Identification of Bmi1-interacting proteins as constituents of a multimeric mammalian Polycomb complex

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The *Bmi1* gene has been identified as a mouse Polycomb group (Pc-G) gene implicated in the regulation of *Hox* gene expression. Here we describe the characterization of a Bmi binding protein Mph1, which shares similarity to *Drosophila* polyhomeotic. Coimmunoprecipitation experiments indicate that Bmi1 and Mph1, as well as the Mel18 and M33 proteins described previously, are constituents of a multimeric protein complex in mouse embryos and human cells. A central domain of Bmi1 interacts with the carboxyl terminus of Mph1, whereas a conserved α -helical domain in the Mph1 protein is required for its homodimerization. Transgenic mice overexpressing various mutant Bmi1 proteins demonstrate that the central domain of Bmi1 is required for the induction of anterior transformations of the axial skeleton. Bmi1, M33, and Mph1 show an overlapping speckled distribution in interphase nuclei. These data provide molecular evidence for the existence of a mammalian Polycomb complex.

[Key Words: Bmi1; polyhomeotic; Mph1/Rae-28; Polycomb; RING finger; subnuclear localization]

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The vertebrate *Hox* genes, as well as their *Drosophila* counterparts, play a key role in the determination of the body plan, as judged from the phenotype of *Hox* loss- and gain-of-function mutant mice (for review, see Krumlauf 1994). Compared to the fly, relatively little is known about the regulation of *Hox* gene expression in vertebrates. In *Drosophila*, the expression pattern of the homeotic genes of the Antennapedia and Bithorax complexes are initiated early in embryonic development by the gap and pair-rule genes (for review, see Bienz and Müller 1995). These genes are expressed transiently, whereas continuous homeotic gene activity is essential for implementing stable determination of cell fates. Additional factors have been identified that are important for the maintenance of homeotic gene expression patterns throughout subsequent *Drosophila* development. These maintenance factors fall into two classes: the Polycomb group (Pc-G), which maintains the repressed state in those cells where the homeotic gene was originally inactive [for review, see Paro (1993) and Pirotta (1995)]; and the Trithorax group (trx-G), which sustains the active state in cells where the homeotic gene was originally expressed (for review, see Kennison 1993). At later

stages of development Pc-G mutants start to express genes of the homeotic clusters outside their normal domains, which results in the transformation of larval segments toward the identity of more posterior ones. Genetic analysis indicates that the Pc-G consists of ~20 members, 7 of which, namely Polycomb (Pc), polyhomeotic (ph), Posterior sex combs (Psc), Enhancer of zeste [E(z)], Polycomb-like (Pcl), extra sex combs (esc), and Sex comb on midleg (Scm), have been characterized at the molecular level [Pirotta (1995) and references therein; Bornemann et al. (1996)]. Genetic and molecular evidence indicates that Pc-G gene products act in large protein complexes. Heterozygous combinations of different Pc-G mutations have a much more severe phenotype (Jürgens 1985) and haplo-insufficient phenotype of some mutants can be rescued by increasing the copy number of another member (Cheng et al. 1994). The Pc protein is localized in large immunologically visible dots in the nucleus (Messmer et al. 1992; Franke et al. 1995) and was found associated in a multimeric complex with at least one other member of the Pc-G, the ph protein (Franke et al. 1992). Furthermore, the ~100 polytene binding sites for the Pc, ph, and Pcl completely coincide and largely overlap with those for Psc (DeCamillis et al. 1992; Franke et al. 1992; Rastelli et al. 1993; Lonie et al. 1994). Pc-G proteins bind to specific regulatory regions,

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so-called Pc-G response elements (PREs), which are necessary for maintaining the repressed state of homeotic genes (Müller and Bienz 1991; Busturia and Bienz 1993; Simon et al. 1993; Chan et al. 1994; Chiang et al. 1995). Little is known, however, about how Pc-G proteins interact molecularly or how they acquire PRE binding site specificity.

There is accumulating evidence that vertebrates use similar mechanisms to maintain *Hox* gene expression boundaries throughout development. Mammalian homologs have been identified for Trx-G and Pc-G members. The murine M33 protein, which shares distinct regions of homology with the *Drosophila* Pc protein (Pearce et al. 1992), is able to rescue in part the Pc mutant phenotype in transgenic flies suggesting that Pc-G gene function has been conserved in evolution (Müller et al. 1995). So far, the most striking example of a functional conservation of a murine Pc homolog has been provided by the *Bmi1* proto-oncogene (Haupt et al. 1991; van Lohuizen et al. 1991b) and the closely related *Mel18* gene (Tagawa et al. 1990). Both gene products share sequence similarity to the *Drosophila* Psc protein and its relative Su(z)2 (Brunk et al. 1991; van Lohuizen et al. 1991a). By analogy to Pc-G loss-of-function mutants, *Bmi1* and *Mel18* null mutant mice display posterior transformation of the axial skeleton which correlates with an anterior shift of several *Hox* gene expression boundaries (van der Lugt et al. 1994, 1996; Akasaka et al. 1996). Moreover, transgenic mice that overexpress the *Bmi1* gene show anterior transformation of the axial skeleton in a *Bmi1* dosage-dependent manner accompanied with a caudal shift of the anterior expression boundary of at least the *Hoxc-5* and *Hoxc-8* genes (Alkema et al. 1995; van der Lugt et al. 1996). The bidirectional transformations of the axial skeleton of mice haplodeficient for the *Mll* gene, which shares similarity to the *Drosophila* *trx* gene, indicates that TrxG function is conserved in evolution as well (Yu et al. 1995).

To gain further insight into the mechanisms of vertebrate homeotic gene silencing we have identified Bmi1 binding proteins. In this report we describe the identification of the *Mph1* gene, the product of which shares sequence homology with the *Drosophila* ph protein. We provide evidence that the Mph-1 protein forms homodimers and can directly interact with the Bmi1 and Mel18 proteins. Coimmunoprecipitation experiments strongly suggest that Bmi1, Mel18, Mph1, and M33 are constituents of a multimeric protein complex. We have mapped the Bmi1–Mph1 interaction domains and correlate this with the phenotype of transgenic mice that overexpress mutant forms of the *Bmi1* gene. Confocal microscopy analysis demonstrated the colocalization of Bmi1, Mph1 and M33 in multiple subnuclear speckles in interphase nuclei. Our results provide molecular evidence for the existence of a mammalian Pc complex.

Results

Identification of Bmi1-interacting proteins

We used the Bmi1 protein as bait in a yeast two-hybrid screen with a 14.5-day mouse embryo cDNA library

(Chien et al. 1991; Chevray and Nathans 1992). DNA sequence analysis showed that four cDNA library plasmids, recovered from independent positive colonies, all contained the same 2009-bp insert encoding a 600-amino-acid protein. An independent screen of the same two-hybrid library using Mel18 as bait resulted in three positive clones, two of which were identical to the cDNA recovered from the Bmi1 two-hybrid screen. The third cDNA corresponded to the same gene but contained a shorter 1081-bp insert.

Mph1 is structurally related to the Drosophila ph protein

Several overlapping cDNA clones from a 16.5-day mouse embryo cDNA library combined with 5' RACE products gave a full-length cDNA clone that contained a single long open reading frame (ORF) encoding a 1012-amino-acid protein. The predicted protein sequence showed significant homology to the *Drosophila* Pc-G family member, *polyhomeotic* (Deatrick et al. 1991; DeCamillis et al. 1992) (Fig. 1). We therefore named the gene *Mph1* (mouse ph). Apart from the length of the 3' untranslated region the *Mph1* cDNA is virtually identical to the *Rae-28* cDNA that was recently identified as a retinoic acid (RA)-inducible cDNA from F9 embryonal carcinoma cells (Nomura et al. 1994). Some RACE-PCR products were derived from a *Mph1* cDNA having a 21- or 156-bp deletion that would lead to a Mph1 protein lacking amino acid residues 153–159 or 153–204, respectively. The latter deletion was also reported for some of the *Rae-28* cDNA clones (Nomura et al. 1994) and may arise from alternative splicing. We refer to this gene as *Mph1/Rae-28*. Although the overall identity of the Mph1/Rae-28 protein to the ph protein was only 30.7%, the Mph1/Rae-28 and ph protein shared significant similarity at the carboxy-terminal region. A 31-amino-acid homology domain, present in the carboxy-terminal half of the proteins, shows 65% identity. Another homology domain, comprising a 66-amino-acid stretch at the extreme carboxyl terminus, showed 62% identity (Fig. 1B). This domain shares weak similarity to a 62- to 67-residue domain present in a diverse set of other proteins, including members of the Ets family of transcription factors (Wasylyk et al. 1993) and a set of cytoplasmic proteins, four of which are involved in sexual differentiation in yeast (Ponting 1995) (Fig. 2). Secondary structure predictions and threading algorithms suggested that the domain has a largely α -helical structure comprised of closely packed helices that are connected with short loops. We refer to this domain as the SEP domain (yeast sterility-, Ets related-, PcG proteins). Although the overall homology between the different domains was rather low, the conserved spacing of hydrophobic residues and the conserved α -helical-predicted structure suggests a common function. The SEP domain of the ph proteins is most closely related to the carboxy-terminal region of two other recently identified *Drosophila* gene products. The first, lethal[3]malignant brain tumor [(3)mbt] is the product of a *Drosophila* tumor suppressor gene (Wislar

Figure 3. Expression pattern of *Mph1/Rae-28*. (A) Expression of *Mph1/Rae-28*, *Bmi1*, and *Mel18* during embryogenesis. Total RNA (10 μ g) from total embryos, embryonic head, or body spanning the last 10 days of embryonic development were separated on agarose gels, transferred to nitrocellulose, and subsequently probed with a *Bmi1*, *Mel18*, and *Mph1/Rae-28* cDNA probe. (B) Expression pattern of *Mph1/Rae-28* in adult tissues. Northern blots containing 5 μ g of Poly(A)⁺ RNA were hybridized with a *Mph1/Rae-28* probe. Two *Mph1/Rae-28* transcripts of ~3.5 and 4 kb were clearly detected in testis after overnight exposure; transcripts in the other tissues were visible after 4 days of exposure. The Northern blots were hybridized with an actin probe to check for the amount of loaded RNA. The 28S and 18S ribosomal bands are indicated as size markers.

highly expressed in testis, whereas low-expression levels were found in all other tissues analyzed (Fig. 3B). Using an interspecific mouse backcross panel the *Mph1/Rae-28* gene was mapped to mouse chromosome 6.3 cM distal to the *Raf1* proto-oncogene (N.G. Copeland, pers. comm.).

Bmi1 and *Mel18* bind to the *Mph1/Rae-28* protein in vivo

To obtain evidence for the association of the *Bmi1* and *Mel18* proteins with the *Mph1/Rae-28* protein in vivo, antisera were raised against purified glutathione *S*-transferase (GST)-*Bmi1*, GST-*Mph1/Rae-28*, and Malc-*Mel18* fusion proteins. We could specifically immunoprecipitate the in vitro-transcribed and -translated *Bmi1*, *Mel18*, and *Mph1/Rae-28* with their cognate antisera. Because the *Mph1/Rae-28* gene is highly expressed in 12.5- to 13.5-day embryos, total embryo protein extracts of these developmental stages were used in coimmunoprecipitation experiments. The two polyclonal antisera

raised against nonoverlapping parts of the *Mph1/Rae-28* protein detected a 120- and 130-kD protein doublet on Western blots containing total embryo extracts (Fig. 4A). The observed mobilities of these proteins are larger than the predicted 105 kD. This could be attributable to an aberrant mobility on SDS-PAGE as has been observed for other proteins including *Bmi1* (van Lohuizen et al. 1991b). Alternatively, the doublet could represent different post-translationally modified forms of the *Mph1/Rae-28* protein or reflect the different spliced isoforms of the *Mph1/Rae-28* gene. Immunoprecipitation with the *Mph*-MN serum followed by probing the Western blot with the *Mph*-SM serum revealed two bands characteristic of the *Mph1/Rae-28* protein (Fig. 4A). The same two bands were also seen when protein extracts are precipitated with polyclonal *Bmi1* or *Mel18* sera (Fig. 4B). The *Mph1/Rae-28* protein was not coprecipitated when the immunoprecipitation was performed with normal rabbit serum, a polyclonal antiserum against the unrelated *Pim1* or *E2F5* proteins. In addition, when *Bmi1* polyclonal serum was used for immunoprecipitations in *Bmi1*^{-/-} embryo protein extracts the *Mph1/Rae-28*-specific bands were not seen (Fig. 4B) demonstrating that the coprecipitation is not attributable to the formation of aspecific protein aggregates in the embryo extracts.

The polyclonal and monoclonal antibodies against *Bmi1* detected three closely migrating proteins of 45–48 kD (van Lohuizen et al. 1991b) in total embryo protein extracts (Fig. 4C). The 45- to 48-kD bands were specific for the *Bmi1* protein, as they are not visible in a protein extract from a *Bmi1*^{-/-} embryo. The different *Bmi1* protein species were also observed when in vitro-translated ³⁵S-labeled *Bmi1* protein is made (van Lohuizen et al. 1991b). The two highest bands most likely represent phosphorylated isoforms of the *Bmi1* protein, as these isoforms were reduced to the single fastest migrating isoform when in vitro-translated *Bmi1* was treated with calf intestine phosphatase prior to SDS-PAGE (data not shown). The strong band around 55 kD is the result of binding of the second antibody to the mouse immunoglobulin heavy chains present in a 13.5-day embryo protein extract. To further substantiate the specificity of the interaction of *Bmi1* with *Mph1/Rae-28* we performed the reciprocal experiment. As shown in Figure 4C the *Bmi1* protein was coimmunoprecipitated using the polyclonal *Mph*-SM antibodies.

M33, *Mph1/Rae-28*, and *Bmi1* are present in a multiprotein complex

We determined whether the murine Pc homolog *M33* interacts with the *Bmi1* and *Mph1/Rae-28* proteins in embryo extracts. As is shown in Figure 5A, a polyclonal antiserum raised against *M33* specifically coimmunoprecipitates both *Mph1/Rae-28* and *Bmi1* from day-12.5 mouse embryo protein extracts. Similarly, we could coimmunoprecipitate human *Bmi1* from human U-2 osteosarcoma (U2-OS) cell extracts (Fig. 5B). The affinity-purified *M33* antiserum recognizes a 74-kD protein on Western blots with total extracts from human U2-OS

Figure 4. In vivo interaction of Mph1/Rae-28 and Bmi1. Immunoprecipitations of embryonic protein extracts with the indicated antibodies were performed under moderate salt conditions, separated on a 9% SDS-polyacrylamide gels, blotted to nitrocellulose filters, probed with different antibodies, and detected by ECL. The input lane contains 40 μ g of total 12.5-day embryo extract. The immunoprecipitations of 250 μ l of embryo extract were performed with the indicated antibodies. The positions of the molecular mass markers (in kD) are indicated. (A) (Left) Western blot probed with the Mph-SM antiserum. Two bands were recognized by the Mph1 antiserum (indicated with arrowheads). Mph1/Rae-28 is specifically precipitated with the Mph1, Bmi1, and Mel18 but not with the Pim1 or normal rabbit serum (NRS). The heavy smear around 55 kD is the result of cross-reactivity with the heavy chains of the primary antibodies. (Right) The two Mph1/Rae-28 bands were also detected on a Western blot probed with Mph-MN serum, raised against a different part of the Mph1/Rae-28 protein. (B) Control experiment (long exposure) showing that Mph1/Rae-28 was not coprecipitated with the Bmi1 polyclonal antiserum in *Bmi1* nullizygous embryo extracts (-/-). Mph1/Rae-28 was also coprecipitated with the Bmi1-F6 monoclonal antibody. (C) Western blot probed with Bmi1-F6 monoclonal antibody. The three Bmi1-specific bands around 45 kD (indicated with arrowheads) were detected in *Bmi1* heterozygous (+/-) embryo protein extracts but are absent in *Bmi1* nullizygous embryo extracts (-/-). Bmi1 is coprecipitated with the Mph1/Rae-28 serum but not with the NRS. The heavy band around 55 kD is the result of cross-reactivity with the heavy chains of the antibodies present in 12.5-day protein extracts.

cells, and the specificity of the antiserum was further demonstrated by its ability to recognize and immunoprecipitate specifically an HA-epitope-tagged M33 expressed in transfected COS cells (data not shown).

In a reciprocal experiment, Bmi1-F6 antiserum could not coprecipitate M33 from protein extracts (data not shown). It is possible that the Bmi1-F6 antibody interferes with M33 binding or, alternatively, that the interaction between the two proteins is indirect, leading more easily to disruption of the complex during the immunoprecipitations. We favor the latter possibility as we have been unable to demonstrate a direct interaction between the two proteins in a yeast two-hybrid experiment and in COS cell cotransfection experiments (data not shown). Our results demonstrate that M33 is present with Bmi1 and Mph1/Rae-28 in a multiprotein complex and suggest that the interaction between Bmi1 and M33 is indirect.

Bmi1, Mph1/Rae-28, and M33 colocalize to subnuclear domains in interphase nuclei

Further support for the in vivo interaction of Bmi1, Mph1/Rae-28, and M33 came from the intracellular lo-

calization of the endogenous Bmi1 and Mph1/Rae-28 proteins in the U2-OS cell line, by indirect immunofluorescence. The Bmi1, Mph1/Rae-28, and M33 antisera specifically detected their closely related human homologs (Alkema et al. 1993; A. Otte, pers. comm.), which are expressed at high levels in U2 OS cells (Fig. 5B; data not shown). Specific staining was found in the nucleus with no staining in the cytoplasm (Fig. 6A–G). All three proteins were excluded from the nucleolus and show a speckled distribution throughout the nucleoplasm. A similar Bmi1 staining pattern was observed in the human erythroid leukemia cell line, K562. Although a speckled Bmi1 distribution was observed in the nuclei of other cell lines, the size and distribution varied and may correlate with expression levels. For instance, overexpression of Bmi1 in NIH-3T3 cells, which have a very low endogenous Bmi1 expression level, led to an increase in size of nuclear speckles (data not shown). Immunofluorescence with the Bmi1 antibody gave weaker staining in mitotic cells (Fig. 6A). During anaphase no Bmi1 staining was observed, whereas the Bmi1 signal reappeared upon chromosome decondensation in late telophase indicating that the large Bmi1-containing complexes reassemble after mitosis. However, the absence of Bmi1 staining could reflect the inaccessibility of Bmi1 for the antibody because of the compact nature of the chromatin at this stage (Messmer et al. 1992). Double-labeling experiments showed that the staining patterns for the Mph1/Rae-28, Bmi1, and M33 proteins completely overlap in interphase nuclei, supporting the in-

Figure 5. In vivo interaction of M33 with Mph1/Rae-28 and Bmi1. Immunoprecipitations of embryonic protein extracts or human U2-OS cell extracts with the indicated antibodies were performed as described in Fig. 4. The positions of the molecular mass markers (in kD) are indicated. (A) The Bmi1 and Mph1/Rae-28 proteins are coprecipitated with the M33 protein. The Bmi1- and Mph1/Rae-28-specific bands are indicated with arrowheads. Immunoprecipitations with the Mph-SM and Bmi1-38 antisera were taken along as positive controls. (B) Bmi1 is coprecipitated with M33 from human U2-OS cell extracts. The Bmi1- and Mph1/Rae-28-specific bands were absent when precipitations were performed with NRS as a negative control.

teraction between these proteins in a multiprotein complex (Fig. 6C,F).

The subnuclear colocalization of the Mph1/Rae-28 and Bmi1 proteins is reminiscent of the speckled distribution of the PML oncoprotein, which is involved in acute promyelocytic leukemia (Dyck et al. 1994; Weis et al. 1994). Like Bmi1 and Mel18, the PML protein contains a RING finger domain that is important for its localization to specific nuclear domains, so-called PML oncogenic domains (PODs). To determine whether Bmi1, Mph1/Rae-28, and PML are constituents of the same subnuclear structure we performed a double-labeling experiment with the PML monoclonal 5E10 (Fig. 6H) and the Mph1/Rae-28 antiserum. Although the PML antibodies recognize similar subnuclear structures they did not colocalize with the ones that contain the Bmi1 and Mph1/Rae-28 protein (Fig. 6I). Other well-characterized subnuclear domains contain coilin and factors involved in pre-mRNA splicing (Fig. 6K) (Fu and Maniatis 1990). As is demonstrated in Figure 6L, Mph1/Rae-28 also does not colocalize with splicing factors.

Localization of Bmi1 to subnuclear domains depends on an intact RING finger

Because of the demonstrated importance of the RING

finger domain for localization of the PML protein to POD domains (Kastner et al. 1992; Borden et al. 1995), we asked whether mutations in the Bmi1 RING finger would affect its subnuclear distribution. Hereto we transiently transfected HA-epitope-tagged Bmi1 constructs in which two central cysteines of the RING finger domain were mutated to phenylalanine (Bmi1 Δ Zn), or HA-epitope wild-type Bmi1 as a control into U2-OS cells. Whereas wild type HA-Bmi1 colocalizes with Mph1/Rae-28 in the subnuclear domains (Fig. 7D,E), HA-Bmi1 Δ Zn is diffusely expressed throughout the nucleoplasm in most cells, with some cells displaying enhanced staining of the mutant protein in large subnuclear regions (Fig. 7A,B). These results indicate that as reported for PML, the RING finger is important for subnuclear localization of Bmi1, and demonstrate that overexpression of mutant or wild-type Bmi1 proteins does not detectably alter the subnuclear distribution of endogenous Mph1/Rae-28.

A central domain of Bmi1 interacts with the carboxyl terminus of Mph1/Rae-28

We identified the Bmi1-Mph1/Rae-28 interaction domains by the yeast two-hybrid system. As shown in Figure 8, the Gal4-DNA-binding domain (DBD)-Bmi1 protein containing amino acid 59–227 was the smallest fusion protein that is still able to interact with the Mph1/Rae-28 protein. This portion of the Bmi1 protein harbors a putative helix–turn–helix–turn–helix–turn (HTHTHT) motif that is conserved in the *Drosophila* Psc protein. Removal of either the carboxy-terminal proline/serine-rich region (amino acid 228–324) or the amino-terminal end of the Bmi1 protein that harbors the RING finger domain did not affect the interaction with the Mph1/Rae-28 protein. The shortest Mph1/Rae-28 clone recovered from the Bmi1/Mel18 two-hybrid screen encoded the carboxy-terminal end of the Mph1/Rae-28 protein starting at amino residue 720. This portion of the Mph1/Rae-28 protein contained the first homology domain, the zinc finger motif, as well as the SEP domain. Deletion of the first Mph1/Rae-28 homology domain no longer gave rise to β -galactosidase-expressing yeast colonies, suggesting that this domain is essential for the interaction with Bmi1. Also deletion of the extreme carboxy-terminal 10 amino acids, which are part of the conserved SEP domain, abrogated Bmi1 binding. These results indicate that the conserved carboxyl terminus of the Mph1/Rae-28 protein is sufficient for the interaction with Bmi1 and Mel18.

Mph1/Rae-28 forms homodimers

The two-hybrid embryo cDNA library was also screened with the carboxy-terminal portion of Mph1/Rae-28 (amino acid 413–1012) as bait. Analysis of 10 positive colonies revealed that 6 contained Mph1/Rae-28 cDNA inserts. Four of these clones were identified previously in the two-hybrid screen using Bmi1 and Mel18 as bait. The

Figure 6. Colocalization of endogenous Bmi1, Mph1/Rae-28, and M33 proteins in U2-OS cells in distinct subnuclear domains. (A) Subnuclear distribution of Bmi1 at different cell cycle stages. Indirect immunofluorescence (see Material and Methods) was detected by confocal laser scanning microscopy. The Bmi1 mAb F6 signal appears in yellow, and nuclear DNA is stained with propidium iodide (red). Bmi1 staining was absent in mitotic cells (cell in right upper corner) and reappeared in late telophase (two middle cells at the bottom). (B–D) Double-labeling experiment using the Bmi1 mAb F6 (B, green) and purified polyclonal Mph-SM serum (D, red). The merge of both pictures (C) shows that the Bmi1 and Mph1/Rae-28 are distributed in a completely overlapping pattern in interphase nuclei. (E–G) Double labelling with Bmi1 mAb F6 (E, green) and affinity-purified rabbit anti-M33 (G, red). The merge in F shows complete colocalization of Bmi1 and M33. (H–J) The distribution of the Bmi1 and Mph1/Rae-28 protein was compared with that of the PML protein. A double-labeling experiment is shown using the mAb 5E10 that recognizes the PML protein (H, green) and the rabbit anti-Mph-SM serum (J, red). Although a similar dotted distribution was observed for the PML protein, the merge (I) shows that the staining patterns do not overlap. The two yellow dots in I do not represent real colocalization of PML and Mph, because the confocal images depicted in H–J represent a summation of signals in different focal planes. (K–M) Mph1/Rae-28 does not colocalize with splicing factors. A double-labeling experiment using monoclonal antiserum SC35, recognizing splicing factor (Fu and Maniatis 1990) (K, green) with rabbit anti-Mph-SM (M, red) reveals no colocalization (merge, L).

TAD-Bmi1 fusion protein gave no blue staining yeast colonies.

The central domain of Bmi1 is required for anterior skeletal transformation in transgenic mice

Previously, we have shown that Eμ-Bmi1 transgenic mice that overexpress the Bmi1 protein by virtue of the immunoglobulin heavy chain enhancer (Eμ), *Pim1* promoter and Moloney murine leukemia virus long terminal repeat (LTR) display anterior transformation of the axial skeleton (Alkema et al. 1995). Using the same promoter and enhancer module we generated several independent transgenic mouse lines that highly expressed different mutant forms of the *Bmi1* gene during embryogenesis (Fig. 9; M. Alkema, H. Jacobs, M. van Lohuizen, and A. Berns, in prep.). The Eμ-BmiΔP and Eμ-BmiΔH lines overexpressed carboxy-terminal truncated forms of the Bmi1 protein lacking the last 87 and 190 amino acids, respectively. The Eμ-BmiΔZn transgenic lines overexpressed a Bmi1 mutant protein, in which two highly conserved cysteine residues of the RING finger region are changed to phenylalanine residues. All lines were analyzed for skeletal transformation and compared to the previously reported Eμ-Bmi1 transgenic mice. The Eμ-BmiΔH transgenic mice did not show skeletal abnormalities. However, both the Eμ-BmiΔP and Eμ-BmiΔZn transgenic mice showed transformation of cervical (C), thoracic (T), lumbar (L), and sacral (S) vertebrae (Table 1). Anterior transformation of the Eμ-BmiΔP and the Eμ-BmiΔZn-21 transgenic mice include thickening of the

two remaining clones appeared to be identical and contained an insert that encoded the carboxy-terminal part of the Mph1/Rae-28 protein starting at amino acid 847. This part of the protein starts carboxy-terminal of the zinc finger motif and still contains the SEP domain, which is conserved between the fly and mouse ph proteins. Deletion of the last 10 amino acids of this domain prevented Mph1/Rae-28 dimerization in a two-hybrid assay (Fig. 8A). These results demonstrate that this SEP-containing carboxy-terminal domain of the Mph1/Rae-28 protein is essential and most likely sufficient for dimerization. *Bmi1* or *Mel18* cDNA-containing plasmids were not found in this screen. This could be a result of an unfavorable conformation of the Gal4TAD-Bmi1/Mel18 fusion protein, as direct testing of a two-hybrid interaction of a Gal4-DBD-Mph1/Rae-28 with Gal4-

Figure 7. Localization of Bmi1 to subnuclear domains depends on an intact RING finger. HA-epitope-tagged Bmi1 (D–F) or HA-Bmi1ΔZn RING finger mutant (A–C) were transiently transfected into U2-OS cells. HA-tagged proteins were detected 3–4 days after transfection, with indirect immunofluorescence using monoclonal antiserum 12CA5, and compared to endogenous Mph1/Rae-28 (C,F). Wild-type HA-Bmi1 (D, green) colocalizes with Mph1/Rae-28 (F, red), as indicated by the merge (E). The same frame contains both an untransfected cell (E, top, red) as a transfected cell (E, bottom, yellow), indicating the specificity of the 12CA5 antiserum. In contrast, the RING finger mutant HA-Bmi1ΔZn displays a diffuse nuclear staining (A, top, two transfected cells); merge in B.

neural arch of C2, transformation of T1 toward the identity of C7 (Fig. 10B,C), transformations of T8 toward the identity of T7, L1 to T13 and S2 to S1 (Fig. 10F,G; Table 1). In the Eμ-BmiΔZn-31 transgenic mice the anterior transformations were restricted to T8 and L6 (Table 1), which could be attributable to a lower expression level of the transgene in these mice (M. Alkema, H. Jacobs, M. van Lohuizen, and A. Berns, in prep.). In contrast to the Eμ-Bmi1 transgenic mice, the ribs often were attached asymmetrically to the sternum in the of Eμ-BmiΔP and the Eμ-BmiΔZn transgenic mice (Fig. 10F) or a rib-pair was attached to one side of the sternum but not to the other (Fig. 10G). In general, the shift in segment identity was similar to that observed in the Eμ-Bmi1 transgenic mice, although the penetrance of the anterior transformation in the Eμ-BmiΔP and the Eμ-BmiΔZn transgenic mice is lower. This could be explained by reduced activity of the mutant Bmi1 protein or by a lower expression level of the mutant Bmi1 transgene, as the penetrance of the anterior transformations was shown to be Bmi1 dose dependent. We favor the latter explanation, as in homozygous Eμ-BmiΔZn-21 transgenic mice the penetrance of the anterior transformations was more pronounced than in the heterozygous transgenic littermates and was similar to those observed in Eμ-Bmi1 homozygous transgenic mice (Table 1; Fig. 10D,H). Our data strongly suggest that overexpression of the central part of the Bmi1 protein, including the HTHHT motif, is sufficient for the respecification of vertebrae identity. These data correlate with the domain of the Bmi1 protein, which was

found to be important for the interaction with the Mph1/Rae-28 protein.

Discussion

Mph1/Rae-28 is a murine homolog of Drosophila ph

We identified Mph1/Rae-28, a murine homolog of the *Drosophila* Pc-G member ph, in a two-hybrid screen using Bmi1 and Mel18 as bait. The Mph/Rae-28 gene was also identified as a RA-inducible cDNA in a mouse embryonal carcinoma cell line (Nomura et al. 1994). Mph1/Rae-28 shows high similarity to ph in the carboxy-terminal portion of the protein containing a single putative zinc finger and a conserved SEP domain. Other structural similarities between Mph1/Rae-28 and ph include the overall size of the protein and the presence of glutamine-rich regions. Glutamine stretches are also found in other transcriptional regulators, including homeotic proteins. The glutamine-rich region in the transcription factor Sp1 has been shown to be important for transcriptional activation via interaction with TAF110 (Emami et al. 1995), which is one of the factors associated with the TATA-binding protein (TBP). The glutamine-rich regions in some Pc-G proteins, such as Mph1/Rae-28, might provide a link to the basal transcription apparatus. Interestingly, Bmi1 expression is induced in the mouse embryo carcinoma cell line P19 upon differentiation by RA (M. van Lohuizen, unpubl.).

Bmi1, Mel18, Mph1/Rae-28, and M33 are constituents of a mammalian Pc complex

Immunoprecipitations of total embryo extracts with Bmi1, Mel18, Mph1/Rae-28, or M33 antibodies showed that Bmi1 and Mel18 interact with Mph1/Rae-28 in vivo and demonstrate that M33 is also present in the precipitated protein complex. The colocalization of the Bmi1, Mph1/Rae-28, and M33 proteins in distinct subnuclear domains in interphase nuclei of U2-OS cells further supports their in vivo interaction. These interactions strongly suggest that the mammalian Pc-G members are constituents of a multimeric protein complex. Other nuclear proteins, associated with distinct functions such as pre-mRNA splicing, RNA transcription (Xing et al. 1993), DNA replication (Newport and Yan 1996), and telomere-associated gene repression (Palladino and Gasser 1994), localize to specific subnuclear domains. We have shown that Bmi1, Mph1/Rae-28, and M33 do not colocalize with splicing factors. Other less well-characterized nuclear substructures, which are more reminiscent of the Bmi1, Mph1/Rae-28, and M33 distribution, have been described using a variety of human autoimmune antisera (Saunders et al. 1991) and monoclonal antibodies. Some of the autoimmune sera detect proteins in interphase nuclei that colocalize with the RING finger protein PML. Although the size and distribution of the PML-containing subnuclear structures is similar to the ones containing Bmi1 and Mph1/Rae-28, they do not colocalize. The *Drosophila* Pc and ph protein show a

Figure 8. Bmi1-Mph1/Rae-28 and Mph1/Rae-28-Mph1/Rae-28 interaction domains. (A) Schematic representation of Gal4-DBD-Bmi1 and Gal4-DBD-Mph1/Rae-28 fusion proteins used to determine the binding domains. The Gal4 DNA-binding domain (amino acids 1–147) and the Gal4 *trans*-activation domain (amino acids 768–881)-encoding part were fused in-frame to the amino terminus of a part of the Bmi1 or Mph1/Rae-28 protein. The characteristic protein motifs of the Bmi1 and Mel18 protein are represented schematically: The shaded box denoted Rnf represents the RING finger domain; the dark-shaded region represents the putative HTHHT region; P/S represents the proline/serine rich region at the carboxyl terminus of the Bmi1 and Mel18 protein; the small dark-shaded region indicates the nuclear localization signals of the Bmi1 and Mel18 protein. The schematically represented protein motifs of the Mph1/Rae-28 protein are described in Fig. 3. The amino- and carboxy-terminal amino acids of the protein parts are indicated. The yeast strain Y190 was transformed with the fusion protein-encoding plasmids, and at least 20 colonies were scored for β -galactosidase activity using a colony lift assay. Specificity of the interaction for combinations of fusion proteins that gave blue staining yeast colonies were confirmed with control Gal4TAD fusion proteins. (B) Summary of the data presented in A. The carboxy-terminal 166 amino acids of the Mph1/Rae-28 protein, including the SEP domain, are sufficient for Mph1/Rae-28 dimerization. Amino acids 720–1012 of the Mph1/Rae-28 protein, including the first homology domain, conserved zinc finger, and the SEP domain, are sufficient for Bmi1 binding. An internal part of the Bmi1 protein, including the HTHHT motif, is essential for Mph1/Rae-28 binding.

similar speckled distribution in embryonic and tissue culture cells (Messmer et al. 1992; Franke et al. 1995). Interestingly, mutations in the Pc chromodomain affect the subnuclear distribution of the ph protein indicating that the localization of ph depends on Pc function (Franke et al. 1995). Whereas it is tempting to speculate that the speckles seen with Bmi1, Mph1/Rae-28, and M33 antisera represent clustered mammalian Pc complexes, it is not yet clear whether the subnuclear domains are functionally important for Pc-G silencing, as a Bmi1 protein with mutated RING fingers is no longer specifically localized to these domains but still is able to induce anterior transformations of the axial skeleton when overproduced in transgenic mice. The transient overproduction of wild-type or RING finger-mutant Bmi1 in U2-OS cells does not lead to major changes in

the distribution of endogenous Bmi1 or Mph1/Rae-28 proteins, although subtle changes might well have escaped detection at this level of resolution. The lack of drastic changes to the Pc-G protein complex when Bmi1 is overproduced or deleted are not unexpected, given the subtle posterior or anterior transformations in Bmi1 knockout or transgenic mice, respectively. In this respect, it will be important in future experiments to analyze the effects on the Pc-G multiprotein complex in Bmi1/Mel18 double knockout mice, or EED mutant mice, which show much more severe morphological transformations (Schumacher et al. 1996).

Functional domains of Mph1/Rae-28 and Bmi1

The carboxy-terminal portion of the Mph1/Rae-28 pro-

Figure 9. The Bmi1 and mutant Bmi proteins of the different transgenic lines are represented schematically. The amino-terminal RING finger domain (Rnf), the putative HTH domain, and the carboxy-terminal proline/serine rich region (Pro/Ser) are shaded. The small dark-shaded regions indicate the nuclear localization signals of Bmi1.

tein with the highest sequence similarity to the ph protein is sufficient for the interaction with the Bmi1 and Mel18 protein. This part of the protein contains a putative zinc finger and the conserved SEP sequence. Homodimerization of some of the members of the Pc-G might be an important feature in building multimeric protein complexes. Our data provide the first evidence for a functional role of the conserved SEP domain in homodimerization. The presence of a similar sequence in the Pc-G member Scm (Bornemann et al. 1996) suggests that it may also play a role in heterodimerization of ph and Scm. The SEP domain is important for Scm function in flies, as in the *Scm^{XF24}* null mutant the carboxy-terminal 49 amino acids of the SEP domain are deleted

(Bornemann et al. 1996). The HLH domain of the c-Myc, MyoD, and E2 class of transcription factors have been shown to be important for homo- and heterodimerization among family members (Murre et al. 1994). Although molecular evidence is currently lacking, a similar role in protein-protein interaction has been suggested for the SEP domain in Ets family members (Wasylyk et al. 1993) and yeast sexual differentiation proteins (Ponting 1995). Mutations in the SEP domain also abolish Bmi1 binding, suggesting that Mph1/Rae-28 dimerization is required for Bmi1 binding.

Comparison of the Bmi1, Mel18, Psc, Su(z)2 (Brunk et al. 1991; van Lohuizen et al. 1991a), and the recently identified *Drosophila* lethal[3]73Ah protein (Irminger

Table 1. Abnormalities of the axial skeleton in *Ep-Bmi* mutant transgenic mice

		wt (n = 40)	<i>Ep-Bmi</i> (n = 19/3)*	<i>Ep-BmiΔP</i> (n = 25)	<i>Ep-BmiΔZN-21</i> (n = 15/3)*	<i>Ep-BmiΔZN-31</i> (n = 10)	<i>Ep-BmiΔH</i> (n = 9)
C2	broadened neural arch	0	19/3*	3	7	0	0
	Ectopical aaa	0	0	0	0/2*	0	0
	Assymetric ribs	0	0	4	5	4	0
T1	sternal rib						
	one side	0	2	5	1	0	0
	both sides	10	0	19	10	10	9
	rudimentary rib						
	one side	0	9	5	2/1*	0	0
	both sides	0	9	1	0	0	0
	no rib						
	one side	0	7	1	3/1*	0	0
	both sides	0	1/3*	0	0/2*	0	0
T2	split costal cartilage						
	one side	0	11	4	2/1*	0	0
	both sides	0	7	0	1	0	0
T8	ribs attached to sternum						
	one side	0	0	7	5	2	0
	both sides	0	19/3*	16	9	4	0
L6	connected ribs						
	one side	0	0	7	3	1	0
	both sides	0	19/3*	5	8/3*	0	0
L5	connected ribs						
	one side	0	0	0	0/2*	0	0
S2	connected to the ilium						
	one side	0	3	10	0/2*	0	0
	both sides	0	17/2*	3	10	0	0
S3	connected to the ilium						
	one side	0	0/1*	0	0/2*	0	0

Newborn mice of the different *Ep-Bmi* transgenic lines were analyzed for malformations of the axial skeleton under a dissecting microscope. The malformations were compared with those reported previously for mice of the *Ep-Bmi33* and *Ep-bmi52* lines (Alkema et al. 1995). Mice that are homozygous for a given genotype are indicated with an asterisk (*). (wt) Wild type. (aaa) anterior arch of the atlas.

Figure 10. Anterior transformations of the axial skeleton in *Ep-Bmi1* mutant transgenic mice (see also table 1). Whole-mount alizarin red/alcian blue-stained skeletons of newborn wild-type (A,E) *Ep-BmiΔP-40* (B,F), *Ep-BmiΔZn-21* (C,G), and homozygous *Ep-BmiΔZn-21* (D,H). (A–D) Lateral view of the occipital, cervical, and upper thoracic region. The exoccipital bone (EO), the atlas (C1) with the anterior arch of the atlas (aaa), the axis (C2), and the first thoracic vertebra (T1) are indicated. The most notable abnormalities in *Ep-BmiΔP-40* and *Ep-BmiΔZn-21* transgenic mice included the thickening of the neural arch of the axis (C2) and transformation of T1 to the identity of C7 (B,C). In the *Ep-BmiΔP-40* transgenic mouse shown in B a residual rib is present on T1, which fuses to the costal cartilage of T2. The T1 and T2 ribs of the *Ep-BmiΔZn-21* transgenic mouse shown in C fuse at the sternum. In the transgenic mice shown in B,C, and D, the dorsal cartilage extension of T2, the processus spinosus, is now present on T3. The penetrance of the transformation is transgene dosage dependent, as shown in D. In homozygous transgenic *Ep-BmiΔZn-21* mice T1 is completely transformed to C7, as no residual rib is present anymore. An extra anterior arch is now also present on the ventral side of C2 ('aaa') and the neural arch has broadened further and is fused to the neural arch of C1. The neural arch of C3 has also broadened and is split at the dorsal side, which can be interpreted as a transformation to C2. (E–H). Ventral view of thoracic region of a wild-type (E), *Ep-BmiΔP-40* (F), *Ep-BmiΔZn-21* (G) and *Ep-BmiΔZn-21* (H) homozygous transgenic mouse. The vertebrosteral ribs are numbered. (F,G) The asymmetric attachment of the ribs to the sternum often found in these mice. (F) The thorax of an *Ep-BmiΔP-40* transgenic mouse, in which the sternabrae acquire a zipper-like structure because of an asymmetric rib attachment. In G (*Ep-BmiΔZn-21*) there is no rib attached to the sternum opposite to the left rib that originates from T2. Note that ossification occurs at the right side at the attachment site of this rib. In both cases (F,G) eight vertebrosteral ribs are present on the left side, whereas there are the expected seven on the right. (H) In *Ep-BmiΔZn-21* homozygous transgenic mice the ribs from T8 are attached to the sternum and a pair of ribs is attached to the first lumbar vertebra (L1).

and Nothiger 1995) revealed two homology regions that may point to domains with functional significance: the RING finger domain at the amino terminus of the Bmi1 protein and part of the putative HTHHT domain in the carboxy-terminal half of the protein. The carboxy-terminal part of the Bmi1 protein contains a region rich in proline, glutamine, and serine residues. Within this region, PEST sequence motifs, implicated in rapid protein degradation, are found. However, they do not seem to confer instability to the Bmi1 protein in view of the ob-

served 10-hr half-life in tissue culture cells (M. van Lohuizen, unpubl.).

Deletion of the RING finger domain and the Pro/Ser rich region did not affect the Bmi1–Mph1/Rae-28 interaction indicating that only the internal part of the Bmi1 protein, including the conserved HTHHT motif, is important for Mph1/Rae-28 binding. It is relevant to note that overexpression of a Bmi1 protein lacking the Pro/Ser rich region or containing mutations in the RING finger domain still induced anterior transformation of

the skeleton, whereas overexpression of a Bmi1 protein that lacks the HTHTHT motif did not lead to skeletal transformation. This suggested that the Bmi1-Mph1/Rae-28 interaction is essential for *Hox* gene regulation. We cannot fully exclude that the Bmi1 RING finger has a role in Pc-G function, as the mutant Bmi1 transgenic mice were made in wild-type *Bmi1* background. The RING finger domain is important for other functions of the Bmi1 protein. In contrast to the E μ -*Bmi1* and E μ -*Bmi1* Δ P transgenic mice the E μ -*Bmi1* Δ Zn transgenic mice were no longer predisposed to lymphomagenesis [M. Alkema, H. Jacobs, M. van Lohuizen, and A. Berns, in prep.]. In this respect, it is of interest that the RING finger is required for proper subnuclear localization of Bmi1. A similar importance for localization to POD domains has been observed for the RING finger of the PML oncoprotein [Borden et al. 1995]. Because the RING finger clearly differs from classical DNA-binding zinc fingers in terms of sequence homology and tertiary structure [Barlow et al. 1994; Borden et al. 1995] we think that the Bmi1 RING finger plays a role in protein-protein rather than protein-DNA interactions. However, it has been reported that the related Mel18 RING finger is essential for DNA binding [Tagawa et al. 1990]. Furthermore, it was shown recently that Mel18 binds to a GACTNGACT target DNA sequence using a random oligonucleotide assay [Kanno et al. 1995]. Although Bmi1 has DNA-binding activity [M. Alkema, unpubl.], we have not been able to demonstrate sequence specific binding using a similar assay. Also the related Psc and Su(z)2 proteins were reported to have only nonspecific DNA-binding activity [Rastelli et al. 1993].

The posterior transformation of the axial skeleton of the *Bmi1* and *Mel18* null mutant mice are very much alike, suggesting functional redundancy [van der Lugt et al. 1994; Akasaka et al. 1996]. The fact that the Mel18 and Bmi1 protein share Mph1/Rae-28 as a binding partner supplies a molecular basis for the similar phenotypes in these mice. However, the subset of *Hox* gene expression boundaries affected in the *Mel18* mutant mice only partially overlap with the subset affected by the absence of Bmi1. Therefore, multimeric Pc-G protein complexes might be formed in different compositions, each having a different efficacy to repress different subsets of *Hox* genes.

Materials and methods

Yeast two-hybrid screen

Yeast strain Y190 [Durfee et al. 1993], which contains two chromosomally located Gal4-inducible reporter genes, *HIS3* and *lacZ*, was transformed with a bait plasmid (pPC97 [Chevray and Nathans 1992]) containing the entire *Bmi1* or *Mel18* ORF or part of the *Mph1/Rae-28* cDNA (encoding amino acids 413–1012) fused to the Gal4 DBD. Production of the Gal4-DBD fusion protein was confirmed with Western blot analysis. The bait-containing strain was subsequently transformed by the lithium acetate method with a pool of plasmids expressing the Gal4 *trans*-activation domain (TA) fused to random sequences of a 14.5-day CD1 mouse embryo cDNA library [Chevray and

Nathans 1992]. One million transformants were selected for growth on plates lacking histidine supplemented with 25 mM 3-aminotriazole. His⁺ colonies were subsequently analyzed for β -galactosidase activity using a colonylift assay. The Bmi1 screen gave 40 His⁺ colonies, 4 of which expressed β -galactosidase; the Mel18 screen yielded 48 His⁺, 3 of which stained blue after a β -gal assay; for the Mph1/Rae-28 screen, 10 of 67 colonies that grew on plates lacking histidine also expressed β -galactosidase. cDNA library plasmids recovered from double-positive yeast colonies were tested for bait specificity by retransformation with different Gal4-DBD fusion plasmids: pPC97–p107, pPC97–BS69, and pPC97 without an insert. To map the interaction domains of Bmi1 and Mph1/Rae-28, *Bmi1* and *Mph1/Rae-28* cDNA fragments were subcloned in the Gal4-DBD and Gal4-TAD vector and cotransformed to Y190. The resulting yeast colonies were then assayed for β -gal activity.

Immunological reagents

To generate antibodies against Bmi1, Mph1/Rae-28, and Mel18 GST-Bmi1, GST-MphSM, GST-MphMN, and Malc-Mel18 proteins were produced in *Escherichia coli* and purified with GST-Sepharose or amylose-Sepharose beads, respectively. pGST-*Bmi1* was constructed by cloning a cDNA fragment encoding amino acids 1–202 in pGEX2T (Pharmacia). pGST-*MphSM* and pGST-*MphMN* were constructed by cloning a cDNA fragment encoding amino acids 413–760 (SM) and amino acids 760–1012 (MN) in pGEX2T. pMalc-*Mel18* was constructed by cloning a cDNA fragment encoding the entire *Mel18* ORF in pMalc. The different purified proteins were used to immunize rabbits and mice (pGST-*Bmi1*). After three rounds of immunization with the different purified fusion proteins polyclonal serum was obtained. The Mph-SM serum was affinity purified using purified Malc-Mph1/Rae-28 protein (amino acids 538–1012) coupled to affigel (Bio-Rad). The polyclonal Bmi1 serum was affinity purified using Ni²⁺ column-purified 6xhis-Bmi1 [Novagen] fusion protein produced in Sf9 insect cells after infection with a recombinant baculovirus. In vitro transcription/translations followed by immunoprecipitation assay confirmed the specificity of the different polyclonal antibodies. Monoclonal antibodies against pGST-Bmi1 were made by fusion of splenocytes of immunized BALB/c mice to Sp2O myeloma cells 1 week after the final boost. Positive tissue culture supernatants were identified in an ELISA assay. Supernatants of 12 ELISA-positive hybridoma cell lines were tested for immunoprecipitation, Western blotting, and immunofluorescence. M33 antibodies were raised against M33 amino acids 298–519, expressed in *E. coli* from a PET23 vector [Novagen]. For the experiments described in this paper we used the monoclonal antibody F6 against Bmi1, and the polyclonal antisera Bmi1-38, Mel18-27, MphSM-91, and MphMN-93.

Immunoprecipitations

Embryo protein extracts were made from 12.5- to 13.5-day murine embryos. Yolk sacs or placentas were used for DNA analysis and subsequent genotyping of the mice as described [Alkema et al. 1995]. Embryos were put in 2 ml of Eppendorf tubes in 1 ml of ELB buffer [Whyte et al. 1988] (containing 5 mM EDTA, 0.5 mM DTT, 1 μ g/ml of chymostatin, aprotinin, antipain, and leupeptin, and 1 mM phenylmethylsulfonylfluoride) and dounced 10 times with a tight Eppendorf potter, sonicated for 20 sec (50% duty cycle in Branson sonifier), and centrifuged for 15 min in an Eppendorf centrifuge at 14,000 rpm. The supernatant of the lysate was used for immunoprecipitations. The protein concentration of the embryonic extracts was measured by the

Bradford method. The lysate was precleared for 1 hr with normal mouse or rabbit serum coupled to protein A-Sepharose beads and 250 μ l of the supernatant (protein concentration 10 mg/ml) was used in immunoprecipitations with 3 μ l of antiserum or 100 μ l of tissue culture supernatant from hybridoma cells. After 1 hr at 4°C immune complexes were collected with 30 μ l of protein A-Sepharose and washed four times (5 min) in 1 ml of ELB buffer and transferred to a fresh tube for a final wash step. After adding 30 μ l two times sample buffer, the immunoprecipitated proteins were separated on a 9% SDS-polyacrylamide gel and transferred to nitrocellulose.

Western blot analysis

Nitrocellulose membrane was blocked in PBST (PBS, 0.05% Tween 20) containing 5% dried milk for 3 hr at room temperature. The membrane was subsequently incubated with the antibody diluted 1:2000–5000 in PBST containing 1% dried milk for 1 hr at room temperature. After washing, the membrane was incubated with horseradish peroxidase-linked swine anti-rabbit IgG or goat anti-mouse IgG as second antibody in PBST containing 1% dried milk for 1 hr at room temperature. Antibodies were detected by enhanced chemiluminescence (Amersham).

Transfection experiments

COS-7 cells were grown in Dulbecco's modified Eagle medium (DMEM) with 10% FCS. For transfections, cells were seeded at a density of 2×10^5 per well in six-well culture dishes. The next day cells were incubated with lipofectamine (GIBCO-BRL) containing 2 μ g of plasmid DNA/well, according to the manufacturers descriptions. After 48 hr total protein extracts were prepared in ELB, as described above.

U2-OS cells were transfected (8 μ g of plasmid DNA+0.5 μ g of puromycin-selectable marker DNA) using standard CaPO_4 precipitation, according to standard procedures. One day after transfection, cells were subjected to puromycin selection (8 μ g/ml), for 36 hr to kill off 95%–99% of untransfected cells. Remaining cells were seeded on multiwell coverslips, recovered for 1 day without puromycin, and processed for immunofluorescence as described below.

Immunofluorescence

The cell line U2OS was grown DMEM with 10% fetal calf serum on multiwell coverslips to ~50% confluence, washed three times in PBS, and fixed in freshly prepared 2% formaldehyde-PBS for 15 min at room temperature. The cells were incubated subsequently in 2×5 min with PBS; 5 min with PBS containing 0.5% Triton X-100; 2×5 min with PBS; 5 min with 0.1 M glycine in PBS, and 2×5 min with PBS; preblocked in blocking solution (PBS containing 5% fetal calf serum, 5% normal goat serum, 0.02% Triton X-100) for 1 hr at room temperature; 2 hr at room temperature with 1:40 diluted Bmi1-F6 monoclonal antibody, 1:100 diluted SC35 monoclonal, 1:40 diluted anti PML 5E10 monoclonal antibody (Stuurman et al. 1992), or 1:20 diluted affinity-purified rabbit polyclonal Mph1/Rae-28 antibody or affinity-purified M33 polyclonal antibody, respectively, in blocking solution; 4×5 min with PBS+0.02% Triton X-100; 1 hr at room temperature with 1:100 diluted FITC-conjugated goat anti-mouse (Jackson Immuno Research Laboratories), or 1:1000 diluted goat anti-rabbit-TRITC in blocking solution. After 4×5 min with PBS+0.02% Triton X-100 specimens were embedded in vectastain (+ or – propidium iodide stain). Images of labeled cells were produced on a Bio-Rad confocal laser scanning microscope with a 63 $\times$ /1.35 oil immersion lens. FITC and

TRITC were recorded separately and then superimposed. When first antisera were omitted or replaced with NRS/NMS as specificity controls, no significant background signal was detected.

Northern blot analysis

RNA from total embryos or adult tissue was isolated by the LiCl-urea method. For Northern blot analysis, 15 μ g of total RNA or 5 μ g of poly(A)⁺ RNA was separated on a 1% agarose gel and transferred to nitrocellulose filters. The *Bmi1* cDNA fragment encoding the ORF, *Mel18* cDNA fragment encoding the ORF, or the longest *Mph1/Rae-28* two-hybrid cDNA clone was labeled by the random priming method and used as a probe. Filters were hybridized as described (Sambrook et al. 1989) and washed at 0.1 $\times$ SSC, 0.1% SDS, at 65°C.

cDNA cloning and sequencing

A partial *Mph1/Rae-28* two-hybrid cDNA clone was used as a probe as described (Alkema et al. 1993) to screen a 16.5-day mouse embryo cDNA library. Sequencing was performed on both strands by the dideoxy chain termination method using a Promega T7 polymerase sequencing kit, with 7-deazadGTP to resolve compression in G/C-rich areas. Initial sequencing was performed using T7, Sp6, and T3 sequence primers. Sequences obtained with these primers were subsequently used to synthesize internal oligonucleotide primers for further sequence analysis.

Skeletal analysis

Whole-mount skeletons of newborn mice were stained with alcian blue 8GS and alizarin red as described (van der Lugt et al. 1994). DNA was isolated from a tissue sample and analyzed by Southern blot analysis to determine the presence of the transgene as described (Alkema et al. 1995). The samples were cleared for 1 week in 1% KOH followed by 1-week clearing steps in 0.8% KOH, 20% glycerol, 0.5% KOH, 50% glycerol. Cleared skeletons were transferred to 50% glycerol, 50% ethanol, for photography and storage. Transgenic mice and embryos were identified by Southern blotting of *EcoRI*-digested tail or extra embryonic tissue DNA, using the *Bmi1* 19.1 probe (Alkema et al. 1995).

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Note

The *Mph1* sequence has been deposited to the GenBank data library under accession no. U63386.

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