in vitro and in vivo. Moreover, Bmi1 regulates the expression of the HoxC5 gene through its effect on
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H2A ubiquitylation. In addition, knockdown of Bmi1 causes cells to grow substantially slower than control cells. Therefore, our studies reveal a critical role for Bmi1 in H2A ubiquitylation, Hox gene expression, and cell proliferation. Consistently, Bmi1 has been reported for H2A ubiquitylation and HoxC13 gene silencing in mouse cells, but the role of Bmi1 in Ring1-dependent H2A ubiquitylation was not (25).

Bmi1 was originally identified in mouse leukemia as an oncogene that functions cooperatively with Myc (26, 27). Subsequent studies demonstrated that Bmi1 is overexpressed in a number of human cancers (21, 22) and that overexpression of Bmi1 specifically promotes cell proliferation (28, 29). Moreover, Bmi1 plays indispensable roles in stem cell maintenance of the hematopoiesis and neuronal systems and in the proliferation of early cerebellar progenitor cells (30–33). Bmi1 has been shown to function through Ink4/Arf locus, which encodes the p16Ink4a and p19Arf cell cycle regulator (28, 34). Here we demonstrate a direct link between Bmi1 and H2A ubiquitylation. Our studies indicate that Bmi1-regulated H2A ubiquitylation may be important for Bmi1-regulated gene expression and cell proliferation. Additional mechanisms may also exist, since Bmi1 and its associated complex could bind and compact chromatin directly (35). Further experiments are needed to investigate the domains of Bmi1 involved in the regulation of H2A ubiquitylation, gene expression, and cell proliferation.

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