Loss- and gain-of-function mutations show a Polycomb group function for Ring1A in mice

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SUMMARY

The products of the Polycomb group (PcG) of genes act as transcriptional repressors involved in the maintenance of homeotic gene expression patterns throughout development, from flies to mice. Biochemical and molecular evidence suggests that the mouse Ring1A gene is a member of the PcG of genes. However, genetic evidence is needed to establish PcG function for Ring1A, since contrary to all other murine PcG genes, there is no known Drosophila PcG gene encoding a homolog of the Ring1A protein. To study Ring1A function we have generated a mouse line lacking Ring1A and mouse lines overexpressing Ring1A. Both Ring1A−/− and Ring1A+/− mice show anterior transformations and other abnormalities of the axial skeleton, which indicates an unusual sensitivity of axial skeleton patterning to Ring1A gene dosage. Ectopic expression of Ring1A also results in dose-dependent anterior transformations of vertebral identity, many of which, interestingly, are shared by Ring1A−/− mice. In contrast, the alterations of Hox gene expression observed in both type of mutant mice are subtle and involve a reduced number of Hox genes. Taken together, these results provide genetic evidence for a PcG function of the mouse Ring1A gene.

Key words: Polycomb, Mouse, Ring1A, Ring1B, Hox, Axial skeleton

INTRODUCTION

The Polycomb group (PcG) of genes were first identified as trans-acting regulators of homeotic gene function in Drosophila (Kennison, 1995). Two clusters of homeotic genes, the Antennapedia complex (ANT-C) and the bithorax complex (BX-C), collectively referred to as the homeotic complex (HOM-C) are responsible for the determination of segmental identities in Drosophila. The ANT-C genes determine and maintain the identity of the head and anterior thoracic segments, whereas the BX-C genes control the identity of posterior thoracic and abdominal segments (Kaufman et al., 1980; Lewis, 1978). The expression of homeotic genes in specific overlapping domains along the anterior-posterior (AP) axis of the embryo is correlated with the physical order of genes in the chromosome (Harding et al., 1985; Lewis, 1978). This principle of colinearity has been conserved in vertebrates (Duboule and Dollé, 1989; Graham et al., 1989). Loss- and gain-of-function mutations in the HOM-C genes cause the cells in those regions where the concentration of the homeotic products is altered, to form structures characteristic of a different segment of the fly. One of these homeotic phenotypes is the transformation of the second and third legs into first leg, as is evident from the presence in the second and third legs of adult males of ectopic sex combs characteristics of the first leg. Mutations in loci outside the HOM-C that cause an extra sex combs phenotype identify the PcG of genes (Jürgens, 1985). In PcG mutants, the initial pattern of expression of HOM-C genes is normal, but later in development a generalized derepression occurs (Simon et al., 1992; Soto et al., 1995; Struhl and Akam, 1985). Thus, the PcG genes function to maintain, rather than to determine, homeotic gene repression. The maintenance of homeotic gene expression is controlled by another set of genes, the trithorax group (trxG), often identified as suppressors of the PcG-induced homeotic phenotypes (Kennison, 1993).

The PcG genes encode a group of structurally heterogenous proteins (Simon, 1995). Recently, plant and vertebrate genes encoding proteins containing regions of homology with Drosophila PcG products have been identified (Gould, 1997; Preuss, 1999; Schumacher and Magnuson, 1997). Mutations in these genes result in homeotic phenotypes and alterations in the expression patterns of homeotic genes (Gould, 1997; Schumacher and Magnuson, 1997). This indicates a conservation of the PcG function throughout evolution. The PcG proteins form large complexes arising from their mutual interactions through evolutionary conserved protein motifs (Kyba and Brock, 1998a,b). The genetic interactions and
Genomic Ring1A consists of two DNA fragments, a 4.2 kb from a mouse 129SVJ genomic library. The targeting vector (Fig. 1A) consists of two DNA fragments, a 4.2 kb BamHI fragment spanning 5' flanking sequences and a 2.0 kb BstEII-KpnI fragment containing the last exon, separated by the mouse phosphoglycerate-kinase (PGK) promoter-driven HPRT minigene (PGK/pDWMI1; Porter et al., 1996). These sequences were then linked to a Herpes simplex virus thymidine kinase gene driven by the MC promoter (Thomas and Capeschi, 1987) (PyTK) for negative selection. The targeting vector was linearized and electroporated into HM-1 embryonic stem (ES) cells as previously described (Porter et al., 1996). Colonies surviving the HAT/ganciclovir selection were transferred into 24-well plates and approximately half of each was processed for PCR analysis. The targeted allele was identified with primers specific for the last exon of Ring1A (TGGGGGCGGAGCGTTACCG, oligo 2) and for the HPRT minigene (AGCTTACCTCTGTAGAATTGTCG, oligo 3). Colonies positive for the mutated Ring1A allele were expanded for further genotyping by Southern blot analysis, using a 5' external fragment (probe in Fig. 1A) under stringent conditions. Chimeric mice generated by the injection of mutated ES cells into Balb/c blastocyst were mated to Balb/c mice and heterozygous animals identified by PCR, using oligos 2, 3 and an additional primer specific from the 3' end of the deleted sequences (TTTGAGCGAAGGTGTGCAC, oligo 1). Mice were also genotyped by Southern blot using a Ring1A cDNA probe. Heterozygous mice were then backcrossed to Balb/c mice.

Generation of transgenic mice

The Ring1A expression construct was obtained by inserting the Ring1A cDNA into a modified version of plasmid pCAGGS (Niwa et al., 1991). The DNA construct was separated from vector sequences by preparative electrophoresis and elution from Elutip columns (Schleicher and Schuell) prior to microinjection in the pronuclei of fertilized oocytes from superovulated (C57BL/10 × Balb/c) F1 females mated with (C57BL/10 × Balb/c) F1 males. Transgenic mice were identified by Southern blot analysis using the Ring1A cDNA as a probe.

Skeletal preparations

Skeletal preparations were stained with Alizarin Red S and Alcian Blue 8GX as previously described (Lufkin et al., 1992). Briefly, carcasses were skinned, eviscerated and fixed overnight in 90-100% ethanol. The cartilage was stained with 0.2 mg/ml Alcian Blue in 75% KOH, air dried and counterstained with Alizarin Red S and Alcian Blue 8GX as previously described (Lufkin et al., 1992). Briefly, 12.5 dpc embryos were isolated, fixed overnight in 90-100% ethanol, dehydrated, and embedded in paraffin wax and sectioned. For in situ hybridization, mouse embryos were fixed in 4% paraformaldehyde and hybridized to digoxigenin-labelled probes as described previously (Schoorlemmer et al., 1997) (PyTK). The Hybridization, embryos were embedded in paraffin wax and sectioned. For Ring1A and PLZF probes we used the cDNAs previously described (Schoorlemmer et al., 1997) and for Ring1B, a 1.4 kb cDNA fragment. In timed pregnancies, non of the day of appearance of vaginal plug was taken as 0.5 dpc (days post coitum). Pregnant females were killed at the desired gestation time and embryos were collected from the decidua. Amniotic fluid was used for genotype analysis.
RESULTS

Generation of Ring1A-deficient mice

To inactivate the Ring1A gene, a targeting vector was designed to replace 2.7 kb of the locus with a HPRT minigene by homologous recombination (Fig. 1A). This deletes the majority of the coding region including the translation initiation start, the RING finger, the homology domain 2 and part of the homology domain 3 (Schoorlemmer et al., 1997), and is expected to result in a null mutant.

The targeting vector was electroporated into ES cells and among 198 clones analyzed 10 were found to have a Ring1A targeted allele. Three of these clones were used to generate chimeric mice, which passed the targeted allele to their offspring. Mice heterozygous for the mutated allele interbred to homozygosity close to the expected segregation ratio (24 +/+, 56 +/- and 20 -/- out of 100 mice born) suggesting that the mutation caused no embryonic lethality. Mice homozygous for the Ring1A mutation showed no Ring1A transcripts detectable by whole-mount in situ hybridization to Ring1A mRNA (Fig. 1D). Moreover, no Ring1A protein was detected either by western blot analysis of total protein extracts or in tissue sections from mutant embryos (Fig. 1E,F). Male and female Ring1A-/- mice appeared normal and were fertile. Because of the singular expression of Ring1A in the boundaries between rhombomeres we examined their fate in mutant mice using PLZF, an established marker for rhombomere boundaries (Cook et al., 1995). The expression pattern of PLZF mRNA in rhombomere boundaries remained unchanged in Ring1A-/- embryos (not shown).

Since the product of the Ring1B gene is structurally related to Ring1A we analyzed its expression in wild-type and Ring1A-/- mice by in situ hybridization and western blot analyses. Ring1B transcripts were found distributed ubiquitously during embryonic development (Fig. 1D), thus overlapping the pattern of Ring1A expression. Moreover, Ring1B expression remained unaffected in Ring1A-deficient mice (Fig. 1D,E), thus making functional redundancy feasible.

Ring1A-deficient mice show skeletal abnormalities

The analysis of skeletal whole mounts of newborn Ring1A-/- mice revealed a number of abnormalities along the AP axis (Fig. 2; Table 1). The most penetrant alteration affected the second cervical vertebra (C2) in all mice, which showed either a broadening of the neural arch, or ectopic points of ossification and aberrant cartilage growth (Fig. 2B,C). Also in the cervical
region, the cartilage of the first cervical vertebra (C1) of about half of the mice failed to close (Fig. 2A,B). In the thoracic region of 19% of the mutant mice the spinous process of the third thoracic vertebra (T3) was the most prominent whilst the process of T2 was greatly reduced, suggesting T3 had taken a more anterior identity (Fig. 2D,E). About 42% of Ring1A−/− mice had eight vertebral ribs (on one or both sides) instead of seven, which is an anteriorisation of the thoracic vertebra T8 into T7 (Fig. 2G,H). In the lumbar region, 45% of the mice showed on one or both sides either a rudimentary rib or ectopic cartilage not attached to the vertebrae, or both (Fig. 2I,J). This abnormality is consistent with an anteriorisation of the lumbar vertebrae 1 into T13. When skeletal whole mounts of 18.5 dpc Ring1A−/− fetuses were examined (Fig. 3), all mice showed splitting of the neural arches of the cervical vertebrae. Moreover, the foramen transversum of C3 to C6 was open (Fig. 3). It is worth noting that the penetrance and expressivity of these defects in the axial skeleton of Ring1A−/− mice ranged from 40 to 100% of that of wild type (wt) mice. Ring1A+/- mice contrasted with the posteriorization of skeletal structures observed in mice with mutation in PcG genes.

**Hox gene expression in Ring1A−/− mice**

The spatially restricted expression patterns of Hox genes are important for establishing positional identity along the AP axis. It has been previously shown that Hox gene expression is affected in loss-of-function mutations of PcG genes in mice (Akasaka et al., 1996; Coré et al., 1997; Takihara et al., 1997; van der Lugt et al., 1996). These data prompted us to examine the pattern of mRNA expression of Hox genes from clusters A (Hoxa3, Hoxa4), B (Hoxb4, Hoxb8) C (Hoxc4, Hoxc5, Hoxc6, Hoxc8) and D (Hoxd4, Hoxd11) in 11.5 dpc Ring1A−/−

### Table 1. Skeletal alterations in mice lacking and overexpressing Ring1A

<table>
<thead>
<tr>
<th>Ring1A deficient mice (%)</th>
<th>Wild type (16)</th>
<th>Ring1A+/− (14)</th>
<th>Ring1A−/− (31)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Newborns</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Abnormal C1</td>
<td>0</td>
<td>21</td>
<td>52</td>
</tr>
<tr>
<td>Abnormal C2</td>
<td>0</td>
<td>57</td>
<td>55</td>
</tr>
<tr>
<td>C2→C1</td>
<td>0</td>
<td>36</td>
<td>45</td>
</tr>
<tr>
<td>T3→T2</td>
<td>0</td>
<td>19</td>
<td></td>
</tr>
<tr>
<td>T8→T7†</td>
<td>0</td>
<td>36</td>
<td>42</td>
</tr>
<tr>
<td>L1→T13</td>
<td>6%</td>
<td>29</td>
<td>45</td>
</tr>
</tbody>
</table>

| 18.5 dpc Vertebral abnormalities | 0 | 62 | 100 |

<table>
<thead>
<tr>
<th>Ring1A transgenic lines (%)</th>
<th>wt (10)</th>
<th>6111 (11)</th>
<th>6081 (21)</th>
<th>6109 (7)</th>
<th>6117 (12)</th>
<th>6117 (3)*</th>
<th>DH (17)**</th>
</tr>
</thead>
<tbody>
<tr>
<td>C2→C1</td>
<td>0</td>
<td>91</td>
<td>95</td>
<td>100</td>
<td>83</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>T8→T7‡</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>24</td>
<td>0</td>
<td>33</td>
<td>12</td>
</tr>
<tr>
<td>T10→T9</td>
<td>0</td>
<td>73</td>
<td>52</td>
<td>71</td>
<td>25</td>
<td>66</td>
<td>88</td>
</tr>
<tr>
<td>L1→T13</td>
<td>0</td>
<td>54</td>
<td>48</td>
<td>0</td>
<td>0</td>
<td>66</td>
<td>70</td>
</tr>
</tbody>
</table>

*Homozygous 6117; **double hemizygous 6117, 6081.

Number of mice analyzed are given in parentheses.

Unilateral and bilateral abnormalities were counted as being positive in the analysis.

C1, atlas; C2, axis; T2, T3, T7, T8, T9, T10, T13, 2nd, 3rd, 7th, 8th, 9th, 10th, 13th thoracic vertebrae; L1, 1st lumbar vertebra.

Penetration of skeletal abnormalities is indicated as percentage of mice analysed.
Ring1A is a PcG gene in mice

5097

embryos showed ectopic expression (nearly ubiquitous) and at much higher levels than in wild-type embryos (not shown).

Anterior transformations of the axial skeleton in mice overexpressing Ring1A

Analysis of the axial skeleton of Ring1A transgenic neonates revealed several alterations along the AP axis in all the lines. These malformations are summarized in Table 1. In the cervical region, the neural arch of C2 was broadened resembling the appearance of the C1 vertebra (Fig. 4A-D). In the thoracic region, anterior transformations were also observed including the transformation of T8 into T7 as indicated by the presence of eight ribs attached to the sternum, instead of seven (Fig. 4E,F). The transitional vertebra of transgenic animals was also transformed anteriorly. In wild-type mice this vertebra is T10 and is defined as the most anterior vertebra to show a lumbar rather than a thoracic articulation. In a large proportion of newborn transgenic animals the dorsal cartilage and the neural arch of T10 took the appearance of T9, thus making T11 the new transitional vertebra (Fig. 4G,H). In the lumbar region, the first vertebra (L1) was anteriorly transformed toward the identity of T13. Either extensive ribs or rib heads were found attached to one or both sides of L1 (Fig. 4L).

As seen in Table 1, the penetrance of the anterior transformations observed in Ring1A transgenic animals was highly variable. These variations suggested a dosage dependence resulting from differences in the expression levels of the transgene. To test this we generated mice homozygous for the 6117 line and double hemizygous for the 6117 and 6081 lines. In both cases, the penetrance and expressivity of the phenotype became more pronounced when compared to that of the 6117 and/or the 6081 hemizygous animals. The anterior transformations of the axial skeleton resulting from the ectopic expression of Ring1A are similar to those observed in bmi1 transgenic mice (Alkema et al., 1995).

Hox gene expression in Ring1A transgenic mice

We analyzed the distribution of Hox transcripts in 11.5 dpc embryos of the transgenic lines 6081 and 6111. As for the Ring1A-/- embryos, we found subtle changes in the expression domains of a reduced number of Hox genes. The changes consisted of a reduction in the signal intensities in the anterior boundaries of expression in the paraxial mesoderm. These included Hoxb8 and Hoxc8 in embryos of the line 6081, but not in embryos of the line 6111, which only showed alterations in Hoxc6 expression in a subset of embryos (data not shown). Overall, the analysis of Hox gene expression in Ring1A-/- mice and in Ring1A transgenic mice suggests that Ring1A can contribute to the regulation of Hox gene expression.

DISCUSSION

Cumulative biochemical and molecular evidence suggests that the mammalian Ring1A and Ring1B genes are members of the PcG of genes. However, genetic evidence is needed to establish PcG function for the Ring1 proteins. Here, we have addressed such question by generating a mouse line with a null mutation in the Ring1A locus and transgenic mice lines that overexpress Ring1A. An additional interest in these mutants is the lack of a Drosophila PcG gene encoding a homolog of the Ring1
The mouse line lacking Ring1A alteration in the paraxial mesoderm, although at low levels, could explain by whole-mount in situ hybridization. The presence of Ring1A mesoderm structures at levels low enough to be easily detected of 9.5 dpc embryos. This suggests that Ring1A protein is readily detectable in the paraxial mesoderm embryos (Schoorlemmer et al., 1997). However, we show that be restricted to the embryonic nervous system of 9.5-11.5 dpc how such a phenotype arises, as a variety of abnormalities in the axial skeleton. It is not evident how such a phenotype arises, as Ring1A mice lack a delay in expression. The penetrance and expressivity of the axial skeleton phenotype of Ring1A−/− mice is that the skeletal transformations are anterior, whereas the ones observed in mice with mutations in the PcG genes bmi1, mell8 and rae28 are posterior (Akasaka et al., 1996; Takihara et al., 1997; van der Lugt et al., 1994). In M33−/− mice, although most skeletal transformations are posterior, they also show the anterior transformation of C2 into C1 (Coré et al., 1997; Katoh-Fukui et al., 1998). Given that PcG genes are thought to be negative regulators of Hox gene expression, the phenotype of PcG mouse mutants has been explained by the posterior prevalence (Duboule, 1991) and by the Hox code models (Kessel and Gruss, 1991) developed to interpret the skeletal transformations of mice with mutations in Hox genes. The former states that the effects of Hox genes expressed more anteriorly are suppressed by the action of Hox genes with more posterior, overlapping, boundaries of expression. The latter states that the combination of Hox genes expressed in a region is what determines its identity. According to these models, the derepression of Hox genes in PcG mutants would lead to posterior transformations of those body parts that now mimic the pattern of Hox expression of more posterior regions. However, the above models may not be optimal to interpret PcG phenotypes. First, they often fail to predict vertebral identities and the direction of homeotic transformation of vertebrae in Hox mutant mice (Boulet and Capocci, 1996; Jeannotte et al., 1993; Ramirez-Solis et al., 1993; Small and Potter, 1993). Second, mutations in individual PcG genes can affect several Hox genes simultaneously, adding to the complexity of the interpretation of skeletal alterations.

The skeletal alterations in Ring1A−/− mice include a delay in the development of cervical vertebrae, and in about half of the mice aberrant neural arches in the C2. Interestingly, the latter...
are reminiscent of those seen in mice heterozygous for a null mutation in the \textit{Mll} locus (Yu et al., 1995), a mammalian homolog of the \textit{Drosophila} \textit{tra} gene. \textit{Mll} heterozygous mice show bidirectional transformation of vertebrae along the axial skeleton and posteriorization of Hox gene expression. A possibility is that the loss of \textit{Ring1A} function affects the proliferation rate of the growth plate cartilage and/or the replacement of chondrocytes by osteoblasts. We are currently examining this possibility.

**\textit{Ring1A} in the regulation of Hox gene expression**

The skeletal phenotypes of \textit{Ring1A}-deficient mice are associated with minor alterations of Hox gene expression in these mice. This observation is most likely due to compensation of the \textit{Ring1A} deficiency by the product of the \textit{Ring1B} gene. A similar compensation effect by PC2 may be operating in \textit{M33}\textsuperscript{−/−} mice, which also show weak alterations of Hox gene expression (Coré et al., 1997). The role of \textit{Ring1A} and \textit{M33} as regulators of Hox genes, therefore, is not as influential as that of \textit{bmi1}, \textit{mel18} and \textit{RAE28} proteins (Akasaka et al., 1996; Takihara et al., 1997; van der Lugt et al., 1996). These results suggest that even small changes in the levels of Hox proteins may lead to changes in the AP patterning of the axial skeleton. Thus, it would be possible that quantitative differences in Hox gene expression, with no alterations of the corresponding spatial expression patterns, could take place in \textit{Ring1A} mutant mice. Consistent with this suggestion is the observation of a reproducible delay in the appearance of in situ hybridization signals for \textit{Hoxc8} in \textit{Ring1A}\textsuperscript{−/−} embryos compared to wild-type embryos. Conversely, in transgenic embryos the hybridization signals for \textit{Hoxb8} and \textit{Hoxc8} appear later than in wild-type embryos (M. L. and M. V., unpublished). A semiquantitative analysis of the expression of these genes in mutant and wild-type mice is being carried out.

In addition, there is the possibility that Hox gene expression is deregulated in \textit{Ring1A} mutant mice very early in development. It is known that early alterations in the expression of some Hox genes can lead to skeletal abnormalities which cannot be compensated for at a later developmental stage, when an appropriate expression pattern of Hox genes is observed. An example is the expression of \textit{Hoxd11} and \textit{Hoxd10} genes in mice with a mutation in the \textit{HoxD} cluster (Zakany et al., 1997). A similar transient alteration of Hox gene expression is the anteriorization of the rostral boundary of \textit{Hoxc9} in the neuroectoderm at early, but not later stages, of development in \textit{M33}\textsuperscript{−/−}\textit{bmi1}\textsuperscript{−/−} mice (Bel et al., 1998).

**Partial similarities of skeletal phenotypes in loss and gain of \textit{Ring1A} function**

The overexpression of \textit{Ring1A} leads to the anteriorization of the identities of a number of vertebrae, which is the type of skeletal alterations conventionally expected for PcG gain-of-function mutations. Thus, an intriguing finding of this study is the partial resemblance between the phenotypes of \textit{Ring1A}-deficient and \textit{Ring1A}-overexpressing mice, as indicated by the common C2 to C1, T8 to T7 and L1 to T13 transformations. A similar scenario is found in \textit{Hoxc8} loss- and gain-of-function experiments, which lead to remarkably similar alterations in the axial skeleton including the anterior transformations of T8 to T7 and L1 to T13 (Le Moellec et al., 1992; Pollock et al., 1992). According to an antipodal model, \textit{Hoxc8} would interact with one or more cofactors to form a complex controlling axial patterning. A functional complex would require a specific concentration of \textit{Hoxc8} protein so that its absence or its presence at higher levels would result in a non-functional complex, and ultimately lead to the same aberrant phenotype. Whether the subtle alterations in the regulation of \textit{Hoxc8} or the deregulation of another unidentified factor(s) acting as in the antipodal model are responsible of the common features in the \textit{Ring1A} phenotypes is not known.

Finally, the phenotype of mice lacking \textit{Ring1A}, in the presence of the more ubiquitous and highly related \textit{Ring1B} protein, is an indication that in some cases, different \textit{PcG} genes, including paralogs, play specific roles within a given regulatory pathway.

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