A Phosphorylated Form of Mel-18 Targets the Ring1B Histone H2A Ubiquitin Ligase to Chromatin

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SUMMARY

Recent studies have shown that PRC1-like Polycomb repressor complexes monoubiquitylate chromatin on histone H2A at lysine residue 119. Here we have analyzed the function of the polycomb protein Mel-18. Using affinity-tagged human MEL-18, we identify a polycomb-like complex, melPRC1, containing the core PRC1 proteins, RING1/2, HPH2, and CBX8. We show that, in ES cells, melPRC1 can functionally substitute for other PRC1-like complexes in Hox gene repression. A reconstituted subcomplex containing only Ring1B and Mel-18 functions as an efficient ubiquitin E3 ligase. This complex ubiquitylates free histone substrates nonspecifically but is highly specific for histone H2A lysine 119 in the context of nucleosomes. Mutational analysis demonstrates that while Ring1B is required for E3 function, Mel-18 directs this activity to H2A lysine 119 in chromatin. Moreover, this substrate-targeting function of Mel-18 is dependent on its prior phosphorylation at multiple residues, providing a direct link between chromatin modification and cell signaling pathways.

INTRODUCTION

Polycomb group (PcG) repressor proteins were originally identified as factors involved in the maintenance of homeobox gene silencing during development in D. melanogaster. Further studies showed these factors to be conserved in a wide range of organisms, with key roles in developmental gene regulation, cell-cycle regulation, and pluripotency in embryonic stem (ES) cells (for recent reviews, see Jorgensen et al., 2006; Schwartz and Pirotta, 2007).

Biochemical and genetic studies revealed that PcG proteins are components of at least two major multiprotein complexes, termed polycomb repressor complex 1 (PRC1) (Saurin et al., 2001; Shao et al., 1999) and PRC2 (Cao et al., 2002; Czermin et al., 2002; Kuzmichev et al., 2002; Muller et al., 2002). Other PcG complexes have also been described (Klymenko et al., 2006; Kuzmichev et al., 2004). Analyses of PRC1 in D. melanogaster (Shao et al., 1999), and subsequently in mammalian cells (Levine et al., 2002), defined the core components Polycomb (PC), posterior sex combs (PSC), polyhomeotic (PH), and RING1 (dRING). There are two or more homologs of each of these core proteins in human and other higher organisms. It remains to be determined whether these homologs have equivalent and/or unique functions.

Early studies demonstrated that purified and reconstituted PRC1 complexes can inhibit transcription and SWI/SNF-mediated chromatin remodeling (Francis et al., 2001; Shao et al., 1999). More recently, PRC1-like complexes, containing the core components RING1/2 and BMI1, were isolated from HeLa cells and shown to function as a ubiquitin E3 ligase that specifically monoubiquitylates histone H2A lysine 119, this activity being attributed to the RING2 (Ring1B) protein (Wang et al., 2004). Monoubiquitylated H2A (H2Aub1) is a relatively abundant histone modification, estimated to comprise 5%–15% of available nucleosomal H2A (West and Bonner, 1980).

Genetic studies revealed that mouse cells deleted for Ring1B and the closely related homolog Ring1A, exhibit global loss of H2Aub1 (de Napoles et al., 2004). Although the function of H2Aub1 remains to be determined, several pieces of evidence suggest that it is important for PRC1-mediated gene repression. First, PRC1 targets, such as Hox genes and the inactive X chromosome in mammals, are enriched for H2Aub1 (Cao et al., 2005;
Second, in D. melanogaster, a point mutation affecting only the RING finger domain of dRING results in a classical polycomb phenotype (Fritsch et al., 2003). Analysis of this mutant protein in vitro demonstrated concomitant loss of H2A E3 ubiquitin ligase activity (Wang et al., 2004).

While, in vitro, the histone ubiquitin ligase activity of mouse Ring1B can be stimulated by other subunits of PRC1 (Cao et al., 2005), subcomplexes containing either human RING1 (Ring1A) or RING2 (Ring1B) with BMI1 exhibited E3 activity equivalent to that of PRC1 purified from HeLa cells (Wei et al., 2006). Since addition of other PRC1 proteins to this latter subcomplex did not enhance its E3 activity, it is likely that the Ring1B/Bmi1 subcomplex comprises a minimal bipartite ubiquitin E3 ligase. Similar synergistic effects have been reported for the RING finger proteins Brca1 and Bard1, in which heterodimerization markedly stimulates the E3 ubiquitin ligase activity of Brca1 (Hashizume et al., 2001; Mallory et al., 2002). Furthermore, like Brca1/Bard1, the ubiquitin ligase function of Ring1B/Bmi1 is enhanced by autoubiquitylation (Ben-Saadon et al., 2006; Chen et al., 2002; Hashizume et al., 2001; Mallory et al., 2002).

Structural analysis of a Ring1B/Bmi1 complex (Buchwald et al., 2006; Li et al., 2006) revealed extensive interactions between the RING finger domains of Ring1B and Bmi1 and that the association with the ubiquitin-conjugating enzyme (E2) maps to a surface on Ring1B (Buchwald et al., 2006). Although Bmi1 is not required for E2 interaction, it has been shown to contribute to the stabilization of the PRC1 complex (Cao et al., 2005; Wei et al., 2006).

In mammals, there are at least four paralogs of the D. melanogaster RING finger protein PSC: Bmi1, Mel-18, MBLR, and NSPc1 (Akasaka et al., 2002; Brunk et al., 1991; van Loohuizen et al., 1991). Evidence suggests that these paralogs may interact with other PcG proteins to form a set of distinct but related complexes. Specifically, MBLR together with Ring1B is implicated in the E2F6 complex (Ogawa et al., 2002), and NSPc1 together with Ring1B and other PRC1 proteins is a component of a BCOR corepressor complex (Gearhart et al., 2006).

Mel-18 protein is 70% identical to Bmi1 at the amino acid level. Genetic experiments suggest that these proteins may have similar or overlapping functions. Mel-18 knockout mice exhibit homeotic transformations and cell-cycle deficiencies similar to those reported for Bmi1 mutants (Akasaka et al., 1996, 1997; van der Lugt et al., 1994), and the Mel-18 protein localizes to PRC1 target genes (Fujimura et al., 2006). Despite this, Mel-18 has not been identified in purified PRC1 or related complexes (Gearhart et al., 2006; Levine et al., 2002; Ogawa et al., 2002; Wang et al., 2004) and, unlike Bmi1, did not enhance the E3 ligase activity of Ring1B in vitro (Cao et al., 2005).

In this study, we investigated the function of this protein and whether it forms part of a functional polycomb repressive complex. We show that Mel-18 is indeed a component of a PRC1-like complex and that it can functionally substitute for Bmi1 in repressing Hox gene expression in ES cells. Furthermore, contrary to previous reports, we show that a holocomplex and a reconstituted Ring1B/Mel-18 subcomplex efficiently ubiquitylate H2A lysine 119. Interestingly, we find that a phosphorylated form of Mel-18 is important for targeting this complex to histone H2A lysine 119. Our results provide insights into the mechanism of action of histone H2A ubiquitin E3 ligase complexes and demonstrate a direct link between chromatin regulation by PcG repressor complexes and cell signaling pathways.

RESULTS

Affinity Purification of a Polycomb-like Complex

To investigate whether, like other polycomb proteins, MEL-18 is present in cells as part of a large multiprotein complex, we expressed and purified human MEL-18 protein, fused to a tandem affinity purification (TAP) tag, from 293T cells. Mass spectrometry analysis identified several proteins that copurify with MEL-18 (Figure 1A). These proteins, namely RING1/2, the short isoform of HPH2a (Yamaki et al., 2002; Tonkin et al., 2002), and CBX8, were previously identified as core PRC1 components (Shao et al., 1999; Levine et al., 2002). Other paralogs of HPH2 were present in substoichiometric amounts, suggesting that multiple MEL-18 complexes might exist in the cell. Importantly, the MEL-18 paralog BM1 was not present in this complex, and, conversely, MEL-18 was not present in a PRC1-like complex isolated using TAP-tagged BMI1 (see Figure S1 in the Supplemental Data available with this article online). Thus, MEL-18 and BM1 proteins are components of similar but mutually exclusive PRC1-like complexes. We subsequently refer to these complexes as MEL-18 PRC1 (melPRC1) and BM1 PRC1 (bmiPRC1).

melPRC1 is also present in mouse ES cells. Nickel-affinity purification of Mel18-FlagHis expressed in BM3 ES cells, which lack endogenously expressed Mel-18 and Bmi1, also “pulled down” Ring1A, Ring1B, and mPh1, homologs of the human PRC1 proteins RING1/2 and HPH1 (Figures S2A and S2B). Copurification of mPh1, rather than mPh2, most likely reflects low levels of mPh2 expression in mouse ES cells (H.K. and M.E., unpublished data).

Regulation of Hox Gene Expression in Embryonic Stem Cells

Microarray-based gene expression profiles identified several Hox genes whose expression is upregulated in cells lacking Mel-18 and Bmi1 (H.K. and M.E., unpublished data). Using RT-PCR, we measured expression of several of these genes in independently derived clones of BM3 that overexpress Mel-18-FlagHis (BM3-1, BM3-2, and BM3-3). In these cells, expression of the Hoxa1, Hoxa5, Hoxd4, and Hoxd8 genes was approximately 5- to 10-fold lower than in the parental BM3 cell line (Figure 1B). Downregulation of gene expression was not a general phenomenon, as expression...
of the Oct-4 gene in the same cells was unaffected by Mel-18. Therefore, like PRC1, melPRC1 may function as a negative regulator of Hox gene expression in ES cells.

**Localization of melPRC1 to Hox Gene Promoters**

To determine whether melPRC1 localizes to the promoters of Hox genes, we performed chromatin immunoprecipitation (ChIP) analysis. As expected, neither Mel-18 nor Ring1B was enriched at the promoters of Hox genes or Oct-4 in parental BM3 cells (Figure 1C and Figure S3). However, in BM3-1 and BM3-3 cells, we observed enrichment of both Ring1B and Mel-18 at the promoters of Hoxa1, Hoxa5, Hoxd4, and Hoxd8, but not at Oct-4 (Figure 1C and Figure S3). Hence, there is a clear correlation between the repression of gene expression and the recruitment of melPRC1 to the promoters of Hoxa1, Hoxa5, Hoxd4, and Hoxd8.

**Ring1B/Mel-18 Complex Is a Ubiquitin E3 Ligase**

Although Bmi1 and Ring1B interact to form a ubiquitin ligase complex that ubiquitylates nucleosomes in vitro, no such activity has been reported for Mel-18. We expressed and purified recombinant Mel-18 and Ring1B proteins in a complex, from Sf9 cells (Figures 2A and 2C). We noted that while Ring1B migrates as a single species, Mel-18 migrates as several distinct bands after polyacrylamide gel electrophoresis (Figure 2A). Subsequent analysis revealed these bands to be different phosphorylated forms of Mel-18 (see below).

Contrary to a previous report (Cao et al., 2005), we found that Ring1B/Mel-18, in the presence of the ubiquitin-conjugating enzyme (E2) UbcH5C, efficiently mono-ubiquitylates nucleosomes in vitro (Figure 2B). Like many other RING domain E3 ligases, Ring1B/Mel-18 also undergoes autopolyubiquitylation (Figure 2B), mainly on Ring1B (data not shown).
We observed efficient ubiquitylation of nucleosomes only when Mel-18 and Ring1B were coexpressed and copurified as a complex (Figure 2D, lane 5). Neither Mel-18 or Ring1B purified individually nor a mixture of the two proteins exhibited strong E3 ligase activity (Figure 2D). This suggests, first, that both Mel-18 and Ring1B are required for the efficient ubiquitylation of nucleosomes and, second, that a factor (or factors)
present during the assembly of the Ring1B/Mel-18 complex in Sf9 cells plays an important role in activating the ubiquitin ligase function of the complex (see below).

**Ring1B/Mel-18 Ubiquitylates Histone H2A**

In vitro, Ring1B/Mel-18 seemed to ubiquitylate nucleosomes on a single histone (Figure 3A, lane 2). Modification was largely monoubiquitylation, although some diubiquitylated histone was also observed. We next investigated ubiquitylation of individual histones in vitro. We found that, whereas Ring1B/Mel-18 ubiquitylates individual nucleosome core histones with similar high efficiency, ubiquitylation of H1, the linker histone, was much less efficient (Figure 3A, lanes 3–8, respectively). Hence it appears that the Ring1B/Mel-18 complex acquires specificity for its histone substrate only in a nucleosomal context.

SDS-PAGE suggested that ubiquitylation of nucleosomes occurred on either histone H2A (H2Aub1) or histone H2B (H2Bub1). To investigate which of these histones is modified by Ring1B/Mel-18, we made nucleosomes with recombinant histone proteins in which either lysine 118 and 119 of H2A or lysine 120 of H2B were substituted with arginine residues (Figure 3B, lanes 2 and 3, respectively). We found that, whereas the Ring1B/Mel-18 ubiquitylated wild-type nucleosomes and those containing H2BK120R mutant with equal efficiency (Figure 3C, lanes 1 and 2 and 5 and 6, respectively), those made with H2AK118/119R were largely unmodified (Figure 3C, lanes 3 and 4).

Comparison of Mel-18/Ring1B with Bmi-1/Ring1B revealed that these complexes exhibit similar specific activity toward H2A on a nucleosomal substrate (Figure S4A). This was also the case when we compared the activity of melPRC1 and bmiPRC1 holocomplexes isolated from 293T cells (Figure S4B). We conclude that Ring1B/Mel-18 is a chromatin E3 ubiquitin ligase with specificity for lysines 118 or 119 of histone H2A. Again, this mirrors the specificity of the bmiPRC1 ubiquitin ligase and, because H2Aub1 is thought to be a repressive modification, is in keeping with the role of melPRC1 as a negative regulator of gene expression.

**The Role of the Ring1B RING Domain in Ubiquitylation**

RING domains are known to confer E3 ligase function. To investigate the role of the Ring1B RING domain in ubiquitylation of H2A, we made cysteine-to-glycine substitutions at residue 87 or 90, representing cysteines 7 and 8 of the RING domain (Figure 4A). We also made mutant protein with an isoleucine-to-alanine substitution at residue 53, which is critical for interactions with the...

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**Figure 3. Ring1B/Mel-18 Complex Exhibits Defined Substrate Specificity**

(A) Ubiquitylation assays were performed using 1 μg Ring1B/Mel-18 complex with various substrates, as indicated. Shown are 1.5 μg nucleosomes (lane 2); 1 μg of individual histones H2A, H2B, H3, and H4 (lanes 3–6); H2AX (lane 7); and H1 (lane 8). I125-labeled products are shown.

(B) Purified reconstituted nucleosomes. Shown are wild-type nucleosome (lane 1), mutant H2AX^{K118/119R} nucleosome (lane 2), and mutant H2B^{K120R} nucleosome (lane 3).

(C) Ubiquitylation of reconstituted nucleosomes by Ring1B/Mel-18 in vitro. Wild-type nucleosomes (lanes 1 and 2), histone H2AX^{K118/119R} mutant (lanes 3 and 4), and histone H2B^{K120R} mutant (lanes 5 and 6) nucleosomes are shown.
ubiquitin-conjugating enzyme Ubc5C in other E3 ligases (Brzovic et al., 2003) (Figure 4A). Although these mutant proteins copurified normally with Mel-18 (Figures 4B and 4D), they were defective for the ubiquitylation of the nucleosome substrate and for autoubiquitylation (Figures 4C and 4E). In addition, these mutants were unable to ubiquitylate free histones (data not shown). We infer that the RING domain of Ring1B plays a central role in conferring
Mel-18 Phosphorylation Linked to H2A Ubiquitylation

E3 ligase activity to the Ring1B/Mel-18 complex, and this most likely involves the recruitment of the ubiquitin-charged E2 to the complex.

**Mel-18 RING Domain Is Important for Recognition of the Nucleosome**

We also made mutant proteins with cysteine-to-glycine substitutions at residues 53 and 56 in the RING domain of Mel-18 (Figure 5A). These mutants also copurified normally in a complex with Ring1B (Figure 5B). However, while Ring1B/Mel-18C53G ubiquitylated nucleosomes with normal efficiency (Figure 5C, lanes 2 and 4), Ring1B/Mel-18C53G did not (Figure 5C, lane 3). Nevertheless, Ring1B/Mel-18C53G was not completely defective, as it retained autoubiquitylation activity and the ability to ubiquitylate free histones (Figure 5C, lane 3, and Figure 5D, lanes 3–8). Therefore, in contrast to Ring1B, the RING domain of Mel-18 is not required for E3 function per se but may function specifically to mediate the interaction with its chromatin substrate.

**Phosphorylation of Mel-18**

Both Mel-18 purified from ES cells as well as recombinant Mel-18 migrate as several distinct bands after SDS-PAGE (Figures 6A and 6B, respectively). These are different phosphorylated forms of Mel-18, most of which disappear upon treatment with alkaline phosphatase (AP) (Figures 6A and 6B). Interestingly, in HeLa cells, the phosphorylated form of MEL-18 is associated with the chromatin fraction (Figure S5) and remains so throughout the cell cycle (Figure S6). We note that the forms of MEL-18 that are phosphorylated to a lesser extent are no longer associated with chromatin during mitosis (Figure S6B). However, it is unclear whether this represents redistribution or degradation of hypophosphorylated MEL-18.

The phosphorylated recombinant Mel-18 protein was subjected to tryptic digest and mass spectrometry. LC-MS/MS analysis led to the positive identification of two phosphopeptides where Ser132 was phosphorylated in one (minor site) and Ser254, Ser260, and Ser265 were phosphorylated in the second (Table S1). Additionally, MALDI-TOF mass spectrometry identified phosphopeptides encompassing residues 263–329 (+1–3 PO4) and 236–329 (+2–8 PO4) (data not shown). This was validated by inspecting the survey scans from the LTQ Orbitrap. The same peptides were observed in high charge states (5+ to 10+) at high mass accuracy (data not shown). The resultant LC-MS/MS spectra from these ions did not yield sufficiently rich fragmentation data to assign phosphorylation sites. However, after elastase digestion of a tryptic digest of Mel-18, shorter overlapping fragments of the same region of the protein were generated, and the resultant spectra permitted the assignment of phosphorylation sites at Ser110, Ser254, Ser258, Ser260, Ser265, Ser278, Thr281, Ser286, and Ser299 (Table S1). These data resolved the orbitrap survey scan result, which detected a tryptic peptide encompassing residues 236–329 with up to eight phosphorylation sites.

The location of phosphorylation sites is summarized in Figure 7A. The majority cluster in the proline/serine-rich domain at the C terminus with two sites, one of which is relatively weak, present in the central domain of the protein. Phosphorylated residues are conserved in human and mouse Mel-18 (Figure S7A), and all of the sites except Ser254 and Ser299 are also conserved in Bmi1 (Figure S7B).

**Phosphorylation of Mel-18 Is Required for Ubiquitylation of Nucleosomes**

We noted that both wild-type Mel-18, purified independently of Ring1B, and the Mel-18C53G mutant protein exist largely in the nonphosphorylated form (Figures 2B and 5B). Since neither of these protein complexes is able to ubiquitylate nucleosomes in vitro, we reasoned that there might be a correlation between the ubiquitin ligase function of Mel-18 and its phosphorylation status.

We assayed the ubiquitin ligase activity of Ring1B/Mel-18 complex treated with AP. Whereas untreated Ring1B/Mel-18 or that treated with buffer alone ubiquitylated nucleosomes with similar high efficiency (Figure 6C, lanes 2 and 3), this was greatly diminished with the AP-treated complex (Figure 6C, lane 4). The E3 function of the MelPRC1 holocomplex purified from 293T cells showed a similar requirement for phosphorylation (Figure 6D). Importantly, the AP-treated Ring1B/Mel-18 complex retained the ability to ubiquitylate free histone proteins and also to carry out autoubiquitylation (Figure 6E, lanes 4–11 compared to lane 3), suggesting that, while E3 function is still intact, the complex was unable to recognize H2A lysine 119 in the context of the nucleosome substrate. Hence, the properties of the unphosphorylated Mel-18 directly mirror those of Mel-18C53G mutant complex and suggest that, while phosphorylation does not enhance the intrinsic ubiquitin ligase function of the complex, it is required to promote recognition of the substrate.

**DISCUSSION**

In this study, we used affinity purification to identify human and mouse PRC1-like complexes containing MEL-18/Mel-18 (melPRC1). We show that, while melPRC1 shares common subunits with the previously described bmiPRC1, it is a functionally distinct complex. We demonstrate that melPRC1 represses Hox gene expression, and this correlates with its presence at the promoters of these genes. Ubiquitylation of H2A lysine 119 is associated with gene repression (Cao et al., 2005; de Napoles et al., 2004; Wang et al., 2004). In accordance, melPRC1 and a reconstituted subcomplex of melPRC1 comprising Mel-18 and Ring1B is an efficient E3 ligase in vitro and ubiquitylates H2A lysine 119 in chromatin. Mutation analysis reveals that while Ring1B plays a direct role in the ubiquitylation reaction, probably by mediating the interaction with the ubiquitin-conjugating enzyme, Mel-18 is critical for targeting the complex to lysine 119 of histone H2A in nucleosomes. Importantly, we find that, unlike bmiPRC1,
nucleosomal targeting of melPRC1 requires prior phosphorylation of Mel-18. Collectively, these results show that the RING finger protein Mel-18 plays a critical role in recruiting polycomb proteins to chromatin and provide a link between polycomb-mediated gene repression and cell signaling pathways.

Figure 5. A Mutation in the RING Domain of Mel-18 Abolishes Ubiquitylation of Nucleosomes, but Not Free Histones
(A) Amino acid sequence of a consensus RING domain and the RING domain of Mel-18. Boxed residues represent the amino acids mutated and correspond to the circled residues of the consensus RING domain.
(B) Wild-type and mutant Ring1B/Mel-18 complexes. Molecular weight markers are indicated.
(C) Mutant Ring1B/Mel-18 complexes were examined for ubiquitylation of nucleosomes. [125I]-ubiquitylated histone H2A (H2Aub1) and autoubiquitylated Ring1B/Mel-18 (asterix) products are indicated.
(D) Mutant Ring1B/Mel-18 complexes were examined for ubiquitylation of individual histones. Reactions were performed using 1 μg of recombinant histones (as indicated). [125I] autoubiquitylated Ring1B/Mel-18 (asterix) product is indicated.
Functional Interchangeability of PRC1-like Complexes

The function of individual PRC1 components in higher organisms is poorly understood. Previous studies demonstrated that while Mel-18 and Bmi1 knockout mice have similar, though not identical, phenotypes (Akasaka et al., 1996, 1997; van der Lugt et al., 1994, 1996), the double knockout mouse embryos exhibit a more profound phenotype (Akasaka et al., 2001). This suggests that while these two proteins have independent functions, they may also exhibit some functional redundancy. This view is supported by our demonstration that Mel-18 can restore repression of Hox gene expression to Mel18/−/−Bmi1/−/−ES cells.

Figure 6. Phosphorylated Form of Mel-18 Directs Ring1B to Chromatin

(A) Mel-18 isolated from ES cells is phosphorylated. Western blot of Mel-18 complex melPRC1 isolated from BM3-3 cells (see Figure S1B). The complex was treated with AP or buffer for 1 hr and immunoblotted for Mel-18 or Ring1B antibody. Phosphorylated and nonphosphorylated forms of Mel-18 are indicated.

(B) Recombinant Ring1B/Mel-18, but not mutant Ring1B/Mel-18(C53G), complex is phosphorylated. Purified Ring1B/Mel-18 and Ring1B/Mel-18(C53G) were treated with AP, AP, Ring1B, and the phosphorylated and nonphosphorylated forms of Mel-18 are indicated.

(C) Dephosphorylation of Mel-18 ablates E3 ligase activity on nucleosomes. Left panel, Ring1B/Mel-18 complex treated, or not, with AP. Right panel, AP-treated and -untreated complex was assayed for ubiquitin ligase activity on nucleosome substrate.

(D) Dephosphorylation of the melPRC1 complex purified from 293T cells ablates E3 ligase activity on nucleosomes. AP-treated complex was assayed for ubiquitin ligase activity on nucleosome substrate.

(E) AP-treated Ring1B/Mel-18 retains the ability to ubiquitylate free histones. AP-treated and -untreated complex was assayed for ubiquitin ligase activity on nucleosome or recombinant histones.
In keeping with this notion, we find that MEL-18 (Mel-18) is part of a multiprotein complex with RING1/2 (Ring1A/B), HPH2a (mPh2), and CBX8 (mPc3), all of which also associate with BMI1 (Bmi1) (Levine et al., 2002; Wang et al., 2004) (Figure S1). However, since MEL-18 and BMI1 components are not found together in the same complex, they probably participate in distinct polycomb repressor complexes (melPRC1 and bmi1PRC1, respectively). This may explain the nonoverlapping functions of Mel-18 and Bmi1 defined in genetic experiments.

Mechanism of H2A Lysine 119 Ubiquitylation

Biochemical analyses of Ring1B/Bmi1 (Cao et al., 2005) and Ring1B/Mel-18 (this study) suggest that the two RING finger proteins synergize to form an active core that is sufficient for the efficient ubiquitylation of H2A lysine 119. The fact that Cao et al. (2005) failed to detect H2A ubiquitylation using Mel-18 in complex with Ring1B may be attributable either to differences in the recombinant Mel-18 protein used in the two studies, for example the levels of phosphorylation at a specific site(s), or alternatively to differences in the assay conditions used.

In both subcomplexes, the Ring1B subunit is essential for ubiquitin transfer. It is generally held that RING finger E3 ligases provide a scaffold, which recruits both the ubiquitin-charged E2 enzyme and the substrate, juxtaposed so that transfer of ubiquitin can occur directly from E2 to substrate (Zheng et al., 2002). Our data suggest that Ring1B performs only the first of these functions, recruitment of the E2-conjugating enzyme. Consequently, mutations in Ring1B that alter the structure of the RING domain or interfere with the recruitment of the E2 abolish E3 function against chromatin or free histone substrates as well as autoubiquitylation. It is of note that we find no significant ubiquitin ligase activity with the Ring1B subunit alone.
On the other hand, a similar mutation (C53G) in the RING domain of Mel-18 abolishes ubiquitylation of nucleosome substrates, but not autoubiquitylation or the ubiquitylation of free histones. This suggests that while Mel-18 is not essential for E3 function per se, it is required to position the E3 ligase for the specific ubiquitylation of H2A lysine 119 in a chromatin context. Furthermore, the failure of the C53G mutant complex to ubiquitylate nucleosomes can be attributed to the fact that this mutant Mel-18 is not phosphorylated (see below). Extrapolating our observations, we suggest that other PSC paralogs, Bmi1, NSPc1, and MBLR, may carry out an equivalent function in targeting the E3 ligase activity of Ring1A/B to H2A lysine 119 in chromatin. The presence of the different PSC paralogs would allow the targeting of different genes by distinct PRC1-like complexes and/or a tight regulation via different cell signaling pathways.

**Regulation of Polycomb Complexes by Phosphorylation**

Recent studies have begun to identify how cell signaling pathways regulate gene repression by PcG proteins. Phosphorylation has been shown to reduce the HMTase activity of the PRC2 complex (Cha et al., 2005). Similarly, it has been suggested that phosphorylation of Bmi1 by MAPKAP kinase 3pK results in dissociation of bmiPRC1 from chromatin (Voncken et al., 2005). Jak-stat signaling has been shown to downregulate genes encoding PRC1 components, and this is important in transdifferentiation of imaginal disc cells in *D. melanogaster* (Lee et al., 2005). Our results provide a first example in which phosphorylation enhances activity of a polycomb complex, specifically stimulating recognition of the nucleosome substrate and ubiquitylation of H2A lysine 119 by the bmiPRC1 E3 ligase.

We identified nine phosphoserine and a single phosphothreonine residue in Mel-18. Sequence analysis reveals that these are consensus sites for multiple serine/threonine kinases, including the casein kinase, cyclin-dependent kinase, and MAPK families. A previous study suggested that phosphorylation of Mel-18 can also be mediated by protein kinase C (Fujisaki et al., 2003). While we cannot rule this out, the majority of sites we identified are not a good consensus for this kinase family. The unambiguous identification of the regulatory kinase for melPRC1 awaits further study.

Bmi1 can also be phosphorylated, but only at mitosis, correlating with its dissociation from chromatin (Voncken et al., 2005). Although this is functionally distinct from phosphorylation of Mel-18, it is possible that some of the sites of phosphorylation overlap. Two of the phosphoserine residues identified in Mel-18 (serines 254 and 299) are not conserved in Bmi1. Consequently, these sites may be good candidates for mediating unique functions of Mel-18.

Mel-18 is phosphorylated in Sf9 cells only when complexed with Ring1B (Figure 2C). It is of note that the C53G mutation in Mel-18, which abrogates phosphorylation, does not do so by disrupting this complex. As C53 is located in the RING domain of Mel-18, away from the majority of phosphorylation sites and at a residue that is highly conserved in Bmi1 paralogs, we speculate that C53G might alter the conformation of Mel-18 so that recognition by the regulatory kinase(s) is impaired.

How, then, does phosphorylation of Mel-18 impact on the H2A ubiquitylation activity of melPRC1? One possible mechanism is that phosphorylation of Mel-18 is required for the complex to bind to the surface of the nucleosome. Alternatively, phosphorylation may induce a conformational switch in Mel-18 already bound to a nucleosome, positioning Ring1B:E2 for transfer of ubiquitin onto H2A lysine 119. The fact that both phosphorylated and unphosphorylated Mel-18 is found in the chromatin fraction of HeLa cell nuclei perhaps favors the latter hypothesis (Figure S5 and Figure 7B). Structural analyses of phosphorylated and unphosphorylated forms of Mel-18/Bmi1 complexed with Ring1B should provide further insight.

In summary, our data suggest that evolution of the PSC homolog Mel-18 in higher organisms has allowed the acquisition of a distinct mode of regulating H2A ubiquitylation via phosphorylation. Although the biological function of Mel-18 phosphorylation remains to be determined, we envisage two possible models, which are illustrated in Figure 7B. First, a Mel-18 kinase may be regulated so as to repress target genes in response to a specific signal. Second, Mel-18 phosphorylation may be a default state, and regulation of a Mel-18 phosphatase could function to derepress target genes. In future work, identification of Mel-18 kinase/phosphatase activities should shed light on the biological function of Mel-18 phosphorylation in gene regulation by polycomb complexes.

**EXPERIMENTAL PROCEDURES**

**Cell Lines and Derivations**

BM3 cells were derived from Mel-18+/−/Bmi1+/− heterozygote crosses using standard methods (H.K. and M.E., unpublished data). Generation of BM3 cell lines stably expressing Mel-18-FlagHis are described in the Supplemental Experimental Procedures. BM3 and BM3 transgenic cell lines were grown in DMEM (GIBCO) supplemented with 16% FCS (Autogen Bioclear, Calne, Wiltshire, UK), l-glutamine, nonessential amino acids, 50 IU/ml penicillin/streptomycin, 2-mercaptoethanol (GIBCO), and 1000 units/ml LIF (Chemicon) at 37 °C and 5% CO2.

HeLa cells were grown in DMEM supplemented with 10% FCS, l-glutamine and 50 IU/ml penicillin/streptomycin at 37 °C and 5% CO2. Cell-cycle synchronization experiments were carried out as described in the Supplemental Experimental Procedures.

293T cells were cultured at 37 °C in a humidified, 5% CO2 atmosphere in DMEM supplemented to contain 10% FCS, 100 IU/ml penicillin, and 100 μg/ml streptomycin. 293T cells stably expressing human Mel-18-TAP, BMI-1-TAP, or TAP alone were generated as described in the Supplemental Experimental Procedures.

**Purification of MEL-18 and BMI1 Complexes by TAP-Tag Purification**

Human MEL-18 and BMI1 coding sequences were amplified from cDNA and cloned to produce an in-frame TAP tag, generating pGM-MEL-18TAP or pQBT (BMI1-TAP). pGM-TAP expressing TAP alone was used as a negative control. 293T TAP, 293T, MEL-18TAP, and
293T-ER QBT cells were harvested and protein complexes purified as described in the Supplemental Experimental Procedures.

**Purification of Recombinant Mel-18/Ring1B**

Full-length mouse Mel-18, Bmi1, and Ring1B were tagged with His$_{12}$ and FLAG or HA and cloned into pDEST8 using the Gateway cloning system (Invitrogen). Mutant Mel-18-FlagHis and Ring1B-FlagHis were generated from the pDEST clones using site-directed mutagenesis kit (Stratagene). Recombinant baculovirus was generated using the Bac-to-Bac baculovirus system (Invitrogen). Wild-type virus vMel-18-HA, or vBmi1-HA, and vRing1B-FlagHis or the reciprocal tagged versions were coinfected in Sf9 insect cells for 60 hr. Complexes were purified as described in the Supplemental Experimental Procedures. Mutant Mel-18-FlagHis and wild-type Ring1B-HA or mutant Ring1B-FlagHis and wild-type Mel-18-HA were coinfected and grown as above. Mutant protein complexes were purified as wild-type complex.

**Ubiquitination Assays**

Unless otherwise stated, reactions (25 µl) were performed as described in Mallory et al. (2002) using 300 ng E1 (Boston Biochem), 300 ng UbH5c (affinity), 1 µg ubiquitin (Sigma), 1 µg purified E3, and 1.5 µg recombinant oligomericosomes, or 2.5 µg of ES cell chromatin. $^{125}$I-labeled products were visualized using the Molecular Dynamics Typhoon Phosphorimagery and ImageQuaNT software. Where indicated, complexes were dephosphorylated by treatment with 2 U of AP (Roche 1 U/µl) in phosphatase buffer for 2 hr at 37°C.

**Identification of Phosphorylation Sites**

Approximately 1 µg of Mel-18 was separated by SDS-PAGE and stained with colloidal Coomassie. Mass spectrometry was carried out as described in the Supplemental Experimental Procedures.

**Western Blot Analysis and Antibodies**

Antibodies used in western blotting were Mel-18 (Abcam) 1:500, Ring1B (Atsuta et al., 2001) 1:500, Ring1A (Schoolermer et al., 1997) 1:500, mPht1 (Miyagishima et al., 2003) 1:10, cyclin A (Santa Cruz), anti-Ring1B (Atsuta et al., 2001), or the appropriate control IgG antibodies overnight at 4°C with rotation. Beads were washed four times with wash buffer 1 and once with final wash buffer. Immunoprecipitated chromatin was eluted from the beads in elution buffer and reverse crosslinked overnight. The precipitated DNA was dissolved in 100 µl of TE. ChIP DNA was analyzed by real-time PCR using SYBR green (Bio-Rad) following the manufacturer’s instructions. Enrichment was normalized to input DNA. Primers utilized are shown in Table S3.

**Supplemental Data**

Supplemental Data include Supplemental Experimental Procedures, seven figures, three tables, and Supplemental References and can be found with this article online at http://www.molecule.org/cgi/content/full/28/1/107/DC1/.

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