Figure 2. ChIP-Seq Demonstrates RING1B Occupancy at PcG Target Loci in ***Eed***$^{+/+}$ and ***Eed***$^{-/-}$ mESCs
(A) Example screen shots for Eed$^{+/+}$

that are not annotated as CpG islands. Interestingly, a high proportion of the nonoverlapping peaks also coincide with CpG islands (Figures 2E and S2C). Co-occurrence of RING1B sites and CpG was shown to be signi cant ($p < 0.001$, co-occurrence of R test; Huen and Russell, 2010). Taken together these results demonstrate extensive overlap of RINGB targets in mESCs lacking H3K27me3 relative to WT cells.

Re-recruitment of PRC1 Activity to PcG Target Loci following Depletion of H2AK119u1

PRC2 occupancy and H3K27me3 can be maintained at an ectopic site following withdrawal of the primary recruitment signal (Hansen et al., 2008), possibly via binding of an aromatic cage in EED to H3K27me3 (Margueron et al., 2009). With this in mind, we considered that although primary recruitment of PRC1 may require PRC2-mediated H3K27me3, H2AK119u1, once established, could function as a signal for maintenance of PRC1 occupancy. To address this, we used the reversible proteasome inhibitor MG132 (Dantuma et al., 2006) to deplete H2AK119u1 in Eed4 cells in the presence or absence of PRC2 and then determined whether restoration of H2AK119u1 occurs following withdrawal of the inhibitor. Secondary effects of MG132 treatment on mESCs are negligible with the described conditions (Szutorisz et al., 2006). As shown in Figure 3A, treatment of cells with MG132 for 6 hr ef ciently depleted global H2AK119u1 (lanes 2 and 6). Allowing cells to recover for 3 days after withdrawal of the inhibitor resulted in restoration of H2AK119u1 in both the presence and the absence of H3K27me3 (lanes 4 and 8, respectively), and this was also the case after only 1 day of recovery (Figure S3A). ChIP analysis demonstrated that H2AK119u1 accumulates appropriately at PcG target loci after recovery, with levels being slightly reduced in the absence of H3K27me3 (Figure 3B). Recruitment of RING1B and EZH2 was retained following MG132 treatment, albeit at a slightly reduced level (Figure S3B).

Expression analysis (Figure 3C) demonstrated that treatment with MG132 derepresses PcG target loci in both the presence and the absence of H3K27me3, and that silencing is restored following withdrawal of the inhibitor. No effect was seen at the Gata1 locus at which represFigur i([PcG-i]Eed... [garbled overlapping text]

Figure 3. H2AK119u1 Is Re-established following Depletion in Eed4 WT and Eed4 cKO mESCs

(A) Western blot for H2AK119u1 and H3K27me3 in histone extracts. CBB: Coomassie brilliant blue. H2AK119u1 is completely depleted after 6 hr with 10 μM MG132 and is then restored when cells are left to recover (recov) for 3 days after inhibitor removal, irrespective of presence of H3K27me3.

(B) ChIP for H2AK119u1 and H3K27me3 in Eed4 WT and Eed4 cKO cells. H3 is shown as a control. Bars show average + SD, n = 3.

(C) Expression analysis (Rel. expr.) of selected loci. For RT-PCR analysis, values were normalized against the average of three housekeeping genes,Hmbs, Gapdh, and Idh1. Bars show average + SD, n = 3.

See also Figure S3.

Figure 4. Proteomic Analysis of MEL-18, RYBP, and CBX7 Complexes in mESCs

(A) Silver-stained SDS polyacrylamide gel of control, MEL-18-Flag, RYBP-Flag, and CBX7-Flag puri cations. PRC1 subunits identi ed by mass spectrometry of excised bands are indicated.

(B) Table showing the PRC1 core subunits copurifying with MEL-18-Flag, RYBP-Flag, and CBX7-Flag, as identi ed by mass spectrometry analysis. [1]Mascot score for speci ed proteins, [2]number of unique peptides identi ed. *The two peptides matched to RING1A are also present in RING1B.

(C) MEL-18-Flag, RYBP-Flag, and CBX7-Flag puri cations analyzed by western blot with the indicated antibodies.

See also Figure S4.

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Figure 5. MEL-18 Interacts with RYBP and CBX7 in Mutually Exclusive Catalytically Active Complexes

(A) CoIP of endogenous RING1B, MEL-18, RYBP, and CBX7 from Eed4 WT mESC nuclear extracts, analyzed by western blot with the indicated antibodies and the appropriate IgG control. Benzonase (Benzo) and ethidium bromide (EtBr) were added where indicated. 10% input and 15% of RING1B, MEL-18, RYBP, CBX7, and the appropriate control CoIP are shown.

(B) Left panel: RING1B/MEL-18, RING1B/MEL-18/RYBP, and RING1B/MEL-18/CBX7 protein complexes analyzed by western blot using antibodies as indicated, or by Simply Blue Safe staining (SBS). Right panel: Ubiquitylation assays performed using indicated concentrations of RING1B/MEL-18 (lanes 3–7), RING1B/

between Eed4 WT (Figure 5A), and Eed4 cKO mESCs (Figure S5A). These results demonstrate mutually exclusive binding of CBX7 and RYBP subunits and de ne the existence of two distinct PRC1-like complexes comprising, on the one hand, RING1B, MEL-18, CBX7, and MPH1 and, on the other, RING1B, MEL-18, and RYBP. We refer to these complexes henceforth as CBX-PRC1 and RYBP-PRC1. CoIP experiments in a mouse broblast cell line also revealed mutually exclusive interaction of CBX7 and RYBP with RING1B/MEL-18 (Figure S5B), indicating that RYBP-PRC1 and CBX-PRC1 coexist in different cell types.

To further investigate the composition of CBX-PRC1 and RYBP-PRC1, we established mESC lines expressing epitope-tagged RYBP or CBX7 and then puri ed the associated proteins. For RYBP (Figures 4A, panel 3 and 4B), we copuri ed RING1A/B and MEL-18. CBX proteins, including CBX7, were not detected at all. We did, however, copurify NSPC1 and MBLR, homologs of MEL-18 that are components of the BCOR and E2F6 complexes, respectively. This nding is consistent with the previously reported association of RYBP with these complexes (

factor YY1 (Garc´a et al., 1999). The latter nding is consistent with a previous study that identi ed YAF2, a close homolog of RYBP, as a YY1 interactor (Kalenik et al., 1997). Subsequent studies identi ed RYBP as having a role in apoptosis (Zheng et al., 2001). At present it is not clear whether these different

Figure 7. RYBP-PRC1 Is Recruited to Xist RNA Territories Independently of H3K27me3 and Is Required for H2AK119 Ubiquitylation in ***Eed***$^{+/+}$ and ***Eed***$^{-/-}$ mESCs

(A) Immuno uorescence analysis of RYBP (green) and H2AK119u1 (red) in 36^{EedTg} and $36^{Eed-/-}$ mESCs induced to express transgenic Xist RNA. DNA was counterstained with DAPI (blue). Graphs illustrate the proportion of cells in which H2AK119u1 foci and RYBP foci colocalize, based on scoring 100 cells on each of three separate slides.

(B) Stable cell lines were established following transduction of Eed$^{+/+}$ and Eed$^{-/-}$ mESCs with scrambled or either of two independent RYBP shRNAs (sh2 and sh3). Acid extracted histones (H2AK119u1 and H3) or nuclear extracts (RYBP, RING1B, and LAMIN B), were prepared and analyzed by western blot.

(C) Model as discussed in text. Key: DNA (black line); nucleosomes with single N terminus of H3 and C terminus of H2A (cylinders); H3K27 trimethylation (Me); H2AK119u1 (Ub); recruitment factors (gray shape with ?).

See also Figure S7.

mammalian homologs of Drosophila PC, speci cally CBX2, 4, 6, and 8, but this has not been tested. We also observed that RYBP-PRC1 has a reduced association with MPH1. Consistent with this, RING1B but not MPH1/2 localizes to Xist RNA territories in Eed-de cient mESCs (Schoeftner et al., 2006). We assume that RYBP-PRC1 excludes other mammalian homologs of Drosophila PH, MPH2 and MPH3, but this also is untested. PH has been reported to interact with PSC (Kyba and Brock, 1998), and therefore to explain near exclusion of MPH1 from RYBP-PRC1, we speculate that RYBP occludes the required interaction surface on MEL-18.

Collectively our observations indicate that RYBP-PRC1 is comprised of three core components, RYBP, RING1B, and MEL-18/BMI-1. Our proteomic studies using epitope-tagged MEL-18 (this study) and BMI-1 (not shown) did not reveal other stoichiometric components, although we cannot rule out the presence of other key components at substoichiometric levels. Additionally, although in mESCs MEL-18 is an abundant PSC homolog, we cannot rule out a signi cant contribution of BMI-1 or other PSC homologs to RYBP-PRC1. Indeed, mESC RYBP complexes included the PSC homologs NSPC1 and MBLR, associated with BCOR and E2F6 complexes, respectively (Gearhart et al., 2006; Ogawa et al., 2002; Sánchez et al., 2007; Trimarchi et al., 2001). Studies in Drosophila have shown that the PRC1-related complex dRAF, comprised of RING1, PSC, and KDM2, plays a central role in global H2AK119u1 (Lagarou et al., 2008). This provides an interesting parallel with mESCs in which the BCOR complex includes a mammalian homolog of KDM2, KDM2B (Gearhart et al., 2006; Sánchez et al., 2007). However, there is no evidence that RYBP in Drosophila participates in PRC1-related complexes.

Recruitment of RYBP-PRC1

Genome-wide analysis of RING1B binding in the absence of H3K27me3 indicates that RYBP-PRC1 and PRC2 are recruited to many of the same target genes. It is intriguing that distribution of RING1B, localizing across CpG islands, resembles that of PRC2, even in the absence of H3K27me3. Indeed a recent study has suggested that unmethylated CpG domains may be suf cient to recruit PRC2 (Mendenhall et al., 2010). Although it is possible that the same signature recruits RYBP-PRC1, our analysis demonstrates sites bound by RING1B only in the presence of H3K27me3 and other sites where H3K27me3 is less important for RING1B targeting. Collectively, these observations suggest some differences in the targeting mechanisms of PRC2 and RYBP-PRC1, or at least in the relative contribution of the two pathways at speci c loci.

Given that RYBP-PRC1 and PRC2 have signi cantly overlapping targets, can RYBP provide clues as to how targeting is mediated? As discussed above, RYBP was previously shown to interact with the transcription factor YY1 (Garc´a et al., 1999). Interestingly YY1 is the mammalian homolog of Drosophila PHO, which in the context of the PHO-RC complex plays a central role in PcG targeting (Klymenko et al., 2006). However, we did not nd YY1 together with PRC1 in proteomic or native immunoprecipitation analyses. Moreover mapping of YY1-binding sites in mESCs reveals no signi cant overlap with PRC2 binding (Squazzo et al., 2006). We therefore conclude that interaction of RYBP with YY1 is unlikely to be relevant, at least in mESCs.

RYBP has a single conserved domain, a Ranbp2 zinc nger (Ranbp2-ZnF). A subset of proteins with this domain are associated with RNA metabolism, and moreover, nuclear magnetic resonance (NMR) studies have demonstrated that the Ranbp2-ZnF in these proteins binds RNA (Nguyen et al., 2011). This is potentially interesting in light of recruitment of RYBP in response to Xist RNA expression and also a series of recent studies suggesting a wider role for noncoding RNA in PcG recruitment (reviewed in Pauli et al., 2011). Arguing against this, comparative analysis indicates that the RYBP Ranbp2-ZnF belongs to a different class and that none of the contact residues for RNA binding are conserved or similar (not shown). A further subset of Ranbp2-ZnF proteins interact with ubiquitin, also characterized at the structural level (Wang et al., 2003), and here RYBP does show greater similarity. Indeed, it has been suggested that RYBP interacts with H2AK119u1 and additionally is subject to self-monoubiquitylation as a consequence of being in complex with RING1B (Arrigoni et al., 2006). This could be argued to point to a role for RYBP-PRC1 in maintaining H2AK119u1 by interacting with pre-existing marks on neighboring nucleosomes. However, our observation that RYBP is not displaced and that H2AK119u1 can be re-established following depletion by MG132 treatment, in both the presence and the absence of H3K27me3, appears to discount this idea. In sum, involvement of RYBP provides some intriguing clues that may help to understand PcG targeting, but further studies are needed to determine which, if any, are relevant.

Interplay of PRCs

Our data suggest that parallel pathways target H2A ubiquitylation to PcG targets in mESCs and on the inactive X chromosome. A model illustrating this is shown in Figure 7C. As discussed, the primary signal that recruits PRC2 and RYBP-PRC1 is unknown. CBX-PRC1 recruitment, on the other hand, is linked to PRC2-mediated H3K27me3. Although RYBP-PRC1 recruitment can occur in the absence of H3K27me3, we cannot rule out that CBX-PRC1 binding is at least partially dependent on RYBP-

Moreover, recent studies have shown that depletion of JARID2,

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