

## Polycomb Group and SCF Ubiquitin Ligases Are Found in a Novel BCOR Complex That Is Recruited to BCL6 Targets†

Micah D. Gearhart,<sup>1</sup> Connie M. Corcoran,<sup>1</sup> Joseph A. Wamstad,<sup>2</sup> and Vivian J. Bardwell<sup>1,2\*</sup>

Department of Genetics, Cell Biology and Development and Cancer Center<sup>1</sup> and Molecular, Cellular, Developmental Biology and Genetics Graduate Program,<sup>2</sup> University of Minnesota, Minneapolis, Minnesota 55455

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**The corepressor BCOR potentiates transcriptional repression by the proto-oncoprotein BCL6 and suppresses the transcriptional activity of a common mixed-lineage leukemia fusion partner, AF9. Mutations in human BCOR cause male lethal, X-linked oculofaciocardiodental syndrome. We identified a BCOR complex containing Polycomb group (PcG) and Skp–Cullin–F-box subcomplexes. The PcG proteins include RING1, RYBP, NSPC1, a Posterior Sex Combs homolog, and RNF2, an E3 ligase for the mono-ubiquitylation of H2A. BCOR complex components and mono-ubiquitylated H2A localize to BCL6 targets, indicating that the BCOR complex employs PcG proteins to expand the repertoire of enzymatic activities that can be recruited by BCL6. This also suggests that BCL6 can target PcG proteins to DNA. In addition, the BCOR complex contains components of a second ubiquitin E3 ligase, namely, SKP1 and FBXL10 (JHDM1B). We show that BCOR coimmunoprecipitates isoforms of FBXL10 which contain a JmjC domain that recently has been determined to have histone H3K36 demethylase activity. The recruitment of two distinct classes of E3 ubiquitin ligases and a histone demethylase by BCOR suggests that BCOR uses a unique combination of epigenetic modifications to direct gene silencing.**

The *BCL6* gene encodes a sequence-specific transcriptional repressor (17, 23, 65) that is highly expressed in germinal center B cells. Germinal centers are maturation sites within lymphoid tissues where antigen-stimulated B cells proliferate, hypermutate their immunoglobulin (Ig) genes, undergo Ig class switch recombination, and give rise to progeny plasma cells that produce antibodies with high affinity for antigen (63). *BCL6* plays a central role in this process, modulating the transcription of genes involved in lymphocyte activation, cell cycle arrest, apoptosis, and differentiation (5, 22, 49, 54, 59–61, 66, 75, 76). Deregulated expression of *BCL6* in germinal center B cells plays an oncogenic role in non-Hodgkin's lymphomas (4, 16), presumably by inhibiting apoptosis and enhancing proliferation.

*BCL6* belongs to a subclass of zinc finger proteins with a POZ/BTB domain at the N terminus and Cys<sub>2</sub>-His<sub>2</sub> zinc fingers at the C terminus (3, 70, 87). *BCL6* can interact with a variety of corepressors via several domains, including the POZ domain, a central repression domain, and the zinc fingers (19, 24, 25, 29, 36, 45, 82). The central domain of *BCL6* recruits the corepressor MTA3 and its associated HDAC-containing chromatin remodeling complex (Mi-2/NuRD) (29). Importantly, MTA3 knockdown in B cells derepresses *BCL6* targets that are upregulated upon differentiation into plasma cells (29). The POZ domain of *BCL6* interacts with NCOR, SMRT, and BCOR in a mutually exclusive fashion (37). In *BCL6*-positive lymphoma cells, peptides that bind to the POZ domain of *BCL6* and block interactions with NCOR, SMRT, and BCOR

cause apoptosis and cell cycle arrest. The peptides do not, however, cause plasma cell differentiation (61). This suggests that the functions of *BCL6* may be segregated among different corepressors, with NCOR, SMRT, and/or BCOR silencing genes involved in apoptosis and cell cycle control and MTA3 silencing genes involved in plasma cell differentiation (29, 51, 61).

While the highly related NCOR and SMRT corepressors are found in complexes containing HDAC3 and the JmjC domain protein JMJD2A (32, 48, 80, 86), the repression mechanisms used by the unrelated corepressor BCOR are less well understood (37). We previously identified BCOR in a yeast two-hybrid screen, and aside from three ankyrin repeats it contains no other recognizable domains. In transient-transfection luciferase reporter assays, BCOR potentiates *BCL6* repression, and BCOR tethered to DNA can repress transcription independently of *BCL6*. Certain isoforms of BCOR, generated by use of an alternative splice acceptor site, can interact with AF9 and suppress its transcriptional activation. In humans, BCOR plays multiple important roles in development, as evidenced by the complex phenotypes seen in oculofaciocardiodental (OFCD) syndrome females heterozygous for mutations in this X-linked gene. Nevertheless, specific target genes regulated by BCOR have not yet been identified.

To help elucidate the mechanisms by which BCOR represses transcription, we purified the BCOR complex and performed biochemical and functional analyses. We found that the BCOR complex contains Polycomb group (PcG) proteins, including a histone H2A ubiquitin E3 ligase and an SCF ubiquitin E3 ligase. BCOR is also able to associate with a JmjC domain histone H3 K36 demethylase-containing protein. We find that the BCOR complex and the mono-ubiquitylated form of histone H2A localize to several *BCL6* targets, including *P53* (*TP53*) and *Cyclin D2* (*CCND2*), in lymphoma cells. The enzymatic activities of the BCOR complex, through epigenetic

\* Corresponding author. Mailing address: Department of Genetics, Cell Biology and Development, 6-160 Jackson Hall, 321 Church St. SE, Minneapolis, MN 55455. Phone: (612) 626-7028. Fax: (612) 626-7031. E-mail: bardw001@umn.edu.

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modifications of chromatin, provide a mechanism for silencing of BCL6 targets that is distinct from the HDAC and chromatin remodeling activities of the SMRT, NCOR, and MTA3 complexes (29, 32, 48, 80).

#### MATERIALS AND METHODS

**Cloning, plasmids, and antibodies.** BCOR, NSPC1, RING1, and RNF2 were cloned from preexisting clones (37, 53) or the expressed sequence tag (EST) clones referenced by accession numbers BI457391, BC002922, and BC012583, respectively. FBXL10 was cloned by PCR amplification from ESTs BM473316 and BC008735 and cDNA from HEK293 cells. Isoform A of human BCOR contains all possible coding exons. Isoform C of human BCOR is generated by use of an alternate splice acceptor site and results in a protein that is shorter by 34 amino acids. BCOR(A) (1-1476) and BCOR(C) (1-1442) mimic a splice acceptor mutation observed in OFCD patients that is presumed to result in an in-frame stop codon six amino acids C-terminal to residues 1476 and 1442 in isoforms A and C, respectively.

N-terminally Flag-tagged and C-terminally hemagglutinin (HA)-tagged BCOR isoform A and N-terminally Flag-tagged human NSPC1 were cloned into pZOME-1N (Cellzome) to create ProtA<sub>2</sub>-TEV-CBP-flag-BCOR(A)-HA- and ProtA<sub>2</sub>-TEV-CBP-flag-NSPC1-encoding retroviruses. All open reading frames or deletions were cloned into a modified version of pGEX-2T (GE HealthCare), T7plink-NTAG, EFplink2 (provided by Richard Treisman, Cancer UK, London, England), or EFplink-Flag (provided by Caroline Hill, Cancer UK, London, England) for bacterial, *in vitro*, or mammalian expression. All nucleotides of inserts were verified by sequencing. Rabbit polyclonal antibodies were raised against BCOR, FBXL10, and NSPC1 using glutathione *S*-transferase (GST) fusions of human BCOR(C) (1035-1230), human FBXL10 (726-817), and human NSPC1 (128-189) and subsequently affinity purified.

**Complex purification.** Stable cell lines (BCOR) or pools (NSPC1 and GFP-puro) of HeLa S3 or HEK293 cells were generated by infection with ProtA<sub>2</sub>-TEV-CBP-flag-BCOR(A)-HA, ProtA<sub>2</sub>-TEV-CBP-flag-NSPC1, and green fluorescent protein (GFP) retroviruses. Cells were cultured in Joklik's medium (Biosource) or minimal essential medium (Invitrogen) with 5% calf serum (Biosource) and 1  $\mu$ g/ml puromycin. Nuclear extracts (26) were supplemented with 0.1% NP-40, 0.1% Tween, 2.0 mM EGTA, and 0.5 mM EDTA and incubated with M2-agarose (Sigma) overnight. Beads were washed in 50 mM Tris pH 8.0, 1.0 mM MgCl<sub>2</sub>, 1.0 mM imidazole, 0.1% NP-40, 20% glycerol, 2.0 mM dithiothreitol (DTT), 2.0 mM phenylmethylsulfonyl fluoride (PMSF), 2.0 mM EGTA, 0.5 mM EDTA, protease inhibitor cocktail (Complete EDTA-free; Roche), and either 350 mM (BE-350) or 700 mM KCl (BE-350). Complexes were eluted with ~30% yields using 2 mg/ml Flag peptide, substituting 2.0 mM CaCl<sub>2</sub> for EGTA and EDTA in the 350 mM KCl buffer (BC-350) and recaptured with calmodulin-Sepharose (GE HealthCare). Calmodulin beads were washed with BC-350 and BC-700 and eluted with ~30% yields in BE-350 for an overall yield of ~10%. For mass spectrometry analysis, eluants were precipitated with 0.02% deoxycholate and 20% trichloroacetic acid overnight, resolved on a 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) gel, and visualized by silver staining (SilverQuest; Invitrogen). Excised bands were reduced, alkylated, trypsinized, and extracted for analysis by matrix-assisted laser desorption/ionization-time of flight analysis (QSTAR XL; Applied Biosystems). Proteins were identified using Mascot (58), and amenable peptides were confirmed by tandem mass spectrometry fragmentation. Alternatively, eluants were resolved on a Superose 6 gel filtration column in BE-350 at a yield of approximately 2% and deoxycholate-trichloroacetic acid precipitated for SDS-PAGE analysis.

**Immunoprecipitations and *in vitro* protein interactions.** Immunoprecipitations and GST pull-down assays were performed as described elsewhere (37) with minor modifications. Approximately 60 million Ramos or HEK293 cells were used for immunoprecipitations of the endogenous BCOR complex (see Fig. 3B, below) with 2 to 4  $\mu$ g of normal rabbit IgG (Santa Cruz Biotechnology) or BCOR antibody. Rabbit TrueBlot horseradish peroxidase (eBiosciences) was used to visualize NSPC1 in immunoprecipitation experiments (see Fig. 2A and 3B, below). Glutathione *S*-transferase fusion proteins were expressed in BL21(DE3) *Escherichia coli*. Protein concentrations were normalized by Coomassie staining on SDS-PAGE gels. Proteins were translated *in vitro* with [<sup>35</sup>S]methionine (Amersham) and the T7 TNT quick-coupled system (Promega). Two  $\mu$ l of radiolabeled proteins was preincubated with 110  $\mu$ l binding buffer (20 mM HEPES pH 7.9, 100 mM KCl, 2 mM EDTA, 0.1% NP-40, 10% glycerol, 0.5% nonfat dry milk, 5 mM DTT) plus 30  $\mu$ l of blocked glutathione agarose (50% slurry) for 30 min at room temperature and centrifuged at 1,000  $\times$  *g* for 3 min to pellet beads. Supernatant was transferred to a clean tube and centrifuged

at 21,000  $\times$  *g* for 10 min. One hundred  $\mu$ l of the high-speed supernatants was transferred to a new tube. Fifteen microliters of GST-fusion beads (a 50% slurry containing a total of approximately 3.0  $\mu$ g of full-length GST fusion protein) was added, and the mixtures were incubated for 1 h at room temperature. The beads were centrifuged at 1,000  $\times$  *g* for 3 min, washed twice with binding buffer and once with binding buffer minus nonfat dry milk, boiled in SDS loading buffer, resolved by SDS-PAGE, and imaged by autoradiography.

**siRNAs transfections.** Nonspecific small interfering RNA (siRNA; Dharmacon D-001210-01) or a pool of siRNAs targeting human FBXL10 (Dharmacon M-014930) was transfected into HEK293 cells at a concentration of 100 nM using Dharmafect 1 lipid reagent and harvested using TRIZOL reagent (Invitrogen).

**Chromatin immunoprecipitation.** Log-phase Ramos cells were cross-linked with 1% formaldehyde for 10 min at 37°C before quenching with 0.125 M glycine for 5 min. Cells were washed twice in phosphate-buffered saline (PBS) containing Complete protease inhibitors (Roche) and 1.0 mM PMSF. Cells were resuspended in cell lysis buffer containing 10 mM HEPES pH 7.9, 0.5% NP-40, 1.5 mM MgCl<sub>2</sub>, 10 mM KCl, 0.4 mM DTT, protease inhibitors (Complete; Roche), and 1.0 mM PMSF for 10 min on ice. Cells were centrifuged for 5 min at 1,500  $\times$  *g* at 4°C and resuspended in nuclear lysis buffer containing 20 mM HEPES pH 7.9, 25% glycerol, 0.5% NP-40, 0.420 M NaCl, 1.5 mM MgCl<sub>2</sub>, 0.5 mM EDTA, protease inhibitors (Complete; Roche), and 1.0 mM PMSF for 20 min on ice. DNA was sheared using a sonic dismembrator (model 500; Fisher Scientific) equipped with a double-stepped microtip at 30% power for 5 s on, 10 s off for a total of 8 min of "on" time to generate fragments between 500 and 6,000 bases. Lysates were centrifuged for 10 min at 21,000  $\times$  *g* at 4°C. Supernatants were diluted into an equal volume of dilution buffer containing 20 mM Tris-HCl pH 7.9, 1% Triton X-100, 2 mM EDTA, 50 mM NaCl, protease inhibitors (Complete; Roche), and 1.0 mM PMSF and precleared for 1 h at 4°C with protein A-Sepharose (GE HealthCare) that had been blocked with 0.5 mg/ml bovine serum albumin and 200  $\mu$ g/ml sheared salmon sperm DNA. Diluted and cleared extracts corresponding to 2  $\times$  10<sup>6</sup> Ramos cells were incubated with each of the following antibodies: no-antibody control, 2  $\mu$ g normal rabbit IgG (Santa Cruz sc-2027), 2  $\mu$ g BCL6 (N3; sc-858; Santa Cruz), 2  $\mu$ g rabbit polyclonal antibodies generated against human BCOR, 4  $\mu$ g SKP1 (610530; BD Biosciences), 4  $\mu$ g mono-ubiquitylated H2A (Ub-H2A; E6C5; Upstate), 25  $\mu$ l RNF2 monoclonal hybridoma (1), or 4  $\mu$ g RYBP (AB3637; Chemicon). Rabbit antibodies against mouse IgG or IgM (Upstate) were added to samples 1 h before immune complexes were recovered using blocked protein A-Sepharose. Beads were washed with low salt (20 mM Tris-HCl pH 8.0, 1% Triton X-100, 2 mM EDTA, 150 mM NaCl), high salt (20 mM Tris-HCl pH 8.0, 1% Triton X-100, 2 mM EDTA, 500 mM NaCl), and LiCl (10 mM Tris, pH 8.0, 1% NP-40, 1% deoxycholic acid, 0.25 M LiCl, 1 mM EDTA), and twice with TE (10 mM Tris-HCl pH 8.0, 1 mM EDTA) before being eluted from beads with freshly prepared elution buffer (100 mM NaHCO<sub>3</sub>, 1% SDS). After cross-links were reversed in the presence of 200 mM NaCl at 65°C for 5 h, samples were treated with RNase and proteinase K and DNA fragments were purified using a PCR Clean-Up column (QIAGEN). Genomic DNA was quantitatively amplified from each sample in duplicate using SYBR Green QPCR reagent (Stratagene) on an MX3000P thermocycler (Stratagene) and the following primers: BCL6, 5'-GCAGTGGTAAAGTCCGAAG C-3' and 5'-AGCAACAGCAATAATCACCTG-3'; Cyclin D2, 5'-GGGGGAG CCGGACCTAATC and 5'-CTCGCCCCCTGACTTGTCTGAC-3'; P53, 5'-GG AGAAAACGTTAGGGTGTG-3' and 5'-GCTTTTGCCTTGTCTCAG-3'; P53 5' region, 5'-GTTTTTCAGCCCTTTCCTCC-3' and 5'-GTGCCCTTCC TCTCCTC-3'.

**H2A ubiquitylation assays.** Eluants from the tagged NSPC1 purification were incubated with rabbit E1, Ubc5c, Flag-tagged ubiquitin, and oligonucleosome substrate as described previously (78).

**TS cell culture and immunofluorescence.** Trophoblastic stem (TS) cells were cultured and prepared for immunofluorescence as described previously (21). Mouse embryonic fibroblasts (MEFs) were irradiated and plated at 2  $\times$  10<sup>6</sup> cells/10-cm dish. TS cells were grown on MEFs in TS medium (RPMI 1640 with 20% fetal bovine serum, 1 mM sodium pyruvate, 10  $\mu$ M  $\beta$ -mercaptoethanol, 2 mM L-glutamine) plus 25 ng/ml FGF4, and 1  $\mu$ g/ml heparin was added on the day of use as described previously (72). Cells were cultured in a 5% CO<sub>2</sub> incubator at 37°C and passaged 1:15 every 4 days. For immunofluorescence experiments, TS cells were grown on glass coverslips for 2 days from a 1:10 passage. Cells were rinsed three times with PBS and fixed with 2% paraformaldehyde in PBS for 15 min at 25°C. Cells were then rinsed three times with PBS and permeabilized with 0.4% Triton X-100. Cells were rinsed three times with PBS and blocked with 0.2% fish skin gelatin in PBS for 30 min at 25°C. Cells were then incubated with primary antibodies in 5% normal goat serum in PBS for 1 h at 25°C in a humid chamber. Dilutions of antibodies were as follows: BCOR, 1:25; RNF2 (1), 1:2. Post-antibody incubation, cells were washed three times for 3 min each with 0.2%

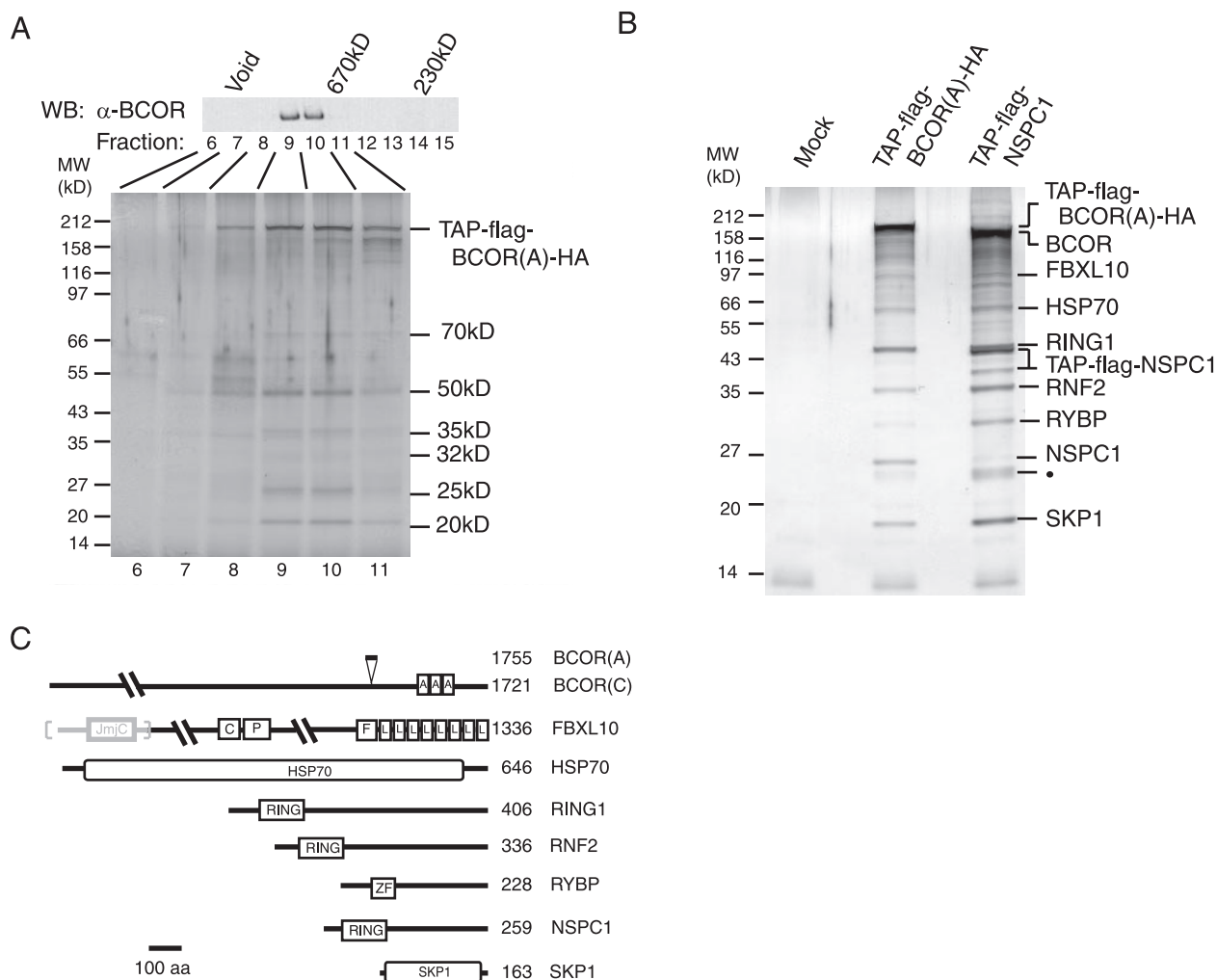


FIG. 1. BCOR and NSPC1 are obligate partners in an ~800-kDa complex. (A) Western blotting and silver stain analysis of size-exclusion fractionation (Superose 6) of the BCOR complex purified from a tagged BCOR HeLa S3 cell line. Bands at 20 kDa, 25 kDa, 32 kDa, 35 kDa, 50 kDa, and 70 kDa and tagged BCOR elute as a tightly migrating ~800-kDa complex but at a 2% yield. (B) Silver stain analysis and protein identifications from purifications of HEK293 cells stably expressing untagged GFP (Mock), TAP-Flag (FL)-tagged BCOR, or TAP-FL-tagged NSPC1. Bands immediately below BCOR and at 40 kDa were identified as BCOR and tagged NSPC1 degradation products, respectively. All identifications were confirmed by tandem mass spectrometry fragmentation except for the four observed peptides from RYBP, which were not amenable to fragmentation. HSP70 has been observed in other PcG complexes (46). The band at 24 kDa (●) was present in both purifications, but insufficient data prevented definitive identification. (C) Schematic diagram of proteins found in the BCOR complex. The amino terminus of FBXL10 (shown in gray) containing the JmjC domain was not detected by mass spectrometry. RYBP has fewer amino acids than NSPC1 but has been shown to run aberrantly at 32 kDa in previous studies (30). The following abbreviations were used: A, ankyrin; C, CXXC; P, PHD; F, F-box; L, leucine-rich repeat; ZF, zinc finger. Two alternatively spliced isoforms of BCOR (A and C) used in this study differ by 34 amino acids.

fish skin gelatin in PBS. Cells were then incubated with appropriate secondary antibodies (GAR-568 1-11036 and GAM-488 A-11029; Molecular Probes) for 30 min at 25°C in a humid chamber. Cells were then washed three times for 3 min each with 0.2% fish skin gelatin in PBS, followed by two rinses with PBS. Cells were finally mounted in PermaFluor antifade reagent (Immunon) and stored at 4°C.

## RESULTS

### Purification and identification of BCOR complex components.

To help reveal BCOR-dependent repression mechanisms, we performed biochemical analyses of the BCOR complex. BCOR-interacting proteins were identified from HeLa S3 and HEK293 cell lines stably expressing multiply epitope-tagged BCOR at an approximately 1:1 ratio to the endogenous protein. Although

these cells do not express BCL6, they can easily be produced in large quantities suitable for biochemical purification. Size-exclusion chromatography of a two-step affinity-purified BCOR complex indicates that HeLa S3-tagged BCOR is in an ~800-kDa complex and coelutes with at least six protein bands (Fig. 1A). Mass spectrometric analysis (see Table S1 in the supplemental material) of copurifying bands after a two-step purification from HEK293 cells identified HSP70 and the PcG proteins NSPC1 (PCGF1), RING1, and RNF2 (Fig. 1C), which are homologs of proteins found in the PcG PRC1 complex (46). We also identified SKP1 (SKP1A, isoform b), a component of Skp1-Cullin-F-box (SCF) E3 ubiquitin ligases (14). Additional bands at 24 kDa, 32 kDa, and 97 kDa were specifically observed in the tagged BCOR

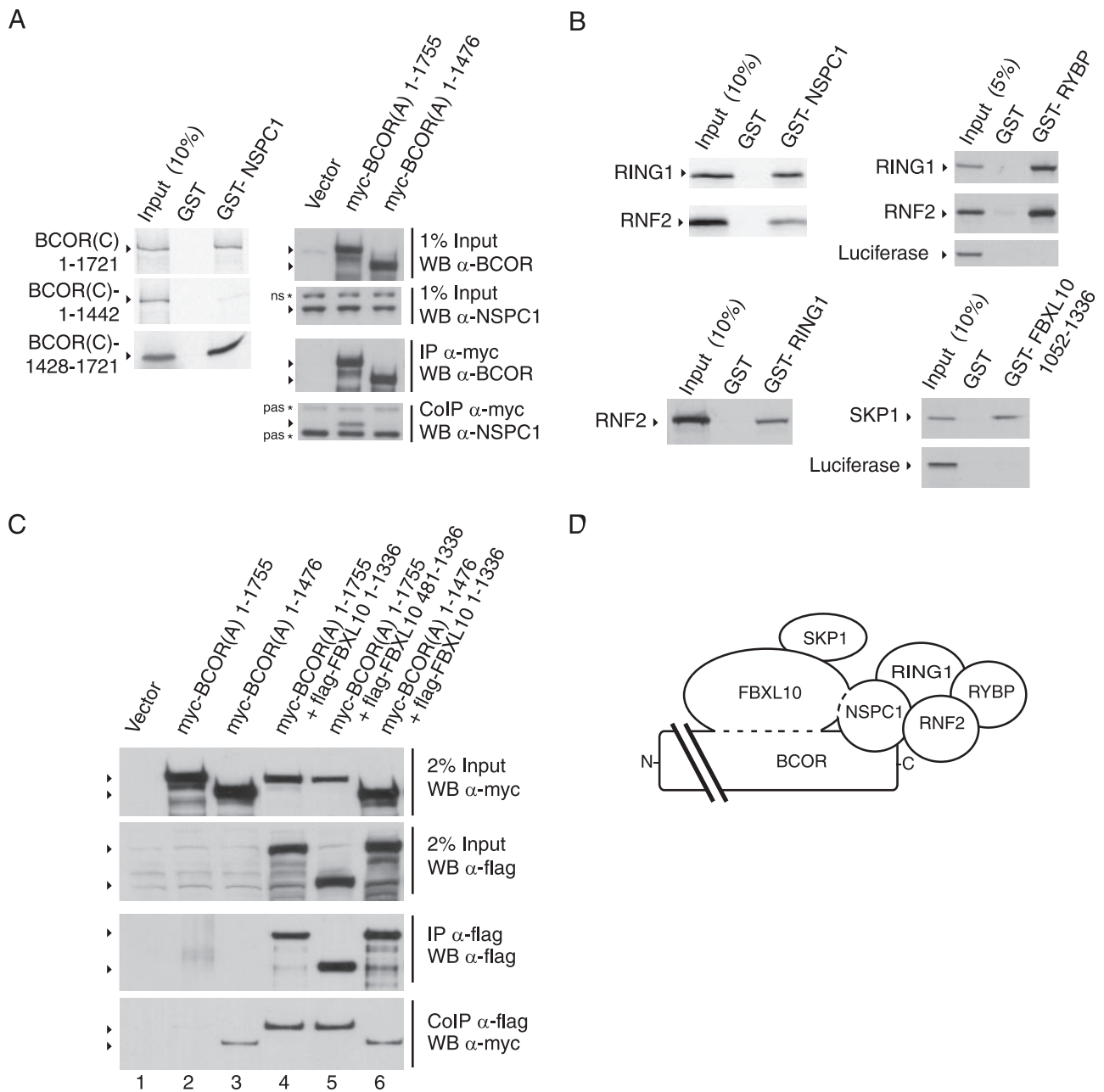


FIG. 2. Mapping of protein-protein interactions within the BCOR complex. (A) The C terminus of BCOR is necessary and sufficient for interaction with NSPC1. Left: A GST fusion of NSPC1 was used to pull down *in vitro*-translated radiolabeled BCOR(C) (1-1721), BCOR(C) (1-1442), or BCOR(C) (1428-1721). Right: Transfected myc-BCOR(A) (1-1755) but not myc-BCOR(A) (1-1476) precipitates endogenous NSPC1 from HEK293 cells. Additional bands in the NSPC1 Western blots (\*) correspond to nonspecific interactions and cross-reactivity with protein A-Sepharose. (B) Upper left: GST-NSPC1 interacts with *in vitro*-translated radiolabeled RING1 and RNF2. Lower left: A weak interaction was observed between GST-RING1 and *in vitro*-translated RNF2. Upper right: GST-RYBP interacts with *in vitro*-translated RING1 and RNF but not luciferase. Lower right: GST-FBXL10 (1052-1336) interacts with *in vitro*-translated SKP1. (C) Cotransfected full-length myc-BCOR(A) (1-1755) but not myc-BCOR(A) (1-1476) coimmunoprecipitates with transfected Flag-FBXL10 (1-1336) and Flag-FBXL10 (481-1336) in HEK293 cells. Full-length BCOR is enriched in the presence of full-length and N-terminally truncated FBXL10 (lanes 4 and 5, respectively) relative to background (lane 2). In contrast, C-terminally truncated BCOR is not enriched relative to background (compare lane 6 to lane 3). We consistently observe that the abundance of full-length BCOR, but not C-terminally truncated BCOR, is reduced upon cotransfection with FBXL10 (compare input lane 2 to lanes 4 and 5 and lane 3 to lane 6). However, endogenous BCOR levels are only modestly increased upon siRNA knockdown of FBXL10 (data not shown; see Fig. 3A). (D) Model of protein-protein interactions in the BCOR complex. FBXL10 is tentatively drawn (hashed lines) to have direct contacts with BCOR and NSPC1, but direct interactions between either molecule have been difficult to dissect.

purifications, but quantities were insufficient for identification. HDACs and AF9 (MLLT3), previously shown to interact with BCOR (37, 69), were not detected by silver staining or immunoblot analysis (not shown), perhaps reflecting a transient or chromatin-dependent association.

NSPC1 is a homolog of the *Drosophila melanogaster* PRC1 component Posterior Sex Combs (Psc) protein (31, 55). Because this homolog of Psc had not been found in other PcG complexes, we hypothesized that NSPC1 is a unique component of the BCOR complex, and we subsequently performed a reciprocal tagging experiment in HEK293 cells. Purification of tagged NSPC1 isolated and identified all of the associated bands observed in the tagged BCOR complex (Fig. 1B). Higher yields in the tagged NSPC1 purification also permitted the identification of the 32-kDa and 97-kDa bands as RING1-YYP1-binding protein (RYBP) and FBXL10 (Fig. 1C). The 24-kDa band remains unidentified. The similarity in constituents between the tagged BCOR and tagged NSPC1 purifications suggests that BCOR and NSPC1 are requisite partners in HEK293 cells and that tagging either BCOR or NSPC1 results in purification of the same complex. Aside from the chaperone protein HSP70, all proteins identified in the BCOR complex fall into two categories: PcG-associated proteins or SCF ubiquitin ligase components.

**In vitro and in vivo determinations of protein-protein interactions within the BCOR complex.** To determine how BCOR and NSPC1 are associated in this complex, we performed GST pull downs and coimmunoprecipitation experiments. We found that in vitro-translated full-length BCOR, but not C-terminally deleted BCOR (1-1442), interacts efficiently with bacterially produced GST-NSPC1 (Fig. 2A, left panel). The C terminus of BCOR (1428-1721) is sufficient to interact with GST-NSPC1 (Fig. 2A, left panel). Endogenous NSPC1 is immunoprecipitated by transfected full-length myc-BCOR, but not by C-terminally truncated myc-BCOR in HEK293 cells (Fig. 2A, right panel). We conclude that the C terminus of BCOR is both necessary and sufficient for interaction with NSPC1.

Like other mammalian Psc homologs (12, 35, 71), GST-NSPC1 interacts with both in vitro-translated RING1 and RNF2 (Fig. 2B, left upper panel). RING1 and RNF2, in turn, interact with each other (Fig. 2B, left lower panel) and RYBP (Fig. 2B, upper right panel) (30). Since we have not detected a direct interaction between BCOR and RING1 or RNF2 (data not shown), we conclude that NSPC1 bridges the interaction from BCOR to RING1 and RNF2, which in turn interact with RYBP (Fig. 2D). Together NSPC1, RING1, RNF2, and RYBP form a PcG subcomplex within the BCOR complex.

Two proteins in the BCOR complex, FBXL10 and SKP1, have not previously been associated with PcG-mediated repression but are presumed components of an SCF ubiquitin E3 ligase. FBXL10 is one of many F-box proteins containing the SKP1-interacting F-box domain (39). In the FBXL subfamily, leucine-rich repeats carboxy terminal to the F-box recognize specific substrates, often dependent on posttranslational modification, and then mediate their ubiquitylation (14). As expected, in vitro-translated SKP1 interacts with a GST-FBXL10 (1052-1336) protein containing the F-box (Fig. 2B, lower right panel). Although we thought the leucine-rich repeats might facilitate additional contacts within the complex, we failed to

detect a strong interaction between GST-FBXL10 (1052-1336) and any of the other components of the BCOR complex (data not shown). However, transfected full-length Flag-FBXL10 can coimmunoprecipitate full-length myc-BCOR but not C-terminally truncated myc-BCOR in HEK293 cells (Fig. 2C). An N-terminally truncated Flag-FBXL10, roughly corresponding to the 97-kDa protein we observed in the purifications, also interacts with full-length myc-BCOR (Fig. 2C). Thus, the C terminus of BCOR is necessary for interaction with NSPC1 and FBXL10 (Fig. 2A, C, and D). However, we do not know whether FBXL10 interacts directly with BCOR or the PcG subcomplex. Direct interactions with FBXL10 have been difficult to dissect in GST pull-down experiments, perhaps because the interaction of FBXL10 with another component(s) of the BCOR complex requires a posttranslational modification.

Full-length FBXL10 contains a JmjC domain, a CXXC zinc finger, a plant homeodomain (PHD), the SKP1-binding F-box, and leucine-rich repeats. However, the 97-kDa band in the tagged-NSPC1 purification contained peptides from only the C-terminal two-thirds of full-length FBXL10; thus, the protein in this band lacks the N-terminal JmjC domain (Fig. 1C). Analysis of EST databases indicates that the FBXL10 gene encodes multiple isoforms due to the use of alternative promoters and splice sites. To determine which isoforms of FBXL10 are expressed in HEK293 cells, we produced an antibody against a region of human FBXL10 immediately following the PHD domain that is distinct from the highly related FBXL11 (JHDM1A). This antibody recognizes one major band at 97 kDa and five additional protein bands ranging from 88 to 172 kDa that are significantly depleted upon treatment with siRNAs against FBXL10 (Fig. 3A). The 97-kDa band corresponds in size to the band observed in the tagged NSPC1 purification.

To determine if the other isoforms of FBXL10 associate with endogenous BCOR, we performed immunoprecipitations in HEK293 cells. We found that BCOR antibodies coimmunoprecipitate all six isoforms of FBXL10 (Fig. 3B, lower panel), including the 164-kDa and 172-kDa isoforms that, based on EST data, must contain the JmjC domain. Since the BCOR complex purification was carried out in HeLa S3 and HEK293 cells, we tested whether a similar BCOR complex is present in B cells. In Ramos cells, a Burkitt's lymphoma B-cell line, endogenous BCOR coimmunoprecipitates NSPC1 (Fig. 3B, upper panel) and all six isoforms of FBXL10 (Fig. 3B, lower panel). This indicates that, like in HEK293 cells (Fig. 1B and 3B, lower panel), both PcG and SCF components are associated with BCOR in B cells. Although the 164-kDa and 172-kDa isoforms of FBXL10 are barely visible in the input, they account for approximately one-fifth of the coimmunoprecipitated FBXL10 in Ramos cells. Thus, a significant fraction of the BCOR complexes contain an isoform of FBXL10 harboring the JmjC H3 K36 demethylase domain (74).

Even though we have not mapped a direct interaction between the BCOR complex and FBXL10, four lines of evidence indicate that the SCF and PcG proteins are in a single complex with BCOR (Fig. 2D). First, bands corresponding in size to SKP1 as well as PcG proteins comigrate with BCOR in the size exclusion column (Fig. 1A). Due to the presence of SKP1, which interacts directly with FBXL10 (Fig. 2B, lower right

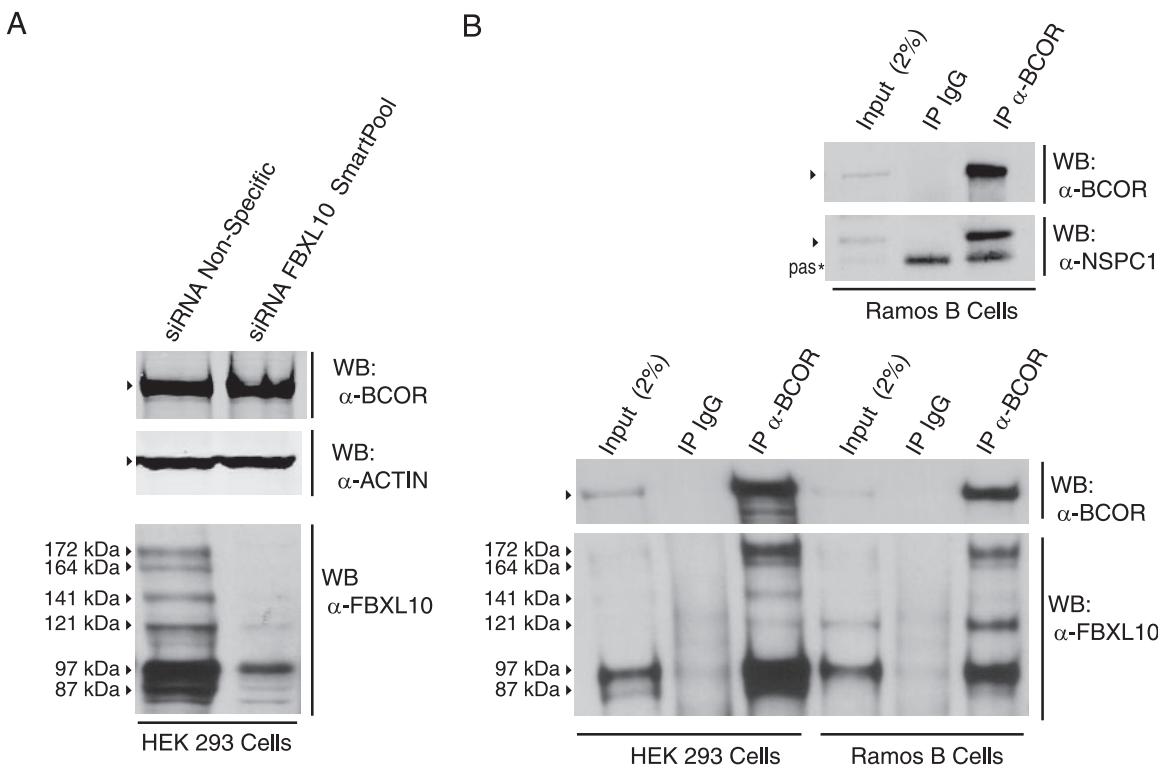


FIG. 3. Six FBXL10 isoforms associate with BCOR in HEK293 and Ramos B cells (A) FBXL10 knockdown and immunoblotting with FBXL10-specific antibodies identifies six FBXL10 isoforms in HEK293 cells. Western assays of total cell extracts blotted with BCOR, actin (Sigma), and FBXL10 antibodies are shown. FBXL10 knockdown results in only a modest increase in BCOR levels. (B) Antibodies to BCOR coimmunoprecipitate all six isoforms of FBXL10 from HEK293 and Ramos nuclear extracts (lower panel). BCOR coimmunoprecipitation of NSPC1 is also seen in Ramos cells (upper panel). The lower band in the NSPC1 Western blot (\*) is due to cross-reactivity with protein A-Sepharose.

panel), we presume that the isoforms of FBXL10 also comigrate with BCOR but are below the limit of detection of silver staining in this analysis. Second, SKP1 and PcG proteins were all identified in the tagged BCOR purification (Fig. 1B; see also Table S1 in the supplemental material). Third, SKP1 and FBXL10 were identified in the tagged NSPC1 purification (Fig. 1B; see also Table S1). Fourth, FBXL10 and BCOR coimmunoprecipitate, indicating that they can be found in the same complex (Fig. 2C and 3B, lower panel). Together our data indicate that BCOR associates in a single complex with both PcG PRC1 proteins and SCF components that, depending on the incorporated FBXL10 isoform, can also contain a JmjC histone demethylase domain.

**The BCOR complex contains E3 ligase activity for histone H2A.** Of the BCOR complex PcG proteins, RNF2 is the only one with a known enzymatic activity: an E3 ligase for the histone protein H2A (12, 78). Ub-H2A is thought to be involved in maintaining a repressed chromatin state (21, 28, 38, 78). Knockdown of the *Drosophila* homolog of RNF2 and RING1, dRing, in S2 cells results in loss of histone H2A ubiquitylation and upregulation of a PcG target gene (78). To confirm this activity in the BCOR complex, purified complex was incubated with E1, E2, Flag-tagged ubiquitin, and nucleosomal substrate. Consistent with the presence of RNF2, the BCOR complex catalyzes the addition of Flag-tagged ubiquitin onto H2A in a concentration-dependent manner (Fig. 4, upper

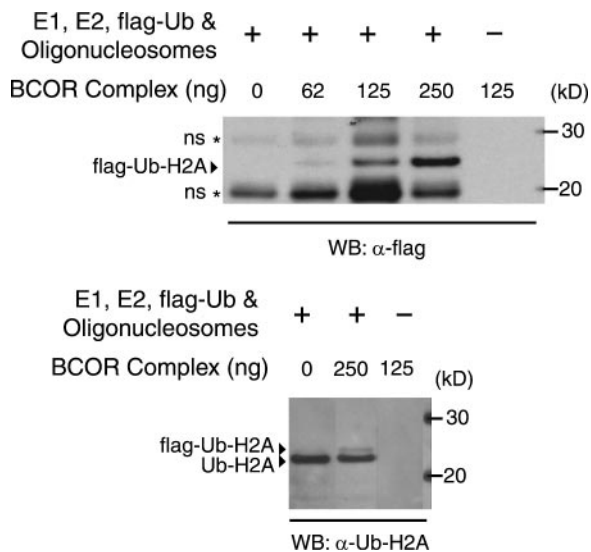


FIG. 4. Role of the BCOR complex in H2A mono-ubiquitylation. The BCOR complex purified from the NSPC1-tagged cell line catalyzes the addition of Flag-ubiquitin (flag-Ub) onto histone H2A in a dose-dependent manner. RNF2 has been previously shown to ubiquitylate H2A, generating the Flag-tagged product (78). The band at 26 kDa is the only band that increases in a BCOR complex-dependent manner and is recognized by antibodies against the Flag epitope (upper panel) and Ub-H2A (lower panel). Additional Flag-ubiquitylated proteins (\*), which are observed even in the absence of the BCOR complex above and below Flag-Ub-H2A, presumably reflect ubiquitin ligase activities present in the oligonucleosome preparation.

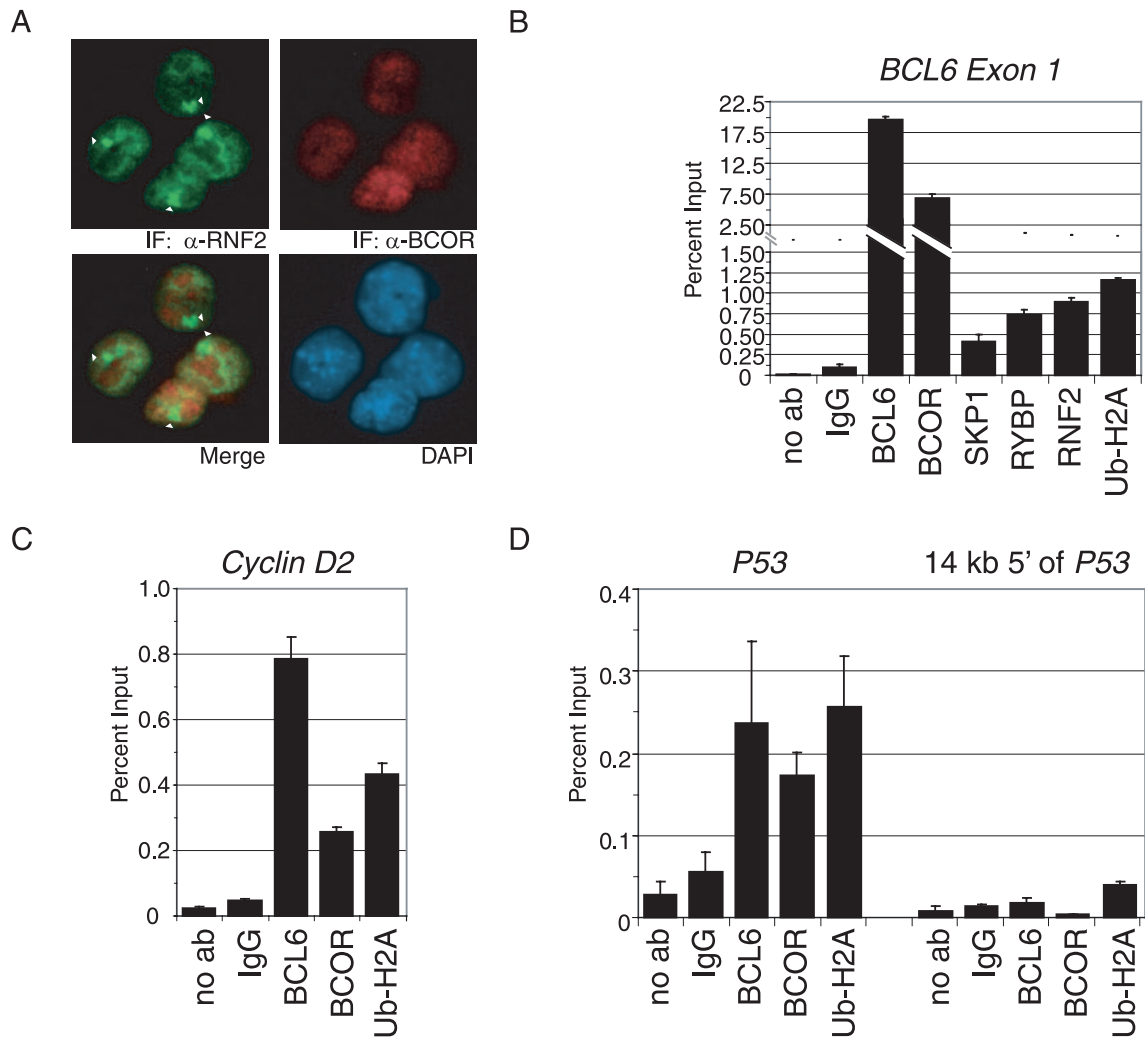


FIG. 5. The BCOR complex occupies promoters of BCL6 target genes. (A) BCOR does not colocalize with the inactive X chromosome. Immunofluorescence of XX trophoblastic stem cells using BCOR and RNF2 (1) antibodies is shown. RNF2 has been shown to localize to Xi (21, 28). The Xi is indicated with white arrows. (B) ChIP experiments show that BCL6, BCOR, BCOR complex components SKP1, RNF2, and RYBP, and the monoubiquitylated form of H2A are associated with the autoregulatory region of the *BCL6* gene. The anti-Ub-H2A antibody (Upstate) is specific for the monoubiquitylated form of histone H2A (12, 77, 78). (C and D) BCL6, BCOR, and the monoubiquitylated form of H2A are present at the promoters of *Cyclin D2* and *P53* but not a region 14 kb 5' of the *P53* gene.

panel). Identity of the Flag-Ub-H2A band was confirmed by Western blotting with antibodies specific for ubiquitylated Ub-H2A (Fig. 4, lower panel). These results show that in addition to the potential H3 K36 demethylase activity, the BCOR complex has E3 ligase activity for histone H2A.

**BCOR does not colocalize to the inactive X chromosome in trophoblastic stem cells.** PcG proteins and H2A mono-ubiquitylation also are associated with the inactive X chromosome (Xi) in mouse XX trophoblastic stem cells (21, 28). To test whether the BCOR complex is involved in X inactivation, we performed immunolocalization experiments in trophoblastic stem cells. BCOR does not localize to the intensely staining Xi labeled by RNF2 antibodies but shows a diffuse nuclear staining (Fig. 5A). This suggests that BCOR is unlikely to play a role in the maintenance of X inactivation in trophoblastic stem cells. The non-Xi diffuse nuclear staining of RNF2 likely re-

flects its association with BCOR as well as other PcG complexes, such as E2F6.com-1 (56).

**The BCOR complex is recruited to BCL6 targets in B cells.** Although BCOR can potentiate BCL6 repression in transient-reporter assays (37), it is not known whether BCOR is recruited to any of the BCL6 target genes. Therefore, we investigated the occupancy of the BCOR complex on endogenous BCL6 target genes (57, 59, 61, 66, 79) in Ramos cells using chromatin immunoprecipitation (ChIP) assays. We found that BCL6, BCOR, SKP1, RYBP, and RNF2 specifically immunoprecipitate a negative autoregulatory region in the first exon of the *BCL6* gene (Fig. 5B), indicating that the entire BCOR complex, including PcG and SCF components, are present at this locus. In addition, ChIP analyses showed that BCL6 and BCOR are present at the BCL6-binding sites of *Cyclin D2* and *P53* but not a region 14 kb 5' of *P53* (Fig. 5C and D). The

higher efficiency of ChIP at the *BCL6* locus relative to *Cyclin D2* and *P53* may be due to cooperative binding of BCL6 to the multiple binding sites in the first exon of this gene (57, 79), as POZ domain-dependent cooperative binding by BCL6 has been reported to three sites in the murine germ line  $\epsilon$  promoter (33). Importantly, our data suggest that BCL6, via BCOR, can target PcG and SCF proteins sequence-specifically to DNA.

**Monoubiquitylated H2A is present together with BCOR at BCL6 targets in B cells.** Because we have not been able to efficiently knock down BCOR or BCOR complex components using RNA interference in cultured B cells, we do not know whether the BCOR complex is essential for repression of BCL6 targets. However, in ChIP experiments using monoclonal antibodies that specifically recognize the ubiquitylated form of H2A (12, 77, 78), we found that Ub-H2A is enriched at *BCL6* exon 1, *Cyclin D2*, and *P53* relative to a region 14 kb 5' of the *P53* gene (Fig. 5B to D). The presence of PcG proteins in the BCOR complex, together with localization of Ub-H2A to BCL6 targets *in vivo*, strongly suggests that the BCOR complex is regulating these genes with its H2A mono-ubiquitylation activity.

## DISCUSSION

We have identified a novel set of proteins that associate with the corepressor BCOR in a single 800-kDa complex that is recruited to a subset of BCL6 targets. The proteins in the BCOR complex include the PcG and PcG-associated proteins NSPC1, RING1, RNF2, and RYBP as well as components of an SCF ubiquitin ligase, SKP1, and FBXL10. Our biochemical and functional analyses indicate that BCOR recruits a unique combination of enzymatic activities to chromatin targets: a PcG E3 ubiquitin ligase for histone H2A, a demethylase for histone H3 K36, and an SCF E3 ubiquitin ligase. Corepressor recruitment by the POZ domain of BCL6 is required for the proliferation and survival of BCL6-positive lymphoma cells (61). However, the relative contributions of the deacetylase and demethylation activities of the NCOR and SMRT complexes (32, 48, 80, 81, 86) versus the histone ubiquitylation and demethylation activities of the BCOR complex to repression of individual BCL6 targets remain to be determined. Our ChIP results indicate that histone H2A ubiquitylation activity of the BCOR complex is present at the *Cyclin D2* and *P53* promoters, suggesting that BCOR likely contributes to the repression of these genes and perhaps to the enhanced proliferation and survival of BCL6-positive lymphoma cells.

Our finding that the BCOR complex contains PcG proteins which may contribute to BCL6-driven oncogenesis is consistent with a growing number of observations indicating a role for PcG proteins in stem cell identity, mammalian development, cell cycle regulation, and cancer (11, 62). PcG proteins are known to be part of a memory system that relies on epigenetic modification of chromatin to ensure faithful transmission of cell identities through cell division (10). In addition to its potential roles in oncogenesis with BCL6 and MLL-AF9, BCOR is clearly an essential corepressor in multiple developmental pathways. OFCD patients, who have mutations in *BCOR*, have severe craniofacial, digital, and cardiac defects. Peripheral blood lymphocytes from female patients show

strongly biased inactivation of the X chromosome carrying the mutant BCOR allele (53), indicating that BCOR is also required for normal hematopoiesis. The diverse phenotypes observed in OFCD patients and the widespread expression of BCOR suggest additional tissue-specific transcription factors recruit the BCOR complex and its associated PcG and SCF proteins.

The recruitment of BCOR complex PcG proteins to target genes by BCL6 in B cells suggests that BCL6 functions as a PcG-targeting factor. Interestingly, BCL6 is related to the *Drosophila* POZ zinc finger transcription factor GAGA, which is thought to play a role in PcG and TrxG protein recruitment to DNA (6, 50, 52). Typically, histone H3 K27 methylation, mediated by the ESC-E(Z) methyltransferase complex, creates a binding site for the chromo domain of the Pc protein in the PRC1 complex (13). Although the BCOR complex, unlike other PRC1-like complexes (46, 56, 78), does not copurify with a chromo domain-containing protein, the complex does contain protein domains with the potential to bind chromatin. CXXC domains like the one found in FBXL10 can bind unmethylated CpG sequences (9, 40, 68). PHDs can bind nucleosomes (8, 27), and in two recent examples this binding was specific to H3 K4-trimethylated nucleosomes (67, 83). These domains of FBXL10 could stabilize the association of the BCL6-recruited complex with chromatin or may allow for BCL6-independent recruitment of the BCOR complex. Similarly, the BCOR-interacting protein AF9 (69) interacts with the chromo domain protein MPc3 (Cbx8) (34), which may facilitate chromatin contacts (7). The BCOR complex may function analogously to the E2F6.com-1 complex recruited by the transcription factor E2F6 (56). Like the BCOR complex, this complex includes RING1, RNF2, and a Psc homolog (MBLR) and also copurifies with the chromatin-binding domain protein (HP1 $\gamma$ ), which may stabilize binding of E2F6.com-1 to chromatin trimethylated at K9 of histone H3 (56).

The interaction of BCOR with PcG proteins as well as an F-box protein with a JmjC domain demethylase is particularly striking. JmjC domain demethylase enzymes are able to remove methyl groups from lysines that have been mono-, di-, or trimethylated (18, 20, 43, 73, 74, 81, 85). The specificity of FBXL11 (JHDM1A) has been thoroughly characterized, and the protein has been found to demethylate mono- and dimethyl lysine 36 of histone H3 (74). FBXL10 (JHDM1B) has been reported to also demethylate H3 K36 (74), but its specificity remains to be fully established. The presence of methylated H3 K36 correlates with transcriptional elongation (44, 47, 84), suppression of intragenic transcription (15, 41, 42), and the defining of actively transcribed regions (2, 64). BCOR complexes containing the demethylase activity of the full-length FBXL10 may therefore additionally contribute to transcriptional regulation by altering H3 K36 methylation at target genes.

The F-box and the leucine-rich repeats at the C terminus of FBXL10 are presumed components of an SCF E3 ubiquitin ligase. Although we see a modest FBXL10-dependent effect on endogenous BCOR abundance (Fig. 3A), the direct target, nature (mono versus poly), and outcome (signaling versus degradation) of the ubiquitylation activity of FBXL10 are unknown. The presence of SKP1 at the *BCL6* locus (Fig. 5B) indicates that SCF ubiquitin ligase components are found on



chromatin with the BCOR complex. Future characterization of the SCF E3 ligase activity of FBXL10 will provide important insights into BCOR complex-dependent gene silencing.

Because deregulated expression of BCL6 plays an oncogenic role in non-Hodgkin's lymphomas and BCL6 can recruit BCOR to promoters involved in apoptosis and cell cycle regulation, we suggest that BCOR and its associated PcG and SCF proteins may also play a role in BCL6-associated lymphomas. Once a definitive functional role for BCOR is established, the enzymatic activities of the BCOR complex may be attractive therapeutic targets for non-Hodgkin's lymphoma.

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