Developmental Cell, Vol. 3, 499–510, October, 2002, Copyright ©2002 by Cell Press

Plzf Mediates Transcriptional Repression of *HoxD* Gene Expression through Chromatin Remodeling

Maria Barna,[1] Taha Merghoub,[1] José A. Costoya,[1] Davide Ruggero,[1] Matthew Branford,[1] Anna Bergia,[2] Bruno Samori,[2] and Pier Paolo Pandolfi[1,3]
[1]Molecular Biology Program
Department of Pathology
Sloan-Kettering Institute
Memorial Sloan-Kettering Cancer Center
New York, New York 10021
[2]Department of Biochemistry
University of Bologna and
INFM
Bologna, 40126
Italy

Summary

The molecular mechanisms that regulate coordinated and colinear activation of *Hox* gene expression in space and time remain poorly understood. Here we demonstrate that Plzf regulates the spatial expression of the AbdB *HoxD* gene complex by binding to regulatory elements required for restricted *Hox* gene expression and can recruit histone deacetylases to these sites. We show by scanning forced microscopy that Plzf, via homodimerization, can form DNA loops and bridge distant Plzf binding sites located within *HoxD* gene regulatory elements. Furthermore, we demonstrate that Plzf physically interacts with Polycomb proteins on DNA. We propose a model by which the balance between activating morphogenic signals and transcriptional repressors such as Plzf establishes proper *Hox* gene expression boundaries in the limb bud.

Introduction

The spatial and temporal order of *Hox* gene activation is colinear with the physical position of the genes along their respective clusters (Gaunt et al., 1989; Dolle et al., 1989; Izpisua-Belmonte et al., 1991). In vertebrates, the transcriptional mechanisms involved in *Hox* gene regulation are largely unknown. The *PLZF* (promyelocytic leukemia zinc finger) gene was identified by virtue of its involvement in chromosomal translocations associated with acute promyelocytic leukemia (APL) (Chen et al., 1993). PLZF is a nuclear protein (Reid et al., 1995) containing at the C terminus nine *Krüppel*-type zinc-finger domains, which recognize specific DNA sequences (Li et al., 1997; Sitterlin et al., 1997). At the N terminus, PLZF contains a BTB/POZ domain, which mediates self-association and transcriptional repression when fused to a heterologous DNA binding region (Bardwell and Treisman, 1994; Dong et al., 1996). PLZF functions as a transcriptional repressor through its ability to recruit, via the BTB/POZ domain, nuclear corepressors such as SMRT, N-CoR, Sin-3, and, in turn, class I and II histone deacetylases to the transcriptional complex (Hong et al., 1997; He et al., 1998; Grignani et al., 1998; Lin et al., 1998; David et al., 1998; Lemercier et al., 2002).

We have generated mice with a null mutation in *Plzf*, which show striking patterning defects in both the limb and axial skeleton, including homeotic transformations of anterior skeletal elements in the developing limb into posterior structures (Barna et al., 2000). These transformations are accompanied by the anteriorization and ectopic expression of each member of the 5′ AbdB *HoxD* gene complex in the developing hindlimb. These results imply that Plzf may act as an upstream regulator of *HoxD* gene expression; however, the molecular mechanisms by which Plzf would restrict the posterior boundaries of expression of the *HoxD* gene complex are still unknown. Several models have been proposed to account for regulation of *Hox* gene expression which illustrate (1) the importance of *cis* elements within these genes which would regulate their transcription in space and time and (2) a higher-order silencing mechanism which would prevent posterior *Hox* genes from being activated at an earlier stage through a repressive chromatin configuration (Dolle et al., 1989; van der Hoeven et al., 1996; Kondo et al., 1998; Kondo and Duboule, 1999). However, no transcription factor with direct repressive activity has been , so far, implicated in this process.

Here we define the role of Plzf in controlling the spatial activation of the AbdB *HoxD* gene complex through binding to *cis* elements within *Hox* genes and recruitment of histone deacetylases as well as Polycomb proteins, in turn favoring the transition from a euchromatic to a heterochromatic chromatin state. These results will be discussed in the context of previous models of *Hox* gene regulation.

Results

Selective Deregulation of Spatial but Not Temporal Expression of the Abdb *HoxD* Complex in *Plzf*$^{-/-}$ Mice

Throughout normal limb development, 5′ *HoxD* genes display restricted patterns of expression which correlate with both the temporal and spatial colinearity of the complex. Whole-mount in situ hybridization of *Plzf*$^{-/-}$ embryos did not reveal any premature temporal activation of 5′ *HoxD* genes, as *Hoxd11* transcripts were not expressed at an earlier time than wild-type embryos (Figure 1A). However, when 5′ *HoxD* transcripts are first expressed in spatially restricted posterior domains of the limb, their expression was anteriorized in *Plzf*$^{-/-}$ embryos, showing a uniform expression of transcripts along the entire extent of the hindlimb bud (Figure 1B; and data not shown). As previously reported (Barna et al., 2000), the *Plzf*$^{-/-}$ phenotype is largely restricted to the hindlimb, which may be due to redundancy of Plzf function in the forelimb because of the presence of a

[3]Correspondence: p-pandolfi@ski.mskcc.org

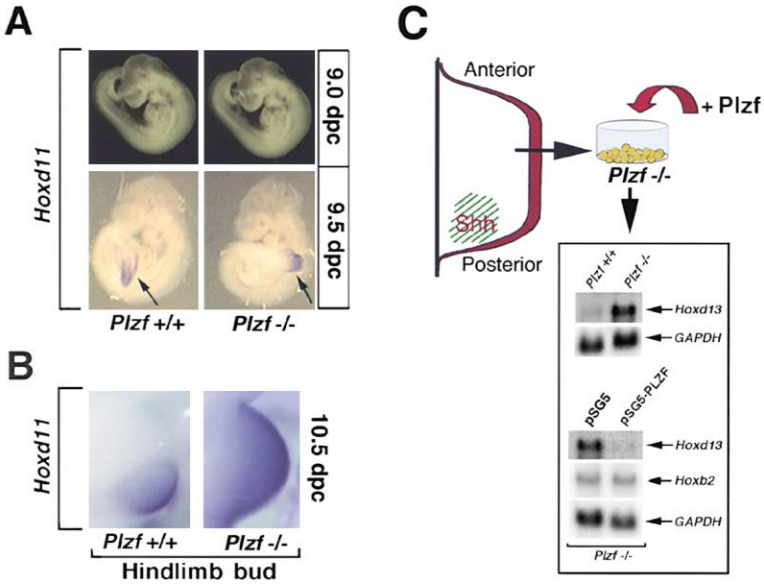

Figure 1. Aberrant *HoxD* Gene Expression in *Plzf*$^{-/-}$ Mice and Its Rescue in *Plzf*$^{-/-}$ Limb Cell Cultures

(A) Expression of *Hoxd11* in *Plzf*$^{-/-}$ and wild-type embryos at 9.0 and 9.5 dpc.

(B) *Hoxd11* expression pattern in the developing hindlimb of *Plzf*$^{-/-}$ and wild-type controls at 10.5 dpc.

(C) Northern blot analysis of *Hoxd13* and *Hoxb2* in *Plzf*$^{-/-}$ and wild-type limb cells as well as in *Plzf*$^{-/-}$ limb cells following transfection of a Plzf expression vector or an empty vector.

Plzf homolog. Similarly, these changes in *Hox* gene expression are limited to the hindlimb. Anteriorization of *HoxD* expression occurred prior to any visible morphological defects in *Plzf*$^{-/-}$ embryos and is consistent with the misexpression of 5′*HoxD* genes at later stages of hindlimb development in *Plzf*$^{-/-}$ mice (Barna et al., 2000). *Plzf* inactivation therefore results in loss of spatial colinear expression of 5′*HoxD* genes, while their temporal sequential activation does not appear to be affected.

Elevated *HoxD* Gene Expression Is Rescued by the Reintroduction of Plzf in Limb Cultures Derived from *Plzf*$^{-/-}$ Embryos

As alterations in limb development may result in the ectopic activation of 5′*HoxD* genes, whose elevated expression are a response to these morphological changes, we sought to test whether the anteriorization of *HoxD* transcripts in *Plzf*$^{-/-}$ embryos was a direct or indirect consequence of Plzf activity. To this end, we utilized low-density primary limb cells from wild-type and *Plzf*$^{-/-}$ embryos to assay the level of expression of one of the 5′*HoxD* genes that showed anteriorized expression in the hindlimb bud, *Hoxd13*. *Plzf*$^{-/-}$ limb cultures showed a marked increase in *Hoxd13* transcripts with respect to wild-type cultures, by Northern blot analysis (Figure 1C; and data not shown). The reintroduction of Plzf in *Plzf*$^{-/-}$ limb cultures (Experimental Procedures) reduced the level of *Hoxd13* transcripts to a comparable level with respect to wild-type limb cultures, while it had no effect on the expression of *Hoxb2*, a *Hox* gene not regulated by Plzf in vivo (Figure 1C). These results therefore suggest that Plzf directly modulates the expression of 5′*HoxD* genes in primary limb cells.

Plzf Binds to Multiple Sites within the 5′*HoxD* Gene Cluster and Mediates Long-Range DNA Looping

To understand the mechanisms by which Plzf mediates *Hox* gene expression, we first analyzed the *Hoxd11* gene for the presence of putative Plzf binding sites (Li et al., 1997; Sitterlin et al., 1997), as *cis* regulatory regions of this gene have been previously identified (Gerard et al., 1993). We identified five putative Plzf binding sites. Two of the binding sites were located in the promoter of *Hoxd11*, one within an intronic sequence, and two within highly conserved regulatory regions, known as region VI and IX, present in the 3′ UTR of the gene (Figure 2A).

We employed limb extracts from *Plzf*$^{-/-}$ and wild-type embryos to perform gel shift retardation assays utilizing labeled oligonucleotide probes spanning the Plzf binding sites present within the *Hoxd11* gene (Plzf binding sites 1–5). A shift is present only in wild-type extracts, but not in extracts from *Plzf*$^{-/-}$ mutants, is competed by a cold oligonucleotide, and is absent with a mutant oligonucleotide in all cases (Figure 2B). As a control, both *Plzf*$^{-/-}$ and wild-type extracts were equally capable of shifting a SP3 oligonucleotide (data not shown). The presence of Plzf in the retarded DNA protein complex was confirmed by Western blot analysis of the proteins eluted from the region of the gel encompassing the shifted band observed utilizing wild-type extracts (Figure 2C). We confirmed that this interaction is direct by the ability of GST-Plzf to bind to an oligo spanning *Hoxd11* RRIX (Figure 2E).

To assess whether Plzf binds to *Hox* regulatory elements in vivo, we performed DNA immunoprecipitation assays to isolate regions of DNA bound by Plzf in primary limb cells. We specifically detected *Hoxd11* RRIX in the DNA that was bound by Plzf in vivo (Figure 2D).

Plzf possesses an evolutionarily conserved BTB/POZ domain that has been shown to mediate homodimerization (Minucci et al., 2000). In order to assess if Plzf through homodimerization could alter the architecture of the *HoxD* locus, we utilized scanning force microscopy (SFM) to visualize stretches of *Hoxd11* genomic DNA in the presence of GST-Plzf. To this end, purified GST-Plzf was incubated with different DNA fragments within *Hoxd11* (Figures 2A and 2G). The samples were deposited on freshly cleaved mica and imaged by SFM. The position of GST-Plzf along the DNA was determined by

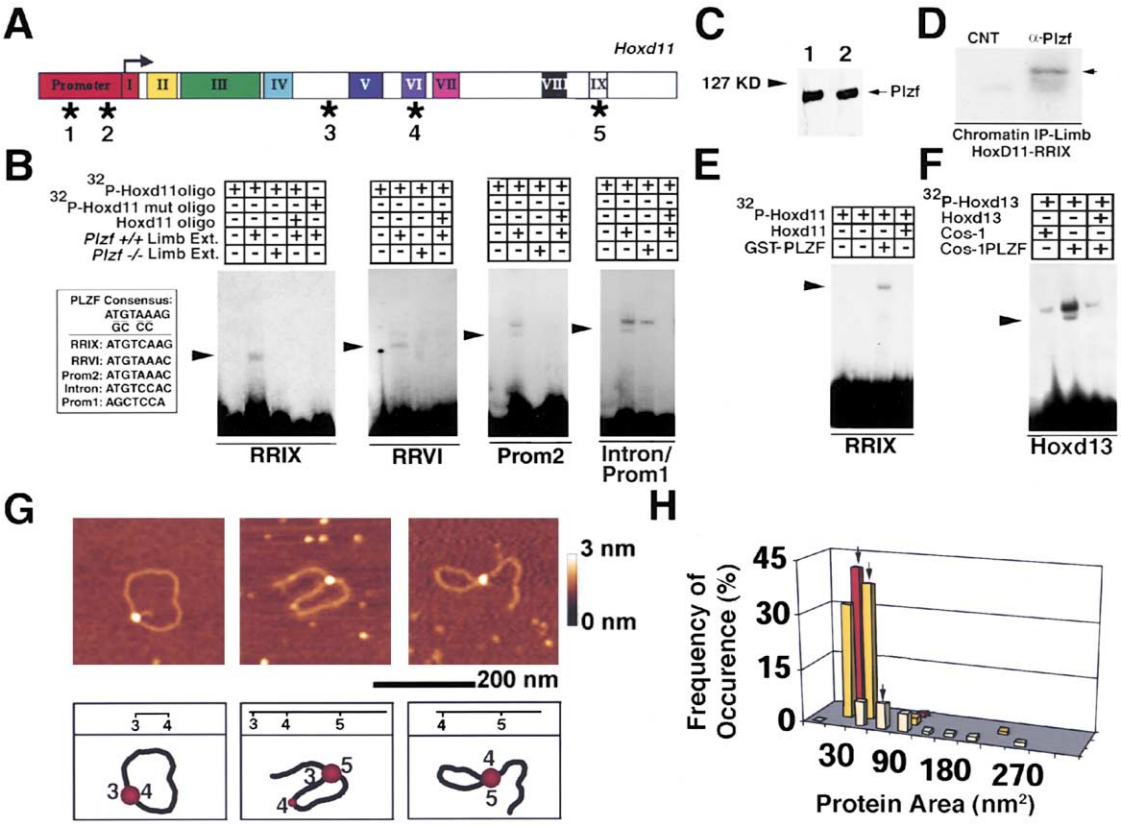

Figure 2. Plzf Binds to Multiple Sites within Regulatory Regions of the *Hoxd11* Gene and Mediates Long-Range Interactions through DNA Looping

(A) Schematic representation of the *Hoxd11* gene, which contains five (1–5) Plzf binding sites (indicated by the star). Evolutionarily conserved regulatory regions within the gene are boxed and numbered.

(B) Electromobility shift analysis with oligonucleotides spanning each of the Plzf binding sites within *Hoxd11* incubated with *Plzf*$^{-/-}$ and wild-type limb extracts.

(C) Western blot analysis of the shifted complex observed in (B) utilizing wild-type limb extracts and an oligonucleotide spanning the Plzf binding site within *Hoxd11* regulatory region IX. Lane 1, COS-1 cells transfected with a Plzf expression vector. Lane 2, proteins from shifted band in (B).

(D) ChIP assays of *Hoxd11* RRIX in mouse limb cells utilizing a Plzf-specific Ab or a preimmune sera control (CNT).

(E) Electromobility shift analysis spanning the Plzf binding site within *Hoxd11* regulatory region IX, utilizing GST-Plzf.

(F) Electromobility shift analysis with oligonucleotides spanning the Plzf binding site within *Hoxd13* (Experimental Procedures) incubated with Cos cell extracts and Cos cell extracts transfected with Plzf.

(G) SFM images of looped complexes of *Hoxd11* DNA fragments mediated by GST-Plzf. Three representative examples are shown. A schematic representation of the corresponding looped structures between different Plzf binding sites within *Hoxd11* is illustrated.

(H) The *x* axis (ticks represent 25 nm^2 intervals) shows statistical analysis of the area protein bound on DNA at loop junctions (light yellow bars); at single DNA binding sites (bright yellow bars); and on the surface of the mica substrate (red bars). The *y* axis shows the frequency in percent at which GST-Plzf and *Hoxd11* complexes are observed. The arrows indicate the highest frequency (percent of complexes observed) of GST-Plzf bound to mica, at single binding sites, and at loop junctions.

measuring the DNA contour length from the center of the protein to each DNA end (data not shown). Surprisingly, we consistently observed complexes between GST-Plzf and *Hoxd11* genomic DNA fragments which resulted in looped structures. In fact, 7%–10% of all complexes (n = 427) were looped, and three representative examples of looped complexes are shown (Figure 2G). Loop formation occurred between different Plzf binding sites within the *Hoxd11* locus, schematically represented in Figure 2G.

To assess whether the DNA looping was mediated by homodimerization of the Plzf protein, we measured the size of the protein molecules either deposited on mica, bound at single DNA sites, or at DNA loop junctions (Nettikadan et al., 1996). The area of protein molecules deposited on mica (n = 1050) is centered on the value of 30 nm^2 that matches the expected value of a monomeric protein. The distribution of the dimensions of Plzf protein molecules on single sites along the DNA chain showed two main peaks: one at about 30 nm^2 and the other at about double this value. The distribution of the dimensions of Plzf protein molecules bound on DNA loop junctions is shifted toward higher values and is centered between 60 and 90 nm^2 (Figure 2H). These results strongly suggest that Plzf tends to bind DNA as a dimer or even as a trimer, mostly when DNA loops are formed.

Thus, Plzf is capable of binding to multiple sites within *Hoxd11* regulatory elements. Furthermore, sequence

analysis of other 5′ *HoxD* genes identified Plzf binding sites in all cases and oligos containing these sites that were all bound by Plzf in gel shift analysis (see Experimental Procedures for a detailed description of these sites and Figure 2F). Moreover, SFM analysis of Plzf bound to *Hoxd11* genomic DNA provides evidence for long-range interactions between distant Plzf binding sites located within *HoxD* regulatory elements mediated by Plzf di- or trimerization.

Plzf Directly Mediates Transcriptional Repression of *HoxD* Gene Expression

To study the transcriptional consequence of Plzf binding to *Hox* gene regulatory elements, we generated two luciferase reporter constructs corresponding to the *Hoxd11* promoter (d11 promoter) and regulatory region IX (RRIX) in which we demonstrated the presence of sequences bound by Plzf (see above). While *Plzf*$^{-/-}$ embryos showed ectopic expression of *Hoxd11* in more anterior regions of the hindlimb, accompanied by homeotic transformations, the forelimbs of these mice were relatively unaffected (Barna et al., 2000). We therefore also tested whether the transcriptional repressive abilities of Plzf differed in the forelimb with respect to the hindlimb. Luciferase activity was assayed following transfection of these constructs in low-density primary limb cultures derived from *Plzf*$^{-/-}$ and wild-type embryos. Strikingly, we observed a 3- to 4-fold increase in the basal activity of the d11 promoter reporter and a 45-fold increase in basal activity of the RRIX reporter in *Plzf*$^{-/-}$ hindlimb cells with respect to wild-type cells (Figure 3A). However, we did not observe any statistically significant difference in the basal activity of the RRIX reporter or the d11 promoter reporter between wild-type and *Plzf*$^{-/-}$ forelimb cells (Figure 3A). Furthermore, the basal activity of both reporter constructs was markedly higher in wild-type forelimbs in comparison to wild-type hindlimbs, suggesting that Plzf is unable to mediate transcriptional repression of these *Hoxd11* reporter constructs in the context of the forelimb (Figure 3A). These results are consistent with the hindlimb-specific phenotype in *Plzf*$^{-/-}$ mice.

We next monitored whether we could rescue the elevated luciferase activity following cotransfection of Plzf expression vector and reporter constructs in *Plzf*$^{-/-}$ limb cells. The RRIX reporter as well as the d11 promoter reporter was repressed in a dose-dependent manner, by increasing concentrations of Plzf. The repression conferred by Plzf on the *Hoxd11* reporter constructs was almost 80% at its highest dose (Figure 3B). Thus, Plzf is essential for the transcriptional repression of *Hoxd11* reporter constructs in the hindlimb.

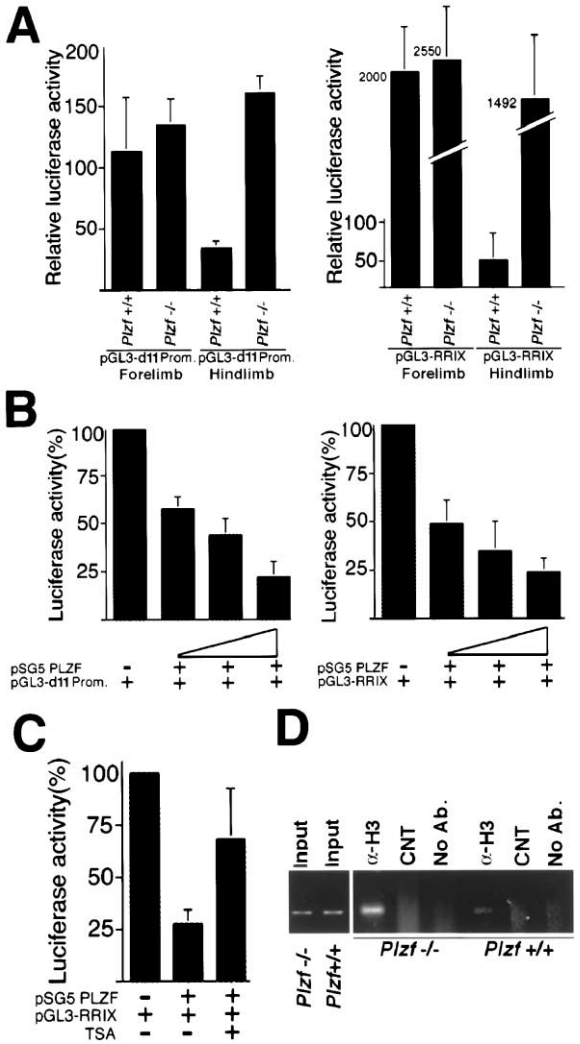

Figure 3. Plzf Mediates Transcriptional Repression of *HoxD* Regulatory Regions

(A) Relative luciferase activity of *Hoxd11* regulatory regions fused to the SV40 minimal promoter corresponding to the *Hoxd11* promoter (pGL3-d11Prom.) and Regulatory Region IX (pGL3-RRIX) in *Plzf*$^{-/-}$ and wild-type hindlimb and forelimb low density cultures.

(B) Rescue of elevated pGL3-d11Prom. and pGL3-RRIX luciferase levels in *Plzf*$^{-/-}$ hindlimb cells following the reintroduction of Plzf in a dose-dependent manner (Experimental Procedures).

(C) *Plzf*$^{-/-}$ limb cells were transfected with the pGL3-RRIX luciferase reporter, and Plzf in the presence or absence of TSA (Experimental Procedures).

(D) ChIP assay of *Hoxd11* RRIX in limb cells utilizing an anti-acetylated Histone H3 antibody, preimmune sera control (CNT), or no antibody (No Ab.). The PCR products from *Plzf*$^{-/-}$ and wild-type samples were run simultaneously on the same gel.

Plzf Mediates Transcriptional Repression of *HoxD* Reporter Constructs and Can Recruit Histone Deacetylases

PLZF forms complexes with nuclear corepressors such as SMRT, N-CoR, and Sin-3, thus recruiting histone deacetylases (HDACs) to the transcription complex, resulting in nucleosome assembly and transcriptional repression (see Introduction). To determine if HDAC-associated enzymatic activity is required for the ability of Plzf to repress the *Hoxd11* reporter constructs, we examined the effect of Trichostatin-A (TSA), a specific inhibitor of HDACs, on this function (Yoshida et al., 1990). TSA significantly reverted the transcriptional repressive activity of Plzf (Figure 3C).

We next determined whether the chromatin state of *HoxD* regulatory regions that were bound by Plzf were altered by the absence of Plzf in vivo. To this end we performed chromatin immunoprecipitation (CHIP) analysis with an anti-acetylated histone H3 antibody in *Plzf*$^{-/-}$

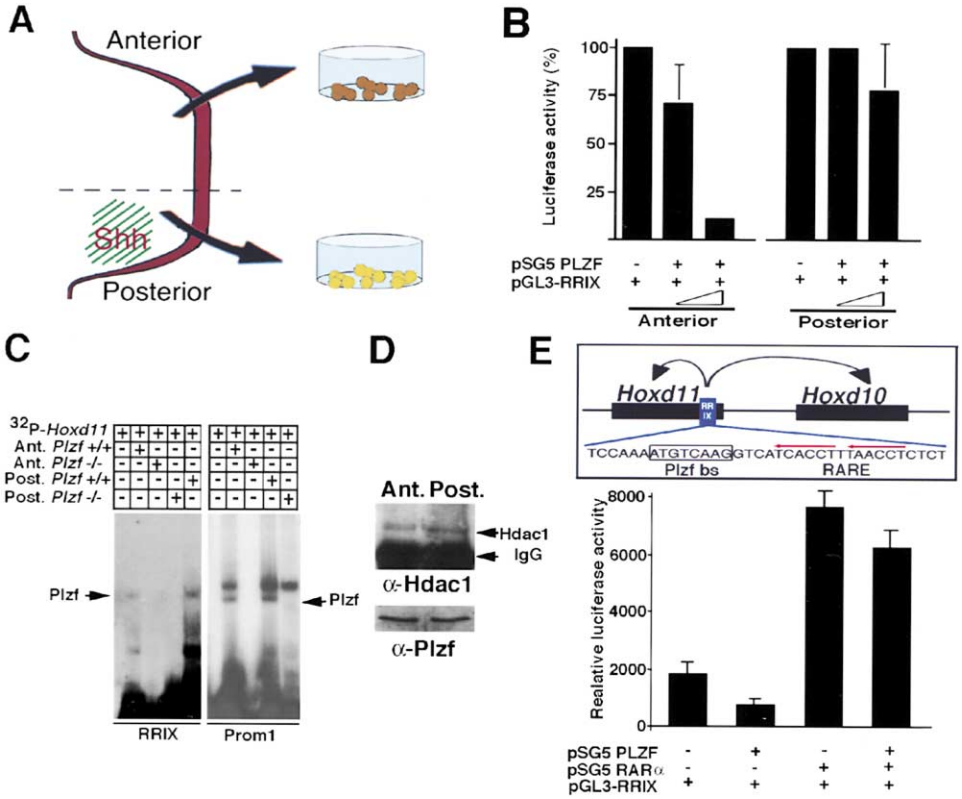

Figure 4. The Transcriptional Repressive Activity of Plzf on *HoxD* Gene Expression in Anterior versus Posterior Micromass Cultures Is Antagonized by Posteriorizing Signals

(A) Anterior and posterior micromass limb cultures were established from 10.5 dpc wild-type embryos (Experimental Procedures).

(B) Transcriptional repression of *Hoxd11* reporter gene constructs by Plzf in anterior versus posterior micromass cultures represented as percent repression of the basal activity of *Hoxd11* luciferase reporter. The activity of the reporter gene without cotransfection of Plzf is shown as 100%.

(C) Electromobility shift analysis spanning the Plzf binding site within *Hoxd11* regulatory region IX (RRIX) and the *Hoxd11* promoter (Prom 1), utilizing cell extracts obtained from anterior and posterior regions of the limb bud from *Plzf*$^{-/-}$ and wild-type embryos. The arrow indicates the shifted band containing Plzf complexes.

(D) Immunoprecipitation of HDAC-1 in anterior and posterior regions of the limb bud with a Plzf antibody.

(E) Schematic representation of the location of the Plzf binding site (Plzf bs) and RARE within Hoxd11 regulatory region IX (RRIX). Cos-1 cells were transfected with the pGL3-RRIX luciferase reporter, Plzf, or RARα in the presence of RA (Experimental Procedures). Similar results were obtained in limb cells (Figure 6G).

and wild-type hindlimb cells. We observed a marked increase of acetylated histones on *HoxD* regulatory regions in the absence of Plzf (Figure 3D). These results therefore implicate HDAC-mediated transcription repression mediated by Plzf in the regulation of *HoxD* gene expression.

Plzf Differentially Mediates Transcriptional Repression in Anterior versus Posterior Limb Micromass Cultures: Balance of *trans*-Acting and *trans*-Repressing Factors

Plzf expression by whole-mount in situ hybridization and immunohistochemistry is concomitant with limb bud formation, and at 10.5 dpc, *Plzf* is present throughout the limb (Barna et al., 2000) (data not shown). Plzf is therefore not restricted in expression to regions of the limb where the *HoxD* genes are not expressed. In order to test whether Plzf functionally possessed differential transcriptional repressive properties in anterior versus posterior regions of the limb, we utilized a limb micromass culture method that mimics the anterior and posterior characteristics of limb bud cells in ex vivo cultures. (Vogel and Tickle, 1993) (see Experimental Procedures). Plzf showed a dose-dependent transcriptional repressive ability in anterior limb micromass cultures (cells corresponding to regions of the limb bud where *HoxD* genes are excluded at 10.5 dpc). In contrast, even at the highest dose of Plzf, *Hoxd11* reporter expression was not repressed in posterior limb micromass cultures (cells corresponding to regions of the limb bud where *HoxD* genes are expressed at 10.5 dpc) (Figures 4A and 4B).

We next sought to determine the molecular basis for this differential repressive ability of Plzf in anterior versus posterior cells. In gel shift experiments, Plzf was bound to *Hoxd11* regulatory elements and was able to recruit HDAC in both anterior and posterior regions of the limb (Figures 4C and 4D). In the limb bud, Shh or

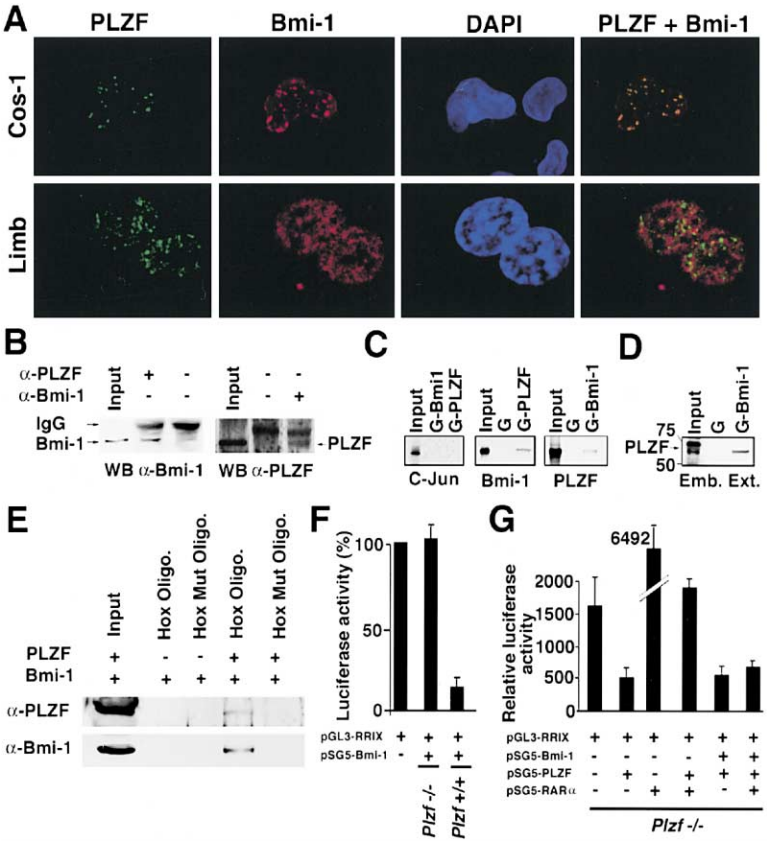

Figure 5. Plzf Tethers the Polycomb Protein Bmi-1 to *Hoxd11* Regulatory Regions, Preventing Transcriptional Activity by *trans*-Acting Factors

(A) Immunofluoresence of Cos-1 transfected cells and primary limb cells with Plzf and Bmi-1 antibodies.

(B) Cos cells were transfected with Plzf and/or Bmi-1, and coimmunoprecipitation experiments were performed with anti-Bmi-1 and anti-Plzf antibodies, or pre-immune sera.

(C–D) In-vitro pull-down assays performed using GST-Plzf, GST-Bmi-1, GST-Jun, or GST (G) alone with in vitro translated ^{35}S-labeled proteins or total body embryo cell extracts.

(E) A biotinylated *Hoxd11* RRIX oligo was incubated with cells transfected with or without Plzf, and subsequent Western blot analysis was performed with a Bmi-1 Ab.

(F) *Plzf*$^{-/-}$ and wild-type limb cells were transfected with the *Hoxd11* RRIX reporter construct and Bmi-1.

(G) *Plzf*$^{-/-}$ limb cells were transfected with the *Hoxd11* RRIX reporter construct and Bmi-1, Plzf, and/or RARα in the presence of RA (Experimental Procedures).

RA has been shown to induce *HoxD* gene expression when ectopically placed in anterior regions of the limb where *HoxD* genes are not normally expressed, although the mechanism of this activation remains unclear (Helms et al., 1994; Johnson and Tabin, 1997). We tested whether the presence of either RA or Shh would alter the ability of Plzf to mediate transcription repression of *Hoxd11* regulatory elements. We also took advantage of the fact that a nuclear hormone receptor, retinoic acid receptor α (RARα)-responsive element (RARE) was present within the RRIX reporter (Figure 4E). This RARE has previously been shown to be important for correct *Hoxd11* expression in vivo (Gerard et al., 1996). Addition of Shh or transfection of RARα in the presence of RA resulted in marked *trans*-activation of *Hoxd11* regulatory elements, which antagonized the *trans*-repressive activity of Plzf (Figures 4E and 5G; and data not shown).

Therefore, posteriorizing signals derived from the developing limb such as RA and Shh can override the *trans*-repressive ability of Plzf. This data provide a possible mechanism for how Plzf differentially mediates transcriptional repression of *HoxD* genes in anterior versus posterior regions of the limb bud.

Plzf Directly Tethers Polycomb on DNA which Antagonizes Posteriorizing Signals in the Limb

Polycomb (PcG) family proteins are transcriptional repressors of *Hox* genes; and they act to maintain correct *Hox* gene expression boundaries through an epigenetic mechanism involving remodeling of the chromatin structure (Pirrotta, 1998, Paro, 1990). Most PcG proteins do not bind to DNA directly. Therefore, how PcG proteins would be tethered on DNA in order to mediate *Hox* gene silencing remains unknown. Mammalian PcG proteins Ring-1, Bmi-1, and hPc2 form a protein complex that is localized to discrete nuclear structures known as PcG bodies associated with heterochromatin (Saurin et al., 1998). PLZF also localizes to discrete nuclear foci of unknown functional significance (Reid et al., 1995). Due to the ability of Plzf to directly mediate transcriptional repression of *Hox* gene expression, we tested whether Plzf would colocalize in the nucleus with PcG bodies. We observed colocalization of Plzf and Bmi-1 in discrete nuclear foci both in transfected cells as well as primary limb cultures, suggesting that Plzf is present within the PcG complex and localizes in the nucleus to PcG bodies (Figure 5A). We next tested whether Plzf would physically interact with one of the mammalian PcG proteins, Bmi-1, in vivo. Using coimmunoprecipitation assays, we determined that Plzf associated with Bmi-1 (Figure 5B). Plzf and Bmi-1 interaction was direct as assessed by GST pull-down assays with purified proteins (Figure 5C). Furthermore, GST-Bmi-1 was able to pull down endogenous Plzf from total embryo extracts (Figure 5D). We next determined whether Plzf and Bmi-1 would physically associate on DNA, utililizing an oligo affinity binding assay (OABA). Bmi-1 was only associated with RRIX when Plzf was present (Figure 5E).

In order to assess the functional relevance of the association of Plzf and Bmi-1, we performed at first *trans*-repression assays on the *Hoxd11* RRIX reporter in *Plzf*$^{-/-}$ and wild-type limb cells. Strikingly, Bmi-1 was

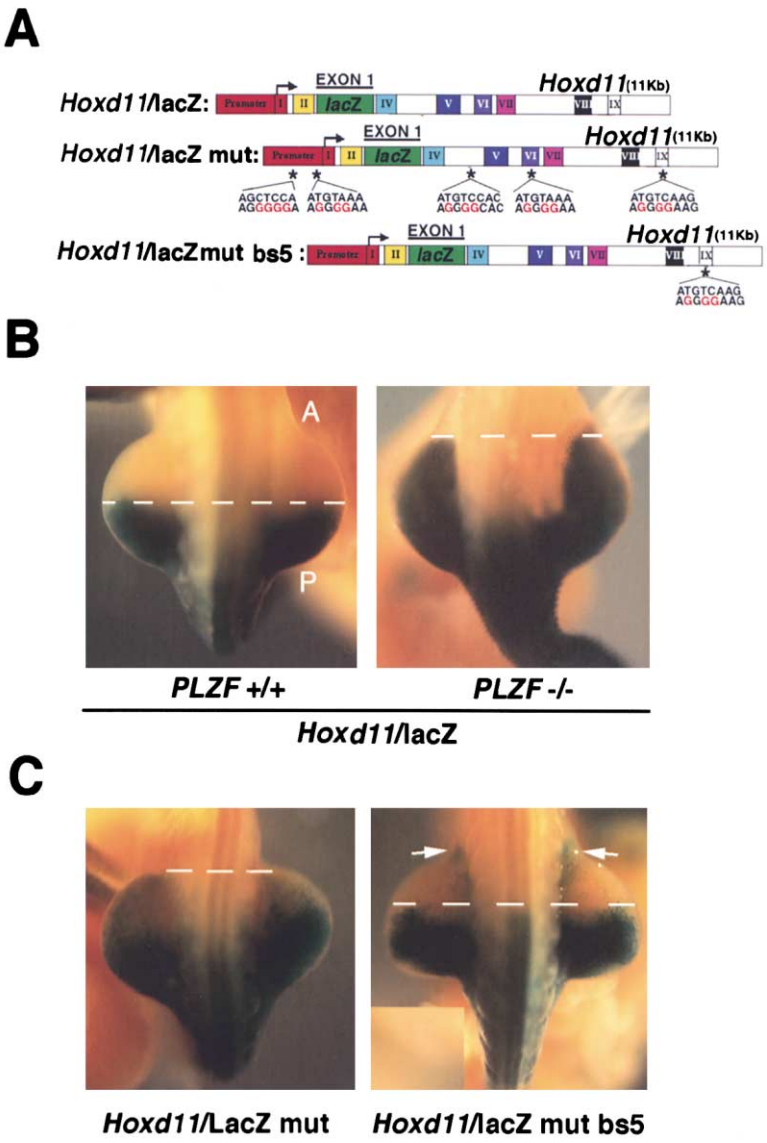

Figure 6. The Effect of Mutations in Plzf Binding Sites within a *Hoxd11*/lacZ Reporter Construct In Vivo

(A) Schematic representation of the *Hoxd11*/lacZ reporter constructs utilized for the generation of transgenic mice.

(B) A stable transgenic line carrying the *Hoxd11*/lacZ transgene was generated and β-gal expression was monitored in 10.5 dpc wild-type and *Plzf*$^{-/-}$ embryos.

(C) Mutations in all five Plzf binding sites within the *Hoxd11*/lacZ transgene (*Hoxd11*/lacZ mut) and a single mutation in the Plzf binding site corresponding to *Hoxd11* Regulatory Region IX (*Hoxd11*/lacZ mut bs5) were generated and β-gal expression was monitored in embryos which expressed the transgene. Arrow indicates β-gal-positive cells within the anterior margins of the limb bud.

able to repress the basal activity of *Hoxd11* RRIX reporter in wild-type, but not in *Plzf*$^{-/-}$ limb cells (Figure 5F). We next tested whether the association between Plzf and Bmi-1 would affect the function of other *trans*-acting factors in the limb. Posteriorizing signals in the limb such as retinoic acid (RA) have been shown to be important in affecting the transcriptional state of *Hox* genes in anterior tissues (Johnson and Tabin, 1997). We took advantage of the fact that a RARE binding site for factor, RARα, was present in close vicinity to the Plzf binding site (see previous paragraphs and Figure 4E). In primary limb cells, RARα acted as a potent transcriptional activator of the HoxD11 RRIX reporter in the presence of RA (Figure 5G). Plzf was not able to mediate transcriptional repression of the HoxD11 RRIX reporter, in the presence of RARα, although it was able to suppress expression to basal level of activity (Figure 5G). Strikingly, cotransfection of Plzf and RARα in the presence of Bmi-1 completely restored the transrepressive potential of Plzf (Figure 5G).

Thus, Plzf directly interacts with Polycomb family members, and this may serve to tether their chromatin remodeling potential to specific *cis*-acting elements within the Hox locus. Furthermore, the interaction between Plzf and Polycomb members such as Bmi-1 may antagonize posterior activating signals in the limb bud such as RA, which act on target nuclear hormone receptors.

Regulation of *Hoxd11* Gene Expression by Plzf In Vivo

Regulation, in vivo, of the *Hoxd11* gene can be faithfully studied in transgenic mice using a 11 kb fragment of *Hoxd11* genomic DNA, known as *Hoxd11*/lacZ which is capable of reproducing the spatially restricted expression pattern of *Hoxd11* in the hindlimb (Gerard et al., 1993). First, we generated a stable line of *Hoxd11*/lacZ transgenic mice to examine the expression of this construct in *Plzf*$^{-/-}$ embryos. While wild-type embryos showed a restricted expression of β-gal to the posterior

mesoderm of the developing hindlimb, *Plzf*$^{-/-}$ embryos displayed β-gal expression throughout the entire anterior-posterior extent of the limb bud (Figures 6A and 6B) that mimicked the anteriorization of *Hoxd11* transcripts in *Plzf*$^{-/-}$ embryos (see Figure 1B).

Mutations in all of the Plzf binding sites within the *Hoxd11*/lacZ reporter (Figure 6A; and Experimental Procedures) resulted in anteriorization of β-gal expression within the wild-type limb bud (Figure 6C) and fully reproduced the expression of the *Hoxd11*/lacZ reporter construct in a *Plzf*$^{-/-}$ background (Figure 6B). Interestingly, a single mutation in the Plzf binding site corresponding to regulatory region IX of *Hoxd11* (see above paragraphs) also resulted in a partial anteriorization of β-gal expression which was never as extensive as when all five Plzf binding sites were mutated (Figure 6C).

Plzf therefore directly acts in vivo to regulate the spatial expression of *Hoxd11* in the limb bud by binding to regulatory elements required for restricted gene expression boundaries. When these Plzf binding sites are mutated, this results in ectopic expression of *Hoxd11* within anterior regions of the developing limb.

Discussion

Plzf in the Control of Spatial Expression of the *HoxD* Gene Complex

Tremendous efforts have been made at understanding the molecular mechanisms that would account for accurate expression of vertebrate *Hox* genes. Taken together, several experimental approaches have highlighted the importance of *cis*-acting regulatory elements as well as a higher order or global mechanism acting on the entire *Hox* cluster in order to account for temporal and spatial control of *Hox* gene expression. For instance, reporter gene constructs have demonstrated the ability of regulatory regions within *Hox* genes to reproduce important patterns of expression of the endogenous genes (e.g., Behringer et al., 1993; Gerard et al., 1993; Beckers et al., 1996). On the other hand, temporal and spatial colinearity of *Hox* gene expression may also be regulated by long-range interactions with a "locus control region" functionally related to the *Globin* gene complex (Hanscombe et al., 1991), or by the progressive transition from an inactive to an active state of chromatin configuration (closed to open) (Duboule 1992; van der Hoeven et al., 1996; Kondo et al., 1998). In fact, large deletions near the 5′ end of the *HoxD* complex result in deregulation in the temporal colinearity of *Hox* gene expression, suggesting that the *HoxD* locus is subjected to a higher order silencing mechanism that would prevent genes from being activated at an early stage (Kondo and Duboule 1999). It is intuitive that, if concomitantly active, local regulatory influences would be subordinate to this more general regulatory system. One question which clearly emerges from this analysis is the identification of specific *trans*-acting factors which would integrate both local and/or global regulatory influences in order to mediate the correct expression of *Hox* genes either in time or in space: Plzf can indeed serve this purpose.

Plzf inactivation results in a loss of the restricted spatial expression of 5′ *HoxD* genes, in that all the 5′ *HoxD* genes of the AbdB complex are ectopically expressed through the limb bud. Furthermore, we have demonstrated that the reintroduction of Plzf in limb cultures derived from *Plzf*$^{-/-}$ embryos represses *HoxD* gene expression. We have demonstrated the importance of Plzf to bind to *Hox* gene regulatory elements in vivo within the context of a *Hoxd11*/lacZ reporter construct in which mutations in all of the Plzf binding sites results in deregulated expression of the transgene. Moreover, given the presence of multiple Plzf binding sites bound by Plzf within the *HoxD* locus and that all 5′ *HoxD* genes are anteriorized in *Plzf*$^{-/-}$ embryos, it is likely that Plzf can regulate the expression of the entire 5′ *HoxD* complex by directly binding to regulatory elements within each gene member of the complex. Furthermore, we have demonstrated that the transcriptional activity of Plzf is, at least in part, linked to the enzymatic activity of HDACs, in turn resulting in chromatin remodeling from an open to a closed heterochromatic status via histone deacetylation. This is of particular relevance as several models of *Hox* gene regulation have predicted the importance of transitions of the chromatin configuration in *Hox* gene regulation; however, the mechanism was largely unknown.

We have visualized DNA loop formation mediated by Plzf molecules bound at distantly located sites. Therefore, in addition to the ability of Plzf to remodel chromatin, its homodimerization status could mediate long-range interactions of DNA elements present within the *HoxD* locus. Long-range interactions between regulatory regions containing different Plzf binding sites may play an important role in coordinating the spatial expression of distant *Hox* genes within the 5′ *HoxD* locus.

Balance between *trans*-Actin and *trans*-Repressing Signals in Regulation of *Hox* Gene Expression in the Limb

We demonstrate that the ability of Plzf to mediate transcriptional repression of *Hox* genes is severely compromised in posterior regions of the limb (where *HoxD* genes are expressed at this stage). Shh or RA has been shown to induce *HoxD* genes in a sequential manner when ectopically placed in anterior regions of the limb where *HoxD* genes are not normally expressed (Helms et al., 1994; Johnson and Tabin, 1997). This suggests that these factors play an important role in the regulation of *HoxD* genes in posterior regions of the limb, although the mechanisms of this activation remain unclear. Here we show that posteriorizing factors such as Shh or RA can overcome Plzf *trans*-repressive ability. Therefore, a competition between Plzf and positively acting posteriorizing factors may ultimately regulate the chromatin state of the 5′ HoxD locus (Figure 7). This is supported by the fact that RARα in its liganded state remodels chromatin through recruitment of histone acetyltransferases (HATs) (reviewed in McEwan, 2000). This activity could be counterbalanced by the ability of Plzf to recruit HDAC, thus favoring a more heterochromatic chromatin state.

Plzf Recruitment of Polycomb: Tilting the Balance between *trans*-Acting and *trans*-Repressing Signals

We show that Plzf directly interacts with PcG members such as Bmi-1 and colocalizes with PcG bodies. Bmi-1

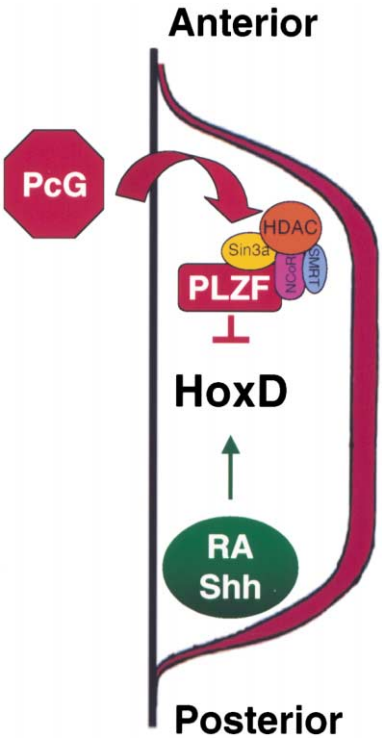

Figure 7. Model for the Role of Plzf in Mediating Transcriptional Repression of the *HoxD* Gene Locus

Plzf represses the expression of genes belong to the *HoxD* cluster in the limb by directly binding to regulatory elements and can recruit histone deacetylases to these sites. Plzf may therefore regulate the spatial colinear activation of AbdB *HoxD* gene complex by chromatin remodeling, changing the chromatin conformation from open to closed. In addition, Plzf can mediate long-distance interactions between *cis* regulatory elements within the *HoxD* locus via homodimerization. The transcriptional repressive ability of Plzf is antagonized in posterior regions of the limb by posteriorizing signals such as RA and Shh. Plzf physically associates with the polycomb protein Bmi-1 on DNA. The association of Plzf with polycomb proteins may prevent the *trans*-acting activity of these morphogens, thereby establishing a gradient of positive and negative transcriptional influences governing the spatial expression of *HoxD* gene expression in the limb bud.

has been previously shown to regulate correct *Hox* gene expression boundaries in vertebrates (van der Lugt et al., 1994; Alkema et al., 1995). The mechanism by which PcG members such as Bmi-1 would act as a *trans*-repressive factor of target *Hox* gene expression remained largely unknown. Our data indicate that Plzf directly binds Hox regulatory elements and can act to tether PcG members on DNA. Plzf directly interacts with Bmi-1, and it may interact with other PcG members as well.

During *Drosophila* embryogenesis the transcriptional activity of gap and pair-rule transcription factors plays a crucial role in the early determination of the expression pattern of *Hox* genes. However, due to the transient expression of the *gap* and *pair-rule* genes, in later stages of embryonic development, the maintenance of expression boundaries of homeotic genes requires the activity of two antagonistic group of genes, *PcG* and the *trithorax* group (*TrxG*) (Kennison, 1993; Simon, 1995; Gaul and Jackle, 1990). Therefore, PcG proteins maintain transcriptional repression of homeotic genes through an epigenetic process thought to involve chromatin remodeling. Like the gap proteins, Plzf specifically binds DNA, thus directly repressing *Hox* gene expression. However, unlike gap, Plzf expression is maintained throughout limb development.

We have tested the functional relevance of the interaction between PLZF and PcG in vertebrate *Hox* gene regulation and demonstrated that recruitment of PcG may play an important role in preventing activation of *Hox* genes by transcriptional activators such as RARα. Therefore, the Plzf and PcG complex might tilt the balance between *trans*-acting and *trans*-repressing factors, favoring maintenance of the transcriptional repressed state (Figure 7). In agreement with this notion, PcG have been already implicated in limiting the accessibility of *trans*-acting factors to DNA (Zink and Paro, 1995). Furthermore, M33 a PcG member that belongs to the same complex as Bmi-1 has been implicated in defining access to RAREs localized in regulatory elements present within *Hox* genes, including *Hoxd11*, as M33-deficient mice show altered RA sensitivity (Core et al., 1997; Bel-Vialar et al., 2000).

Although our data underscore the importance of Plzf/PcG interactions in the limb, the homeotic transformations in axial skeletal structures observed in both Plzf and Bmi-1 null mice (van der Lugt et al., 1994, 1996; Alkema et al., 1995; Barna et al., 2000) suggest that this interaction may also be important in regulating *Hox* gene expression patterns in other embryonic structures.

Implications for Aberrant *HOX* Gene Expression and Polycomb Function in Leukemogenesis

Deregulated function and/or expression of specific *HOX* genes have being directly implicated in leukemogenesis (reviewed in Look, 1997). In APL, as a consequence of translocations between chromosome 11 and 17, the *PLZF* gene fuses to the *RARα* gene resulting in two fusion genes which are coexpressed in the leukemic blast: PLZF-RARα and RARα-PLZF (reviewed in Rego and Pandolfi, 2001). Both proteins can act as dominant-negative PLZF mutants (He et al., 2000). It will be therefore important to determine the expression pattern of potential target *HOX* genes whose correct expression is dependent on PLZF activity in APL blasts. Inactivation of PLZF function and the consequent aberrant expression of *HOX* genes could in turn participate in leukemogenesis.

In APL, PLZF-RARα can also act as a potent and RA-insensitive transcriptional repressor of RARα through aberrant nuclear corepressor/HDAC associations, thus rendering the APL blasts unresponsive to the differentiating effects of RA (Grignani et al., 1998; He et al., 1998; Lin et al., 1998). Surprisingly, however, PLZF-RARα can exert this role, at least in part, epigenetically since RA can readily induce its physical degradation (Rego et al., 2000). The fact that Bmi-1 and Plzf physically interact may provide a mechanism underlying this aberrant epigenetic repressive ability by PLZF-RARα. The recruitment of BMI-1 to the leukemogenic transcription complex may prevent proper transcriptional activation of RARα target genes even in the absence of the fusion protein.

Experimental Procedures

Whole-Mount In Situ Hybridization

Whole-mount in situ hybridizations were carried out as previously described (Barna et al., 2000).

Northern Blot Analysis

Total RNA was prepared from limb cultures using Trizol reagent (GIBCO-BRL). For Northern blot analysis, denatured total RNA (10 μg) was hybridized with the mouse *Hoxd13*, *Hoxb2*, and *GAPDH* fragments as a probe.

Cell Culture and Transfection

Hindlimb buds were dissected from embryos and the ectoderm removed after soaking the limbs in 2% trypsin (GIBCO, 1:250) in calcium and magnesium free Hanks buffered salt solution (GIBCO), pH 7.4, for 20–30 min. The mesenchyme was disaggregated and the cells centrifuged. Low-density monolayer layer cultures were obtained by resuspending cells in CMRL medium (GIBCO) with fetal bovine serum (FBS) and seeding at a concentration of 1,000,000 primary cells in a 35 mm dish. High-density micromass limb bud cultures of 10.5 dpc mouse embryos were prepared as described (Vogel and Tickle 1993). FGF-4 (gift of Dr. Lee Niswander) was added to posterior limb bud cells at a concentration of 1 μg/ml to maintain polarizing activity (Vogel and Tickle 1993). Primary limb bud cells were transfected using lipofectamine plus reagent (GIBCO). The luciferase reporter plasmids (400 ng) were cotransfected with increasing concentrations of the Plzf expression vector or an empty vector (72, 180, and 360 ng). The RRIX reporter plasmid (400 ng) was cotransfected with Bmi-1 (424 ng), RARα (424 ng), Shh (480 ng). For cells transfected with a RARα expression plasmid, RA was supplemented in the media at a concentration of 30 ng/ml. In cases where multiple expression plasmid were used, the plasmids were cotransfected at a 1:1 molar ratio. The TK-β-galactosidase expression vector or TK-Renilla (25 ng) was also cotransfected in order to normalize for transfection efficiency. Forty-eight hours later, cells were harvested and prepared for FACS sorting or determination of luciferase activity with a luminometer according to the manufacturer's instructions (Promega). For the TSA experiments, limb cells were treated with the drug at a concentration of 200 nM for 48 hr, and the medium containing the drug was replaced every 12 hr.

Plasmid Construction

A genomic fragment of 480 bp corresponding to the regulatory element located in region IX of the mouse *Hoxd11* gene (Gerard et al., 1993) was amplified by polymerase chain reaction using two specific primers (5′ AAGATGCACAGCAGCTCATG 3′ and 5′ ACTG CAGCTCTTCATTACAG 3′). This fragment was subcloned into the pCR 2.1 and cloned into pGL3-Promoter vector (Promega) digested with *Xho1* and *Sac1*. This gave the pGL3-RRIX construct. A genomic fragment of 1.5 kb corresponding to the promoter region of the mouse HoxD-11 gene was released from the pGemE/ElacZpA construct (Gerard et al., 1993) using *PstI* and *SalI* and cloned into pSP72 vector (Promega) digested with the same restriction enzyme sites. The cloned fragment was subsequently excised with *HindIII* and *SacI* and cloned into pGL3-Basic vector (Promega) digested with the same restriction enzymes. This gave the pGL3-Promoter construct.

The *Hoxd11*/lacZ mut construct was generated by introducing mutations in all five Plzf binding sites within the pGemE/ElacZpA construct (Gerard et al., 1993) utilizing the QuikChange Site-Directed Mutagenesis Kit (Stratagene) following the manufacturer's suggested protocol employing oligonucleotides containing the desired mutation (underlined) in Plzf binding site 1 (5′ GGGCTCCAACACAG TGAAAGGGGGAAGAGACTTGAACACAGAAG3′); Plzf binding site 2 (5′GCACTCCGCAGAGAAATAGGGGAATCAGGGCTCCCTGCGC 3′); Plzf binding site 3 (5′ GTGTCCGGGCGTGAACACAGGGGCAC GCCGCACTCTACTGTGC3′); Plzf binding site 4 (5′GGGGAACATGG TAAAGGGGAACATCCCTTTCCAATTTTACTGCC3′), and Plzf binding site 5 (5′GTATGCCTTTGAACTTCCAAAAGGGGAAGGTCATCAC CTTTAACCTCTC3′). The insertion of desired mutations was verified by sequencing. The *Hoxd11*/lacZ bs5 mut construct carrying a mutation in Plzf binding site 5 was created by recloning fragments of genomic DNA carrying a mutation in this binding site into the pGemE/ElacZpA construct.

Electrophoretic Mobility Shift Assay

Hindlimbs were removed from embryos, trypsinized, and homogenized in 400 mM NaCL, 10 mM HEPES (pH 7.9), 0.1 mM EGTA, 0.5 mM DTT, 5% Glycerol, 0.5 mM PMSF, and 1% Triton. Lysates were ultracentrifuged at 35,000 × g for 30 min at 4°C. The supernatant was collected and utilized for electrophoretic mobility shift assay (EMSA). The following oligonucleotides were employed in EMSA reactions spanning Plzf binding sites within Hoxd11: RRIX (5′ CTT CCAAAATGTCAAGGTCATCACCTTTAACCTCT 3′), RRVI (5′ GGG AACATGGTAAATGTAAACATCCCTTTC 3′), Intron (5′ GGGCGTG AACACATGTCCACGCCGCACTCT3′), Promoter 1 (5′ CCAACACAG TGAAAGCTCCAAGAGACTTGA 3′), and Promoter 2 (5′ GCAGA GAAA TATGTAAATCAGGGCTCCCTG 3′). Binding reaction mixes for gel retardation assays were carried out as previously described (Zhong et al., 1999). The shifted band observed utilizing RRIX oligos was cut out of the gel and analyzed by SDS-PAGE, followed by Western blot analysis utilizing an anti-Plzf antibody (Barna et al., 2000). Plzf consensus binding sites (underlined) shifted in EMSA assays performed in Cos-7 cells transfected with Plzf were identified in all the members of the Abdb Hox gene cluster: *Hoxd13* (5′ AAGAC CAGGTCAAGTTCTATACGCCTGATG3′), (5′GACCAGCAGGTCAAA ATCTGGTTCCAGAAC3′); *HoxD12* (5′ CCGGGCGTGAACACATGTC CACGCCCGCAC 3′); *HoxD10*(5′ACAACAAAAGAGCTAAAAGGAGA CCAGCCG3′), (5′CTCACCGACAGGCAGGTCAAGATTTGGTTT3′); *HoxD9*(5′GGAGCCCTATTCTATGTAAATGTCCCTCAT 3′). (Zappavigna et al., 1991; Renucci et al., 1992; Gerard et al., 1996).

DNA Immunoprecipitation Assay

Single cell suspensions of limbs derived from *Plzf*$^{-/-}$ and wild-type embryos were prepared. Chromatin immunoprecipitation assays were performed using the chromatin immunoprecipitation assay kit (Upstate Biotech) according to the manufacturer's directions. PLZF Ab or rabbit preimmune sera were utilized. Alternatively, an anti-acetylated Histone H3 antibody was employed (Upstate Biotech). The PCR reaction was carried out with primers spanning the *Hoxd11* regulatory region IX: 5′ AAGATGCACAGCAGCTCATG 3′ and 5′ GTC TGGATGTATGAGCCTG 3′. The PCR product was run on an agarose gel and subjected to Southern blot analysis with the internal oligo: 5′ GAATAATTAGGCGCCTTAAAGT 3′.

Scanning Force Microscopy

Hoxd11 genomic DNA fragments were incubated with GST, GST-PLZF, and PLZF proteins for half an hour on ice in binding buffer (400 mM KCL, 200 mM HEPES, pH 7.5, 10 mM $MgCl_2$, 1 mM EGTA, 5 mM DTT). The reaction mixtures were diluted 10-fold with 10 mM $MgCl_2$ just before the deposition on freshly cleaved mica. The samples were prepared with three different protein to DNA molar ratios, 2, 4, and 8, such that the initial concentration of DNA was 40 nM and that of the protein ranged from 80 to 320 nM. After a deposition time of about 3 min, the surface was rinsed with water and dried with a gentle flux of nitrogen. Imaging was performed in tapping mode with PointProbe noncontact silicon probes (NanoSensors, Germany) on a NanoScope IIIa scanning force microscope system equipped with a multimode head and a type E piezoelectric scanner (Digital Instruments). Images were recorded with a 10 ± 15 mm linear scanning speed at a sampling density of 4 ± 9 nm^2 per pixel. Raw SFM images were processed only for background removal (flattening) using the microscope manufacturer's image-processing software. DNA molecule lengths were measured from the SFM images using ALEX, a software package written for probe microscopy image processing (Rivetti et al., 1996).

GST Pull-Down Assays

In vitro pull-down analysis was performed using GST-fused PLZF and Bmi-1 proteins and GST alone (from pGEX5T; Pharmacia) as previously described (Zhong et al., 1999). Purified GST proteins were used in the protein binding experiments using either total body embryo cell extracts or in vitro translated ^{35}S-labeled proteins. Western blot analysis was carried out for the GST pull-down assay on

total body embryo extracts with a Plzf monoclonal antibody (Oncogene) at 2 μg/ml concentration.

Indirect Immunofluorescence

Indirect immunofluoresence was carried out as previously described (Zhong et al., 1999). Anti-PLZF (Barna et al., 2000) was used at a dilution of 1:200 and anti-Bmi-1 (kind gift of M. van Lohuizen) at 1:50.

Oligo Affinity Binding Assay

The oligo affinity binding assay (OABA) was performed as previously described (Zhong et al., 1999). Biotinylated RRIX oligo and mutated RRIX (100 ng; sequences as in EMSA assays; GeneLink) and Cos-1 cell lysates transfected with Bmi-1 alone or PLZF and Bmi-1 (70 μg) were utilized.

Production of Transgenic Mice

For injection, the pGemE/ElacZpA (Gerard et al., 1993), *Hoxd11*/lacZ mut, and *Hoxd11*/lacZ bs5 mut constructs were excised with *NsiI*, and a fragment of 10.7 kb was injected in all cases. A $Plzf^{-/-}$ transgenic line carrying the *Hoxd11*/lacZ transgene was obtained injecting this construct into $Plzf^{+/-}$ eggs. In addition, all the constructs were tested in transient assays using F1 eggs. Eleven embryos expressing the *Hoxd11*/lacZ transgene were obtained in these transient assays, and all embryos showed a posteriorly restricted expression pattern in the hindlimb as previously described (Gerard et al., 1993). Four embryos expressing the *Hoxd11*/lacZ mut transgene were obtained, and two embryos showed an anteriorization of β-gal expression throughout the hindlimb that was never observed in the *Hoxd11*/lacZ transgene. Eight embryos expressing the *Hoxd11*/lacZ bs5 mut transgene were obtained, and three embryos showed a partial anteriorization of β-gal expression in the hindlimb that was never observed in the *Hoxd11*/lacZ transgene.

X-Gal Staining

Dissected embryos were placed in fixative solution (0.2% glutaraldehyde, 5 mM EDTA, 2 mM $MgCl_2$, 1× PBS) for 2 hr. The embryos were washed in detergent rinse (2 mM $MgCl_2$, 0.2% NP-40, 1× PBS) two times for 15 min on an orbital shaker. The embryos were next incubated in staining solution (5 mM potassium ferricyanide, 5 mM ferrocyanide, 2 mM $MgCl_2$, 2 mg/ml X-gal [dissolved in DMSO], 1× PBS) overnight at 30°C.

Acknowledgments

We thank Lee Niswander for useful discussions and for critical reading of the manuscript; Denis Duboule and Mathew Gerard for the *Hoxd11* genomic region, the *Hoxd11*-LacZ expression vector, and for suggestions; Maarten van Lohuizen for advice and the Bmi-1 antibody; Willy Mark, Joanne Ingenito, and the whole staff of the transgenic facility for injections of multiple constructs. P.P.P. is a Scholar of the Leukemia and Lymphoma Society (LLS). T.M. is supported by a fellowship from LLS. J.A.C. is supported by a FPI fellowship of the Spanish Ministry for Education Culture and Sports. This work was supported by the NIH (CA-71692 awarded to P.P.P.) and MURST (Biotechnology Program and PRIN to B.S.).

Received: July 8, 2002
Revised: August 8, 2002

References

Alkema, M.J., van der Lugt, N.M., Bobeldijk, R.C., Berns, A., and van Lohuizen, M. (1995). Transformation of axial skeleton due to overexpression of bmi-1 in transgenic mice. Nature *374*, 724–727.

Bardwell, V.J., and Treisman, R. (1994). The POZ domain: a conserved protein-protein interaction motif. Genes Dev. *8*, 1664–1677.

Barna, M., Hawe, N., Niswander, L., and Pandolfi, P.P. (2000). Plzf regulates limb and axial skeletal patterning. Nat. Genet. *25*, 166–172.

Beckers, J., Gerard, M., and Duboule, D. (1996). Transgenic analysis of a potential Hoxd-11 limb regulatory element present in tetrapods and fish. Dev. Biol. *180*, 543–553.

Behringer, R.R., Crotty, D.A., Tennyson, V.M., Brinster, R.L., Palmiter, R.D., and Wolgemuth, D.J. (1993). Sequences 5′ of the homeobox of the Hox-1.4 gene direct tissue-specific expression of lacZ during mouse development. Development *117*, 823–833.

Bel-Vialar, S., Core, N., Terranova, R., Goudot, V., Boned, A., and Djabali, M. (2000). Altered retinoic acid sensitivity and temporal expression of Hox genes in polycomb-M33-deficient mice. Dev. Biol. *224*, 238–249.

Chen, Z., Brand, N.J., Chen, A., Chen, S.J., Tong, J.H., Wang, Z.Y., Waxman, S., and Zelent, A. (1993). Fusion between a novel Kruppel-like zinc finger gene and the retinoic acid receptor-alpha locus due to a variant t (11;17) translocation associated with acute promyelocytic leukaemia. EMBO J. *12*, 1161–1167.

Core, N., Bel, S., Gaunt, S.J., Aurrand-Louis, M., Pearce, J., Fisher, A., and Djabali, M. (1997). Altered cellular proliferation and mesoderm patterning in Polycomb-M33-deficient mice. Development *124*, 721–729.

David, G., Alland, L., Hong, S.H., Wong, C.W., DePinho, R.A., and Dejean, A. (1998). Histone deacetylase associated with mSin3A mediates repression by the acute promyelocytic leukemia-associated PLZF protein. Oncogene *16*, 2549–2556.

Dolle, P., Izpisua-Belmonte, J.C., Falkenstein, H., Renucci, A., and Duboule, D. (1989). Coordinate expression of the murine Hox-5 complex homoeobox-containing genes during limb pattern formation. Nature *342*, 767–772.

Dong, S., Zhu, J., Reid, A., Strutt, P., Guidez, F., Zhong, H.J., Wang, Z.Y., Licht, J., Waxman, S., Chomienne, C., et al. (1996). Amino-terminal protein-protein interaction motif (POZ-domain) is responsible for activities of the promyelocytic leukemia zinc finger-retinoic acid receptor-alpha fusion protein. Proc. Natl. Acad. Sci. USA *93*, 3624–3629.

Duboule, D. (1992). The vertebrate limb: a model system to study the Hox/HOM gene network during development and evolution. Bioessays *14*, 375–384.

Gaul, U., and Jackle, H. (1990). Role of gap genes in early Drosophila development. Adv. Genet. *27*, 239–275.

Gaunt, S.J., Krumlauf, R., and Duboule, D. (1989). Mouse homeogenes within a subfamily, Hox-1.4, -2.6 and -5.1, display similar anteroposterior domains of expression in the embryo, but show stage- and tissue-dependent differences in their regulation. Development *107*, 131–141.

Gerard, M., Duboule, D., and Zakany, J. (1993). Structure and activity of regulatory elements involved in the activation of the Hoxd-11 gene during late gastrulation. EMBO J. *12*, 3539–3550.

Gerard, M., Chen, J., Gronemeyer, H., Chambon, P., Duboule, D., and Zakany, J. (1996). In vivo targeted mutagenesis of a regulatory element required for positoning the *HoxD-11* and *HoxD-10* expression boundaries. Genes Dev. *10*, 2326–2334.

Grignani, F., De Matteis, S., Nervi, C., Tomassoni, L., Gelmetti, V., Cioce, M., Fanelli, M., Ruthardt, M., Ferrara, F.F., Zamir, I., et al. (1998). Fusion proteins of the retinoic acid receptor-alpha recruit histone deacetylase in promyelocytic leukaemia. Nature *391*, 815–8.

Hanscombe, O., Whyatt, D., Fraser, P., Yannoutsos, N., Greaves, D., Dillon, N., and Grosveld, F. (1991). Importance of globin gene order for correct developmental expression. Genes Dev. *5*, 1387–1394.

He, L.Z., Guidez, F., Tribioli, C., Peruzzi, D., Ruthardt, M., Zelent, A., and Pandolfi, P.P. (1998). Distinct interactions of PML-RARalpha and PLZF-RARalpha with co-repressors determine differential responses to RA in APL. Nat. Genet. *18*, 126–135.

He, L., Bhaumik, M., Tribioli, C., Rego, E.M., Ivins, S., Zelent, A., and Pandolfi, P.P. (2000). Two critical hits for promyelocytic leukemia. Mol. Cell *6*, 1131–1141.

Helms, J., Thaller, C., and Eichele, G. (1994). Relationship between retinoic acid and sonic hedgehog, two polarizing signals in the chick wing bud. Development *120*, 3267–3274.

Hong, S.H., David, G., Wong, C.W., Dejean, A., and Privalsky, M.L.

(1997). SMRT corepressor interacts with PLZF and with the PML-retinoic acid receptor alpha (RARalpha) and PLZF-RARalpha oncoproteins associated with acute promyelocytic leukemia. Proc. Natl. Acad. Sci. USA *94*, 9028–9033.

Izpisua-Belmonte, J.C., Falkenstein, H., Dolle, P., Renucci, A., and Duboule, D. (1991). Murine genes related to the Drosophila AbdB homeotic genes are sequentially expressed during development of the posterior part of the body. EMBO J. *10*, 2279–2289.

Johnson, R.L., and Tabin, C.J. (1997). Molecular models for vertebrate limb development. Cell *90*, 979–990.

Kennison, J.A. (1993). Transcriptional activation of Drosophila homeotic genes from distant regulatory elements. Trends Genet. *9*, 75–79.

Kondo, T., and Duboule, D. (1999). Breaking colinearity in the mouse HoxD complex. Cell *97*, 407–417.

Kondo, T., Zakany, J., and Duboule, D. (1998). Control of colinearity in AbdB genes of the mouse HoxD complex. Mol. Cell *1*, 289–300.

Lemercier, C., Brocard, M.P., Puvion-Dutilleul, F., Kao, H.Y., Albagli, O., and Khochbin, S. (2002). Class II histone deacetylases are directly recruited by BCL6 transcriptional repressor. J. Biol. Chem. *277*, 22045–22052.

Li, J.Y., English, M.A., Ball, H.J., Yeyati, P.L., Waxman, S., and Licht, J.D. (1997). Sequence-specific DNA binding and transcriptional regulation by the promyelocytic leukemia zinc finger protein. J. Biol. Chem. *272*, 22447–22455.

Lin, R.J., Nagy, L., Inoue, S., Shao, W., Miller, W.H., Jr., and Evans, R.M. (1998). Role of the histone deacetylase complex in acute promyelocytic leukaemia. Nature *391*, 811–814.

Look, A.T. (1997). Oncogenic transcription factors in the human acute leukemias. Science *278*, 1059–1064.

McEwan, I.J. (2000). Gene regulation through chromatin remodeling by members of the nuclear receptor superfamily. Biochem. Soc. Trans. *28*, 369–373.

Minucci, S., Maccarana, M., Cioce, M., De Luca, P., Gelmetti, V., Segalla, S., Di Croce, L., Giavara, S., Matteucci, C., Gobbi, A., et al. (2000). Oligomerization of RAR and AML1 transcription factors as a novel mechanism of oncogenic activation. Mol. Cell *5*, 811–20.

Nettikadan, S., Tokumasu, F., and Takeyasu, K. (1996). Quantitative analysis of the transcription factor AP2 binding to DNA by atomic force microscopy. Biochem. Biophys. Res. Commun. *226*, 645–649.

Paro, R. (1990). Imprinting a determined state into the chromatin of Drosophila. Trends Genet. *6*, 416–421.

Pirrotta, V. (1998). Polycombing the genome: PcG, trxG, and chromatin silencing. Cell *93*, 333–336.

Rego, E.M., He, L.-Z., Warrell, R.P., Jr., Wang, Z.-G., and Pandolfi, P.P. (2000). RA and As_2O_3 treatment in transgenic models of APL unravel the distinct nature of the leukemogenic process induced by the PML-RARα and PLZF-RARα oncoproteins. Proc. Natl. Acad. Sci. USA *97*, 10173–10178.

Rego, E.M., and Pandolfi, P.P. (2001). Analysis of the molecular genetics of Acute Promyelocytic Leukemia in mouse models. Semin. Hematol. *38*, 54–70.

Reid, A., Gould, A., Brand, N., Cook, M., Strutt, P., Li, J., Licht, J.D., Waxman, S., Krumlauf, R., and Zelent, A. (1995). Leukemia translocation gene, PLZF, is expressed with a speckled nuclear pattern in early hematopoietic progenitors. Blood *86*, 4544–4552.

Renucci, A., Zappavigna, V., Zakany, J., Izpisua-Belmonte, J.C., Burki, K., and Duboule, D. (1992). Comparison of mouse and human HOX-4 complexes defines conserved sequences involved in the regulation of Hox-4.4. EMBO J. *11*, 1459–1468.

Rivetti, C., Guthold, M., and Bustamante, C. (1996). Scanning force microscopy of DNA deposited onto mica: equilibration versus kinetic trapping studied by statistical polymer chain analysis. J. Mol. Biol. *264*, 919–932.

Saurin, A.J., Shiels, C., Williamson, J., Satijn, D.P., Otte, A.P., Sheer, D., and Freemont, P.S. (1998). The human polycomb group complex associates with pericentromeric heterochromatin to form a novel nuclear domain. J. Cell Biol. *142*, 887–898.

Simon, J. (1995). Locking in stable states of gene expression: transcriptional control during Drosophila development. Curr. Opin. Cell Biol. *7*, 376–385.

Sitterlin, D., Tiollais, P., and Transy, C. (1997). The RAR alpha-PLZF chimera associated with Acute Promyelocytic Leukemia has retained a sequence-specific DNA-binding domain. Oncogene *14*, 1067–1074.

van der Hoeven, F., Zakany, J., and Duboule, D. (1996). Gene transpositions in the HoxD complex reveal a hierarchy of regulatory controls. Cell *85*, 1025–1035.

van der Lugt, N.M., Domen, J., Linders, K., van Roon, M., Robanus-Maandag, E., te Riele, H., van der Valk, M., Deschamps, J., Sofroniew, M., van Lohuizen, M., et al. (1994). Posterior transformation, neurological abnormalities, and severe hematopoietic defects in mice with a targeted deletion of the bmi-1 proto-oncogene. Genes Dev. *8*, 757–769.

van der Lugt, N.M., Alkema, M., Berns, A., and Deschamps, J. (1996). The Polycomb-group homolog Bmi-1 is a regulator of murine Hox gene expression. Mech. Dev. *58*, 153–164.

Vogel, A., and Tickle, C. (1993). FGF-4 maintains polarizing activity of posterior limb bud cells in vivo and in vitro. Development *119*, 199–206.

Yoshida, M., Kijima, M., Akita, M., and Beppu, T. (1990). Potent and specific inhibition of mammalian histone deacetylase both in vivo and in vitro by trichostatin A. J. Biol. Chem. *265*, 17174–17179.

Zappavigna, V., Renucci, A., Izpisua-Belmonte, J.C., Urier, G., Peschle, C., and Duboule, D. (1991). HOX4 genes encode transcription factors with potential auto- and cross-regulatory capacities. EMBO J. *10*, 4177–4187.

Zhong, S., Delva, L., Cenciarelli, C., Gandini, D., Zhang, H., Fagioli, M., Kalantry, S., and Pandolfi, P.P. (1999). A RA-dependent tumor-growth suppressive transcription complex is the target of the PML-RARα and T18 oncoproteins. Nat. Genet. *23*, 287–295.

Zink, D., and Paro, R. (1995). Drosophila Polycomb-group regulated chromatin inhibits the accessibility of a trans-activator to its target DNA. EMBO J. *14*, 5660–5671.