

YY1 DNA binding and interaction with YAF2 is essential for Polycomb recruitment

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ABSTRACT

Polycomb Group (PcG) proteins are crucial for epigenetic inheritance of cell identity and are functionally conserved from *Drosophila* to humans. PcG proteins regulate expression of homeotic genes and are essential for axial body patterning during development. Earlier we showed that transcription factor YY1 functions as a PcG protein. YY1 also physically interacts with YAF2, a homolog of RYBP. Here we characterize the mechanism and physiologic relevance of this interaction. We found phenotypic and biochemical correction of *dRYBP* mutant flies by mouse YAF2 demonstrating functional conservation across species. Further biochemical analysis revealed that YAF2 bridges interaction between YY1 and the PRC1 complex. ChIP assays in HeLa cells showed that YAF2 is responsible for PcG recruitment to DNA, which is mediated by YY1 DNA binding. Knock-down of YY1 abrogated PcG recruitment, which was not compensated by exogenous YAF2 demonstrating that YY1 DNA binding is *a priori* necessary for Polycomb assembly on chromatin. Finally, we found that although YAF2 and RYBP regulate a similar number of Polycomb target genes, there are very few genes that are regulated by both implying functional distinction between the two proteins. We present a model of YAF2-dependent and independent PcG DNA recruitment by YY1.

INTRODUCTION

Polycomb group (PcG) proteins were initially identified by genetic studies in *Drosophila* as proteins that maintain stable transcriptional repression necessary for proper development (1). There are at least 16 PcG proteins in

Drosophila, with numerous mammalian counterparts, and these proteins regulate expression of homeotic genes during development (2–4). PcG proteins are organized into multiple complexes including, Polycomb Repressive Complex 1 (PRC1) and Polycomb Repressive Complex 2 (PRC2). In *Drosophila*, PRC1 core components consist of Polycomb (Pc), Polyhomeotic (Ph), Posterior sex combs (Psc) and Sex combs extra/dRing1 (Sce/dRing1). PRC1 contains H2A lysine 119 ubiquitinase and SUMO E3 ligase activities; several PRC1 variants are also associated with H3K36 demethylase activity (5–8) [reviewed in (9–11)]. PRC2 consists of Enhancer of zeste [E(z)], Extra sex combs (Esc), Suppressor of zeste 12 [Su(z)12] and the protein p55, and mediates H3 lysine 27 methylation and H3 deacetylation activities (9–11). Biochemical, genetic and genomic analyses revealed the complexity and diversity of the mammalian PcG counterparts (11–13), identified other components associated with both PRC1 and PRC2 in substoichiometric amounts (14–16), and identified many PcG target genes (17–19). Combinations of the various isoforms of the core components constitute various subfamilies of PRC1 and PRC2 (5,13,16,20).

Other PcG complexes include the Pho-repressive complex in *Drosophila* composed of Pho and dSfmbt (21). No enzymatic activity has been observed for this complex, though it can site-specifically bind to DNA due to the Pleiohomeotic (Pho) protein. Other complexes include the RAF complex containing Psc, dRING and dKDM2, and the PR-DUB complex containing Calypso and Asx (5,9,22). Finally, Pho and YY1 can recruit the INO80 chromatin remodeling complex to DNA (21,23). INO80, is recruited by YY1 to active genes suggesting that YY1 uses INO80 not only to activate promoters but also to gain access to target promoters (23). Recent studies showed that in *Drosophila*, null mutants of *dINO80* are late embryonic lethal and show homeotic transformation reminiscent of *Scr*, *Antp*, *Ubx* or *Abd-B* gene loss of function (24) indicating that YY1–INO80 interaction might yet be another mechanism of PcG recruitment.

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In addition, PcG proteins can mediate long-distance DNA interactions to control gene expression (10,25,26).

The mechanism(s) of transcriptional silencing by PcG proteins is poorly understood. Chromatin compaction, covalent modification of histone proteins and direct interactions with RNA polymerase have been proposed as silencing mechanisms (9–11). Two histone modification marks, H3K27me3 and H2AK119ub, contributed by PRC2 (EZH2) and PRC1 (RING1/2), respectively, are believed to be important for the repression mechanism. Most studies show positive correlation of PcG binding and the histone marks at binding sites, but evidence of H3K27me3-independent PcG localization have also been reported (16,27).

How PcG proteins are recruited to DNA, particularly in mammals, remains enigmatic. Studies in *Drosophila* showed that sequence-specific DNA-binding protein Pho, binds to PRE sequences and recruits PcG proteins to DNA (28,29). However, progress in mammalian systems has been hampered by poor characterization of mammalian-PREs and candidate transcription factors that bind to these sequences. Recent studies identified Jarid2, which is conserved from flies to mammals, as a potential candidate for recruitment (30). Jarid2 binds DNA, colocalizes with EZH2, and the methylation status of H3 lysine 27 regulates its transcriptional activity. Jarid2 may also recruit PRC1 in embryonic stem cells (ESCs) (14,27,31,32).

Studies from our laboratory showed that YY1, the mammalian homolog of Pho, can functionally compensate for Pho in *pho* mutant flies, bind to *Drosophila* PREs and recruit PcG proteins to DNA (33,34). Furthermore, we found that the 25 amino acid YY1 REPO (REcruitment of POLycomb) domain is necessary and sufficient for PcG-mediated transcriptional repression *in vivo* and for recruitment of PcG proteins to DNA leading to methylation of H3 lysine 27 (35). These studies establish YY1 as a transcription factor that can recruit PcG proteins to DNA, resulting in PcG-specific histone modification and transcriptional repression. Three studies in mammals identified mammalian 'PRE-like' sequences that bind PcG proteins (36–38). These sequences contain clusters of YY1-binding sites suggesting that YY1 can recruit PcG proteins to DNA in mammals in a similar manner as Pho in *Drosophila*. Consistent with the previous findings, two additional Hox PRE-like elements (*HOXC* and *HOXB*) identified by Woo and colleagues were shown to require YY1 for their repressive activity (39). While YY1 clearly can recruit PcG complexes to DNA, the manner of YY1 interaction with PcG complexes and its mechanism of recruiting PcG complexes to DNA are still unclear.

Two homologous proteins, YAF2 and RYBP, were identified as YY1 interacting proteins (40,41). Functionally, RYBP associates with a subset of PRC1 complexes named PRC1L4 (16) and was shown to be involved in the repressive function of *hoxD11.12*, one of the three mammalian 'PRE-like' sequences (37). Chromatin immunoprecipitation (ChIP)-seq studies in mouse ESCs showed that the association of RYBP-PRC1 complexes to genomic loci is independent of

H3K27me3 and PRC2, but is able to mediate H2A ubiquitylation (20). Recently, comprehensive proteomic and genomic analysis of mammalian PRC1 complexes revealed a RYBP-PCGF-RING1B complex as the functional PRC1 responsible for H2A K119ub1 (13). YAF2 was first identified by its ability to bind to YY1 (40). YAF2 can also interact with the RING proteins. Interestingly, *in situ* hybridization studies in mouse embryos showed distinct differences in spatial and temporal expression of YAF2, RYBP and the RING proteins (42). Though several studies have demonstrated that RYBP is associated with the PRC1 complex, much less is known about the functional relevance of YAF2 and PcG recruitment. Previously we showed that YAF2 can interact with the REPO domain of YY1 and is involved in PcG recruitment (43). Even though RYBP and YAF2 physically interact with PcG proteins, a precise mechanism of recruitment of these proteins to DNA is still unclear.

In the present study, we demonstrate that mouse YAF2 (mYAF2) can perform phenotypic and biochemical rescue of *dRYBP* mutants in *Drosophila*. We performed physical interaction studies between YAF2, YY1 and other PcG proteins, and propose a mechanism of interaction of various components of the PcG complex for recruitment to DNA. ChIP assays and knock-down studies show that YAF2 is required for PcG recruitment *in vivo*, and that YY1 DNA binding is essential for this phenomenon. Gene expression arrays in HeLa cells demonstrate that although YAF2 and RYBP regulate a similar number of Polycomb target genes, there are very few genes that are regulated by both implying functional distinction between the two proteins.

MATERIALS AND METHODS

Fly stocks and transgenesis

The *dRYBP*^{KG08683} stock containing a P-element insertion into the *dRYBP* gene causing loss of function was purchased from the Bloomington Stock Center (stock number 14968), hereafter referred to as *dRYBP*^l (44). The *ry*⁵⁰⁶ parent stock and balancer stocks were kindly provided by Nancy Bonini and Amita Sehgal (University of Pennsylvania). The *BGUZ* reporter stock was kindly provided by Jürg Müller (European Molecular Biology Laboratory, Heidelberg, Germany) (45). All cultures were maintained at 25°C on commercially available medium. Transgenic injections of *hsp70*-driven (pRy-derived) constructs were performed by Genetic Services Inc. (Cambridge, MA, USA). Transgene incorporation was determined by correction to *ry*⁺ phenotype. Transgene positive strains were crossed with balancer stocks and maintained as balanced stocks.

Fly genotyping

Adult flies were harvested by anesthetizing with CO₂ and genomic DNA was extracted in 50 µl buffer (10 mM Tris-HCl pH 8.0, 1 mM EDTA, 25 mM NaCl and 0.1 mg/ml proteinase K) by disruption of adult tissues using a sterile, nuclease-free, aerosol-barrier pipette tip and incubation at

37°C for 30 min. Proteinase K was inactivated at 98°C for 5 min. DNA was used immediately for PCR analysis.

PCR of extracted genomic DNA was performed in 20 μ l reactions using AmpliTaq (Invitrogen), 200 μ M dNTPs, and primers according to the following scheme: *hsp70-FlagYAF2* transgene (predicted size of 571-bp product, 5' CGCATATGGGGATCCCCATGGGCGACAAGAA GAG 3' and 5' CGCCGGGCTCGAGTTAGTGAGA CTCTCCG 3'), wild-type *dRYBP* allele (predicted size of 400-bp product, 5' CCGGCGAGGATTTCTTGTC ATAA 3' and 5' GAAATATCGATAGCCTGGCATTG A 3') and *dRYBP^I* allele (predicted size of 567 bp, 5' CCG GCGAGGATTTCTTGTCATAA 3' and 5' ATTAAC AATGAACAGGACCTAAC 3'). The reactions were subjected to 35 cycles of 95°C for 30 s, 66°C for 30 s and 72°C for 60 s in a BioRad MiniOpticon thermocycler. The PCR products were analyzed by agarose gel electrophoresis and stained with ethidium bromide for visualization.

Yeast two hybrid assay

Yeast (*Saccharomyces cerevisiae* strain AH109, Clontech) was cotransformed with plasmids expressing GAL4 DNA-binding domain (DBD) fusions from pGBK17 and GAL4 activation domain (AD) fusions from pGADt7. Plasmid constructs were prepared using routine subcloning protocols and verified by sequence analysis. Introduction of the plasmids into yeast cells was performed with Frozen EZ Yeast Transformation II Kit, (Zymo Research) according to the manufacturer's protocol. Transformants were selected for plasmid uptake on Trp/Leu drop-out medium (Clontech) and colonies were grown in selective medium Trp/Leu/His/Ade drop-out medium (Clontech) to assay for activation of the *HIS3* and *ADE2* transgenic reporter genes. The viable yeast colonies were transferred two to three times on selective medium. Plates were scanned using an Epson Perfection V500 photo scanner.

BGUZ repression assay

Processing of embryos for BGUZ protein expression was performed as described previously (33,35,43,45). Briefly, embryos from 1 h egg-lays were fixed with formaldehyde at Hour 6 after the egg-lay. The fixed embryos were stained with X-gal to detect LacZ activity in embryonic tissues (33,45).

Drosophila embryo ChIP assays

Processing of *Drosophila* embryos for ChIP assays was performed as described previously (34,35). Immunoprecipitations (IPs) were performed using the following antibodies: anti-Pho (kind gift from Dr Judith Kassis, NIH), anti-Gal4 DBD (Santa Cruz sc-577), anti-Flag M2 (Sigma F-3165) anti-Polycomb (Santa Cruz sc-25762) and anti-H3K27me3 (Millipore 07-449). Embryos from the crosses as indicated in the figures were heat shocked (37°C for 45 min) at 3 h after the start of the egg lay and maintained at 25°C until Hour 6. At that time, the embryos were fixed with 2% formaldehyde, washed, sonicated and equal amounts of chromatin were taken for IP using the above-mentioned antibodies. The immunoprecipitated chromatin was subjected to crosslink

reversal and detection by qPCR with primers for PRE_D listed in Supplementary Table S1 (35,46).

Plasmids, transfections and co-immunoprecipitation assays

Gal4DBD and Flag-tagged pcDNA3.1(+) plasmids were generated by cloning the Flag and the Gal4-DBD N-terminal sequences in-frame with the respective cDNAs (as mentioned in the figure legends). Transfections were performed in HEK293-T cells using Fugene HD (Roche, now Promega Inc.) and 10 μ g of total DNA. Cells were harvested ~72 h post-transfection and nuclear extracts were prepared following the method described in the NE-PER kit (Pierce Endogen). Total protein concentrations were estimated using the Bradford method (47) and 750 to 1000 μ g of nuclear extract was taken for overnight IP with either Flag M2-beads (Sigma, F-2426) or Gal4 antibody (Santa Cruz, sc-577). The beads were washed, boiled and loaded on a denaturing gel and probed for the presence of the interacting partner by western blot.

HeLa ChIP and RT-PCR assays

YAF2 and YY1 were knocked down in HeLa cells using siRNA oligonucleotides (Ambion) for either 48 h or 96 h. Knock-down efficiency was determined by qRT-PCR and western blot analyses. We raised a rabbit polyclonal antibody against full length YAF2 (Cocalico Biologicals), the antibody was affinity purified and confirmed for detection of YAF2 by western blot and ChIP assays. For ChIP, cells were cross-linked with formaldehyde and ChIP assays were performed as described earlier (35,46) with certain modifications. For ChIP assays in HeLa cells, 200 μ g of chromatin was taken for each IP with the following antibodies: YY1 (Santa Cruz, H-414), Ring1B (Active Motif, 39663), Ring1 (Abcam, ab32644), Bmi-1 (Millipore, 17-664), H3K27me3 (Millipore, 07-449) and EZH2 (Millipore, 17-662). Purified DNA was taken for qPCR with primers for *MYTD1*, *EIF3S10*, *hoxA2* or *hoxD11.12* listed in Supplementary Table S1 (37,48). ChIP assays were repeated three to five times with qPCR performed in triplicate for each assay. RT-PCR assays were performed with the primers listed in Supplementary Table S1 for transcripts encoding YAF2, YY1, HoxA2 and HoxD13, using Actin as a control.

Gene expression analysis using RT² profiler PCR arrays

The differential regulation of Polycomb target genes by YAF2 and RYBP was determined using the Pathway focused expression array containing 84 primer sets to genes regulated by PcG proteins, and 12 control primer sets (catalog #PAHS-505Z; SABiosciences, Qiagen). Total RNA from control and YAF2 or RYBP knock-down HeLa cells were reverse transcribed using the RT² First Strand Kit according to the manufacturer's protocol. One microgram of total RNA was taken for PCR array to determine target gene expression. The knock-down efficiency of YAF2 or RYBP was determined separately using gene specific primers and western blots. Genes showing a 2-fold or greater differential regulation were taken for analysis. Arrays were repeated three times for each condition and showed reproducible data.

RESULTS

The *Drosophila* *dRYBP*¹ mutant allele disrupts Polycomb-mediated silencing

Previously we showed that full-length YY1 and the isolated YY1 REPO domain (YY1 residues 205–226) silence a PcG-dependent reporter gene *in vivo* (33,35). Mammalian YAF2, identified as a ligand for the YY1 REPO domain, was recruited to DNA when the REPO domain was tethered to DNA by the GAL4 DBD (43). We predicted that PcG-mediated silencing in *Drosophila* would require the recruitment of the *Drosophila* homolog of mammalian YAF2 and RYBP, dRYBP. The role of RYBP in PcG-mediated silencing led us to hypothesize that the *dRYBP*¹ mutant would show loss of PcG silencing (derepression).

To test this prediction, we performed reciprocal crosses among flies bearing a PcG-responsive *BGUZ* reporter plus a GAL4 DBD-tagged YY1 REPO domain transgene (*GAL4REPO*) driven by the *hunchback* (*hb*) promoter, with *dRYBP*¹ flies (Figure 1A). The *BGUZ* reporter is composed of a *lacZ* coding sequence driven by upstream *ultrabithorax* (*ubx*) promoter and *bx*d enhancer elements. The upstream region also contains GAL4-binding UAS sequences to tether test proteins to the reporter. The reporter construct is expressed throughout the fly embryo in the absence of PcG proteins recruited by effector proteins bound to the GAL4 UAS. In those tissues where PcG proteins can be recruited to the reporter, transcription is silenced and LacZ staining is not detected (45). The *hunchback* promoter delivers a pulse of anterior expression such that when controlling expression of a PcG protein, *lacZ* expression of the *BGUZ* reporter is repressed in the anterior half of the embryo (diagrammed in Figure 1A). Control embryos containing the transgenic *BGUZ* reporter gene show LacZ staining at ~6h of development (Figure 1B, top panel). A fusion protein containing the YY1 REPO domain fused to the Gal4 DBD silenced the *BGUZ* reporter when expressed from the *hunchback* promoter and in a wild-type (*dRYBP*^{+/+}/*dRYBP*^{+/+}) background (second panel, Figure 1B). However, mutation of the fly RYBP gene (*dRYBP*¹) caused derepression of LacZ expression in embryos when the *dRYBP*¹ allele was inherited from the female (third panel, Figure 1B). Derepression was not observed when *dRYBP*¹ males were crossed to females (*BGUZ*; *hb-GAL4REPO*) suggesting maternal contribution of dRYBP to the egg is more important than that contributed by zygotic expression. This observation indicates a requirement for normal expression of dRYBP to mediate silencing of *BGUZ* and argues for an *in vivo* role of YAF2/dRYBP proteins in PcG-mediated silencing. As expected, *BGUZ* crosses with *ry506* showed no impact on LacZ expression (fourth panel), and a hemizygous *BGUZ*; *hbGAL4DBD-REPO* embryo showed the same staining pattern as a homozygous embryo (panels 2 and 5). Additionally, *BGUZ* expression was unaffected by a *dRYBP*¹ background (panel 6).

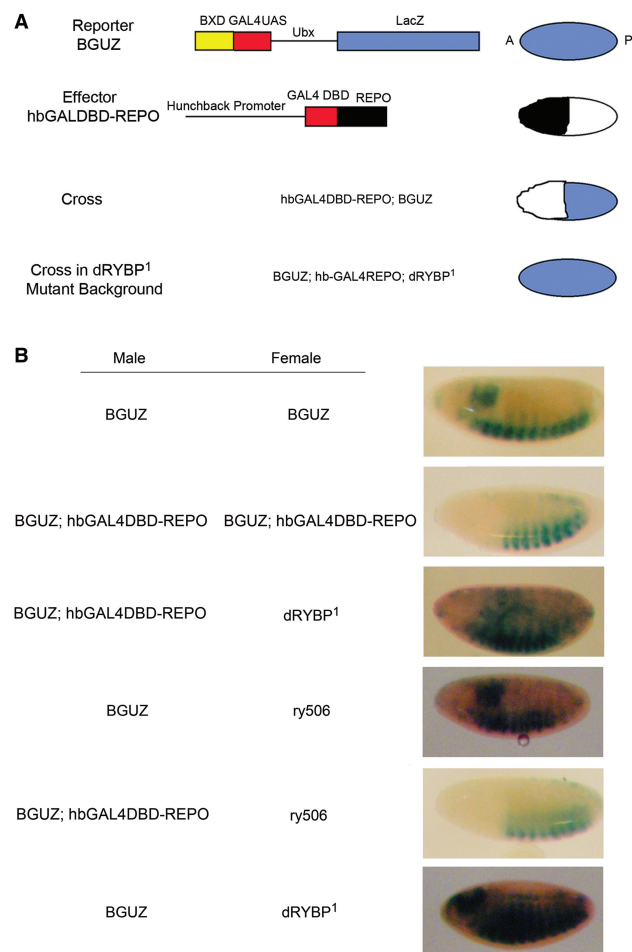


Figure 1. REPO domain silencing of *BGUZ*, a PcG-dependent reporter, requires a wild-type *dRYBP* background. (A) Maps of transgenic constructs and expected results. The *bx*d enhancer, *GAL4* UAS-binding sites, and *Ubx* promoter sequences are shown driving the *lacZ* gene. The *hunchback* promoter driven *GAL4DBD-REPO* expressing transgene is shown below. Expected fly embryo LacZ staining patterns controlled by *hbGAL4DBD-REPO* in a wild-type or *dRYBP*¹ mutant background are indicated on the right. (B) *dRYBP* is required for PcG repression in *Drosophila*. The panels depict representative *Drosophila* embryos stained for expression of LacZ using X-Gal. The crossing strategy for each embryo is depicted on the left with male and female genotypes indicated. The top panel represents *BGUZ*, the transgenic reporter gene where there is uniform staining of the embryos. The second panel represents the cross between *hbGAL4DBD-REPO* flies on the *BGUZ* background and shows anterior repression by the absence of LacZ stain. The third panel represents embryos that are crossed between *hbGAL4DBD-REPO* and the *dRYBP*¹ mutant flies where the absence of dRYBP results in loss of anterior repression. Additional controls crossed are shown in panels 4–6. *hbGAL4DBD-REPO* indicates *hunchback*-driven expression of *GAL4DBD-REPO* (GAL4DBD with YY1 201–226), *dRYBP*¹ designates the mutant *dRYBP* allele. Anterior is oriented toward the left, ventral is oriented down.

Phenotypic and biochemical rescue of *dRYBP*¹ mutants by YAF2

Sequence similarity between YAF2 and RYBP proteins (Figure 2A) led us to ask whether mammalian YAF2 could function in place of dRYBP. dRYBP was reported to physically interact with Polycomb (Pc), Pho and dRING/Sex combs extra (Sce), and *Drosophila* mutants homozygous for the *dRYBP*¹ allele are sublethal (some

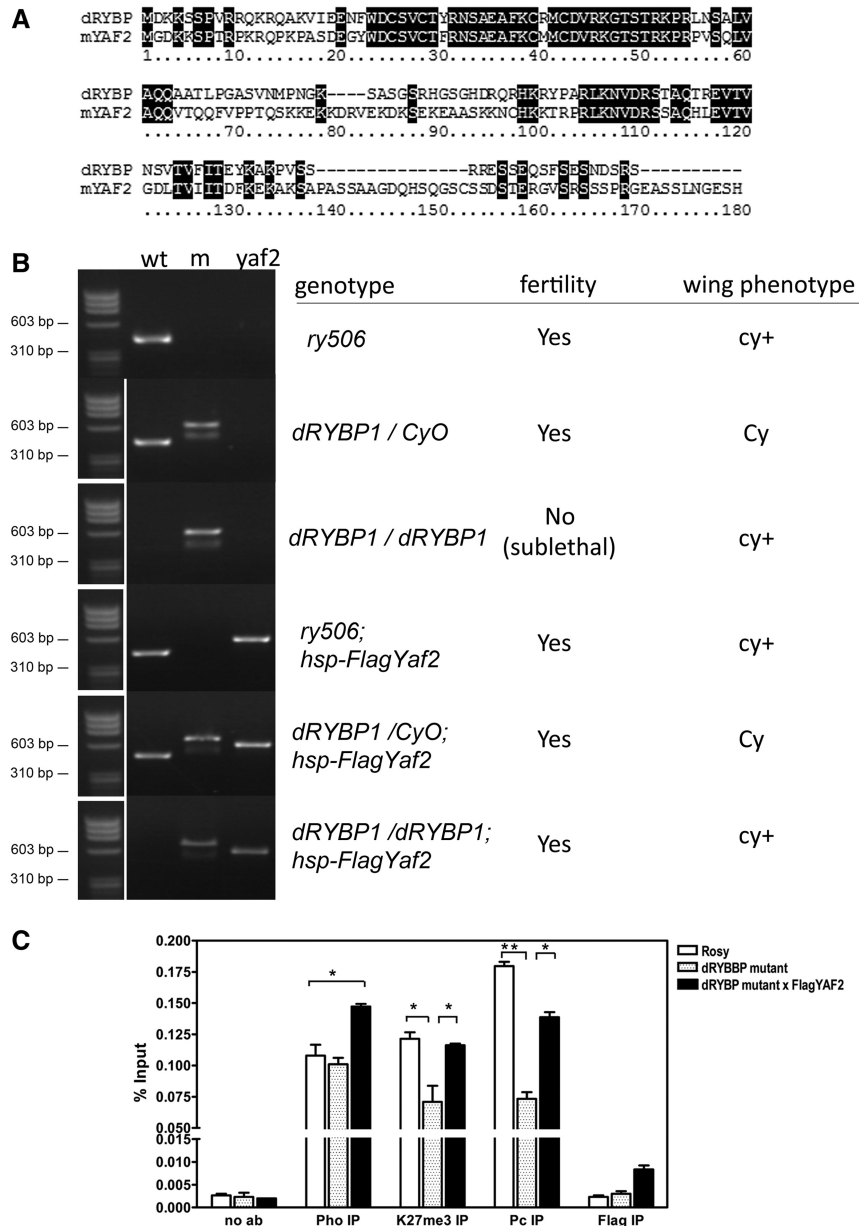


Figure 2. Mouse YAF2 can substitute for dRYBP. (A) CLUSTALW comparison of dRYBP and mYAF2 proteins highlighting the conserved residues suggestive of functional conservation of the two proteins across species. The residues are numbered according to the mouse YAF2 sequence. (B) Genotyping PCR for the wild-type *dRYBP* allele (WT), *dRYBP*¹ allele (m) and transgenic *hsp-FlagYAF2* expression construct in *Drosophila* strains. The marker lanes are reproduced for each panel for size reference of PCR products. Genotypes and phenotypes are located to the right of each panel. The fertility/viability and the observed wing phenotype of the flies are indicated. (C) ChIP assays were performed at the endogenous *ultrabithorax PRE_D* locus using the antibodies shown on the x-axis. The PRE_D sequence lies between *Drosophila melanogaster* genomic sites 12 589 500 to 12 590 299 (version 3.1). Enrichment in wild-type *rosy*⁵⁰⁶ flies is indicated by white bars, whereas the dotted bar shows a reduction in the enrichment of Pc and H3K27me3 in the absence of dRYBP in the mutant flies. This loss of PcG binding is recovered upon overexpression of mYAF2 by heatshock induction of the *hspFlagYAF2* transgene in the mutant flies as shown by the black bars. The error bars indicate the standard deviation of three independent replicates of quantitative PCR analysis. Fly strains: Rosy (*ry*⁵⁰⁶, wild-type), white; *dRYBP*¹/*CyO* (*dRYBP*¹ mutant), gray; *dRYBP*¹/*CyO*; *hsp-FlagYAF2* (corrected mutant), black. Single and double asterisks denote *P* < 0.05 and *P* < 0.001, respectively.

organisms survive to adulthood) and sterile (44). We hypothesized that if YAF2 could also interact with Pho and dRING/Scs, it would rescue the phenotypes of the *dRYBP*¹ mutation. Independent transgenic lines expressing Flag-tagged YAF2 under control of the *hsp70* promoter were crossed into a *dRYBP*¹/*CyO* background. Progeny flies were scored for the presence of *dRYBP* alleles by phenotypic and genotypic analyses. Phenotypic

analysis utilized linkage of *dRYBP* alleles with *cy* alleles. The *CyO* balancer chromosome contains a *dRYBP*⁺ allele and is linked to *cy* wings. Flies lacking this chromosome (i.e. *dRYBP*¹/*dRYBP*¹) will have *cy*⁺ wings. *CyO*/*CyO* flies die during early embryogenesis and are not detected in the adult population. Rescued *dRYBP*¹/*dRYBP*¹ mutants were predicted to be present in a 1:2 ratio to *dRYBP*¹/*CyO*. Determination of the wing phenotype of 128

offspring from a cross of *dRYBP¹/CyO*; *hsp70-flagYAF2* gave 42 *cy⁺* (predicted 42.67) and 86 *Cy* (predicted 85.33, $X^2 P = 0.92$ to 0.94, respectively). Genotypes using allele-specific PCR primers on 40 randomly chosen flies (20 each *Cy* and *cy⁺*) verified the predicted genotypes (Figure 2B). *dRYBP¹/dRYBP¹*; *hsp70-FlagYAF2* flies were tested for fertility by mating *cy⁺* males with *cy⁺* virgin females. These crosses gave rise to viable and fertile progeny lacking the wild-type *dRYBP⁺* allele determined by PCR genotyping. Genotypes and phenotypes are summarized in Figure 2B. Together, these results indicate that mouse YAF2 can rescue the sublethality and sterility phenotypes of the *dRYBP¹* allele.

We previously showed that loss of dRYBP resulted in reduction of DNA recruitment of PcG proteins Pc and E(z) and reduced H3K27me3 mark as measured by ChIP assay at the endogenous *Ultrabithorox PRE_D* sequence (28,43,49). Here we asked if addition of Flag-YAF2 would restore PcG protein binding and H3K27me3 to wild-type levels. *dRYBP¹/CyO*; *hsp70-FlagYAF2* heat-shocked embryos were compared with *dRYBP¹/CyO* and to the transgene parent strain (*ry⁵⁰⁶*) as a reference. Insufficiency of dRYBP resulted in the reduction of the H3K27me3 histone mark and Pc levels at the endogenous *PRE_D* sequence compared with *ry⁵⁰⁶* embryos (Figure 2C). However expression of Flag-YAF2 (Supplementary Figure S1) restored both H3K27me3 and Pc levels to that observed in wild-type embryos

(i.e. *dRYBP⁺/dRYBP⁺* embryos) (Figure 2C). These results indicate that YAF2 can functionally substitute for dRYBP PcG function *in vivo*.

The YY1 REPO domain and carboxy terminal region binds to YAF2

We previously showed that YAF2 is recruited to DNA in *Drosophila* embryos by the YY1 REPO domain (43). To verify this physical interaction in mammalian cells, various YY1 deletion constructs and full-length YAF2 were transiently expressed in HEK293-T cells and tested for co-immunoprecipitation (co-IP) with antibodies against the fusion tags. We predicted that YY1 constructs with an intact REPO domain (YY1 205–226) would interact with YAF2. As expected, GAL4-tagged, full-length YY1 (1–414) bound to Flag-YAF2 (Figure 3A, lane 1). However, GAL4YY1 (1–200) was unable to bind to YAF2, whereas GAL4YY1 (1–256) bound strongly (Figure 3A, lanes 2 and 3). The GAL4 DBD failed to bind to YAF2 (Figure 3A, lane 4). We previously showed that deletion of the YY1 REPO domain resulted in a protein that was unable to silence transcription, recruit PcG proteins to DNA, or bind to YAF2 in a yeast two-hybrid assay (43). Based on these studies, we predicted that the YY1 REPO deletion protein would be unable to interact with YAF2. However, GAL4 YY1ΔREPO deletion protein interacted with YAF2 in a

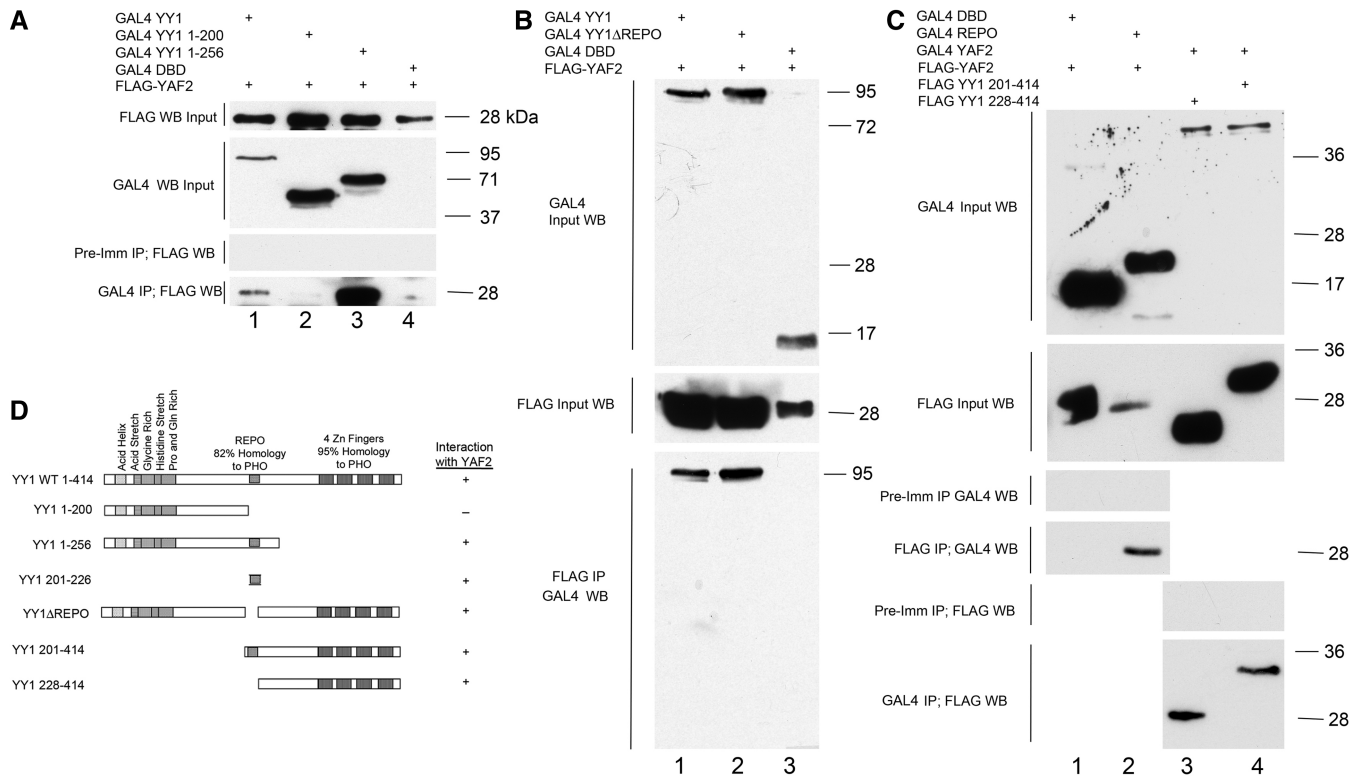


Figure 3. YY1 interacts with YAF2. (A–C) Co-IP of full-length YAF2 and YY1 deletion constructs were performed with nuclear extracts from HEK293-T cells transiently co-transfected with the expression plasmids indicated above each lane. Upper panels show direct western blot analysis of nuclear extracts as input samples for both GAL4DBD and Flag-tagged proteins. The lower panels show co-IP proteins with the indicated antibodies and detection of the interacting partner by western blot analysis using the indicated antibody (WB). (D) Diagram of YY1 mutants and summary of interaction with YAF2.

co-IP assay (Figure 3B, lanes 1–3). In addition, a construct containing only the REPO domain (YY1 amino acids 201–226) fused to the GAL4 DBD interacted with YAF2 (Figure 3C, lanes 1 and 2). This suggested that YAF2 interacted with the YY1 REPO domain as well as sequences downstream of amino acids 226. Indeed, further analysis using YY1 (228–414) identified a second YY1 region of interaction with YAF2 (Figure 3C, lanes 3 and 4). These results are summarized in Figure 3D. Previous reports of YY1–YAF2 interactions and YY1–RYBP interactions also mapped interacting regions to the zinc finger domain of YY1 (41). Thus, our results support the YY1 REPO domain as a YAF2-interacting domain and indicate a second site of interaction located between YY1 residues 228 and 414.

The YAF2 C-terminal region interacts with YY1 and with RING proteins

Having verified that the REPO domain of YY1 can interact with YAF2, we sought to define which region of YAF2 interacts with the REPO domain. Based on sequence alignment of mouse YAF2 and RYBP proteins, we prepared YAF2 N-terminal (residues 1–101) and C-terminal (residues 102–179) fragments and performed co-IP experiments with full-length YY1. A strong interaction signal was observed for YY1 with full-length YAF2 and the C-terminal fragment of YAF2 (Figure 4A). A very weak signal was observed with the N terminal fragment of YAF2 (Figure 4A), and this segment failed to support transcriptional repression in *Drosophila*

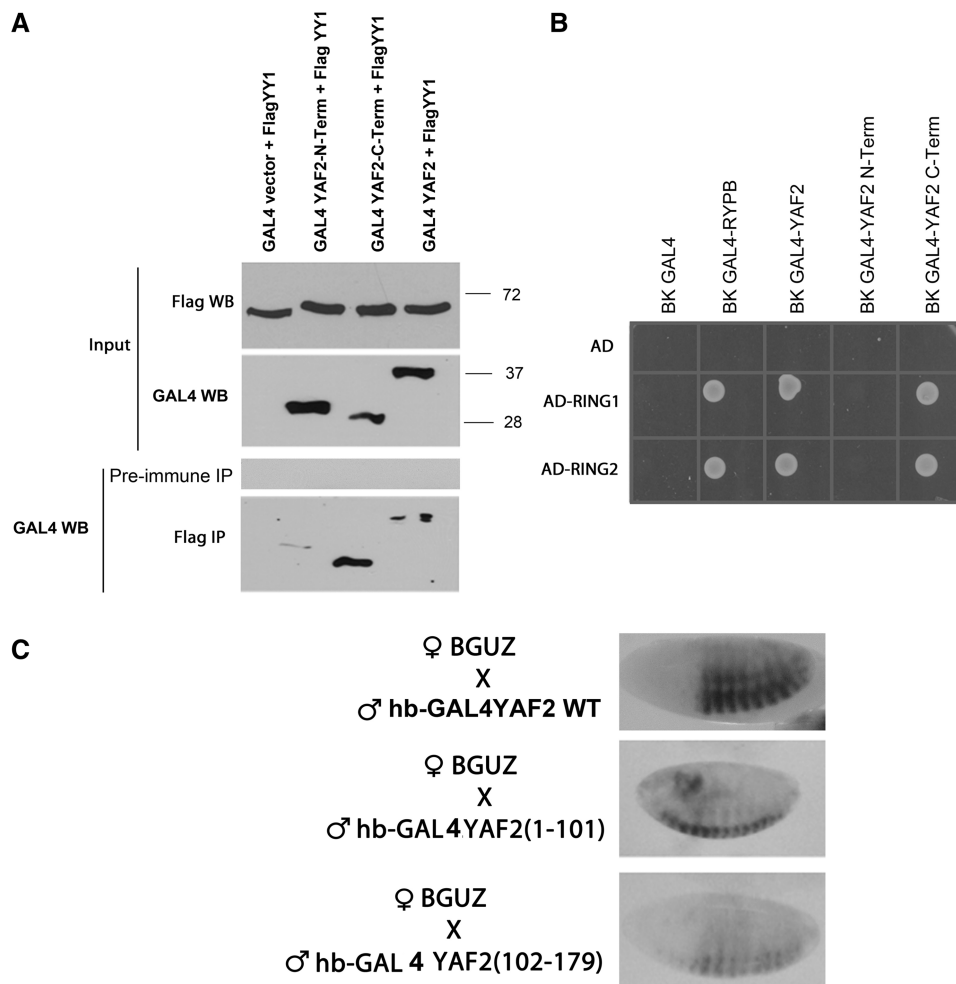


Figure 4. The C-terminal region of YAF2 interacts with YY1 and RING proteins and can silence a PcG reporter gene *in vivo*. (A) Co-IP of full-length YY1 and YAF2 deletion constructs was performed with nuclear extracts from HEK293-T cells transiently co-transfected with the expression plasmids indicated above each lane. The upper panels depict direct western blot analysis of nuclear extracts as input samples for GAL4DBD and Flag-tagged proteins. The lower panels show co-IP proteins with the indicated antibodies followed by western blot analysis using the indicated antibody (WB). (B) Yeast two hybrid detection of YAF2, RYBP and YAF2 deletion proteins with RING1 and RING2 proteins. GAL4 DBD vector expression constructs (BK) and GAL4 AD vector expression constructs (AD) were cotransformed into *S. cerevisiae* strain AH109 and grown on Trp/Leu/His/Ade drop out medium. (C) Representative *Drosophila* embryos stained for expression of LacZ using X-Gal. The crossing strategy for each embryo is depicted on the left with male and female genotypes indicated. *BGUZ* is the transgenic reporter gene. *Hb-GAL4YAF2(1–101)* and *hb-GAL4YAF2(102–179)* indicates *hunchback*-driven expression of GAL4YAF2 deletion proteins YAF2(1–101) and YAF2(102–179), respectively. The middle panel shows the absence of anterior repression by *hb-GAL4YAF2(1–101)* whereas the *hb-GAL4YAF2(102–179)* and *hb-GAL4YAF2* wild-type embryos show repression demonstrating that the C-terminal region of YAF2 is involved in PcG repression *in vivo*. Anterior is oriented toward the left, ventral is oriented down.

(see below, Figure 4C). Thus we believe this weak co-IP signal is not reflective of an interaction required for transcriptional regulation. These data indicate that the C-terminal region of YAF2 strongly interacts with YY1.

A yeast two-hybrid assay was used to test for interactions among YAF2/RYPB and RING proteins. Vectors expressing bait GAL4 DBD fusion proteins (full-length YAF2, full-length RYPB) and prey GAL4 AD fusion proteins (RING1 or RING2) were cotransformed into *S. cerevisiae* strain AH109 (Clontech) and challenged with selective media. Growth on the selective medium is possible only when the two proteins being tested interact and activate the transcription of nutritional reporter genes. As shown in Figure 4B, both YAF2 and RYPB interacted with the mammalian Ring proteins (RING1 and RING2) as well as with dRING/Sce (data not shown). This Ring-interaction region was mapped to the same C-terminal region (YAF2 102–179) that binds YY1. These results indicate that in addition to interacting with YY1, the YAF2 C-terminal region also interacts with the RING proteins.

Based on the physical interaction with the RING proteins, we tested whether the C-terminal region of YAF2 silenced a PcG-dependent reporter gene. We predicted that the residues of YAF2 that interact with the RING proteins (102–179) would likewise silence transcription of the *BGUS* reporter gene. Indeed, fly strains expressing *hb*-driven *GAL4YAF2* wild-type or *GAL4YAF2(102–179)* silenced expression of LacZ in anterior tissues as judged by absence of staining (Figure 4C). In contrast, *GAL4YAF2(1–101)* did not silence expression of LacZ in anterior tissues. Thus the C-terminal domain of YAF2 can physically interact with RING proteins and can functionally silence a PcG-dependent reporter gene. As this region also interacts with the YY1 REPO domain, YAF2 may bridge YY1 with the PRC1 complex.

YAF2 bridges interactions between YY1 and RING proteins

To determine if YY1 might interact with RING1 or RING2 independently of YAF2, we performed co-IP studies. We observed no physical interaction of YY1 with either RING1 or RING2 (Figure 5A), whereas YAF2 interacted with both the RING proteins, verifying our observations with the yeast two-hybrid system (see above). The co-IP results were supported by yeast two-hybrid assays indicating that the isolated REPO domain also was not able to interact with RING proteins (Figure 5B). To test if YAF2 acts as a bridge protein between YY1 and the PRC1 components, we performed co-IP studies of YY1 and RING1 in the presence and absence of YAF2. We found YY1 alone did not interact with RING1 but in the presence of YAF2 RING1 co-immunoprecipitated with YY1 (Figure 5C). Our results suggest a bridging function of YAF2 between the DNA-binding transcription factor YY1, and RING1, a component of the PRC1 complex.

Drosophila Polycomb (Pc) interacts with RING proteins and YY1-REPO domain

Co-IP studies performed with *Drosophila* Pc and RING proteins indicated a strong physical interaction between them substantiating the fact that these proteins physically interact to form the PRC1 complex (50) (Figure 6A). The mammalian counterpart of Pc is CBX, of which there are four isoforms. Surprisingly, YY1 also interacted with Pc (Figure 6A). Recent work by Gao and colleagues (13), indicated that RYPB and CBXs are mutually exclusive in that they do not copurify. We tested for the formation of a ternary complex between YY1, YAF2 and Pc proteins by performing co-IP studies. We found Pc interacted with both YAF2 and YY1 separately, but the interaction of Pc with YAF2 was reduced in the presence of YY1 (Figure 6B). If the interaction between Pc and YAF2 is weak, then increasing the stringency of binding conditions would further inhibit their interactions. This was tested by increasing the salt concentration in the IP buffer from the usual 200 mM to 1 M. As expected the interaction was abrogated with high salt (Figure 6B, right panel). Under similar conditions, we were still able to detect interactions between Pc and YY1 suggesting that Pc more strongly interacts with YY1 compared with YAF2 (Figure 6B, right panel). Our data also show that this interaction is mediated by the REPO domain of YY1, adding another functional feature of the REPO domain (Figure 6C).

YAF2 is essential for Polycomb recruitment in mammals

To investigate whether YAF2 is involved in PcG recruitment in mammals, we knocked-down YAF2 in HeLa cells and performed ChIP assays at mammalian PRE sequences. RNAi knock-down led to dramatic loss of YAF2 transcripts and protein levels, but caused little change in the expression levels of other PcG proteins (Figure 7A). We then assayed for PcG recruitment at DNA sites previously shown to bind to SUZ12 (*MYT1D* and *EiF3S10* genes) (48), to Bmi-1 (the *HoxA2* gene provided as a positive control by Millipore; cat# 17-664) and by various PcG proteins to a recently reported mammalian ‘PRE-like’ sequence (*HoxD11.12* gene) (37). YAF2 knock-down resulted in reduced recruitment of PcG proteins and reduced H3 K27 methylation on all PRE sequences 48 h post knock-down (Figure 7B). We performed gain-of-function studies by overexpressing YAF2 in HeLa cells followed by ChIP assay for PcG binding. We found increased YAF2 expression caused increased YY1 binding but PcG binding did not exceed that observed in cells treated with empty vector (Figure 7C). This may indicate rate-limiting abundance of unidentified tertiary factors needed to further raise PcG DNA binding in response to increased YY1 DNA binding. Two of the mammalian PREs (*MYT1D* and *EiF3S10*) (48) also showed reduction of YY1 binding after YAF2 knock-down (Figure 7B). Although the mechanism for this is not clear, it suggests that YAF2 may augment YY1 DNA binding at some genomic locations. This is supported by elevated YY1 binding at the *MYT1D* site after YAF2 overexpression (Figure 7C).

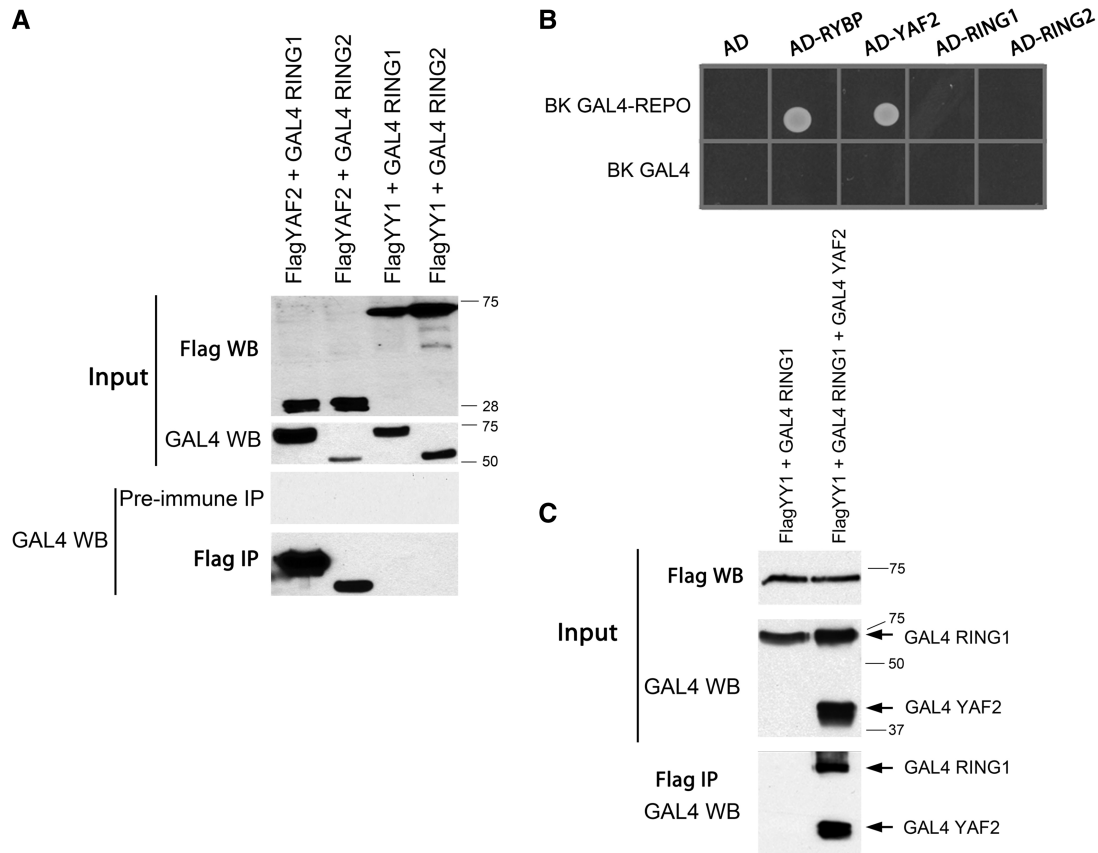


Figure 5. YAF2 acts as a bridge protein between YY1 and the RING proteins. (A) Co-IP of full-length YAF2 or YY1 and full-length RING1 and RING2 proteins were performed with nuclear extracts from HEK293-T cells transiently cotransfected with the expression plasmids indicated above each lane. The upper panels depict direct western blot analysis of nuclear extracts as input samples for GAL4DBD and Flag-tagged proteins. The lower panels show that YAF2 co-immunoprecipitates with both RING1 and RING2 but there is no enrichment of the RING proteins with YY1 (Flag IP). (B) Yeast two-hybrid detection of the YY1 REPO domain with YAF2 and RYBP or RING1 and RING2 proteins shows that YY1 REPO domain interacts with RYBP and YAF2 but not with the RING proteins. GAL4 DBD vector expression constructs (BK) and GAL4 AD vector expression constructs (AD) were cotransformed into *S. cerevisiae* strain AH109 and grown on Trp/Leu/His/Ade drop-out medium. (C) Co-IP of RING1 with YY1 was observed only in the presence of YAF2. Flag-tagged YY1 was co-transfected with Gal4RING1 in the absence or presence of Gal4YAF2. IP was performed with Flag antibody and western blot analysis was performed with GAL4 antibody. The upper two panels represent the input samples and the lower panel represents the IP fraction.

YY1 is essential for PcG assembly on the chromatin

Studies from our laboratory showed that in mammals YY1 is the functional homolog of Pho as it can recruit PcG proteins to DNA and can rescue Pho mutants (33). Though PREs in mammals are not well-defined, YY1-binding sites have been identified at various Hox loci (37,39,51) that are also enriched in PcG proteins suggesting that YY1 binds to DNA and facilitates PcG recruitment.

Our current studies indicate that YAF2 interacts with both PRC1 components and YY1, possibly acting as a bridge protein. If YY1 DNA binding is *a priori* necessary for PcG recruitment, then reduced levels of YY1 would result in lost PcG binding that should not be rescued by YAF2. Indeed, knock-down of YY1 in HeLa cells (Figure 8A) resulted in a dramatic reduction of PcG DNA binding (Figure 8B) with concomitant increased gene expression of the *HoxA2* and *HoxD13* genes (Figure 8C). Earlier we showed that reduced PcG binding upon loss of YAF2 can be reversed by

overexpressing YAF2. However, lost PcG recruitment due to loss of YY1 was not rescued by excess YAF2 at most of the sites (Figure 8B). Interestingly, overexpression of YAF2 increased YAF2 DNA binding even in the absence of YY1. YAF2 contains a zinc-finger domain that could be involved in DNA binding irrespective of YY1, but this binding may not be sufficient for recruitment of PRC1 or PRC2 complexes. Further studies are being directed toward investigating whether YAF2 can bind to DNA by itself.

YAF2 and RYBP regulate distinct Polycomb target genes

Recent studies showed that RYBP is involved in PcG assembly on the chromatin (13,37,52). We sought to analyze the effects of loss of YAF2 and RYBP on Polycomb target gene expression in HeLa cells using the pathway-focused PCR array provided by SABiosciences, Qiagen. Hierarchical clustering of 84 Polycomb target gene expression patterns after YAF2 or RYBP knock-down is shown in Figure 9A. We used 2-fold regulation

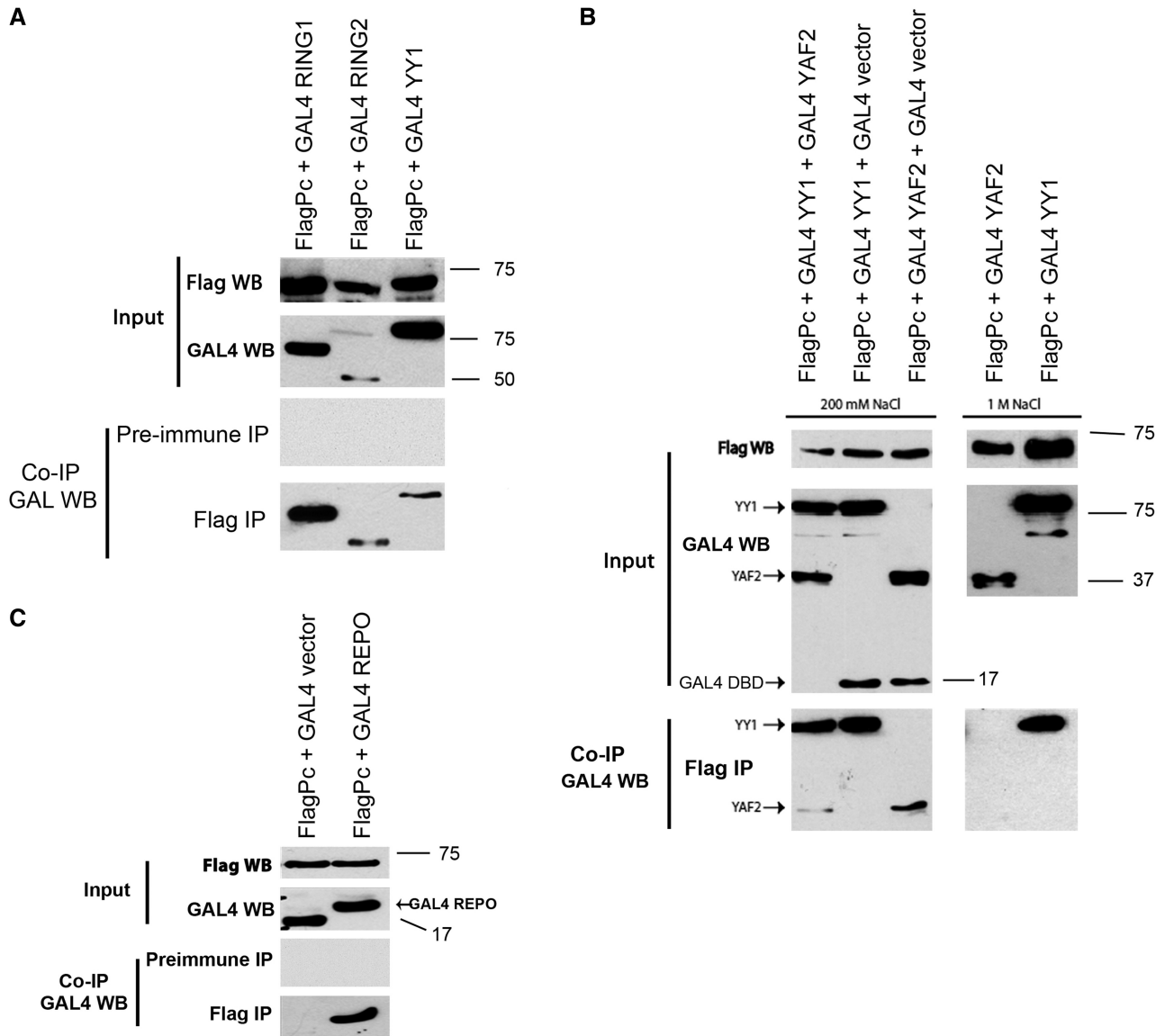


Figure 6. *Drosophila* Pc interacts with RING, YAF2 and the YY1 REPO domain. (A) Co-IP assay was performed with nuclear extracts from HEK293-T cells transiently co-transfected with the expression plasmids indicated above each lane. The upper panels depict direct western blot analysis of nuclear extracts as input samples for GAL4DBD and Flag-tagged proteins. The lower panels show that Pc interacts not only with RING1 and RING2 but also YY1 (Flag IP). (B) Co-IP assay was performed with nuclear extracts from HEK293-T cells transiently co-transfected with the expression plasmids indicated above each lane. Both YY1 and YAF2 coimmunoprecipitate with Pc, but the interaction of Pc is stronger with YY1 compared with YAF2, as determined by a ternary co-IP. With increasing salt concentration, the interaction between YAF2 and Pc is lost suggesting a lower-affinity interaction. However, the interaction between YY1 and Pc remains intact at high salt. (C) *Drosophila* Pc interacts with the YY1 REPO domain. pcDNA3-GAL4DBD plasmid was used as the vector control.

as the cut-off for analyzing the genes. Though the total number of genes that showed altered expression upon knock-down of YAF2 or RYBP was similar, more genes were upregulated upon RYBP knock-down than YAF2. On the contrary, YAF2 knock-down resulted in more genes downregulated than RYBP (Supplementary Tables S2–S9). As shown in Figure 9B, knock-down of RYBP for 48 h and 96 h (Supplementary Figure S2) resulted in upregulation of about 50 Polycomb target genes (Supplementary Tables S6 and S7). Of these, 34 genes are common between the two time points, and 25 can be functionally classified into three broad groups including a distinct category of developmentally

important transcription factors (Supplementary Table S10). Of the 34 genes, 22 were shown earlier to be regulated by PRC2 and Bmi-1 (53) in human ESCs (Supplementary Table S11).

Knock-down of YAF2 resulted in downregulation of 23 genes at 48 h (with a 2-fold cut-off). When compared with the downregulated genes at 96 h, there was a significant reversal in the fold regulation of 19 of these genes (Supplementary Tables S4 and S5 and the array data). Although there were only a handful of genes downregulated by RYBP knock-down, (Supplementary Tables S8 and S9) most of them were downregulated by YAF2 knock-down as well (Figure 9B). The PCR array

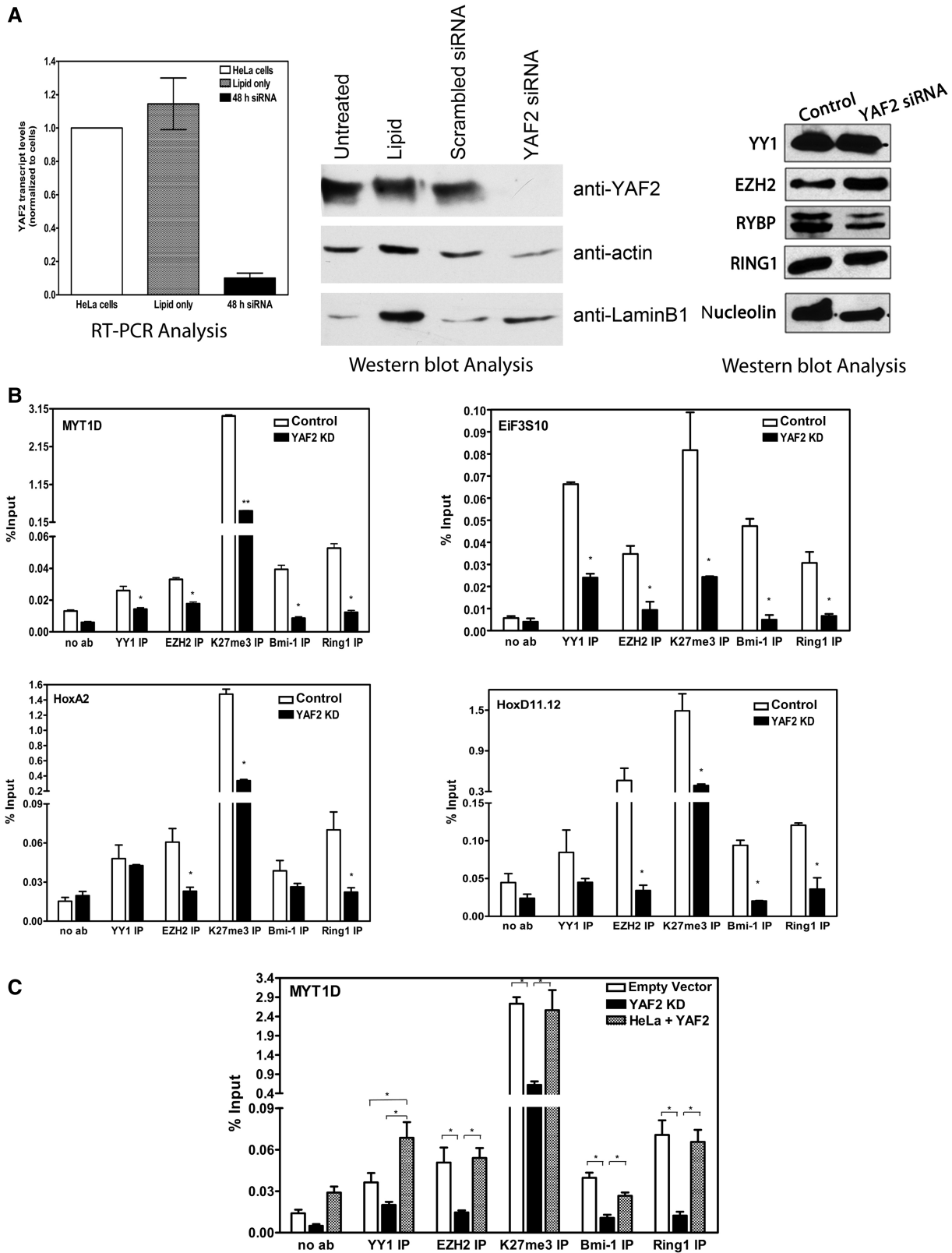


Figure 7. YAF2 is required for PcG recruitment in HeLa cells. (A) Knock-down of YAF2. qRT-PCR of YAF2 transcript levels upon 48 h knock-down of YAF2 using siRNA in HeLa cells is shown on the left. Levels of YAF2 transcripts were normalized to actin. The graph shows the fraction of residual transcript in cells treated with Lipofectamine 2000 only and with YAF2 siRNA when compared with control HeLa cells. YAF2 western blot data of untreated, vehicle control, scrambled siRNA, and YAF2 siRNA treated cells are shown in the middle panel with actin and laminB1 as controls. The right panel shows the expression levels of various Polycomb components upon knock-down of YAF2 for 48 h. Nuclear extracts were made from control and YAF2 siRNA-treated HeLa cells and western blot analysis was performed with antibodies against YY1, EZH2, RYBP and RING1 proteins. Nucleolin (sc-8031, Santa Cruz) was used to normalize for loading errors. (B) ChIP-qPCR analysis to monitor enrichment of PRC1

(continued)

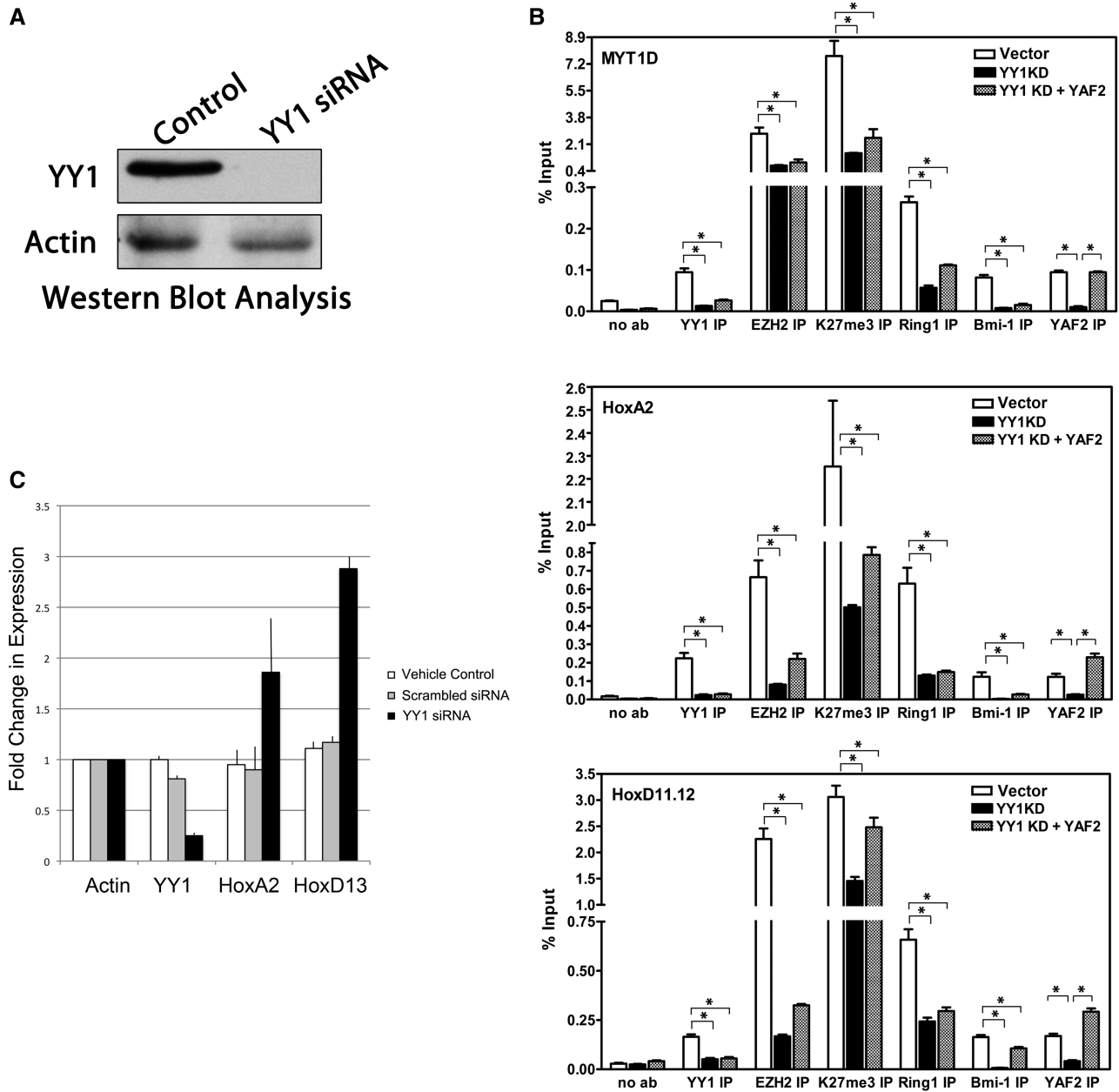


Figure 8. YY1 DNA binding is essential for PcG recruitment. (A) Western blot analysis to monitor the levels of YY1 upon knock-down with siRNA. Whole-cell extracts were prepared from HeLa cells transfected with YY1-siRNA after 96 h followed by western blot analysis to detect YY1 levels. The membrane was stripped and re-probed with actin antibody (A 1978, Sigma) to normalize for loading errors. (B) ChIP-qPCR analysis to monitor the enrichment of YY1, PRC1 and PRC2 components at ‘PRE-like’ sequences upon loss of YY1. The open bars represent control HeLa cells transfected with vector control and the black bars represent HeLa cells in which YY1 has been knocked down. Overexpression of YAF2 does not compensate for the loss of YY1 in recruiting Polycomb proteins (dotted bar) indicating that YY1 is essential for PcG binding. The x-axis indicates the antibodies used for IP. The error bars indicate the standard deviation of three independent replicates of quantitative PCR analysis and the data are represented as percentage of input. Asterisks denote $P < 0.05$. (C) YY1 knock-down results in increased *hoxA2* and *hoxD13* gene expression. qPCR is shown for actin, YY1 *hoxA2* and *hoxD13* transcripts in cells treated with vehicle control, scrambled siRNA or YY1 siRNA.

Figure 7. Continued

and PRC2 components at ‘PRE-like’ sequences upon knock-down of YAF2 for 48 h. The open bars represent lipid control HeLa cells and the black bars represent cells where YAF2 has been knocked down. The x-axis indicates the antibodies used for IP. The error bars indicate the standard deviation of three independent replicates of quantitative PCR analysis and the data are represented as percentage of input. (C) ChIP-qPCR analysis at the *MYT1D* site shows that overexpression of YAF2 results in increased recruitment of PcG proteins at ‘PRE-like’ sites (dotted bar). The x-axis indicates the antibodies used for the IP. The error bars indicate the standard deviation of three independent replicates of quantitative PCR analysis and the data are represented as percentage of input. Single and double asterisks denote $P < 0.05$ and $P < 0.001$, respectively.

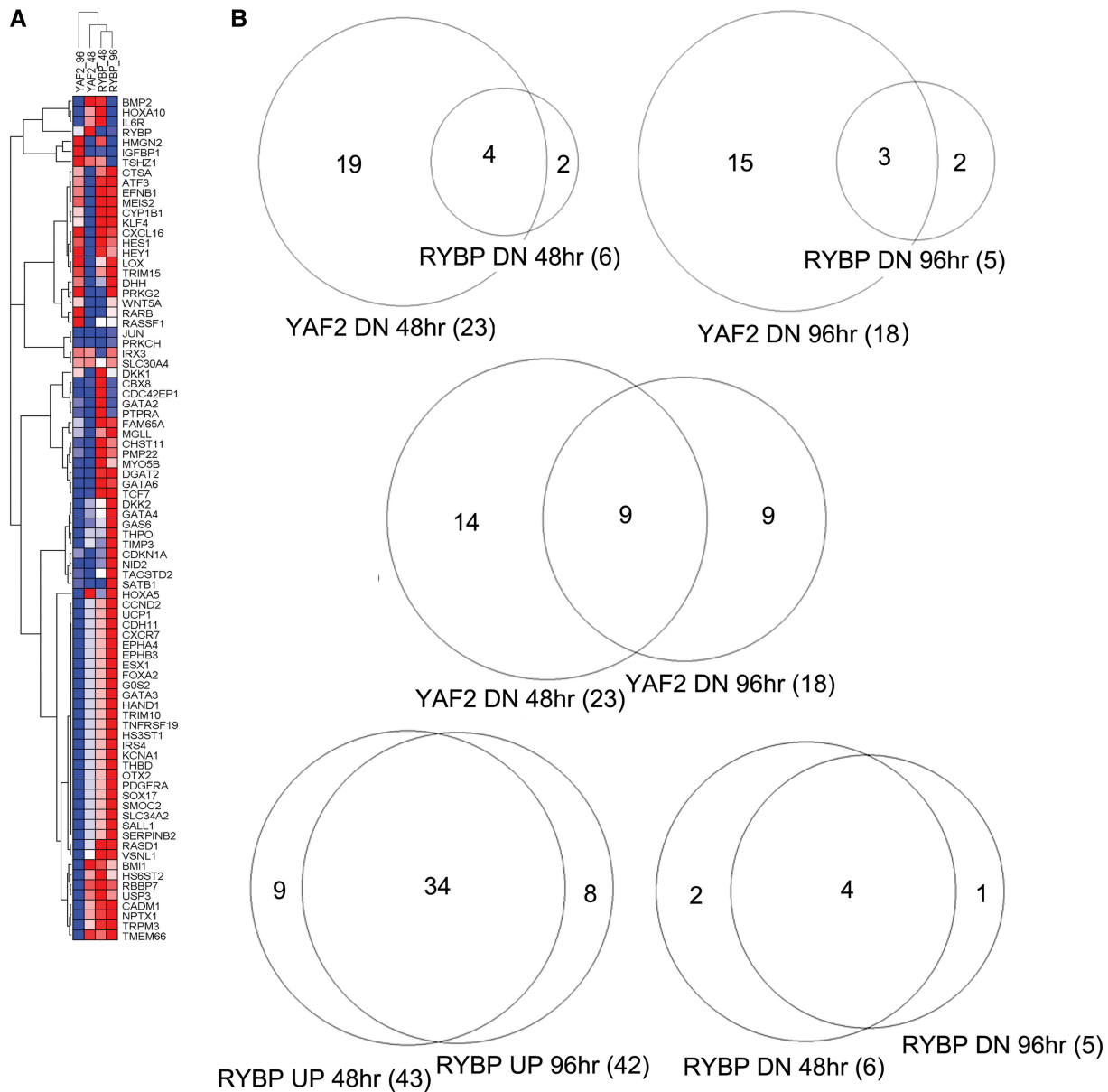


Figure 9. Effect of YAF2 and RYBP knock-down on Polycomb target gene expression. (A) Hierarchical clustering of gene expression of PcG regulated genes on RT² profiler PCR arrays upon knock-down of YAF2 and RYBP for 48 h and 96 h. Results show only partial overlap of genes regulated by YAF2 and RYBP. Red color represents increased expression and blue color represents decreased expression. (B) Venn diagrams show the overlap of genes regulated upon knock-down of RYBP for 48 h and 96 h and those between RYBP and YAF2.

analysis suggests that most Polycomb target genes are differentially regulated by RYBP and YAF2 suggesting distinct roles of the homologs in vertebrates.

DISCUSSION

Based on sequence similarity and the fact that PcG functions are conserved across species, we hypothesized that YAF2 would function as a PcG protein in flies. Our results confirm this prediction and showed PcG repression by YAF2. Mammalian YAF2 was also able to repress transcription *in vivo* and to rescue the *dRYBP¹/dRYBP¹* phenotype, yielding flies that are viable and fertile. Our results confirmed that YAF2 is involved in PcG-mediated

repression and that there is sufficient functional conservation of these gene products to rescue the *dRYBP¹* defect in flies. Mechanistically we showed that YAF2 can not only perform genetic corrections, it can also compensate biochemically for RYBP. Our ChIP assays indicated that YAF2 expression can restore PcG DNA recruitment in *dRYBP¹* embryos. Thus, in the absence of *Drosophila* RYBP, mYAF2 can act as a mediator of PcG function.

We explored the biochemical mechanism of targeting of PcG proteins to chromatin. Our results indicate that YAF2 provides a connecting link between the silencing activities associated with various PRC1 core components to the sequence-specific DNA-binding transcription factor YY1 (Figure 10). This work suggests a mechanism for

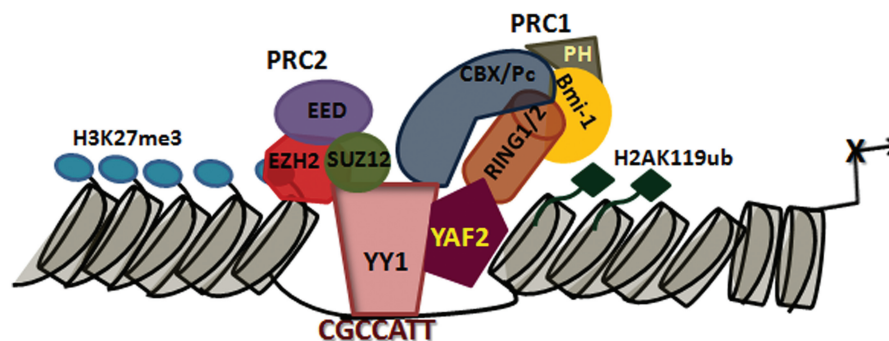


Figure 10. Model of PcG recruitment by YY1. The diagram represents proposed mechanisms of PcG recruitment by YY1. In all cases YY1 binds to DNA. YY1 may then interact with YAF2 to recruit the PRC1 complex. Alternatively, in the absence of YAF2, YY1 may recruit the PRC1 complex via interaction with CBX/Pc. Either of these recruitment pathways would lead to ubiquitination of H2A on lysine 119. In addition, our published work showed that YY1 interacts with EZH2 and SUZ12 (PRC2 components) (59) thereby recruiting the PRC2 complex resulting in trimethylation of histone H3 at lysine 27.

YY1 recruitment of PRC1 components to the DNA via bridging functions of YAF2.

We show the formation of a ternary complex of YY1, YAF2 and RING1 only in the presence of YAF2 suggesting that YY1 bound to the DNA interacts with the PRC1 complex via YAF2 thereby providing a mechanism for YAF2/RYP bridging YY1 to the core PcG-complex. Co-IP and yeast two hybrid assays indicated that the C-terminal region of YAF2 physically interacts with the YY1 REPO domain as well as the RING proteins, consistent with its role as a bridge protein between YY1 and the PRC1 complex. This C-terminal region of YAF2, when tethered to DNA can functionally silence a PcG-dependent reporter gene by interacting with the RING proteins, apparently bypassing the need for YY1 (in this assay YAF2 residues were bound to DNA through the GAL4 DBD). The ability of YAF2/RYP to interact with a multiplicity of PcG-PRC1 complexes would provide a mechanism for recruitment to sites in the genome occupied by YY1. It is noteworthy that at least one report indicates interactions between YY1 and RING1/2 (54) without exogenous YAF2 or RYP added to the experimental system. However since the proteins were isolated from transfected HEK293-T cells, it is possible that endogenous YAF2 or RYP proteins remain bound with the tagged-recombinant proteins during purification. The presence of either YAF2 or RYP could mediate the observed co-purification of YY1 and RING proteins. Our wash buffer contains 1% Triton X-100, whereas Garcia-Tunon used a buffer containing 0.1% NP-40, thus presenting a technical difference that could explain differential co-purification of proteins from the same cell type. In addition their experiments were with whole-cell extracts whereas ours were with nuclear extracts.

Recent studies in vertebrates have shown that PRC1 composition is highly complex based on various assortments of family members. Each combination results in formation of a specific subtype of the PRC1 complex (13,55–57). CBX, the mammalian counterpart of *Drosophila* Polycomb (Pc) has four isoforms that form different PRC1 complexes in mouse ESCs for the maintenance of pluripotency as well as during differentiation

(20,52). High-throughput studies have shown that RYP and CBXs form mutually exclusive PRC1 complexes (13). To reduce the complexity involving isoforms and to address the mechanistic aspect of recruitment, we performed co-IP studies between the fly Pc protein, and YAF2, the RING proteins and the YY1 REPO domain. Strong interaction was detected between Pc and RING1/2 as core components of the PRC1 complex. Interestingly, Pc interacted with YY1 full-length and the YY1 REPO domain. This was unexpected as we anticipated the REPO domain would only interact with YAF2. To determine the possibility of a ternary complex formation, we performed co-IP with Pc, YY1 and YAF2. Pc interaction with YAF2 was reduced in the presence of YY1 suggesting a competitive interaction of YY1 and YAF2 for Pc.

These results suggest a number of mechanisms of Polycomb recruitment by YY1 (Figure 10). First, we envision that many PRE sites bind to YY1 and that interaction with YAF2/RYP results in recruitment of PRC1 complexes via the RING proteins. Several studies showed that YY1-binding sites are present in mammalian ‘PRE-like’ sequences (36,37,39,58). Second, at promoters where RYP or YAF2 are not present, YY1 may directly interact with Pc/CBX to recruit the PRC1 complex to DNA. Finally, our recent report indicating direct physical interaction of YY1 with EZH2 and SUZ12 (59) demonstrates that YY1 recruits PcG proteins to DNA by interacting with the PRC2 complex as well. In flies, biochemically, Pho has been reported to interact with components of both PRC1 and PRC2 in co-IP and GST pulldown assays (58,60). These results provide mechanistic models for YY1-dependent recruitment of PcG complexes (Figure 10). In all cases, YY1 provides the DNA-binding specificity and it interacts with YAF2/RYP or Pc/CBX components of PRC1, or with components of the PRC2 complex (EZH2, SUZ12). Further studies are required to identify which CBX isoform(s) interacts with YY1 in context of mammalian subcomplexes and ESCs (Figure 10).

Our ChIP analyses in HeLa cells showed that knock-down of YAF2 resulted in abrogation of PcG protein binding at sites that were previously identified as ‘PRE-like’ sequences (37,48). Although our analysis of PcG regulated gene expression arrays indicate some overlap

of YAF2 and RYBP function, they seem to regulate in an opposite manner. Prolonged knock-down of YAF2 results in the reversal of 19 of the 23 downregulated genes suggesting a compensatory mechanism for the absence of YAF2. It can be envisioned that RYBP may take over the role of YAF2 in recruiting PcG complexes. Given that YAF2 and RYBP expression patterns differ in mouse embryos (42) and in human tissues, and the fact that they have contrasting effects on *hGABP/E4TF1*-dependent transcription (61), it is likely that genome-wide ChIP-seq studies will reveal a distinct set of promoters bound separately by YAF2 and RYBP. The differential regulation by YAF2 and RYBP suggests a mechanism for fine-tuning the regulation of gene expression by PcG proteins in mammals and adds another layer of complexity to mammalian developmental processes as compared with flies, which contain a single RYBP gene. Further studies are needed to dissect the distinct molecular function(s) of YAF2 and RYBP and determine whether they themselves bind to DNA and have non-Polycomb functions.

SUPPLEMENTARY DATA

Supplementary Data are available at NAR Online.

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REFERENCES

- Lewis, E.B. (1978) A gene complex controlling segmentation in *Drosophila*. *Nature*, **276**, 565–570.
- Simon, J.A. and Kingston, R.E. (2009) Mechanisms of Polycomb gene silencing: knowns and unknowns. *Nat. Rev. Mol. Cell. Biol.*, **10**, 697–708.
- Kerppola, T.K. (2009) Polycomb group complexes - many combinations, many functions. *Trends Cell. Biol.*, **19**, 692–704.
- Kassis, J.A. and Brown, J.L. (2013) Polycomb group response elements in *Drosophila* and vertebrates. *Adv. Genet.*, **81**, 83–118.
- Lagarou, A., Mohd-Sarip, A., Moshkin, Y.M., Chalkley, G.E., Bezstarosti, K., Demmers, J.A. and Verrijzer, C.P. (2008) dKDM2 couples histone H2A ubiquitylation to histone H3 demethylation during Polycomb group silencing. *Genes Dev.*, **22**, 2799–2810.
- Farcas, A.M., Blackledge, N.P., Sudbery, I., Long, H.K., McGouran, J.F., Rose, N.R., Lee, S., Sims, D., Cerase, A., Sheahan, T.W. *et al.* (2012) KDM2B links the Polycomb Repressive Complex 1 (PRC1) to recognition of CpG islands. *Elife*, **1**, e00205.
- Wu, X., Johansen, J.V. and Helin, K. (2013) Fbxl10/Kdm2b recruits polycomb repressive complex 1 to CpG islands and regulates H2A ubiquitylation. *Mol. Cell*, **49**, 1134–1146.
- He, J., Shen, L., Wan, M., Taranova, O., Wu, H. and Zhang, Y. (2013) Kdm2b maintains murine embryonic stem cell status by recruiting PRC1 complex to CpG islands of developmental genes. *Nat. Cell Biol.*, **15**, 373–384.
- Lanzuolo, C. and Orlando, V. (2012) Memories from the Polycomb group proteins. *Ann. Rev. Genet.*, **46**, 561–589.
- Mateos-Langerak, J. and Cavalli, G. (2008) Polycomb group proteins and long-range gene regulation. *Adv. Genet.*, **61**, 45–66.
- Simon, J.A. and Kingston, R.E. (2013) Occupying chromatin: Polycomb mechanisms for getting to genomic targets, stopping transcriptional traffic, and staying put. *Mol. Cell*, **49**, 808–824.
- Aloia, L., Di Stefano, B. and Di Croce, L. (2013) Polycomb complexes in stem cells and embryonic development. *Development*, **140**, 2525–2534.
- Gao, Z., Zhang, J., Bonasio, R., Strino, F., Sawai, A., Parisi, F., Kluger, Y. and Reinberg, D. (2012) PCGF homologs, CBX proteins, and RYBP define functionally distinct PRC1 family complexes. *Mol. Cell*, **45**, 344–356.
- Li, G., Margueron, R., Ku, M., Chambon, P., Bernstein, B.E. and Reinberg, D. (2010) Jarid2 and PRC2, partners in regulating gene expression. *Genes Dev.*, **24**, 368–380.
- Levine, S.S., Weiss, A., Erdjument-Bromage, H., Shao, Z., Tempst, P. and Kingston, R.E. (2002) The core of the Polycomb repressive complex is compositionally and functionally conserved in flies and humans. *Mol. Cell. Biol.*, **22**, 6070–6078.
- Trojer, P., Cao, A.R., Gao, Z., Li, Y., Zhang, J., Xu, X., Li, G., Losson, R., Erdjument-Bromage, H., Tempst, P. *et al.* (2011) L3MBTL2 protein acts in concert with PcG protein-mediated monoubiquitination of H2A to establish a repressive chromatin structure. *Mol. Cell*, **42**, 438–450.
- Ringrose, L. (2007) Polycomb comes of age: genome-wide profiling of target sites. *Curr. Opin. Cell Biol.*, **19**, 290–297.
- Schwartz, Y.B., Kahn, T.G., Nix, D.A., Li, X.Y., Bourgon, R., Biggin, M. and Pirrotta, V. (2006) Genome-wide analysis of Polycomb targets in *Drosophila melanogaster*. *Nat. Genet.*, **38**, 700–705.
- N gre, N., Hennetin, J., Sun, L.V., Lavrov, S., Bellis, M., White, K.P. and Cavalli, G. (2006) Chromosomal distribution of PcG proteins during *Drosophila* development. *PLoS Biol.*, **4**, e170.
- Tavares, L., Dimitrova, E., Oxley, D., Webster, J., Poot, R., Demmers, J., Bezstarosti, K., Taylor, S., Ura, H., Koide, H. *et al.* (2012) RYBP-PRC1 complexes mediate H2A ubiquitylation at polycomb target sites independently of PRC2 and H3K27me3. *Cell*, **148**, 664–678.
- Klymenko, T., Papp, B., Fischle, W., Kocher, T., Schelder, M., Fritsch, C., Wild, B., Wilm, M. and Muller, J. (2006) A Polycomb group protein complex with sequence-specific DNA-binding and selective methyl-lysine-binding activities. *Genes Dev.*, **20**, 1110–1122.
- Scheuermann, J.C., de Ayala Alonso, A.G., Oktaba, K., Ly-Hartig, N., McGinty, R.K., Fraterman, S., Wilm, M., Muir, T.W. and M ller, J. (2010) Histone H2A deubiquitinase activity of the Polycomb repressive complex PR-DUB. *Nature*, **465**, 243–247.
- Cai, Y., Jin, J., Yao, T., Gottschalk, A.J., Swanson, S.K., Wu, S., Shi, Y., Washburn, M.P., Florens, L., Conaway, R.C. *et al.* (2007) YY1 functions with INO80 to activate transcription. *Nat. Struct. Mol. Biol.*, **14**, 872–874.
- Bhatia, S., Pawar, H., Dasari, V., Mishra, R.K., Chandrashekar, S. and Brahmachari, V. (2010) Chromatin remodeling protein INO80 has a role in regulation of homeotic gene expression in *Drosophila*. *Genes Cell*, **15**, 725–735.
- Bantignies, F., Roure, V., Comet, I., Leblanc, B., Schuettengruber, B., Bonnet, J., Tixier, V., Mas, A. and Cavalli, G. (2011) Polycomb-dependent regulatory contacts between distant hox loci in *Drosophila*. *Cell*, **144**, 214–226.
- Schuettengruber, B. and Cavalli, G. (2013) Polycomb domain formation depends on short and long distance regulatory cues. *PLoS One.*, **8**, e56531.

27. Pasini,D., Cloos,P.A., Walfridsson,J., Olsson,L., Bukowski,J.P., Johansen,J.V., Bak,M., Tommerup,N., Rappsilber,J. and Helin,K. (2010) JARID2 regulates binding of the Polycomb repressive complex 2 to target genes in ES cells. *Nature*, **464**, 306–310.
28. Fritsch,C., Brown,J.L., Kassis,J.A. and Muller,J. (1999) The DNA-binding Polycomb group protein pleiohomeotic mediates silencing of a *Drosophila* homeotic gene. *Development*, **126**, 3905–3913.
29. Brown,J.L., Mucci,D., Whiteley,M., Dirksen,M.-L. and Kassis,J.A. (1998) The *Drosophila* polycomb group gene pleiohomeotic encodes a DNA binding protein with homology to the transcription factor YY1. *Mol. Cell*, **1**, 1057–1064.
30. Herz,H.M., Mohan,M., Garrett,A.S., Miller,C., Casto,D., Zhang,Y., Seidel,C., Haug,J.S., Florens,L., Washburn,M.P. *et al.* (2012) Polycomb repressive complex 2-dependent and -independent functions of Jarid2 in transcriptional regulation in *Drosophila*. *Mol. Cell Biol.*, **32**, 1683–1693.
31. Landeira,D., Sauer,S., Poot,R., Dvorkina,M., Mazzarella,L., Jorgensen,H.F., Pereira,C.F., Leleu,M., Piccolo,F.M., Spivakov,M. *et al.* (2010) Jarid2 is a PRC2 component in embryonic stem cells required for multi-lineage differentiation and recruitment of PRC1 and RNA Polymerase II to developmental regulators. *Nat. Cell Biol.*, **12**, 618–624.
32. Peng,J.C., Valouev,A., Swigut,T., Zhang,J., Zhao,Y., Sidow,A. and Wysocka,J. (2009) Jarid2/Jumonji coordinates control of PRC2 enzymatic activity and target gene occupancy in pluripotent cells. *Cell*, **139**, 1290–1302.
33. Atchison,L., Ghias,A., Wilkinson,F., Bonini,N. and Atchison,M.L. (2003) The YY1 transcription factor functions as a PcG protein in vivo. *EMBO J.*, **22**, 1347–1358.
34. Srinivasan,L. and Atchison,M.L. (2004) YY1 DNA binding and PcG recruitment requires CtBP. *Genes Dev.*, **18**, 2596–2601.
35. Wilkinson,F.H., Park,K. and Atchison,M.L. (2006) Polycomb recruitment to DNA in vivo by the YY1 REPO domain. *Proc. Natl Acad. Sci. USA*, **103**, 19296–19301.
36. Sing,A., Pannell,D., Karaiskakis,A., Sturgeon,K., Djabali,M., Ellis,J., Lipshitz,H.D. and Cordes,S.P. (2009) A vertebrate Polycomb response element governs segmentation of the posterior hindbrain. *Cell*, **138**, 885–897.
37. Woo,C.J., Kharchenko,P.V., Daheron,L., Park,P.J. and Kingston,R.E. (2010) region of the human HOXD cluster that confers polycomb-group responsiveness. *Cell*, **140**, 99–110.
38. Cuddapah,S., Roh,T.Y., Cui,K., Jose,C.C., Fuller,M.T., Zhao,K. and Chen,X. (2010) A novel human polycomb binding site acts as a functional polycomb response element in *Drosophila*. *PLoS One*, **7**, e36365.
39. Woo,C.J., Kharchenko,P.V., Daheron,L., Park,P.J. and Kingston,R.E. (2013) Variable requirements for DNA-binding proteins at Polycomb-dependent repressive regions in the human HOX clusters. *Mol. Cell Biol.*, **33**, 3274–3285.
40. Kalenik,J.L., Chen,D., Bradley,M.E., Chen,S.J. and Lee,T.C. (1997) Yeast two-hybrid cloning of a novel zinc finger protein that interacts with the multifunctional transcription factor YY1. *Nucleic Acids Res.*, **25**, 843–849.
41. Garcia,E., Marcos-Gutierrez,C., del Mar Lorente,M., Moreno,J.C. and Vidal,M. (1999) RYBP, a new repressor protein that interacts with components of the mammalian Polycomb complex, and with the transcription factor YY1. *EMBO J.*, **18**, 3404–3418.
42. Kaneko,T., Miyagishima,H., Hasegawa,T., Mizutani-Koseki,Y., Isono,K.-i. and Koseki,H. (2003) The mouse YAF2 gene generates two distinct transcripts and is expressed in pre- and postimplantation embryos. *Gene*, **315**, 183–192.
43. Wilkinson,F., Pratt,H. and Atchison,M.L. (2010) PcG recruitment by the YY1 REPO domain can be mediated by Yaf2. *J. Cell Biochem.*, **109**, 478–486.
44. Gonzalez,I., Aparicio,R. and Busturia,A. (2008) Functional characterization of the dRYBP gene in *Drosophila*. *Genetics*, **179**, 1373–1388.
45. Muller,J. (1995) Transcriptional silencing by the polycomb protein in *Drosophila* embryos. *EMBO J.*, **6**, 1209–1220.
46. Basu,A. and Atchison,M.L. (2010) CtBP levels control intergenic transcripts, PHO/YY1 DNA binding, and PcG recruitment to DNA. *J. Cell Biochem.*, **109**, 478–486.
47. Zor,T. and Selinger,Z. (1996) Linearization of the Bradford protein assay increases its sensitivity: theoretical and experimental studies. *Anal. Biochem.*, **236**, 302–308.
48. Squazzo,S.L., O'Green,H., Komashko,V.M., Krig,S.R., Jin,V.X., Jang,S.-w., Margueron,R., Reinberg,D., Green,R. and Farnham,P.J. (2006) Suz12 binds to silenced regions of the genome in a cell-type-specific manner. *Genome Res.*, **16**, 890–900.
49. Ringrose,L., Rehmsmeier,M., Dura,J.-M. and Paro,R. (2003) Genome-wide prediction of Polycomb/Trithorax response elements in *Drosophila melanogaster*. *Dev. Cell*, **5**, 759–771.
50. Satijn,D.P., Gunster,M.J., van der Vlag,J., Hamer,K.M., Schul,W., Alkema,M.J., Saurin,A.J., Freemont,P.S., van Driel,R. and Otte,A.P. (1997) RING1 is associated with the polycomb group protein complex and acts as a transcriptional repressor. *Mol. Cell Biol.*, **17**, 4105–4113.
51. Cao,R. and Zhang,Y. (2004) SUZ12 is required for both the histone methyltransferase activity and the silencing function of the EED-EZH2 complex. *Mol. Cell*, **15**, 57–67.
52. Morey,L., Aloia,L., Cozzuto,L., Benitah,S.A. and Di Croce,L. (2013) RYBP and Cbx7 define specific biological functions of polycomb complexes in mouse embryonic stem cells. *Cell Rep.*, **3**, 60–69.
53. Bracken,A.P., Dietrich,N., Pasini,D., Hansen,K.H. and Helin,K. (2006) Genome-wide mapping of Polycomb target genes unravels their roles in cell fate transitions. *Genes Dev.*, **20**, 1123–1136.
54. Garcia-Tunon,I., Guallar,D., Alonso-Martin,S., Benito,A.A., Benitez-Lazaro,A., Perez-Palacios,R., Muniesa,P., Climent,M., Sanchez,M., Vidal,M. *et al.* (2011) Association of Rex-1 to target genes supports its interaction with Polycomb function. *Stem Cell Res.*, **7**, 1–16.
55. Luis,N.M., Morey,L., Di Croce,L. and Benitah,S.A. (2012) Polycomb in stem cells: PRC1 branches out. *Cell Stem Cell*, **11**, 16–21.
56. Morey,L., Pascual,G., Cozzuto,L., Roma,G., Wutz,A., Benitah,S.A. and Di Croce,L. (2012) Nonoverlapping functions of the Polycomb group Cbx family of proteins in embryonic stem cells. *Cell Stem Cell*, **10**, 47–62.
57. O'Loughlen,A., Munoz-Cabello,A.M., Gaspar-Maia,A., Wu,H.A., Banito,A., Kunowska,N., Racek,T., Pemberton,H.N., Beolchi,P., Lavial,F. *et al.* (2012) MicroRNA regulation of Cbx7 mediates a switch of Polycomb orthologs during ESC differentiation. *Cell Stem Cell*, **10**, 33–46.
58. Wang,L., Brown,J.L., Cao,R., Zhang,Y., Kassis,J.A. and Jones,R.S. (2004) Hierarchical recruitment of Polycomb-group silencing complexes. *Mol. Cell*, **14**, 637–646.
59. Pan,X., Papasani,M., Hao,Y., Calamito,M., Wei,F., Quinn,W.J.I., Basu,A., Wang,J., Hodawadekar,S., Zaprazna,K. *et al.* (2013) YY1 controls Igk repertoire and B cell development, and localizes with condensin on the Igk locus. *EMBO J.*, **32**, 1168–1182.
60. Mohd-Sarip,A., Cleard,F., Mishra,R.K., Karch,F. and Verrijzer,C.P. (2005) Synergistic recognition of an epigenetic DNA element by Pleiohomeotic and a Polycomb core complex. *Genes Dev.*, **19**, 1755–1760.
61. Sawa,C., Yoshikawa,T., Masuda-Suzuki,F., Delehouzee,S., Goto,M., Watanabe,H., Sawada,J.-i., Kataoka,K. and Handa,H. (2002) YEAF1/RYBP and YAF-2 are functionally distinct members of a cofactor family for the YY1 and E4TF1/hGABP transcription factors. *J. Biol. Chem.*, **277**, 22484–22490.