Proteomics Analysis of Ring1B/Rnf2 Interactors Identifies a Novel Complex with the Fbxl10/Jhdm1B Histone Demethylase and the Bcl6 Interacting Corepressor*

Carmen Sánchez‡§, Inés Sánchez‡¶, Jeroen A. A. Demmers∥, Patrick Rodriguez**, John Strouboulis**‡‡, and Miguel Vidal‡§§

Ring1B/Rnf2 is a RING finger protein member of the Polycomb group (PcG) of proteins, which form chromatin-modifying complexes essential for embryonic development and stem cell renewal and which are commonly deregulated in cancer. Ring1B/Rnf2 is a ubiquitin E3 ligase that catalyzes the monoubiquitylation of the histone H2A, one of the histone modifications needed for the transcriptional repression activity of the PcG of proteins. Ring1B/Rnf2 was shown to be part of two complexes, the PRC1 PcG complex and the E2F6.com-1 complex, which also contains non-PcG members, thus raising the prospect for additional Ring1B/Rnf2 partners and functions extending beyond the PcG. Here we used a high throughput proteomics approach based on the single step purification, using streptavidin beads, of in vivo biotinylated Ring1B/Rnf2 and associated proteins from a nuclear extract from erythroid cells and their identification by mass spectrometry. About 50 proteins were confidently identified, all of which had not been identified previously as subunits of Ring1B/Rnf2 complexes. We found that histone demethylases LSD1/Aof2 and Fbx110/Jhdm1B, casein kinase subunits, and the BcoR corepressor were among the new interactors identified. We also isolated an Fbx110/Jhdm1B complex by biotinylation tagging to identify shared interacting partners with Ring1B/Rnf2. In this way we identified a novel Ring1B-Fbx110 complex that also includes Bcl6 corepressor (BcoR), CK2α, Skp1, and Nsp1/Pcgf1. The putative enzymatic activities and protein interaction and chromatin binding motifs present in this novel Ring1B-Fbx110 complex potentially provide additional mechanisms for chromatin modification/recruitment to chromatin and more evidence for Ring1B/Rnf2 activities beyond those typically associated with PcG function. Lastly this work demonstrates the utility of biotinylation tagging for the rapid characterization of complex mixtures of multiprotein complexes achieved through the iterative use of this simple yet high throughput proteomics approach. *Molecular & Cellular Proteomics 6:820–834, 2007.

In multicellular organisms, cell identity is controlled, at least in part, by epigenetic events, including DNA methylation and post-translational modifications of histones that lead to chromatin structure regulation (1). These modifications are carried out by protein complexes recruited through DNA sequences and/or specific recognition of modified histones. The Polycomb group (PcG) of proteins, first identified genetically as regulators of Hox genes in the fly Drosophila melanogaster (for a review, see Ref. 2), is an example of such a complex. PcG functions cover many aspects of vertebrate development and tissue homeostasis by preventing the inappropriate activation of many transcription factor-coding genes and other genes involved in cell signaling and cell proliferation (3–7). Currently it is believed that PcG proteins play a role in setting the balance between proliferation and differentiation in normal development. Deregulation of PcG proteins disrupts such a balance and often leads to cell transformation and cancer (8).

One of the principle modes of PcG action is through the post-translational modification and binding of histones. One of the modifications, the trimethylation of Lys-27 of nucleosomal histone H3 (9–11) is linked to transcriptional silencing (12), and it is carried out by the SET domain-containing PcG members Ezh1 and Ezh2. Another important histone modification associated with repressed loci is the monoubiquitylation of Lys-119 of histone H2A, a process that needs several

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PcG RING finger proteins, although the critical ubiquitin E3 ligase component is Ring1B/Rnf2 (13–16). These two PcG histone modification activities occur in biochemically distinct protein complexes that do not share subunits. The Polycomb repressing complex 2 (PRC2) methylates H3 Lys-27, and it is recruited, at least in part, through YY1 (17, 18), the only PcG protein that specifically binds to DNA. H2A ubiquitylation is carried out by PRC1 (13, 19), which is targeted to chromatin through the recognition of H3 Lys-27 trimethylation by chromodomain-containing PcG subunits (18, 20), such as M33/Cbx2 or Pc2/Cbx4 (21). Once bound to chromatin, PcG complexes repress transcription by one or more mechanisms, including interference with nucleosome remodeling by the Swi/Snf complex chromatin remodeling (19) and/or with transcription initiation (22).

Histone H2A monoubiquitylation depends critically on Ring1B/Rnf2; this may explain the embryonic lethality observed in constitutive loss-of-function Ring1B/Rnf2 knock-out mice (23). Correlative evidence shows that this histone modification is associated with transcriptional repression; however, the molecular basis (for instance cross-talk between histone modifications) underlying this function is not known. Understanding these mechanisms requires the identification of new Ring1B/Rnf2 partners and the characterization of the complexes they form. These new complexes would also explain additional chromatin-targeting mechanisms unveiled in studies on the inactivation of mammalian X chromosome (silenced as a gene dosage compensation strategy of female cells) showing that Ring1B/Rnf2 targeting and H2A monoubiquitylation occur independently of H3 Lys-27 modifications (24). One such complex may be the E2F6.com-1 complex (25), which contains a subset of PcG proteins, transcription factors, and an H3 Lys-9 histone methyltransferase.

In the present study we aimed for a simple yet high throughput approach that would identify as many Ring1B/Rnf2 partners as possible. To this end we combined the isolation of complexes using a biotinylation tagging approach based on the very high affinity of streptavidin for biotin coupled to the nuclear pellets were resuspended in 10–12 ml of 100 mM Heng buffer (20 mM HEPES, pH 7.9, 100 mM KCl, 10 mM MgCl2, 0.25 mM EDTA, and 0.2% Igepal) and incubated with nuclear extracts for 1 h at 4 °C and nuclei were incubated with rotation for 20 min at 4 °C. The homogenates were ultracentrifuged at 40,000 rpm for 1 h at 4 °C, and supernatants were aliquoted, snap frozen in liquid nitrogen, and stored at −80 °C.

**Analytical Gel Filtration**—Nuclear extracts (0.8–1.5 mg in 200 μl) were size-fractionated on an AKTA FPLC apparatus with a Superose 6 10/30 column (Amersham Biosciences). Individual fractions were concentrated by precipitation with 100% trichloroacetic acid prior to analysis by Western immunoblotting.

**Binding to Streptavidin Beads**—Preparative (for mass spectrometry analysis) or analytical pulldowns were performed with 15–20 or 1 mg of nuclear proteins, respectively, after adjusting the ionic strength to 150 mM KCl with Heng buffer without KCl. Paramagnetic streptavidin beads (Dynabeads M-280, Dynal), 20 μl/mg of protein, were washed three times in PBS, then blocked by incubating for 1 h at room temperature in PBS with 200 ng/μl purified chicken serum albumin (Sigma-Aldrich), and incubated with nuclear extracts for 1 h at 4 °C on a rotating wheel. The beads were washed six times in 20 mM HEPES, pH 7.9, 200 mM KCl, 20% glycerol, 0.25 mM EDTA, and 0.2% Igepal + PMSF. Bound material was eluted by boiling for 5 min in Laemmli protein sample loading buffer and resolved either on continuous SDS-polyacrylamide gels or in precast 4–12% bis-Tris NuPAGE gels (Invitrogen) in MOPS buffer.

**Western Blotting**—Proteins resolved by PAGE were blotted onto ProTRAN nitrocellulose membranes (Schleicher & Schuell). For detection of biotinylated proteins the membranes were blocked for 1 h in TBS containing 0.5% BSA and 0.05% Igepal and subsequently incubated for 1 h at room temperature with streptavidin–horseradish peroxidase (HRP) conjugate (NEL 750, PerkinElmer Life Sciences; dilution: 1:15,000). For immunoblotting, the filters were blocked for 1 h in TBS containing 0.5% BSA, 0.5% Igepal, and 5% skimmed dry milk and then incubated overnight at 4 °C with the indicated antibodies diluted in...
**Ring1B/Rnf2-associated Proteins**

TBS-BSA-Igepal. After washes, the membranes were incubated with HRP-conjugated goat anti-rabbit (Bio-Rad) antibodies or HRP-conjugated goat anti-mouse IgG (Dako) antibodies diluted in TBS for 1 h at room temperature. HRP conjugates were detected using an enhanced chemiluminescence kit (ECL Plus, Amersham Biosciences).

**Coimmunoprecipitation**—Nuclear extracts (1 mg of protein) diluted in Heng buffer without KCl to a final concentration of KCl of 150 mM were incubated with protein G-Sepharose (Amersham Biosciences) for 1 h at 4 °C. The precleared extracts were then incubated with the indicated antibodies. After 2 h at 4 °C, protein G-Sepharose beads (20 μl of 50% packed volume) were added, and the incubation continued for 1 h at 4 °C on a rotating wheel. The beads were then washed three times in 40 mM HEPES, pH 7.9, 0.2 M NaCl, and 0.1% Igepal. Immunoprecipitated proteins were eluted in 2× Laemmli buffer prior to SDS-PAGE and nitrocellulose blotting onto nitrocellulose for Western blot analysis.

**Antibodies**—Ring1A, Ring1B, and Ring1 and YY1 binding protein (RYBP) antibodies have been described previously (27, 35, 36). For YAF2, a rabbit antiserum was raised against a GST-YAF2 protein produced in E. coli as described previously (27). Other antibodies were: anti-BcoR (A01, Abnova; and a gift from V. Bardwell, Minneapolis, MN), anti-Fbxl10/Jhdm1B (a gift from Y. Zhang, Chapel Hill, NC), anti-Nsp1/Pcgf1 (a gift from V. Bardwell, Minneapolis, MN), anti-Skp1 (ab105465, Abcam), anti-Bmi1/Pcgf4 (M20, Abnova), anti-H1p 2/1c (2MOD-1G6, Euromedex), anti-Pc3/Cbx8 (a gift from K. Helin, Copenhagen, Denmark), anti-Aos2/LSD1 (ab17721, Abcam), anti-CtBP1 (E-12, Santa Cruz Biotechnology), and anti-Ck2α (C-18, Santa Cruz Biotechnology).

**Mass Spectrometric Analysis**—SDS-PAGE gel lanes were cut into slices using an automatic gel slicer and subjected to in-gel reduction with dithiothreitol, alkylation with iodoacetamide, and digestion with trypsin (Promega, sequencing grade) essentially as described previously (37). Nano-LC-MS/MS was performed on either a CapLC system (Waters, Manchester, UK) coupled to a Q-TOF Ultima mass spectrometer (Waters, Manchester, UK) or an LTQ mass spectrometer (Thermo) equipped with a nanospray source. Pep-Technologies) and then searched against the National Center for Biotechnology Information non-redundant (NCBI) database (release date, March 3, 2006; taxonomy, *Mus musculus*). The peptide tolerance was typically set to 0.1 M acetic acid; B = 80% (v/v) acetonitrile, 0.1 M acetic acid) in 70 min and at a constant flow rate of 200 nl/min using a splitter. The column eluate was directly sprayed into the ESI source of the mass spectrometer. Mass spectra were acquired in centroid mode; the fragmentation of the peptides was performed in data-dependent mode.

**RESULTS**

**In Vivo Ubiquitylation Assays**—Human embryonic kidney 293T cells were harvested 48 h later in 300 μl of 1% SDS in PBS. After boiling and vigorous vortexing, extracts were supplemented with Triton X-100 (up to 1.5% final concentration) in Tris-buffered saline. Cell extracts were precleared with protein G-Sepharose (Amersham Biosciences) and then incubated overnight with anti-FLAG antibodies (Sigma) at 4 °C. Immunocomplexes were isolated using protein G beads for 1 h at 4 °C with continuous rotation, and the beads were washed twice with 40 mM HEPES, pH 7.9, 0.20 M NaCl, and 0.1% Igepal before transfer to fresh tubes for a final wash. Immunoprecipitated proteins were eluted in 2× loading buffer and separated on a 15% SDS-polyacrylamide gel prior to Western blot analysis with anti-HA and anti-FLAG antibodies. For loading controls, total cell extracts were separated on 10% SDS-polyacrylamide gels for Western blot analysis with anti-Myc and anti-GST antibodies.

**Immunofluorescence**—U2-OS cells grown on glass coverslips were washed three times in PBS, fixed in freshly prepared 4% paraformaldehyde for 10 min at room temperature, and then permeabilized with PBS containing 0.05% Triton X-100 for 5 min. After 5-min PBS washes, the cells were incubated in blocking solution (PBS containing 10% goat serum, 2% BSA, and 0.1% Tween 20) for 30 min at room temperature. The fixed cells were incubated with mouse (anti-Ring1B) and rabbit antibodies diluted in blocking solution for 1 h at room temperature. Coverslips were washed five times for 5 min in PBS with 0.1% Tween 20 and then incubated with goat-anti-mouse IgG coupled to Alexa Fluor 488 and goat anti-rabbit IgG coupled to Alexa Fluor 647 (Molecular Probes) diluted 1:200 in blocking solution. After washes in PBS/Tween, the cells were mounted and analyzed in a confocal microscope (Leica TCS-SP2).

**In Vivo Biotinylation of Ring1B/Rnf2**—Most PcG complex purifications have been carried out in HeLa cells, which are known to express low levels of PcG products (38). Taking this into account together with the fact that the conditional Ring1B/Rnf2 gene knock-out includes a hematopoietic phe-
expressed Ring1B/Rnf2 protein is distinguished from the en-
size-fractionated on a Superose 6 column. The ectopically
extracts of both non-transfected and transfected MEL cells
2
proteins were similar to those of the endogenous protein to
choose a cell clone in which levels of ectopic Ring1B/Rnf2
acid peptide tag fused in-frame to its 5
a tagged Ring1B/Rnf2 cDNA bearing a biotinylable 23-amino
E. coli
MEL cell line that expresses the
levels of PcG proteins.

We stably transfected a derivative of the (proerythroblastic)
MEL cell line, which grows in suspension and expresses higher
of the Ring1B/Rnf2 complex. To this end we chose the MEL
notype2 led us to use a hematopoietic cell line for the isolation
of the Ring1B/Rnf2 complex. A schematic representation of the Ring1B/Rnf2
complexes. A schematic representation of the Ring1B/Rnf2 protein showing the homology domains and the RING finger motif
together with the N-terminal fusion of the BirA ligase recognition peptide tag sequence is shown. This Ring1B/Rnf2 modified cDNA
was stably introduced into MEL cells expressing the E. coli BirA biotin
ligase, which biotinylates the lysine residue underlined in the peptide
tag sequence. Ring1B/Rnf2 complexes were isolated from nuclear extracts by binding to streptavidin beads, and polypeptides were
eluted and separated by SDS-PAGE. The entire lane was cut into 24
gel slices, and proteins were trypsinized and subjected to LC-MS analysis.

Identification of Ring1B/Rnf2-interacting Proteins—To de-
terminate the identity of proteins associated with biotinylated
Ring1B/Rnf2, complexes were isolated from nuclear extracts of transfected cells by binding to streptavidin beads. After
elution, the copurified proteins were resolved by SDS-
PAGE and identified by mass spectrometry (Fig. 1). We con-
sidered for further analysis only polypeptides that were iden-
tified by at least two different peptides with Mascot scores
above a cutoff value of 50. Background proteins (carboxyl-
ases and their coenzymes; ribosomal and splicing factors;
cytoskeletal proteins such as actins, tubulins, and keratins; etc.) identified in a similar analysis of nuclear extracts from
BirA-expressing MEL cells were excluded from the list of
interacting proteins. According to these criteria, around 50
proteins were identified in two independent experiments (Ta-
ble I). Fig. 2C shows an example of an SDS-PAGE gel with
nuclear proteins coeluted with biotinylated Ring1B/Rnf2 to-
gether with the identified proteins shown next to their corre-
sponding gel slices. In cases where the same proteins were
found in multiple slices, perhaps due to breakdown and/or
overloading, the gel slice indicated in the figure is the one
containing the largest number of proteolytic peptides.

Proteins identified by mass spectrometry in our experi-
ments as copurifying with biotinylated Ring1B/Rnf2 were
classified as components of the previously described PRC1
and E2F6.com-1 complexes or as newly identified Ring1B-
associated proteins (Fig. 2C). Components of the PRC1 and
E2F6.com-1 complexes previously to contain Ring1B/
Rnf2 were identified by a large number of peptides. Specifi-
cally we found the murine homologs of all of the human PRC1
PcG proteins, i.e. Polycomb paralogs Pc3/Cbx8, Pc2/Cbx4,
and M33/Cbx2; polyhomeotic paralogs Phc1, Phc2, and
Phc3; Ring1A/Ring1, and Scmh1 (19). Non-PcG proteins
found in PRC1, such as the heat shock 70-kDa protein 8
(Hspa8) and the ATPase Snf2h, were also identified (19). Other
known Ring1/Ring1B interactors found here included Cbx7

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G. Calés, M. Romáu-Trufero, L. Paróu, I. Serrano, M. Endoh, H.
Koseki, and M. Vidal, manuscript in preparation.
and Pcgf3, which are Polycomb and Bmi1/Pcgf4 paralogs, respectively. Cbx6, another Polycomb paralog about which very little is known, was found in one of the experiments. With regard to the E2F6.com-1 complex (25), all of its previously described components (Mga, Euhmt1, L3mbtl2, MBLR/Pcgf6, Myn, E2F6, DP1, HP1γ/Cbx3, YAF2, and the Ring1A/Ring1 and Ring1B/Rnf2 proteins) were identified in our analysis as well. The mass spectrometry results from the streptavidin pulldowns were confirmed by immunoblot analysis with antibodies against selected protein members of the two complexes (Fig. 3A). We first tested some of the known Ring1A/Rnf2 interactors in the PRC1 and E2F6.com-1 complexes, such as Bmi1/Pcgf4, PC3/Cbx8, YAF2, and HP1γ/Cbx3. In all cases, the proteins that were identified by mass spectrometry were confirmed, thus validating our strategy.

Significantly a number of proteins not associated previously with Ring1B/Rnf2 or with the PRC1 and E2F6.com-1 complexes were also identified in our analysis. Of those, the most interesting ones included two histone H3 demethylases, LSD1/Aof2 (40) and Fbx10/Jhdm1B (41); the transcriptional repressor BcOR (42); and the subunits of casein kinase 2, CK2α1, CK2α2, and CK2β, all confidently identified by a large number of peptides. In addition, Skp1, a component of the SCF (Skp/cullin/F-box) ubiquitin ligase complex and an interactor of F-box-containing proteins such as Fbx10/Jhdm1B (43), was also identified in one of the two experiments by peptides covering much of its sequence. We confirmed these interactions initially by streptavidin pulldowns followed by immunoblot analysis (Fig. 3B). These interactions were further validated by coimmunoprecipitation of nuclear extracts from non-transfected MEL cells (Fig. 3C). Anti-Ring1B antibodies specifically immunoprecipitated Fbx10/Jhdm1B, BcOR, LSD1/Aof2, Skp1, and CK2α1. The low levels of immunoprecipitated LSD1 by the Ring1B/Rnf2 antibody indicate that the
Ring1B epitope is not readily accessible by the antibody in the Ring1B-LSD1 complex because immunoprecipitation using anti-LSD1/Aof2 antibodies clearly brings down an appreciable amount of Ring1B. Other reciprocal coimmunoprecipitations with anti-Skp1 and anti-CK2α antibodies also precipitated complexes containing Ring1B/Rnf2. Antibodies against

| Acc. number | Protein | Mass | Exp. 1 Scoreb Peptidesc Coveraged | Exp. 2 Scoreb Peptidesc Coveraged |
|-------------|---------|------|-----------------------------------|-----------------------------------|
| gi57012597  | Bcor    | 194.108 | 3,017 49 33 2.512 43 28.7 |
| gi37360622  | Fbx10/Jhdmi1B | 151.474 | 1,597 32 24.7 1.267 24 18.2 |
| gi71059923  | Ring1B/Rnf2 | 38.056 | 983 12 40.2 1.124 13 44.6 |
| gi6692607   | Mga     | 330.670 | 950 21 7.7 727 15 6.9 |
| gi7304947   | Ptc3/Cbx8 | 39.893 | 918 15 50 1.175 19 66.3 |
| gi1661134   | Hspa8   | 71.021 | 846 26.5 903 16 25.5 |
| gi12585200  | Ptc2/Cbx4 | 60.943 | 815 14 27.6 425 7 16.9 |
| gi1262300   | Ck2α1   | 45.322 | 760 13 34.5 1.059 16 45.8 |
| gi9055280   | Phc2    | 90.542 | 670 11 13.3 824 12 18 |
| gi6681271   | Phc1    | 106.891 | 663 11 14.5 613 8 13.2 |
| gi6753540   | Ck2α2   | 41.360 | 571 9 27.1 799 12 39.7 |
| gi38614119  | Bmi1/Pcgf4 | 37.254 | 553 7 26.5 314 4 14.5 |
| gi28879003  | Wdr68   | 39.415 | 533 8 28.4 |
| gi23273371  | M33/Cbx2 | 55.170 | 451 8 20.6 331 7 16.4 |
| gi20307063  | Nspc1/Pcgf1 | 29.801 | 433 9 31.2 389 9 35.6 |
| gi12857499  | Pcgf5   | 28.148 | 416 7 39 595 11 47.9 |
| gi21961373  | Scmh1   | 79.532 | 362 8 12.6 313 5 9.5 |
| gi21410118  | L3mbtl2 | 80.045 | 345 5 7.3 541 9 14.5 |
| gi51315882  | LSD1    | 93.306 | 314 7 8.9 226 5 7.3 |
| gi31044444  | Pcgf10  | 28.713 | 313 6 21.2 |
| gi4024385   | Yaf2    | 19.985 | 302 4 38.5 132 2 26.8 |
| gi58476686  | Mblr/Pcgf6 | 40.365 | 289 7 19.3 459 8 23.8 |
| gi23396011  | Phc3    | 106.171 | 263 6 6.7 277 3 6.5 |
| gi19548760  | Mel18/Pcgf2 | 38.383 | 255 5 12.3 162 4 8.8 |
| gi20379598  | Parafibromin | 60.653 | 252 6 10.7 293 6 12.8 |
| gi16654827  | Wdr5    | 37.136 | 245 5 15.9 462 6 22.8 |
| gi14028669  | Snf2H   | 122.311 | 232 6 4.7 |
| gi57617008  | Cbx6    | 73.456 | 187 4 8.5 |
| gi20544151  | Hp1/y/Cbx3 | 20.969 | 245 3 23.5 |
| gi72679289  | Usp7   | 104.377 | 191 4 6 |
| gi23530395  | Ckβ2   | 25.268 | 169 4 20.9 410 7 41.9 |
| gi5032027   | Rbbp4  | 47.911 | 160 3 7.1 232 4 8.5 |
| gi13277792  | Rbbp7  | 48.102 | 160 3 7.1 232 4 8.5 |
| gi1583224   | Skp1  | 18.803 | 150 3 19.6 148 3 13.5 |
| gi21450179  | Cbx7   | 18.098 | 150 3 19.6 184 3 23.4 |
| gi25091206  | Setdb1 | 146.453 | 146 4 2.4 98 2 1.8 |
| gi9937986   | Kaiso  | 74.708 | 142 4 5.8 |
| gi22596218  | Mll1   | 61.478 | 139 3 6 104 2 3.8 |
| gi12963679  | Lcp1   | 66.320 | 139 3 6.7 150 4 7.1 |
| gi37805246  | Zipf87 | 83.409 | 136 3 4.3 74 1 1.2 |
| gi52350624  | Ring1A/Ring1 | 39.893 | 136 3 16.9 306 4 14.1 |
| gi58477297  | Wdr58  | 37.918 | 166 3 9.4 |
| gi30851445  | mAM    | 139.819 | 114 3 2.2 60 2 1.4 |
| gi33468903  | Hp1bp3 | 61.046 | 108 4 7 |
| gi20610077  | Matri3 | 95.085 | 102 3 4 264 6 8.7 |
| gi19116244  | Ctbp2  | 46.399 | 117 2 4.8 |
| gi395281    | Dp1    | 45.488 | 102 2 5.6 104 2 5.6 |
| gi22137748  | Xrcc5  | 83.671 | 87 2 2.6 175 4 6.9 |
| gi12006108  | Ira1   | 56.381 | 62 2 3.5 78 2 3.3 |
| gi22902242  | E2f6   | 26.908 | 51 1 4.6 400 7 29 |
| gi110731    | Myn    | 18.234 | 60 2 14.4 |
| gi59807665  | Ehtm1  | 135.382 | 81 2 1.7 |
| gi3023934   | Hdac2  | 55.837 | 79 2 5.1 |
| gi27369714  | Rbpb5  | 59.745 | 59 2 2.8 |
| gi22478874  | Mta1   | 81.432 | 52 1 1.4 100 2 3.1 |

a NCBI accession number.
b Mascot protein score.
c Number of different peptides.
d Fraction of protein recovered in peptides.
Ring1B/Rnf2-associated Proteins

Fbxl10 and BcoR were not effective in immunoprecipitation (data not shown) and could not be used further.

In addition to the proteins above, our mass spectrometric analysis identified a number of proteins as copurifying with Ring1B/Rnf2, although we have not yet confirmed the direct or indirect association of these proteins with Ring1B/Rnf2. Among these were WD domain-containing proteins identified previously as members of chromatin-related complexes such as the closely related Rbpb4 and Rbpb7 proteins, Rbpb5, and Wdr5 (44, 45) and WD proteins of unknown function such as Ira1, Wdr68, and Wdr58. We also identified two previously unknown proteins, Pcgf3 and Pcgf5, together with Nspc1/Pcgf1 (31), which are three RING finger proteins related to the Pcg products Bmi1/Pcgf4 and Mel18/Pcgf2. The histone H3 Lys-9 methyltransferase Setdb1, together with its activating partner, mAM (46), was also identified in the analysis, although the sequence coverage of the corresponding peptides was relatively low, possibly indicating non-stoichiometric amounts or a low abundance complex. Another interesting putative new interactor is the ubiquitin protease encoded by the Usp7 gene, which has been shown in flies to interact genetically with PcG genes (47). These and other new proteins shown in Table I and Fig. 2 were not studied any further here but will be the subject of future investigations. Altogether these results provide evidence for a variety of previously unknown Ring1B/Rnf2-interacting proteins that are potential subunits of several multiprotein complexes.

Fbxl10/Jhdm1B-Ring1B/Rnf2-associated Proteins—To start investigating further the new Ring1B/Rnf2 complexes we decided to perform a similar analysis using a biotin-tagged version of one of the new components identified by mass spectrometry. We focused on histone demethylases because they were not known previously to be Ring1B/Rnf2 interactors. Although the two histone demethylases had been confirmed to be part of Ring1B/Rnf2 complexes we chose to tag Fbxl10/Jhdm1B because of its high Mascot value, corresponding to a larger number of matching peptides that spanned a larger fraction of the protein.

Tagged Fbxl10/Jhdm1B-expressing MEL cell clones were established in a fashion identical to that described for Ring1B/Rnf2. Nuclear proteins bound to Fbxl10/Jhdm1B were isolated using streptavidin beads and identified by mass spectrometry. Identified proteins that are shared between the Ring1B/Rnf2 and Fbxl10/Jhdm1B complex(es) are shown in Table II. Of these, most showed high score and sequence coverage values, consistent with their presence as relatively abundant components of the copurified complex(es). The maximum number of peptide matches corresponded to the BcoR corepressor (42) just as for peptides copurified with biotinylated Ring1B/Rnf2, suggesting that it is an important subunit of the Fbxl10/Jhdm1B-Ring1B/Rnf2 complexes. Other subunits shared with PRC1 included a subset of Ring1B/Rnf2-interacting Pcg RING finger proteins like Ring1A/Ring1 and Bmi1/Pcgf4 and its paralog Nspc-1/Pcgf1 (48). Notably neither MBLR/Pcgf6, a component of E2F6.com-1; Mel18/Pcgf2; nor any of the other Pcgf proteins were identified. YAF2, a direct interactor of Ring1B/Rnf2 (27, 49) and a member of E2F6.com-1, also copurified with biotinylated Fbxl10/Jhdm1B. Of the PRC1 chromodomain-containing components, only P3/Cbx8 was found in these complexes. Additional subunits were CK2α1 and CK2α2, the catalytic subunits of CK2, and the F-box-binding protein Skp1. Other subunits were Wdr5, Rbpb4, and HP1γ/Cbx3, histone-binding proteins shared by many other chromatin complexes (25, 44, 45). Finally CtBP1 and CtBP2, subunits of a number of corepressor complexes (50), were also identified, although the matching peptides covered a reduced portion of the total sequence, suggesting a non-stoichiometric association. Altogether the data confirm that the Fbxl10/Jhdm1B-
Ring1B/Rnf2 complex(es) we identified here for the first time are biochemical entities distinct from other previously known Ring1B/Rnf2 complexes. In addition, the lack of LSD1/Aof2 among the Fbxl10/Jhdm1B interactors suggests that these two histone demethylases are subunits of different Ring1B/Rnf2-containing complexes.

Using various available antibodies we validated the interactions of many of the proteins identified by mass spectrometry analysis as copurifying with Fbxl10/Jhdm1B. Fig. 4A shows immunoblots of streptavidin pulldowns confirming the association of Fbxl10/Jhdm1B with proteins that are also present in Ring1B/Rnf2 complexes. One of the components in Table II, the corepressor CtBP1, had also been identified by just one peptide in the mass spectrometry analysis of the proteins bound to biotinylated Ring1B/Rnf2 proteins. Therefore, it was not included in Table I, which only contains proteins identified by two or more peptides. Western blot analysis for LSD1/Aof2 showed no specific association to Fbxl10/Jhdm1B confirming the mass spectrometry data.

The mass spectrometric analysis identified Fbxl10 and BcoR as major Ring1B/Rnf2 partners. To further test interactions among these proteins we used an in vitro binding assay. Sequences encoding Ring1B/Rnf2 and HP1γ/Cbx3 were fused to the GST gene, and the hybrid genes were expressed in E. coli. Conversely the sequences encoding the entire Fboxl10/Jhdm1B and BcoR proteins were transcribed and translated in vitro in the presence of [35S]methionine. The GST fusion proteins, immobilized on glutathione-Sepharose, were incubated with [35S]Fbxl10/Jhdm1B or [35S]BcoR. After washing, the bound proteins were analyzed by SDS-PAGE. Fig. 4B shows specific binding of [35S]Fbxl10/Jhdm1B to GST-Ring1B but not to GST-HP1γ/Cbx3 or GST alone. In contrast, [35S]BcoR did not show any specific binding to these GST

### Table II

| Acc. number | Protein | Mass | Score | Peptides | Coverage |
|------------|---------|------|-------|----------|----------|
| gi|6792788 | BcoR | 190,043 | 2,702 | 101 | 31.6 |
| gi|3760622 | Fbxl10/Jhdm1B | 151,474 | 838 | 34 | 15.2 |
| gi|2239142 | Ring1B/Rnf2 | 38,265 | 425 | 11 | 20.4 |
| gi|1262300 | CK2α1 | 45,322 | 393 | 6 | 18.7 |
| gi|53165 | HP1γ/Cbx3 | 19,911 | 305 | 4 | 24.3 |
| gi|1661134 | Hspa8 | 71,021 | 294 | 4 | 6.5 |
| gi|1583224 | Skp1 | 18,803 | 236 | 5 | 19.6 |
| gi|2494893 | Rbp4 | 52,194 | 236 | 3 | 10.4 |
| gi|7304947 | Ptc3/Cbx8 | 39,893 | 205 | 3 | 13.8 |
| gi|20307063 | Nspc1/Pcgf1 | 29,801 | 178 | 2 | 16.2 |
| gi|2239144 | Ring1A/Ring1 | 39,893 | 178 | 6 | 12.2 |
| gi|6753540 | CK2α2 | 41,360 | 166 | 2 | 8.9 |
| gi|14250247 | Wdr5 | 22,466 | 153 | 2 | 13.1 |
| gi|192205 | Bmi1/Pcgf4 | 30,022 | 114 | 3 | 11.2 |
| gi|40254385 | YAF2 | 19,985 | 105 | 3 | 13.4 |
| gi|2909779 | CtBP1 | 48,084 | 103 | 2 | 3.2 |
| gi|3452507 | CtBP2 | 46,309 | 103 | 2 | 4.8 |

* NCBI accession number.
* Mascot protein score.
* Number of different peptides.
* Fraction of protein recovered in peptides.

**Fig. 4.** Ring1B/Rnf2 interactors also associated with Fbxl10/Jhdm1B complexes. A, immunoblot analysis of streptavidin-bound material from nuclear extracts from control MEL cells (BirA) or from cells expressing biotin-tagged Fbxl10/Jhdm1B (BirA + Fbxl10). Antibodies used in detection by Western blot (indicated to the right of the panel) were against selected proteins copurified with biotinylated Fbxl10/Jhdm1B and identified by mass spectrometry. In, input; B, streptavidin-bound. B, in vitro binding of [35S]Fbxl10/Jhdm1B to the indicated GST fusion proteins. Input (ln) represents ¼ of total [35S]-labeled protein used in the interaction assay. [35S]Fbxl10/Jhdm1B is detected on the gel at the left, and the recombinant proteins, stained with Coomassie Blue, are detected on the gel at the right. Size of molecular mass markers (in kDa) are indicated on the left. IP, immunoprecipitate; WB, Western blot.
fusion proteins (data not shown) suggesting that it forms part of the complex through association with other subunits.

Because Ring1B/Rnf2 acts as a histone E3 ubiquitin ligase, we asked whether specific subunits with a likely role in ubiquitylation, namely Nspc1/Pcgf1 and Fbxl10/Jhdm1B, could promote histone H2A ubiquitylation in vivo. Cells were transfected with plasmids expressing FLAG-H2A and HA-ubiquitin and with plasmids expressing Nspc1/Pcgf1 or Fbxl10/Jhdm1B. A Ring1B/Rnf2 plasmid was used as a control. Histone H2A was isolated by immunoprecipitation with anti-FLAG antibodies under denaturing conditions, and ubiquitylated conjugates were visualized with anti-HA antibodies. Fig. 5 shows that the low levels of monoubiquitylated H2A (H2A-Ub) due to endogenous ubiquitin (FLAG-H2A-Ub) and by ectopic ubiquitin (FLAG-H2A-HAUb). Bottom panels in A and B are Western blots of whole-cell extracts analyzed with anti-Myc antibodies or anti-GST (for GST-Bmi1/Pcgf4) to test for transfection efficiencies. Positions of molecular size markers (kDa) are indicated on the left. L chain, light chain of anti-FLAG antibodies used for immunoprecipitation; IP, immunoprecipitate; WB, Western blot.

Ring1B/Rnf2-associated Proteins

Fig. 5. Components of Fbxl10/Jhdm1B-Ring1B/Rnf2 complexes and H2A monoubiquitylation in vivo. A, Nspc1/Pcgf1; B, Fbxl10/Jhdm1B. Human embryonic kidney 293 cells were transfected with the indicated combinations of plasmids: 0.5 μg (HA-ubiquitin, FLAG-H2A, Myc-Nspc1/Pcgf1, Myc-Ring1B/Rnf2, GST-Bmi1, and Myc-Ring1B1/Rnf70C) or 1.0 μg (Myc-Fbxl10/Jhdm1B). Cells were harvested 48 h after transfection, and extracts were immunoprecipitated with anti-FLAG antibodies under denaturing conditions. Bound proteins were analyzed by Western blot using anti-FLAG antibodies, to visualize FLAG-H2A, or with anti-HA antibodies, to visualize HA-ubiquitin conjugates. In A, better resolution made it possible to distinguish between FLAG-H2A forms modified by endogenous ubiquitin (FLAG-H2A-Ub) and by ectopic ubiquitin (FLAG-H2A-HAUb). Bottom panels in A and B are Western blots of whole-cell extracts analyzed with anti-Myc antibodies or anti-GST (for GST-Bmi1/Pcgf4) to test for transfection efficiencies. Positions of molecular size markers (kDa) are indicated on the left. L chain, light chain of anti-FLAG antibodies used for immunoprecipitation; IP, immunoprecipitate; WB, Western blot.

We then studied the cell localization of some of the novel Ring1B/Rnf2 partners using the osteosarcoma U2-OS cell line in which Ring1B/Rnf2 is localized in the nucleoplasm and also in speckled structures (27) termed Polycomb bodies, which correspond to pericentromeric heterochromatin domains (52).
Skp1 together with a mouse antibody against Ring1B/Rnf2 allowed double labeling for colocalization studies. We observed signals for Fbxl10/Jhdm1B, BcoR, and Nspc1/Pcgf1 in a fine granular pattern throughout the nucleoplasm. Colocalization within this granular pattern was not very obvious. At the same time, we did not observe colocalization of these proteins with Polycomb bodies as seen for Ring1B/Rnf2 (Supplemental Fig. 1). This absence of colocalization with Polycomb bodies has also been observed with other well known Ring1B/Rnf2-interacting partners such as RYBP (27) and may indicate the partitioning of distinct Ring1B/Rnf2 protein interactions within discrete nuclear subcompartments.

**DISCUSSION**

In this study, we used an efficient *in vivo* biotinylation tagging approach to identify a number of new interacting partners of Ring1B/Rnf2, a key PcG protein member. Some of these novel Ring1B/Rnf2 partners contain domains associated with protein modification by phosphorylation, demethylation, or ubiquitylation, thus providing new clues to the basis of PcG function.

New interactors were isolated using a strategy based on the *in vivo* biotinylation of Ring1B/Rnf2 and their binding to streptavidin. Because of the much higher affinity of the binding of streptavidin to biotin, compared with other epitope tag (FLAG and HA) approaches, multimeric complexes containing biotinylated Ring1B/Rnf2 can be efficiently isolated in a single step. As also seen before, the higher affinity and simpler purification procedure ensures the isolation of complexes found at low concentration and of components loosely associated to complexes that otherwise might have been lost in more conventional serial purification steps or even tandem affinity purifications (53). The combination of such a purification strategy and mass spectrometric proteomics analysis results in a very sensitive, high throughput approach leading to the identification of a large number of polypeptides. Most contaminants that bind to streptavidin can be removed by filtering proteins identified in a parallel purification using extracts from cells that express BirA but not the tagged protein. Our conservative analysis, including only proteins identified with high confidence, revealed 50 polypeptides as copurifying with tagged Ring1B/Rnf2 of which at least 20 proteins, including proteins of no known function, had not been reported previously to interact with Ring1B/Rnf2. As expected, these proteins are subunits of a mixture of Ring1B/Rnf2 complexes. In addition, using the new Ring1B/Rnf2 interactor Fbxl10/Jhdm1B we showed that the biotinylation tagging approach is simple enough so that once a new interactor is identified it can in turn be tagged and affinity-purified, and a new screen can be rapidly initiated so that the complexes mixture can begin to be solved as we have done with the new interactor Fbxl10/Jhdm1B. The identity of the new interactors, a selected subset of which is also depicted diagrammatically in Fig. 6A, makes it unlikely that these complexes are variants of previously identified Ring1B/Rnf2 complexes. Taking the previously known information on Ring1B complexes together with our new data, we propose a (minimum) number of putative Ring1B/Rnf2 complexes shown schematically in Fig. 6B.

**Known Ring1B/Rnf2 Complexes**—Previous work in a number of laboratories has reported the participation of Ring1B/Rnf2 in several multiprotein complexes. The first such complex to be described was PRC1 (Fig. 2C), which was isolated from human tissue culture cells expressing FLAG-tagged M33/Cbx2 or Bmi1/Pcgf4 and contained many PcG protein subunits (19). All of the previously described PRC1 components were also identified in our analysis, thus confirming the presence of PRC1 in erythroid cells. A second Ring1B-containing complex is the E2F6.com-1 complex isolated from HeLa cells expressing a FLAG-tagged derivative of the E2F6 repressor (which is not a PcG member). The E2F6.com-1 complex contained, in addition to Ring1B/Rnf2, DNA-binding proteins (the heterodimers E2F6-DP1 and Mga-Max), the heterodimeric histone H3 Lys-9 methyltransferase EuHMTase/Euhmt1-G9a/Emht2 and the H3 trimethylated Lys-9-binding protein HP1γ (25). All of these subunits were also identified in our characterization of Ring1B/Rnf2 complexes thus also confirming the presence of the E2F6.com-1 complex in erythroid cells.

Ring1B/Rnf2, together with a subset of PRC1 components, was also found in complexes isolated from different nuclear fractions. For instance, Ring1B/Rnf2 copurified with Ring1A/Ring1, Bmi1/Pcgf4, Phc2, and two other unidentified proteins in a complex isolated following histone E3 ligase activity of a preparation derived from the insoluble nuclear fraction of HeLa cells (13). Also Ring1B/Rnf2, together with Ring1A/Ring1, Bmi1/Pcgf4, Phc2, Pc3/Cbx8, and other polypeptides, was found in the complex(es) isolated from a preparation of centromeric chromatin with an anti-centromere protein-A (CENP-A) antibody (54). We did not identify any centromeric proteins in our analysis perhaps due to the fractionation properties of such a complex, which may remain insoluble in the conditions we used to extract nuclear proteins.

Perhaps unexpectedly, Ring1B/Rnf2 was also identified as a subunit of a complex containing components typically associated with transcriptional activation such as the histone H3 Lys-4 methyltransferase Mll1 and the histone H3 Lys-9 deacetylase MOF1. This Mll1-MOF1 complex was isolated from cells expressing FLAG-tagged variants of Wdr5 or Rbbp5, two WD domain-containing proteins that associate with histones. However, we consistently failed to detect Mll1, MOF, or any other subunits of these activating complexes (45). Although some components of this Mll1-MOF complex, such as Mga, Myn (the human ortholog of Max), E2F6, and DP1, were identified the fact that they are also part of the E2F6.com-1 complex led us to conclude that a form of Ring1B/Rnf2-containing Mll1-MOF complex is not present in the cells we used.

**New Ring1B/Rnf2 Complexes Containing Histone Demethylases**—Among the new Ring1B/Rnf2 interactors iden-
fied here are LSD1/Aof2 and Fbxl10/Jhdm1B, two histone H3 demethylases (40, 41). LSD1/Aof2 catalyzes a flavin adenine dinucleotide-dependent amino oxidase demethylation of Lys-4 of histone H3, and it is a subunit of a Ring1B/Rnf2 complex different from the one containing Fbxl10/Jhdm1B. The LSD1/Aof2-Ring1B/Rnf2 complex(es) remain to be characterized fully, but preliminary evidence suggests it is a new entity not identified previously perhaps because it contains a relatively small fraction of Ring1B/Rnf2 (and most likely LSD1/Aof2 too) in the cell. In fact, this histone demethylase has been identified in a variety of subsets of the Braf-HDAC (BHC) repressing complexes (55–58), but our analysis of Ring1B/Rnf2 complexes did not detect either Braf35 or BHC80, two of the components of core BHC complexes (55, 58). Thus, our data suggest that the LSD1/Aof2-Ring1B/Rnf2 complex is a novel complex not related to BHC.

BcoR and Fbxl10/Jhdm1B are among the most abundant Ring1B/Rnf2 interactors identified with the highest confidence, and their association has been validated by coimmunoprecipitation studies; hence we call this the Fbxl10-BcoR complex. Fbxl10/Jhdm1B is a multidomain protein whose jumonji C domain demethylates Lys-36 of histone H3. The Fbxl10/Jhdm1B protein has a CXXC zinc finger motif, plant homeodomain, F-box domains, and five leucine repeats (41). Fbxl10/Jhdm1B has not been associated to any other previously described complexes, and only a heterodimer made of its paralog Fbxl11/JmjD1A and an unidentified subunit was purified in a search for histone demethylase complexes (41). None of the peptides identified in the proteins that copurify with biotin-Ring1B/Rnf2 matched Fbxl11/JmjD1A most likely due to its low expression levels in the hematopoietic cell line we used in our studies. BcoR was the protein with the highest Mascot score in the Ring1B/Rnf2 and Fbxl10/Jhdm1B protein complex purifications. It is a little known protein with ankyrin repeats as the only identifiable protein motifs that is mutated in Lenz microphthalmia with a role in early embryogenesis (59). It is a transcriptional repressor identified by its direct association with Bcl6, a transcriptional factor frequently translocated in lymphomas (42). However, our identification of BcoR as part of a Fbxl10/Jhdm1B-Ring1B/Rnf2 complex associated with histone demethylation and ubiquitination activities strongly suggests alternative mechanisms for the molecular basis of its functions.

Other components not previously identified as Ring1B/Rnf2

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**Fig. 6.** Ring1B/Rnf2 complexes and select new interactors. A, schematic representation of selected new subunits of murine Ring1B/Rnf2 complexes indicating their protein domains as determined by the Simple Modular Architecture Research Tool (SMART) program. Putative functions related to protein modification or chromatin recruitment are indicated below the protein motif. B, schematic representation of the previously identified Ring1B/Rnf2 complexes PRC1 and E2F6.com-1 and of new complexes proposed in this study. The subunits depicted and stoichiometry cannot be accurate because these complexes have been inferred from a combination of mass spectrometry and immunoidentification studies. A subset of components is indicated for clarity, and the unknown components are shown by a question mark. PHD, plant homeodomain; JmjC, jumonji C; LRR, leucine-rich repeat; H3K4, H3 Lys-4; H3K36, H3 Lys-36; SWIRM, SWI3, RSC8, and Moira domain; S_TKc, serine/threonine protein kinases catalytic domain.
interactors or subunits of other PcG complexes include Skp1, an F-box-interacting protein (43); the subunits of casein kinase 2; and the Bmi1/Pcgf4 paralog Nspc1/Pcgf1, a transcriptional repressor (31) not known to be part of any previously characterized complex. Some Fbxl10-BcoR components are shared with other Ring1B/Rnf2 complexes, such as the PRC1 subunits Ring1A/Ring1, Bmi1/Pcgf4, and Pc3/Cbx8 (19) or the E2F6.com-1 subunits Ring1A/Ring1 and HP1γ/Cbx3 (25). The assembly of Fbxl10-BcoR complex(es), the associations among its various subunits, and its functional significance remain to be characterized but are presently under investigation.

Additional New Ring1B/Rnf2 Complexes—ENL/Mllt1, a paralog of Af9/Mllt3 (both encoded by genes that translocate with Mllt1 in leukemias), was identified in our analysis among the proteins that copurified with Ring1B/Rnf2 but not with Fbxl10/Jhdm1B. Because of sequence conservation it is likely that ENL/Mllt1 binds Pc3/Cbx8 as has been described for Af9/Mllt3 (60, 61). Moreover the fact that Af9/Mllt3-Pc3/Cbx8 are able to form a trimeric complex together with Ring1B/Rnf2 may explain why ENL/Mllt1 was copurified with Ring1B/Rnf2 (60) and strongly suggests that they form part of a distinct Ring1B/Rnf2 complex. Sequence coverage and peptide numbers for these proteins as shown in Table I would indicate that the Ring1B/Rnf2-Pc3/Cbx8-ENL/Mllt1 may form a multiprotein complex of lower abundance and may also contain additional, as yet uncharacterized components that are very likely included in Table I. A clue as to the functional activity of these complexes may be found in the phenotypes of Af9/Mllt3-deficient mice, namely the anteriorization of the rostral boundary of expression of a Hox gene and the alterations of the axial skeleton (62), which are reminiscent of those seen in PcG mutants.

Regulatory Roles Suggested by the Composition of the New Ring1B/Rnf2 Complexes—The most interesting finding of this study is that the newly identified interactors significantly expand the putative regulatory activities involving Ring1B/Rnf2 and provide new clues to its recruitment to chromatin and the molecular basis of its functions.

Besides its ubiquitin E3 ligase activity for histone H2A Lys-119, Ring1B/Rnf2 had up to now only been associated with a histone H3 Lys-9 methyltransferase activity in the context of the E2F6.com-1 complex (25). In addition, we also identified in our analysis the ets related gene ERG associated protein with SET domain (ESET)/Sethb1-mAM H3 Lys-9 trimethyltransferase complex as copurifying with Ring1B/Rnf2 complexes, although we did not characterize this further. We also provide important evidence that Ring1B/Rnf2 forms distinct complexes with the histone demethylases LSD1/Aof2 and Fbxl10/Jhdm1B, which demethylate H3 Lys-4 and H3 Lys-36, respectively (63, 64). Whereas methylation of these histone H3 lysine residues has been associated with transcriptional activation (65), there is recent evidence implicating histone demethylases in repression (66). Taken together, Ring1B/Rnf2 complexes have the capacity to ubiquitinate H2A Lys-119, to methylate H3 Lys-9 (Ehmt1-Ehmt2 and maybe Setdb1), and to demethylate H3 Lys-4 (Lsd1/Aof2) and/or H3 Lys-36 (Fbxl10/Jhdm1B) most likely in combinations of (distinct and/or overlapping) activities and presumably binding to different gene target subsets.

Histone H2A monoubiquitylation by complexes containing Ring1B/Rnf2 requires the presence of the PcG RING finger protein Bmi1/Pcgf4 (15, 51). In contrast, if the related Mel18/Pcgf2 protein associates with Ring1B/Rnf2, the complex no longer ubiquitylates nucleosomal H2A at least in vitro (51). Thus, the exchange of RING finger partners appears to be a regulatory strategy to modulate this repressive modification of histone H2A. It would be of interest to know whether the E2F6.com-1 complex, which contains MBLR/Pcgf6 instead of Bmi1/Pcgf4, has an H2A E3 ligase activity. Interestingly the Fbxl10-BcoR complex we identified also contains Bmi1/Pcgf4 and Nspc1/Pcgf1 (but not MBLR/Pcgf6 or Mel18/Pcgf2). Although we cannot discriminate yet whether they heterodimerize independently to Ring1B/Rnf2 in different subcomplexes or are all part of a single complex, one implication of our observations is that these apparent specific associations between Ring1B/Rnf2 and other RING finger protein in different complexes may modulate their overall H2A ubiquitin E3 ligase activity and specificity.

On the other hand, the fact that F-box proteins serve as specificity factors for a family of ubiquitin protein ligases composed of Skp1, RING finger E3 ligases, and other proteins (43) suggests that the Fbxl10-BcoR complex may also be involved in ubiquitylation reactions. Because H2A monoubiquitylation does not require, at least in vitro, an F-box protein, it is possible that the targets of these ubiquitylation reactions are substrates not yet known. Substrate recognition by F-box proteins such as Fbxl10/Jhdm1B usually occurs through binding of the phosphorylated target protein to the leucine repeats of the F-box protein (67). Therefore, the presence of the catalytic subunits of the CK2 among the components of the Fbxl10-BcoR complex may be functionally relevant in such a hypothetical ubiquitylation modification. In a more general way, CK2 may regulate the activity of the Fbxl10-BcoR and other Ring1B/Rnf2 complexes by modifying proteins with which the complexes (even subunits of the complexes) may associate; some of these proteins, like Bmi1/Pcgf4, MBLR/Pcgf6, or Nspc1/Pcgf1, are known to be phosphoproteins (31, 68–70).

Among the new Ring1B/Rnf2 partners are proteins that may facilitate chromatin targeting of the various (sub)complexes. The previously known complexes contain subunits that may account for Ring1B/Rnf2 recruitment by means of binding to methylated Lys-27 or Lys-9 of histone H3 as well as through DNA-binding proteins (25). The new complexes may use BcoR corepressor as a recruiting module through binding of the sequence-specific DNA-binding protein Bcl6 (42). An additional targeting possibility could be through the CXXC zinc
finger of Fbx10/Jhd1B given the sequence conservation with the DNA-binding zinc finger of Mi1 (71). Finally new motifs able to interact with modified histone tails, such as Wdr5 (72) or the plant homeodomain finger (73) of Fbx10/Jhd1B, may also contribute to the recruitment of Ring1B/Rnf2 complexes to specific targets in chromatin.

In summary, we have widened the set of multiprotein complexes containing the Polycomb group protein Ring1B/Rnf2. The new interactors contain protein motifs whose enzymatic activities and binding properties would expand the regulatory potential and gene target diversity of Ring1B/Rnf2 complexes in terms of recruitment to and modification of chromatin. More generally, we have shown that the efficient isolation with streptavidin of biotin-tagged proteins and their associated partners followed by mass spectrometry identification constitutes a high throughput approach for the interpretation of molecular function of proteins that, like Ring1B/Rnf2, are shared by a variety of protein complexes.

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Addendum—While this manuscript was under revision, Gearhart et al. (Gearhart, M. D., Corcoran, C. M., Wamstad, J. A., and Bardwell, V. J. (2006) Polycomb group and SCF ubiquitin ligases are found in a novel BCOR complex that is recruited to BCBL6 targets. Mol. Cell. Biol. 26, 6880–6889) reported the purification of a complex from human tissue culture cells containing a subset of the subunits we identified as copurifying with Fbx10 and Ring1B. The authors also show that such a complex plays a role in H2A monoubiquitylation.

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‡‡ Present address: Inst. of Molecular Oncology, Biomedical Sciences Research Center Alexander Fleming, P. O. Box 74145, 16602 Varkiza, Greece.

§§ To whom correspondence should be addressed. Tel.: 34-91-837-3112; Fax: 34-91-536-0432; E-mail: mvidal@cib.csic.es.

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