RYBP, a new repressor protein that interacts with components of the mammalian Polycomb complex, and with the transcription factor YY1

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This paper is dedicated to the memory of Eugenia Spanopoulou, a colleague and friend.

The products of the Polycomb group (PcG) of genes are necessary for the maintenance of transcriptional repression of a number of important developmental genes, including the homeotic genes. A two-hybrid screen was used to search for putative new members of the PcG of genes in mammals. We have identified a new Zn finger protein, RYBP, which interacts directly with both Ring1 proteins (Ring1A and Ring1B) and with M33, two mutually interacting sets of proteins of the mammalian Polycomb complex. Ring1 binds RYBP and M33 through the same C-terminal domain, whereas the RYBP–M33 interaction takes place through an M33 domain not involved in Ring1 binding. RYBP also interacts directly with YY1, a transcription factor partially related to the product of the *Drosophila pleiohomeotic* gene. In addition, we show here that RYBP acts as a transcriptional repressor in transiently transfected cells. Finally, RYBP shows a dynamic expression pattern during embryogenesis which initially overlaps partially that of Ring1A in the central nervous system, and later becomes ubiquitous. Taken together, these data suggest that RYBP may play a relevant role in PcG function in mammals.

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Introduction

The inheritable silencing of sets of genes is responsible, at least in part, for the maintenance of the determined state of the wide variety of cell types found in multicellular organisms. Genetic analysis in *Drosophila* originally identified a number of genes, the Polycomb group (PcG) of genes, whose products are essential for the maintenance, but not the initiation, of the transcriptionally repressed state of developmentally relevant genes (reviewed in Bienz and Muller, 1995; Kennison, 1995; Simon, 1995; Pirrotta, 1997, 1998). Thus, in *Drosophila* PcG-mutant embryos, while patterns of homeotic gene expression are established

correctly, expression outside their normal boundaries occurs in later development, resulting in homeotic transformations (Duncan, 1982; Jürgens, 1985; Struhl and Akam, 1985; Wedeen *et al.*, 1986). Members of the PcG of genes have also been found in plants (Goodrich, 1998; Grossniklaus *et al.*, 1998), in worms (Holdeman *et al.*, 1998; Korf *et al.*, 1998; Stankunas *et al.*, 1998) and in vertebrates (van Lohuizen *et al.*, 1991; Pearce *et al.*, 1992; Ishida *et al.*, 1993; Nomura *et al.*, 1994; Reijnen *et al.*, 1995; Hobert *et al.*, 1996b; Shumacher *et al.*, 1996; Gunster *et al.*, 1997; Satijn *et al.*, 1997b; Coulson *et al.*, 1998; Stankunas *et al.*, 1998; van de Vosse *et al.*, 1998). Loss-of-function mutations of PcG genes in these organisms result in morphological transformations, consistently accompanied by derepression of some homeotic genes, among other alterations (van der Lugt *et al.*, 1994; Akasaka *et al.*, 1996; Coré *et al.*, 1997; Takihara *et al.*, 1997). This indicates a conservation of the PcG-silencing system throughout evolution. PcG function, however, is still best understood in the regulation of homeotic *Drosophila* genes.

The PcG genes encode a structurally diverse group of proteins, which form large complexes arising from their mutual interaction through protein motifs conserved from flies to man (Kyba and Brock, 1998a,b). PcG functions through multiprotein complexes and this is consistent with the observation that *Drosophila* embryos with mutations in two or more PcG genes show more severe phenotypes than those with mutations in single genes (Jürgens, 1985; Adler *et al.*, 1991; Cheng *et al.*, 1994). PcG proteins are associated with 80–100 sites in polytene chromosomes, showing a partially overlapping distribution pattern (Zink and Paro, 1989; DeCamillis *et al.*, 1992; Martin and Adler, 1993; Rastelli *et al.*, 1993; Sinclair *et al.*, 1998). The diversity of chromosomal regions PcG proteins associate with suggests that many other genes, in addition to the homeotics, are targets of PcG function. Immunoprecipitation studies using *in vivo* cross-linked chromatin show that PcG complexes are associated with DNA sequences known as Polycomb response elements (PREs) (Strutt and Paro, 1997; Strutt *et al.*, 1997; Orlando *et al.*, 1998). These sequences had been identified previously by their ability to confer heritable repression of reporter constructs in transgenic flies in a PcG-dependent manner (Busturia and Bienz, 1993; Simon *et al.*, 1993; Chiang *et al.*, 1995; Gindhart and Kaufman, 1995). As in polytene chromosomes, PcG complexes at different DNA sites show a heterogeneous distribution (Strutt and Paro, 1997). This agrees with the distinctive phenotypes associated with mutations in individual PcG genes (Soto *et al.*, 1995). How the PcG complexes are targeted to DNA sequences is, mostly, unknown, particularly because of the lack of evidence for DNA binding activity of most PcG proteins. Two exceptions are the mammalian Mel-18 protein and the *Drosophila pleiohomeotic* (*pho*) gene, which has

been shown to encode a protein partially related to the ubiquitous transcription factor YY1 (Brown *et al.*, 1998). In addition, PHO binding sequences have been identified in several PREs (Mihaly *et al.*, 1998), which may be responsible, in part, for some PcG complex targeting. The mechanism(s) by which PcG proteins determine inherited gene repression through cell divisions is not known, although it is thought that the formation of looped DNA structures, through the association of PcG complexes bound at nearby sites along the chromosome, is important for interfering with the ability of genes to respond to activators (Pirrotta *et al.*, 1995).

Understanding how the various subsets of PcG proteins are formed at the different genomic sites requires a better knowledge of the identity of PcG members as well as the characterization of the mutual interactions among the various PcG proteins. We have searched for putative new PcG proteins among interactors of the murine Ring1 proteins, Ring1A and Ring1B, two RING finger proteins identified by their ability to bind the mammalian homologs of Polycomb (PC), M33 and Pc2 (Satijn *et al.*, 1997a; Schoorlemmer *et al.*, 1997). In contrast to other mammalian PcG proteins, no known *Drosophila* PcG gene encoding a homolog of Ring1 proteins has yet been identified. Available evidence, however, makes Ring1 proteins likely PcG members. First, Ring1B interacts in a two-hybrid assay in yeast cells with the mammalian homologs of polyhomeotic and Posterior sex combs, PH2 and Bmi1, respectively (Hemenway *et al.*, 1998). Secondly, Ring1 proteins are components of the nuclear PcG complexes and co-immunoprecipitate from cell extracts together with Pc2, PH1 and Bmi-1 (Satijn *et al.*, 1997a). Finally, we have identified a *Drosophila* protein structurally related to Ring1A and Ring1B, which binds to >100 sites on polytene chromosomes, most of which are also Pc sites (S.Pimpinelli and M.Vidal, in preparation). Here we describe a new Zn finger protein, RYBP, which binds both Ring1A and Ring1B as well as M33, although through different domains. We show that RYBP also interacts with YY1, and that acts as a repressor when tethered to a reporter promoter in a tissue culture cell system. All these properties suggest that RYBP plays a relevant role in PcG silencing.

Results

Isolation of cDNAs coding for proteins that interact with Ring1A

A yeast two-hybrid system was used to isolate cDNAs encoding proteins that interact with Ring1A. A fusion between the LexA protein (amino acids 1–202) and the full-length Ring1A protein was used as bait to screen a library of mouse embryo cDNAs fused to the DNA sequence encoding the activation domain of GAL4. Approximately 2×10^6 yeast transformants were screened, of which 33 were scored as positive. Restriction analysis showed 11 types of cDNAs. Twelve of these cDNAs encoded the same uninterrupted open reading frame (ORF) for a protein which showed similarity to YY1-associated factor 2 (YAF-2), a human protein previously identified in a search for YY1-interacting proteins (Kalenik *et al.*, 1997). Because of its ability to bind both Ring1A and YY1 (see below), we termed this protein RYBP (Ring1

A

1	50
RYBP	MGDKKSPTRP KRQAKPAAD GFWDGCVCTE RNSAEAFKCS ICDVRKGT
YAF-2	MGDKKSPTRP KRHAKPSSDE GYWDGCVCTE RNSAEAFKCM MCDVRKGT
1	50
51	100
RYBP	TRKPRINSQL VAQVVAQQA TPTPPKKEKK EKVEKPDKEK PEKDKDISPS
YAF-2	TRKPRPVSQL VAQVVTQQFV PPTQSKKEKK DKVEK---EK SEKE-----T
51	92
101	150
RYBP	VTKKNTNKKT KPSDILKDP PSEANSIQSA NATTKTSETN HSSRPRLKNV
YAF-2	TTSKKNSHKKT ----- RPRRLKNV
93	109
151	200
RYBP	DRSTAQQLAV TVGNVTVIIT DFKEKTRSSS TSSSTVTSSA GSEQNQSSS
YAF-2	DRSSAQHLEV TVGDLTVIIT DFKEKTKSPP ASSA-----A SADQHSQSGS
110	159
201	227
RYBP	GSESTDGSS RSSTPKGDMS AVNDESF
YAF-2	SSDNTERGMS RSSSPRGEAS SLNGESH
160	186

B

Fig. 1. Amino acid sequences of RYBP and the related protein YAF-2. (A) Alignment of the deduced amino acid sequences encoded by the mouse RYBP and human YAF-2 cDNAs. Sequences were aligned using the PILEUP algorithms (Genetics Computer Group, University of Wisconsin, Madison, WI). The residues which are conserved between RYBP and YAF-2 are marked with a dot, while dashes represent gaps introduced to maximize the alignment. The Zn finger motifs are shown underlined. (B) Schematic representation of RYBP and YAF-2 proteins. Black boxes represent the Zn finger domains, shaded and striped boxes represent conserved sequence motifs and open boxes represent non-conserved regions. The approximate percentage of sequence identity (similarity in parenthesis) is given.

and YY1 binding protein). This cDNA contained two ATG codons at its 5' end, separated by a single codon, of which the second codon lies in a perfect Kozak consensus sequence and aligns with the estimated initiation codon of YAF-2 (Figure 1A). In order to isolate a full-length cDNA we performed both 5' and 3' RACE on total mouse brain RNA. Whereas we could not extend further the 5' end of the original clone, 3' RACE yielded a major product of 3.4 kb, consistent with the 4.0 kb transcript identified by Northern blot in total RNAs from both embryonic and adult tissues (not shown). Sequencing of the 3' RACE product showed a RYBP in-frame ORF with a stop codon following 26 codons not present in the original cDNA. Additional searches of DDBJ/EMBL/GenBank databases for homologs of RYBP identified a number of human and mouse expressed-sequence tag

Fig. 2. *In vivo* association of Ring1A and Ring1B proteins with RYBP. Proteins immunoprecipitated from E12.5 embryo extracts with the indicated antibodies were separated by SDS-PAGE (10% gels) and visualized by chemiluminescence after Western blot analysis. For controls, embryo extracts were incubated with a mixture of pre-immune sera. The position of molecular weight markers (in kDa) is indicated. Embryo extract lanes contain 100 µg of protein. The thick bands at ~55 kDa are the immunoglobulins present in the immunoprecipitates. (A) Western blot of anti-Ring1A, anti-Ring1B and pre-immune IgG immunoprecipitates probed with anti-RYBP antibodies. Lane 3 is 15% of anti-RYBP immunoprecipitated material from embryo extracts, as an indication of the amount of RYBP co-immunoprecipitated by anti-Ring1B antibodies. Lane 6 is a total extract from 293T cells transfected with a plasmid expressing HA-tagged RYBP. (B) Western blot of anti-RYBP and pre-immune IgG immunoprecipitates probed with anti-Ring1B antibodies. Lane 8 is 15% of anti-Ring1B immunoprecipitated material from embryo extracts, as an indication of the amount of Ring1B co-immunoprecipitated by anti-RYBP antibodies.

(EST) cDNA clones which overlapped (and extended) only the 3' end of our original cDNA clone. The deduced amino acid sequence of these clones coincided with the sequence from our 3' RACE cDNA. We then performed RT-PCR on total mouse brain RNA using for reverse transcription an oligonucleotide complementary to the 3' untranslated region of EST clone AA623880, followed by PCR amplification using the same primer and an oligonucleotide that spanned the putative initiation codon. We obtained a 780 bp cDNA almost identical to the original cDNA that included the 26 C-terminal codons. We found minor differences with the original clone, which probably reflect the different mouse strains from which the cDNAs were derived (NIH, C57BL10) and resulted in a conservative F95Y change. Finally, we analysed extracts of tissue culture cells transfected with a eukaryotic expression plasmid driving the 780 bp RT-PCR RYBP cDNA and extracts of a E12.5 mouse embryo by Western blot. We found that the mobilities of the polypeptides immunoreactive with anti-RYBP antibodies were substantially identical (Figure 2A, lanes 5 and 6). Taken together these data indicate that the 780 bp cDNA identified encodes the full ORF (227 amino acids) of the RYBP protein.

The RYBP and YAF-2 proteins have an N-terminal C_2C_2 Zn finger motif and display blocks of similarity throughout their sequences (Figure 1B). The region of highest similarity comprises the Zn finger which is 80% identical between the two proteins. Another block of homology is found at the C-terminal end (45% identity and 87% similarity). In RYBP, these two large conserved

regions are separated by non-conserved sequences (Figure 1B). The two proteins have a high content of basic residues (41/227 for RYBP) and an unusual abundance of serine and threonine residues at the C-terminal region (31/84 for RYBP).

***In vivo* interaction of RYBP and Ring1 proteins and its expression during embryogenesis**

To obtain evidence for the association of the RYBP and Ring1 proteins in mammalian cells, we performed immunoprecipitation studies using extracts from E12.5 mouse embryos. Figure 2A shows that polyclonal rabbit anti-Ring1A and rabbit anti-Ring1B antibodies specifically co-immunoprecipitated a 32 kDa protein recognized by anti-RYBP antibodies. The mobility of this protein is identical to that of the major band recognized by anti-RYBP antibodies on Western blots of total embryo extracts, and very similar to that of the 227 amino acid polypeptide encoded by the RYBP cDNA. Most likely, the different mobilities arise from the extra 21 amino acids (encoded by polylinker and HA tag sequences) fused to the transfected RYBP protein. No 32 kDa protein was coprecipitated by normal rabbit IgG or rabbit anti-glutathione *S*-transferase (GST) antibodies. The observed mobilities of these proteins correspond to proteins larger than the predicted 29 kDa, suggesting either post-translational modifications or aberrant mobilities on SDS-PAGE.

In the reciprocal experiment (Figure 2B), rabbit anti-RYBP antibodies co-immunoprecipitated specifically an anti-Ring1B immunoreactive protein of identical mobility to the major band detected in total embryo extracts with anti-Ring1B antibodies. We did not attempt to identify Ring1A among the proteins co-immunoprecipitated by anti-RYBP antibodies because its mobility coincides with that of the IgG heavy chains. In addition to their reactivities with total extracts from embryos, the specificity of these polyclonal antibodies was further checked by their ability to immunoprecipitate their cognate antigens obtained after *in vitro* transcription and *in vitro* translation of the corresponding cDNAs (data not shown). As Ring1A has been found associated with protein complexes containing Bmi-1, as well as polyhomeotic- and Polycomb-related proteins (Satijn *et al.*, 1997a), we conclude that RYBP is also a constituent of PcG complexes.

Additional evidence for the functional relationship between Ring1 proteins and RYBP came from the expression patterns of their transcripts as detected by whole-mount *in situ* hybridization to mouse embryos (Figure 3). At E9.0, RYBP transcripts were mostly detected in cells of the developing central nervous system, as is the case for Ring1A transcripts (Schoorlemmer *et al.*, 1997). Additional sites of RYBP expression in which Ring1A transcripts are not found included the first branchial arch, forelimb buds, tail bud and hindgut. From E9.5 onwards, RYBP was expressed ubiquitously, in contrast with the restricted expression of Ring1A (Figure 3 and data not shown). Despite the wide distribution of RYBP transcripts in most embryonic tissues, expression in the hindbrain appeared, as is the case for Ring1A, in stripes between each rhombomere (Schoorlemmer *et al.*, 1997), suggesting that in certain tissues at least, a concerted action of Ring1A and RYBP may be important.

Fig. 3. *In situ* hybridization analysis of RYBP mRNA in mouse embryos. Lateral views of whole-mount preparations of E9.0 (A) and E9.5 (B) mouse embryos hybridized with a RYBP cRNA probe. (A) At E9.0, expression of RYBP is mostly restricted to the CNS with highest levels in the developing spinal cord (sc) and forebrain (fb). The distribution of RYBP transcripts in the hindbrain (hb) appears segmented. Transcripts for RYBP are also detected in the first branchial arch (ba), the distal part of the forelimb bud (lb), tail bud (tb) and hindgut (hg). (B) By E9.5, RYBP is ubiquitously expressed with the main exception of the heart (h). Higher mRNA levels are detected in the hindbrain region (hb), the first branchial arch (ba) and forelimb bud (lb). (C) Flat-mount preparation of the hindbrain region of a E9.5 mouse embryo hybridized with a RYBP cRNA probe. RYBP expression is restricted to the boundaries between rhombomeres (r1–r6) and the dorsal aspect of the hindbrain (hb).

RYBP binds to the M33 binding domain of Ring1A

To determine whether RYBP and Ring1A interact directly, and to define the Ring1A domain which binds RYBP, we used an *in vitro* protein binding assay. Sequences encoding the entire RYBP protein (amino acids 1–227) were fused to the GST gene, for expression of the resulting hybrid protein in *Escherichia coli*. As a source of Ring1A protein, the entire Ring1A (amino acids 1–377) or truncated derivatives were transcribed and translated *in vitro* in the presence of ^{35}S -labelled methionine. The GST–RYBP fusion protein, immobilized on glutathione (GSH)–Sepharose, was incubated with labelled Ring1A proteins, and after washing, the proteins bound to the beads were analysed by SDS–PAGE. As shown in Figure 4A, both the full-length and an N-terminal-truncated Ring1A protein lacking amino acids 1–200 were able to bind efficiently to GST–RYBP (~40% of input Ring1A), but not to GST (lanes 3 and 7). Thus, RYBP binds to the same C-terminal half of Ring1A we had previously shown to bind M33 (Schoorlemmer *et al.*, 1997). On the other hand, the binding of Ring1B to GST–RYBP occurred with similar efficiency to that of Ring1A (data not shown). The region of Ring1A that binds RYBP comprises two blocks of sequences conserved with Ring1B (Schoorlemmer *et al.*, 1997). The deletion of either of the two blocks, in Ring1A derivatives lacking amino acids 233–277 or 274–377, resulted in proteins unable to bind RYBP (lanes 11 and 15). Even deletion of part of the last homology block, in derivative Ring1A (201–354), resulted in a protein which is unable to bind to GST–RYBP (lane 19). None of the Ring1A proteins showed significant binding to GST alone. Surprisingly, identical results were obtained for binding to M33, using a GST–M33 fusion protein (amino acids 333–519 of M33) containing the Ring1 binding domain of M33 (Schoorlemmer *et al.*, 1997). Only the truncated Ring1A protein lacking amino acids 232–277 showed some ability to bind to GST–M33 (lane 10), although far less efficiently than intact Ring1A (4 and 29% of input proteins bound to GST–M33, respectively). These results indicate that Ring1A and RYBP interact directly, and that this binding occurs through a large C-terminal domain,

involving non-contiguous Ring1A sequences, which also binds M33.

RYBP also interacts with M33

Evidence that both RYBP and M33 recognized the same Ring1A region suggests that RYBP and M33 could compete directly for binding to Ring1A. We tested this possibility by assaying GST–Ring1A binding to M33 in the presence of increasing amounts of RYBP. We found no clear inhibition of Ring1A–M33 interaction (data not shown), which could indicate that RYBP and M33 contacted different sites of the C-terminal region of Ring1A. Alternatively, it was possible that RYBP and M33 interacted with each other. To test this, we used an *in vivo* GST pull-down assay in which a GST–RYBP or GST proteins were co-expressed with a hemagglutinin (HA)-tagged M33 in tissue culture cells. GST fusion proteins were then purified from cell extracts using GSH–Sepharose beads and proteins bound to them were detected by Western blot with an anti-HA monoclonal antibody. We found that HA–M33 was retained on GSH beads when co-expressed with GST–RYBP, but not when co-expressed with GST alone (Figure 5A, lanes 1 and 2).

Additionally, we showed that the interaction of RYBP and M33 is direct, as indicated by the specific binding *in vitro* of [^{35}S]M33 to purified GST–RYBP (Figure 5B, lanes 9 and 10). A rough indication of the region of M33 that binds RYBP was obtained by using truncated [^{35}S]M33 proteins. Deletion of the N-terminal region, in the M33 318–519 variant, abolished the interaction with GST–RYBP (Figure 5B, lane 16), whereas deletion of the C-terminal region, in the M33 1–119 and the 1–317 variants, did not affect the interaction (lanes 13 and 19, respectively). As M33 binds Ring1A through a conserved domain in the C-terminal region (Figure 4), we conclude that M33 binds RYBP and Ring1A through separate domains.

Interaction of RYBP and YY1

RYBP is highly related to the YY1-interacting protein YAF-2, although they also have non-conserved sequences

Fig. 4. Ring1A interacts with RYBP and M33 through a large C-terminal domain. **(A)** Intact Ring1A and the indicated truncated variants were synthesized *in vitro*, radiolabelled with [35 S]methionine and mixed with bacterially produced GST, GST-RYBP or GST-M33 (amino acids 333–519) (5 μ g) immobilized on GSH–Sepharose. After incubation and washes, the bound proteins were separated by SDS–PAGE (12% gels) and visualized by PhosphorImager analysis. Input represents 10% of the total 35 S-labelled protein used in the interaction assays. Sizes of molecular weight markers (in kDa) are indicated on the left. **(B)** Diagram of intact and truncated Ring1A proteins and summary of binding data. The black box represents the RING finger domain. Conserved motifs between Ring1A and Ring1B proteins are indicated by shaded and striped boxes, whereas open boxes represent non-conserved regions. Amino acids are numbered in the various truncated Ring1A proteins. The interaction between each Ring1A protein and GST–RYBP or GST–M33 is designated by +, while +/- indicates a weak interaction and – indicates no interaction.

(Figure 1). Since the YAF-2 region that binds YY1 has not been determined, we wished to test whether RYBP would also interact with YY1. Using an *in vivo* GST pull-down assay, we found that a Myc-tagged YY1 polypeptide was retained on GSH beads when co-expressed with GST–RYBP, but not when co-expressed with GST alone (Figure 6A, lanes 10 and 11), indicating that RYBP and YY1 are able to interact specifically in tissue culture cells. In order to delineate the region of YY1 that binds RYBP we analyzed the association of a number of truncated YY1 proteins with co-expressed GST–RYBP in tissue culture cells. The deletion of N-terminal sequences of YY1, in YY1 (200–414), YY1Δ90–221 or YY1 (274–414) resulted in proteins able to bind to GST–RYBP (Figure 6A, lanes 14–16), whereas the deletion of the C-terminal half of YY1, in YY1 1–200, abolished such binding (Figure 6A, lane 13). The deletion of part of the second Zn finger, together with the third and fourth Zn fingers, in the YY1 (1–333) derivative, did not affect binding to GST–RYBP. On the other hand, and because of the partial similarity between YY1 and PHO we tested the ability of GST–RYBP to interact with PHO protein in tissue culture cells. We found that a HA-tagged PHO polypeptide became bound to GSH beads when co-expressed with GST–RYBP (Figure 6A, lane 17) but not when co-expressed with GST alone (not shown). As the only regions of similarity between YY1 and PHO are a short spacer sequence, not required for RYBP binding as indicated by the YY1Δ90–221 derivative, and the four Zn fingers, we conclude that the first one and half Zn fingers of YY1 are sufficient to bind RYBP.

RYBP domains that bind Ring1, YY1 and M33

Here we have shown that RYBP is a multi-interacting protein which binds, at least, Ring1A, M33 and YY1. As the disposition of contact sites for these proteins can be a major determinant of the functional capabilities of RYBP, we used an *in vitro* interaction assay to define the regions which bind Ring1A, YY1 and M33. The various GST–RYBP proteins used and the observed interactions are summarized in Figure 7B. A common feature of RYBP binding is that the Zn finger was not required for these interactions, as indicated by the full binding activity of a RYBP derivative lacking amino acids 1–42 (Figure 7A, lanes 4, 13 and 22). Similarly, the deletion of the C-terminal stretch of 50 amino acids of RYBP did not affect the binding to any of the proteins (lanes 5, 14 and 23). The deletion of amino acids 119–227 did not affect binding to YY1 or M33 (lanes 12 and 21), but completely abolished the binding to Ring1A (lane 3). Conversely, a truncated RYBP protein consisting of amino acids 158–207 was able to bind Ring1A, but not YY1 or M33 (lanes 6, 15 and 24). Deletion of a subset of these amino acids, in RYBPΔ(161–173), resulted in a derivative which retained its ability to bind YY1 and M33 (lanes 16 and 25) but had no Ring1A binding activity (lane 7). Finally, a truncated RYBP protein consisting of amino acids 119–227 was found to bind all three proteins (lanes 8, 17 and 26). We conclude that YY1 and M33 interact with at least two independent domains of RYBP, whereas Ring1A binds to a short C-terminal region of RYBP not involved in binding to either YY1 or M33. A summary of RYBP interactions is shown in Figure 10.

Fig. 5. *In vivo* and *in vitro* RYBP-M33 interactions. (A) *In vivo* GST pull-down assay. Human kidney 293T cells were cotransfected with a plasmid expressing HA-tagged M33 and a plasmid expressing GST alone or GST fused to full-length RYBP. Proteins from cell extracts bound to GSH-Sepharose were analysed by Western blot with monoclonal anti-HA antibody 12CA5. Total extracts from transfected cells as well as from non-transfected cells were immunoblotted with monoclonal anti-HA antibody 12CA5 or with anti-GST antibodies. (B) *In vitro* GST pull-down assay. Intact M33 and the indicated truncated variants were synthesized *in vitro*, radiolabelled with [³⁵S]methionine and mixed with bacterially produced GST or GST-RYBP (5 µg) immobilized on GSH-Sepharose. After incubation and washes, the bound proteins were resolved by SDS-PAGE (12% gels) and visualized by PhosphorImager analysis. Input represents 10% of the total ³⁵S-labelled protein used in the interaction assays. Sizes of molecular weight markers (in kDa) are indicated on the left. (C) Diagram of intact and truncated M33 proteins and summary of binding data. The shaded box represents the chromodomain and the black box the conserved domain which binds Ring1 proteins. The interaction between each M33 protein and GST-RYBP is designated by +, while - indicates no interaction.

Differential cell localization of intact RYBP and of a RYBP protein unable to bind Ring1

In the human osteosarcoma U2-OS cells, PcG proteins are found associated with large nuclear speckled structures, named PcG bodies (Saurin *et al.*, 1998). Exceptions are the ENX1 and EED proteins, the homologs of *Drosophila* Enhancer of zeste and extra sex combs, respectively

(Sewalt *et al.*, 1998; van Lohuizen *et al.*, 1998). We have shown previously that part of Ring1A is also concentrated to PcG bodies in U2-OS cells (Schoorlemmer *et al.*, 1997). Here we extend that observation to the Ring1B protein (Figure 8A-D). However, in these cells, RYBP was detected distributed throughout the nucleoplasm, in contrast to the speckled Ring1A signal (Figure 8E-H). One possibility is that in U2-OS cells, RYBP is interacting with other protein(s) that would prevent its association with PcG bodies.

To test the relevance of the RYBP-Ring1 interaction to the localization of RYBP in the cell, we generated populations of U2-OS cells stably transfected with an HA-epitope-tagged RYBP construct lacking amino acids 160-174, which does not bind Ring1A *in vitro*, and with a HA-epitope-tagged wild-type RYBP construct. Analysis of transfected cells with anti-HA and anti-Ring1B antibodies revealed a variety of staining patterns of cell nuclei which could be grouped according to the expression levels of RYBP proteins (estimated from immunofluorescence signal intensities). In cells that expressed low levels of both wild-type and truncated HA-RYBP proteins, the signal of Ring1B was speckled, whereas that of transfected RYBP proteins was seen throughout the nucleoplasm (Figure 8I-K). In most cells that expressed intermediate levels of wild-type RYBP, however, the RYBP signal was detected both in the nucleoplasm and in speckles which also contained Ring1B (Figure 8M-O). Finally, among cells that expressed the highest levels of HA-RYBP, a new staining pattern was observed in which both RYBP and Ring1B signals were distributed throughout the cell nucleus, with no evidence of speckled signals (Figure 8Q-S). The frequency of these highly expressing HA-RYBP cells in the cell population was found to decrease with time in culture, suggesting that such high levels of HA-RYBP are harmful to cells. A contrasting result was obtained with cells transfected with truncated HA-RYBP, as the colocalization of HA and Ring1B in speckled signals, or their diffuse distribution in the nucleus occurred only in a minority of the cells that expressed the highest levels of truncated HA-RYBP. These results are summarized in Table I. We found similar results using anti-HA and anti-M33 antibodies (data not shown). These results suggest that the intranuclear localization of RYBP varies with its expression levels and that at a given concentration, a fraction of intact RYBP, and much less efficiently, a fraction of the truncated RYBP protein, associates with PcG bodies. We do not know whether the stability of both intact and truncated RYBP proteins is the same, although they both seem to be expressed at similar levels in transiently transfected cells (data not shown). If this was the case, the higher efficiency of wild-type RYBP to colocalize with and relocalize Ring1B and M33 could be related, at least in part, to its ability to interact directly with Ring1A and Ring1B proteins.

RYBP is a transcriptional repressor

Because of its interaction with components of mammalian PcG complexes known to act as repressors in transiently transfected mammalian cells, we investigated the transcriptional activity of RYBP. The coding sequence of RYBP was fused to amino acids 1-147 of the GAL4 DNA binding domain (pGAL4-RYBP), and the transcriptional

Fig. 6. Identification of a RYBP interacting domain in YY1. **(A)** *In vivo* GST pull-down assays. Human kidney 293T cells were cotransfected with plasmids expressing tagged-intact and truncated human YY1 polypeptides, and with plasmids expressing either GST fused to full-length RYBP or GST alone. Proteins in total extracts or bound to GSH-Sepharose were analyzed by Western blot with monoclonal anti-Myc antibody 9E10 and with monoclonal anti-HA antibody 12CA5 as indicated. Sizes of molecular weight markers (kDa) are indicated on the left. **(B)** Schematic representation of YY1 and PHO and of various truncated YY1 cDNAs used. The spacer region and the four Zn fingers conserved between YY1 and PHO are indicated by the black and grey boxes, respectively. Numbers indicate the amino acids encoded by the various transfected cDNAs. The interaction between each YY1 or PHO protein and GST-RYBP is designated by +, while - indicates no interaction.

activity of the fusion protein was assessed on the pG5tkCAT reporter plasmid, in which chloramphenicol acetyltransferase (CAT) expression is directed by a herpes simplex virus thymidine kinase (tk) promoter containing five GAL4 binding sites located 120 bp upstream of the TATA box. Co-transfection of pGAL-RYBP and pG5tkCAT into NIH 3T3 cells resulted in a marked repression of CAT in a dose-dependent manner (Figure 9B). Repression was dependent on the presence of the GAL4 binding sites since CAT expression was little affected when a promoter-reporter construct lacking the GAL4 binding sites (ptkCAT) was used (Figure 9B). To discard the possibility that the repression activity is related to steric interferences with the promoter, we used a plasmid in which the GAL4 binding sites are located 1.6 kb upstream of the TATA box. CAT expression was still repressed in the presence of GAL4-RYBP, although slightly less efficiently than when expressed from pG5tkCAT (Figure 9C). The activity of this repression at a distance was comparable with that of GAL4-Ring1A.

To define the region(s) in RYBP responsible for transcriptional repression we assayed the ability of a number of truncated RYBP proteins fused to the GAL4 DNA binding domain to repress transcription from pG5tkCAT. The GAL4-RYBP 1-118 derivative, which lacks the C-terminal half, showed very little activity, whereas the complementary truncated protein, GAL4-RYBP 119-227, repressed expression of pG5tkCAT with an efficiency similar to that of the GAL4-RYBP protein. The strong

repression activity of the GAL4-RYBP 42-207 derivative further confirms that the Zn finger is not required for repression. Additionally, the C-terminal stretch of 20 amino acids is also dispensable for repression. The GAL4-RYBP 158-207 derivative, containing the Ring1A interacting domain, showed very little repression activity, although its deletion, in pGAL4-RYBPΔ161-173, resulted in a protein with lower repressor activity than that of the intact GAL4-RYBP protein. The differences in repression activity could not be related to differences in expression levels (data not shown). Thus, a domain comprising amino acids 119-207 is responsible for RYBP transcriptional repression and although the Ring1A binding domain is not sufficient for repression, it may be necessary.

Discussion

Genetic evidence in *Drosophila* suggests that the PcG of genes comprises some 30-40 members. So far, only 14 of them have been identified and a dozen characterized at the molecular level. The identification of additional members and the characterization of their biochemical interactions are, therefore, important for understanding PcG function. In this study we have identified a new murine protein which binds both Ring1A and Ring1B proteins. We show that this protein, termed RYBP, also interacts with the mammalian homolog of PC, M33, a known interactor of Ring1 proteins. In addition, we present evidence for the direct association of RYBP with the

Fig. 7. RYBP binds Ring1A through a region different from the region which contacts YY1 and M33. **(A)** *In vitro* GST interaction assays. Intact and truncated RYBP cDNAs were fused to GST sequences and expressed in bacteria. GST alone or GST-RYBP fusion polypeptides were immobilized on GSH-Sepharose and incubated with *in vitro* translated [³⁵S]Ring1A (lanes 1–9), [³⁵S]YY1 (lanes 10–18) or [³⁵S]M33 (lanes 19–27). Bound proteins were resolved by SDS-PAGE (12% gels) and visualized by PhosphorImager analysis. Input represents 10% of the total ³⁵S-labelled protein used in the interaction assays. Sizes of molecular weight markers (in kDa) are indicated on the left. Lower bands in YY1-GST-RYBP interactions are most likely to be truncated products from the *in vitro* synthesis of YY1. **(B)** Schematic representation of RYBP, showing the truncated derivatives used in the *in vitro* assays. The black box represents the Zn finger. Numbers indicate the amino acids corresponding to the various regions of RYBP fused to GST. The interactions between Ring1A, YY1 or M33 with the GST-RYBP fusion polypeptides are designated by +, while – indicates no interaction.

transcriptional factor YY1, and for the repressor activity of RYBP.

RYBP is a constituent of a mammalian PcG complex

We identified RYBP in a yeast two-hybrid screen for murine embryonic cDNAs encoding proteins that interact with Ring1A. A search of protein databases showed that RYBP is related to no known PcG protein but to YAF-2, a polypeptide previously identified by its ability to bind YY1 (Kalenik *et al.*, 1997). Immunoprecipitation of total mouse embryo extracts with RYBP and Ring1A and Ring1B antibodies showed that RYBP and Ring1 proteins interact *in vivo*. In tissue culture cells known to have large nuclear PcG bodies, however, RYBP was detected throughout the nucleoplasm, although upon transfection an increased level of its concentration led to its colocalization with Ring1B and M33 in PcG bodies. The functional significance of the lack of association of RYBP with nuclear compartments which contain proteins that bind directly to RYBP is presently unclear. Nevertheless, since these speckled structures, not found in *Drosophila* embryos (Buchenau *et al.*, 1998), have not yet been related to PcG function, we reasoned that the observed distribution of RYBP in the cell nucleus is not necessarily incompatible with a role in PcG function.

Apart from a potential Zn finger of the C₂-C₂ variety in their N-terminal region, no other characterized protein motif is found in the protein sequences of RYBP or YAF-2. Among PcG proteins, both mammalian PH proteins, PH1 and PH2, and *Drosophila* PH, have a single Zn finger of the C₂-C₂ type (DeCamillis *et al.*, 1992; Nomura *et al.*, 1994; Gunster *et al.*, 1997), and *Drosophila* sex combs on midleg (SCM) proteins have three (Bornemann *et al.*, 1996). However, the spacing between the pairs of cysteines in the Zn fingers of PH-related proteins and in the Zn fingers of RYBP/YAF-2 is different. The significance of any of these Zn fingers is not yet clear, and for RYBP, at least, we found that it is dispensable for all the protein-protein interactions described here, as well as for its repressor activity.

In situ hybridization analysis to RYBP mRNA showed that in mouse embryos up to E9.0, RYBP is expressed in a tissue-specific pattern similar, although not identical, to that of Ring1A (Schoorlemmer *et al.*, 1997). These data reinforce the notion of tissue-specific PcG complexes, for which evidence is already available in *Drosophila* (Soto *et al.*, 1995). Particularly revealing is the striped expression in the hindbrain which strongly suggests a functional relationship to Ring1A, and perhaps, to hindbrain segmentation. Later on, however, RYBP is found expressed ubiquitously, as is the case for M33 (Pearce *et al.*, 1992; Schoorlemmer *et al.*, 1997).

Fig. 8. Distribution of RYBP and Ring1 proteins in normal and transfected human osteosarcoma U2-OS cells using indirect immunofluorescence. Cell nuclei are stained blue with DAPI. (A–H) Localization of endogenous Ring1A, Ring1B and RYBP in non-transfected cells. Double labelling with affinity-purified rabbit anti-Ring1B antibodies (A, red) and with mouse serum anti-Ring1A (B, green). Anti-Ring1B antibodies were absorbed with GST–Ring1A–Sepharose to discard any cross-reaction with Ring1A. The merge of the two images (C) shows the colocalization of both Ring1 proteins in discrete nuclear speckled structures. Double labelling with affinity-purified rabbit anti-RYBP antibodies (E, red) and mouse serum anti-Ring1A (F, green). The merge of the two images (G) shows a diffuse nuclear signal for RYBP, in contrast with the speckled signal of Ring1A. (I–T) Localization of transfected RYBP and endogenous Ring1B in a population of cells stably transfected with a plasmid expressing HA-tagged RYBP cDNA. Cells were double labelled with affinity-purified rabbit anti-Ring1B antibodies (I, M and Q, red) and with mouse monoclonal anti-HA antibody 12CA5 (J, N and R, green). In cells expressing low levels of HA-RYBP (I–L), the merged images (K) show speckled and diffuse nuclear localizations for Ring1B and RYBP, respectively. In cells that expressed higher levels of HA-RYBP (M–P), the signals of RYBP and Ring1B colocalize in speckles structures, although RYBP is also present in the nucleoplasm (O). Finally, in the cells that expressed the highest levels of HA-RYBP (Q–T), the merge of the two images (S) shows a diffuse green signal because of the intensity of HA-RYBP staining (O), but Ring1B signal (Q, arrow) is also evenly distributed throughout the nucleoplasm.

RYBP binding to YY1 and targeting of PcG complexes to DNA

While PcG function is known to act through specific DNA sequences, the way that PcG complexes are targeted to these DNA sequences has remained elusive. On an individual basis only two PcG proteins have been shown to bear DNA binding activity. One is the murine Mel-18 protein (Kanno *et al.*, 1995; Tetsu *et al.*, 1998), although its *Drosophila* homolog, PSC, does not bind DNA. The

other is the *Drosophila* PHO protein, which is partially related to the mammalian transcription factor YY1 (Brown *et al.*, 1998). Our observation that RYBP interacts with YY1 and also with PHO does not prove a functional equivalence between YY1 and PHO, but supports a role for YY1 and RYBP in attracting PcG proteins to DNA in mammals. One caveat to this suggestion is that co-immunoprecipitation experiments performed to identify cellular complexes containing YY1 and RYBP have

Table I. Changes in the cellular distribution of Ring1B and RYBP in transfected U2-OS cells

Nuclear localization		Levels of transfected HA-RYBP and HA-RYBPΔ(161–173)		
Ring1B	RYBP	low	medium	high
Speckled	diffuse	100 (100)	15 (80)	0 (68)
Speckled	speckled and diffuse	0 (0)	81 (18)	53 (22)
Diffuse	diffuse	0 (0)	4 (2)	47 (10)

Localization of Ring1B and HA-RYBP was determined by indirect immunofluorescence using anti-Ring1B and anti-HA antibodies. Levels of transfected RYBP proteins were estimated from the signal intensities. A minimum of 40–60 cells per each of the three expression levels was scored. Numbers indicate the percentage of cells transfected with intact HA-RYBP which showed the specified nuclear distribution patterns of Ring1B and HA-RYBP. Numbers in brackets correspond to cells transfected with the HA-RYBP deleted variant, HA-RYBPΔ(161–173), which does not bind Ring1.

Fig. 9. RYBP represses transcription in transiently transfected mammalian cells. (A) Schematic representation of the reporter constructs used. pG5tkCAT contains five GAL4 binding sites immediately upstream of the (–105 to +51) HSVtk promoter in plasmid pBLCAT2 (here termed ptkCAT). pG5-1.6-tkCAT contains five GAL4 binding sites placed 1.6 kb upstream of the same HSVtk promoter. (B) Dose-dependent repression by GAL4-RYBP fusion protein. NIH 3T3 cells were cotransfected with 1.5 µg of pG5tkCAT or ptkCAT, together with 50 ng of pCMVlacZ and increasing amounts of pGAL4-RYBP, which expresses the RYBP cDNA as a fusion with the GAL4 DNA binding domain. The total amount of effector plasmid (0.5 µg), was kept constant by addition of the plasmid expressing only the GAL4 DNA binding domain. CAT protein levels were determined 40 h after transfection and normalized to β-galactosidase protein levels. Results are expressed as normalized CAT levels relative to those obtained in the presence of 0.5 µg of the GAL4 expressing vector. The results shown are an average of three experiments with standard deviation indicated. (C) Repression at a distance by GAL4-RYBP and GAL4-Ring1A. NIH 3T3 cells were cotransfected with pG5-1.6-tkCAT (1.5 µg) and pCMVlacZ (50 ng) together with GAL4-RYBP or GAL4-Ring1A expression vectors (0.5 µg). Normalized CAT levels are expressed relative to those obtained in the presence of the GAL4 DNA binding domain alone. (D) Mapping the transcriptional repression domain of RYBP. NIH 3T3 cells were transfected with 1.5 µg of pG5tkCAT and 50 ng of pCMVlacZ together with plasmids expressing GAL4 DNA binding domain alone or fused to various regions of RYBP (0.5 µg) indicated by the boxes, where the stippled box represents the Zn finger motif. Fold repression is expressed as the ratio of normalized CAT protein values in the presence of GAL4-DNA binding domain expression plasmid over normalized CAT protein values in the presence of a given effector. Values represent the averages of three experiments with standard deviation indicated.

yielded negative results. We have shown, however, that a fraction of YY1 and PHO can associate with RYBP when expressed in tissue culture cells. Evidence indicates that recruiting PcG complexes to the DNA through PHO requires other unidentified DNA binding proteins (Brown *et al.*, 1998). It is possible that such interactions in

physiological conditions may be inherently unstable or that particular extraction conditions are required for the YY1-RYBP complex to remain stably associated following cell lysis. It is worth noting that, in contrast to Ring1A-RYBP, YY1-RYBP complexes were unstable in buffers of increasing ionic strength. In addition, the

possibility that a hypothetical involvement of YY1 in targeting PcG complexes to DNA depended on its direct interaction with PcG proteins can not be excluded. Evidence for this is that individual PcG proteins are capable of interacting with several different PcG proteins (Alkema *et al.*, 1997a; Hashimoto *et al.*, 1998; Hemenway *et al.*, 1998).

PcG complexes form through multiple PcG protein-protein interactions

The presence of multiple protein-protein interacting domains in most PcG proteins permits their association in homo and/or heteromeric structures. For example, in interaction assays in yeast, mammalian PH1 binds to itself as well as to PH2, Bmi-1 and Mel-18 (Alkema *et al.*, 1997a; Gunster *et al.*, 1997). In turn, Bmi-1 interacts with itself and with M33 and Ring1A and Ring1B (Hashimoto *et al.*, 1998; Hemenway *et al.*, 1998; Satijn and Otte, 1999). Similar associations have been described for *Drosophila* PcG proteins. In general, these interacting surfaces are part of highly conserved domains in PcG proteins (Kyba and Brock, 1998a,b), and provide a variety of contacts which may help the self-organization and regulation of PcG complexes. A summary of interactions among mammalian PcG proteins is illustrated in Figure 10.

Here we have shown that RYBP is able to bind Ring1 and M33 proteins. Interaction assays *in vitro* revealed that M33 interacts with RYBP and Ring1A through separate domains in the N- and C-terminal regions of M33, respectively. Additionally, we have shown that the binding of Ring1A and M33 to RYBP takes place through separate regions of RYBP. The relative disposition of the contact sites in these proteins makes possible the formation of complexes containing RYBP, M33 and Ring1A, although we have not yet obtained evidence for such a ternary complex. In contrast, RYBP and M33 bind to a site(s) of Ring1A which depends on the integrity of its C-terminal region, which suggests that RYBP and M33 could compete for its binding to Ring1A. An indication of the different activities of the Ring1 and M33 binding sites is suggested by the differential ability of RYBP, with or without the Ring1A interacting domain, to associate with PcG bodies in tissue culture cells. Thus, the relative affinities for particular domains, together with the relative concentrations of PcG proteins in the cell, are the basis for the potential heterogeneity of PcG complexes.

Transcriptional repression by PcG proteins

It is interesting to note that many PcG proteins act as transcriptional repressors when recruited to reporter constructs. These include *Drosophila* PC, PSC and its mammalian homologs (Bunker and Kingston, 1994; Cohen *et al.*, 1996; Schoorlemmer *et al.*, 1997), as well as the mammalian EED and RING1 proteins (Denisenko and Bomszyk, 1997; Satijn *et al.*, 1997a; Schoorlemmer *et al.*, 1997). Our data show that the RYBP protein is also a repressor when tethered to promoters by means of a DNA binding domain. The region of RYBP responsible for repression lies in the C-terminal half of the molecule and, in contrast to M33 (Schoorlemmer *et al.*, 1997), the region that binds Ring1 proteins is not sufficient for full repression. These differences were observed using the same reporter construct and the same cell line, suggesting

Fig. 10. Schematic representation of the mammalian PcG proteins and the domains involved in their direct interactions. Circled names represent PcG proteins in interactions that have been detected between endogenous proteins, whereas plain text indicates interactions identified either in transfected mammalian tissue culture cells, in two-hybrid assays in yeast cells or *in vitro* (Alkema *et al.*, 1995, 1997b; Hobert *et al.*, 1996a; Gunster *et al.*, 1997; Satijn *et al.*, 1997a; Schoorlemmer *et al.*, 1997; Hashimoto *et al.*, 1998; Hemenway *et al.*, 1998; Sewalt *et al.*, 1998, 1999; van Lohuizen *et al.*, 1998; Satijn and Otte, 1999; M. Vidal, unpublished observations; this paper). Mel-18 and Enx-2 are two PcG members very closely related to Bmi-1 and Enx-1, respectively, which have not been depicted in this figure due to the scarce information available regarding their interacting domains. Discontinuities in PH1 and Enx-1 indicate that only the relevant regions of these proteins have been drawn.

that repression occurs through various mechanisms, in agreement with the differential responses of various promoters to different PcG proteins (Bunker and Kingston, 1994).

Does the PcG proteins' ability to affect the function of transcriptional activators in transfected tissue culture cells relate in any manner to PcG silencing? It is difficult to answer this question because this repression is, in molecular terms, virtually unknown. Initially it was believed that PcG repression was mediated by the packaging of chromatin in a compact structure which hindered the access of activators to DNA. Recently a genetic interaction between *Pc* and a gene that encodes a component of complexes with histone deacetylase activity has been shown in *Drosophila* (Kehle *et al.*, 1998). However, improvements in crosslinking chromatin immunoprecipitation experiments suggest that *in vivo* binding of PC, PSC and PH proteins to DNA in *Drosophila* tissue culture cells occurs in a much more localized manner than

previously thought (Strutt and Paro, 1997; Strutt *et al.*, 1997). This would argue against spreading of PcG complexes as a way of gene repression. A recent report shows that binding of PC to *Drosophila* embryos changes during embryogenesis. Whilst in early embryos, when transcription of the homeotics starts, PC is associated with PREs, later on, at the time of derepression in Pc mutants, PC is also detected in sequences adjacent to PREs and, most importantly, bound to core promoters (Orlando *et al.*, 1998). The discovery of PcG–promoter association strongly suggests that, at some point, PcG proteins may contact enhancer proteins or even components of the basal transcription machinery. It is precisely the possibility of such interactions that would explain, in part, the ability of PcG proteins to repress reporter constructs in transfected cells, and why it depends on a variety of structurally unrelated repression domains.

Materials and methods

Plasmids

Plasmid manipulations were performed according to established procedures and when PCR fragments were involved their sequences were verified by sequencing. DNA binding domain and activation domain fusion proteins were expressed in yeast from the plasmids pBTM116 (a gift of P.Bartel and S.Fields) and pGAD10 (Clontech), respectively. Plasmids for transfection studies in mammalian cells were CsCl-purified or isolated using Qiagen columns. The CAT reporter plasmids are as follows: pG5tkCAT, containing five GAL4 binding sites upstream of the –110 to +56 (relative to the transcription initiation site), herpes simplex virus thymidine kinase (HSVtk) promoter (Shi *et al.*, 1991); ptkCAT, also called pBLCAT2 (Luckow and Schütz, 1987), which uses the same minimal HSVtk promoter but lacks GAL4 binding sites; pG5-1.6-tkCAT contains five GAL4 binding sites located 1.6 kb upstream of the minimal HSVtk promoter and was a gift of D.Lepreux. pCMVlacZ, which contains the enhancer and promoter of the immediate early promoter of cytomegalovirus in front of the lacZ gene of *E.coli*. The GAL4 DNA binding domain chimeras were constructed by subcloning of the indicated cDNAs into pSG424 (Sadowski and Ptashne, 1989). For epitope-tagging of M33 and RYBP, the 5' ends of their coding sequences were fused to sequences encoding either the influenza HA-epitope, recognized by the monoclonal antibody 12CA5 (Niman *et al.*, 1983), or the Myc-epitope recognized by the monoclonal antibody 9E10 (Evan *et al.*, 1985) in the expression vectors pSG5 (Green *et al.*, 1988), pCS2 (Rupp *et al.*, 1994) or in the human β -actin expression vector pH β Apr-1-neo (Gunning *et al.*, 1987). For expression in eukaryotic cells of GST or GST-RYBP proteins, the pEBG plasmid (Spanopoulou *et al.*, 1996) which provides the GST at the N-terminus of the fusion proteins was used. A human YY1 cDNA was obtained from T.Shenk and the PHO cDNA was obtained by manipulation of *Drosophila* LD34133 clone purchased from Research Genetics. Details of plasmid constructions are available upon request.

Yeast two-hybrid screen and interaction assays

A GAL4 activation domain-tagged cDNA library from E11.5 mouse embryo RNA constructed in the leucine-selectable plasmid pGAD10 (Clontech), was introduced by LiAc transformation into the *Saccharomyces cerevisiae* L40 strain (Hollenberg *et al.*, 1995) expressing the fusion protein LexA–full-length Ring1A(1–377) from the tryptophan-selectable expression vector pBTM116 (Vojtek *et al.*, 1993). After overnight recovery in yeast complete medium (Trp⁺Leu⁺Ura⁺), the transformants were plated on selective medium for histidine prototrophy (Trp⁺Leu⁺Ura⁺His⁺Lys⁺). His⁺ clones exhibiting β -galactosidase activity on filters were isolated and further analysed. Library plasmids were rescued on *E.coli* strain HB101 (leuB⁺) and selected for leucine prototrophy on minimal plates. These plasmids were then retransformed into L40 along with pBTM116Ring1A(1–377) or plasmids expressing irrelevant LexA fusion proteins, such as LexA–lamin and Lex–daughterless (gifts from S.Hollenberg).

Cell lines, transfections and repression assays

NIH 3T3 cells were obtained from P.Rodriguez-Viciana; U2-OS cells were obtained from ATCC. Human kidney 293T cells were obtained

from E.Spanopoulou. U2-OS cells and 293T cells were propagated in DMEM–10% fetal calf serum, while NIH 3T3 cells were grown in DMEM–10% newborn calf serum. All transfections were performed using Lipofectamine (Gibco-BRL), according to the manufacturer's instructions. Human kidney 293T cells (1.8×10^6 per 6 cm dish) received 2 μ g of plasmid and were harvested 40 h after transfection. For repression assays, NIH 3T3 cells were transfected and extracts analysed exactly as previously described (Schoorlemmer *et al.*, 1997). For stable transfection, U2-OS cells (0.6×10^6 per 10 cm dish) were given 3 μ g of pH β Apr-1-neo plasmid. Next day, fresh medium was added, and 48 h later cells were trypsinized and plated to various densities on 10 cm dishes. Next day, medium containing 0.9 mg/ml G418 was added, and cultures refed every 3 days. Plates containing isolated colonies were trypsinized and the cells pooled to obtain populations of transfected cells.

Immunological reagents

To generate antibodies against RYBP, Ring1B and Ring1A, GST–RYBP, GST–Ring1B, MBP–Ring1B and GST–Ring1A fusion proteins were produced in *E.coli* BL21(DE3). pGST–RYBP was constructed by cloning a cDNA fragment encoding amino acids 81–145 in pGEX-4T1 (Pharmacia) This fragment of RYBP is not conserved in YAF-2, and therefore was chosen to generate anti-RYBP specific antibodies. pGST–Ring1B and pMALC–Ring1B were constructed by subcloning a truncated Ring1B cDNA containing amino acids 132–326 in pGEX-4T1 and pMALc2 (New England Biolabs), respectively. pGST–Ring1A was constructed by cloning a cDNA fragment obtained by PCR corresponding to amino acids 200–300 in pGEX-4T1. This fragment of Ring1A is not conserved in Ring1B (Schoorlemmer *et al.*, 1997), and was chosen to generate antibodies that would not recognize Ring1B. Expression and purification of the fusion proteins was as previously described, except for the addition of 0.2 mM Zn₂NO₃ to buffers. For immunization, recombinant proteins were further purified by SDS–PAGE. Polyclonal mouse anti-Ring1A antiserum was prepared as described (Ou *et al.*, 1993). Affinity chromatography columns were prepared by coupling purified bacterially expressed proteins to CNBr-activated Sepharose (Sigma Chemical Co.). The rabbit GST–RYBP antiserum was absorbed with GST–Sepharose and anti-RYBP antibodies isolated by affinity chromatography on GST–RYBP–Sepharose. Rabbit antibodies anti-Ring1B were isolated from anti-GST–Ring1B antiserum by affinity chromatography on MBP–Ring1B affinity column. Rabbit anti-Ring1A and anti-M33 antibodies were described before (Schoorlemmer *et al.*, 1997). Monoclonal anti-HA antibody 12CA5 was obtained from Boehringer Mannheim.

Immunoprecipitations and in vivo GST pull-down assay

For immunoprecipitations, extracts were prepared from E12.5 embryos (Alkema *et al.*, 1997a) by homogenization in a lysis buffer containing 0.05 M HEPES pH 7.9, 0.25 M NaCl, 2 mM EGTA, 1.0 mM dithiothreitol (DTT), 0.1% Nonidet P-40 (NP-40), 0.2 mM Zn₂NO₃ and protease inhibitors (EDTA free Complete®, Boehringer Mannheim), followed by brief sonication. The homogenates were spun at 4°C for 15 min in an Eppendorf centrifuge at 12 000 r.p.m. The supernatant of the lysates was precleared for 1 h with protein G–Sepharose. The precleared lysates were then incubated with anti-Ring1A, anti-Ring1B or anti-RYBP antibodies (2–3 μ g). After 1 h at 4°C, protein G–Sepharose (20 μ l of a 50% packed volume) was added and the incubation continued for 1 h at 4°C with continuous rotation. The beads were washed in lysis buffer three times, and transferred to fresh tubes for a final wash. Immunoprecipitated proteins were eluted in Laemmli's buffer, separated on a 10% SDS–polyacrylamide gel and transferred to nitrocellulose for Western blot analysis.

In vivo GST pull-down assays (Chatton *et al.*, 1995) were performed on tissue culture extracts made from transfected 293T cells. Cells were scraped using 0.4 ml of lysis buffer/6 cm dish. Cell lysates were sonicated and spun in an Eppendorf centrifuge at 12 000 r.p.m. for 15 min at 4°C. Aliquots of the supernatant of the lysates were mixed with 20 μ l of GSH–Sepharose (50% packed volume) previously incubated with 0.5% non-fat dried milk. After incubation for 1 h at 4°C with continuous rotation, the beads were washed in lysis buffer as before, and bound proteins eluted and separated by SDS–PAGE prior to Western blot analysis.

Western blot analysis

Proteins were separated by SDS–PAGE and transferred to nitrocellulose membranes (Schleicher & Schuell). After overnight incubation at 4°C in TBST (Tris-buffered saline, 0.05% Tween 20) containing 5% non-fat dried milk, membranes were subsequently incubated with the indicated antibodies diluted in TBST for 1 h at room temperature. After washing,

membranes were incubated with horseradish peroxidase-coupled goat anti-rabbit IgG antibodies (Nordic) or horseradish peroxidase-coupled goat anti-mouse IgG antibodies (BioRad) in TBST for 1 h at room temperature. Bound antibodies were detected by chemiluminescence (SuperSignal, Pierce).

Immunofluorescence

Cells growing on glass coverslips were washed three times in phosphate-buffered saline (PBS) and fixed with freshly prepared 2% paraformaldehyde for 10 min at room temperature. The cells were washed 2× 5 min in PBS and permeabilized with PBS containing 0.5% Triton X-100 for 5 min at room temperature. After two 5 min PBS washes, the cells were incubated for 10 min in 0.1 M glycine in PBS. The cells were washed in PBS and incubated in blocking solution (PBS containing 1% non-fat dried milk, 5% horse serum, 2% bovine serum albumin and 0.1% Tween-20) for 30 min at room temperature. The fixed cells were then transferred to blocking solution without milk containing rabbit or mouse antibodies for 1 h at room temperature. Coverslips were washed 3× 5 min in PBS/0.1% Tween 20. The cells were then incubated with goat anti-rabbit IgG coupled to Texas Red (Jackson Immunoresearch Laboratories) and donkey anti-mouse IgG coupled to Cy2 (Amersham) diluted 1:100 in blocking solution for 1 h at room temperature. After 4× 5 min wash in PBS-Tween, cells were mounted and analysed in a fluorescent microscope equipped with a CCD camera. Fluorescent signals were then processed using image analysis software.

In vitro transcription translation and GST protein binding assay

Intact or truncated cDNAs were subcloned in the pCITE4-1 vector (Novagen). RNA was synthesized with 500 ng of supercoiled plasmids and translated in the presence of 40 µCi of [³⁵S]Met (10 mCi/ml, 800 Ci/mmol, New England Nuclear) using a rabbit reticulocyte lysate (Promega Co.). For the *in vitro* GST pull-down assay, 15 µl of GSH-agarose (Pharmacia) and bacterial protein extracts containing either GST alone or GST-RYBP fusions were mixed and rotated at 4°C for 30 min. Agarose beads were washed three times with 0.02 M HEPES-KOH pH 7.9, 0.1% NP-40, 0.15 M NaCl, 1 mM DTT and protease inhibitors. Immobilized GST proteins were then resuspended in 200 µl of the same buffer containing 1–3 µl of the *in vitro* translation mixtures and incubated for 1 h at 4°C with rotation. The beads were washed twice with 1 ml of buffer, transferred to fresh tubes and washed once more. After adding 20 µl of loading buffer, bound proteins were separated in a 10% SDS-polyacrylamide gel. Dried gels were analysed using a PhosphorImager (Molecular Dynamics).

In situ hybridization

Sense and antisense probes were obtained from pBluescript plasmids (Stratagene) containing full-length RYBP cDNAs. After linearization, *in vitro* transcription was performed using T3 (sense) or T7 (antisense) RNA polymerase and digoxigenin-labelled rUTP (Boehringer Mannheim). Whole mount *in situ* hybridization was performed on E8.5–10.5 embryos as previously described (Wilkinson, 1992).

Accession numbers

The murine RYBP (accession No. AF101779) cDNA sequence has been deposited in the DDBJ/EMBL/GenBank database.

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