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The Drosophila Polycomb group gene *Sex combs extra* encodes the ortholog of mammalian Ring1 proteins

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Abstract

In *Drosophila*, the Polycomb group (PcG) of genes is required for the maintenance of homeotic gene repression during development. Here, we have characterized the *Drosophila* ortholog of the products of the mammalian *Ring*1/*Ring*1*A* and *Rnf*2/*Ring*1*B* genes. We show that *Drosophila Ring* corresponds to the *Sex combs extra* (*Sce*), a previously described PcG gene. We find that *Ring*/*Sce* is expressed and required throughout development and that the extreme *Pc* embryonic phenotype due to the lack of maternal and zygotic *Sce* can be rescued by ectopic expression of *Ring*/*Sce*. This phenotypic rescue is also obtained by ectopic expression of the murine *Ring*1/*Ring*1*A*, suggesting a functional conservation of the proteins during evolution. In addition, we find that Ring/Sce binds to about 100 sites on polytene chromosomes, 70% of which overlap those of other PcG products such as Polycomb, Posterior sex combs and Polyhomeotic, and 30% of which are unique. We also show that Ring/Sce interacts directly with PcG proteins, as it occurs in mammals.

Keywords: *Drosophila Ring*; *Sex combs extra*; *Ring1A*; *Ring1B*; Polycomb group

1. Introduction

Genetic analysis in *Drosophila* has unveiled a repression function required for proper regulation of the homeotic genes that determine segmental identities. A large number of genes, collectively known as the Polycomb group of genes (PcG), participate in such a repressive activity (reviewed in Kennison (1995), Orlando (2003) and Pirrotta (1998)). Thus, mutations in the PcG genes lead to homeotic phenotype associated to the indiscriminate expression of genes from the bithorax complex (BX-C) and/or Anntenapedia complex (ANT-C) (Simon et al., 1992; Soto et al., 1995; Struhl and Akam, 1985). Subsequently, PcG related genes have been identified in plants and in vertebrates, and mutations in these genes are, among others, associated to homeotic phenotypes (reviewed in Gould (1997), Preuss (1999) and Schumacher and Magnuson (1997)). The PcG are thought to be required for the maintenance of transcriptionally repressed states of the Hox genes, but not for the initiation of their repression. Other transcriptional repressors of the gap and pair rule groups, transiently expressed during development, are responsible for this initiation of repression.

The molecular mechanism(s) of PcG function is (are) unknown. Several lines of evidence, however, indicate that PcG products work together in multimeric protein complexes in which individual PcG proteins interact with other PcG proteins through conserved domains (Hashimoto et al., 1998; Kyba and Brock, 1998a,b; Satijn and Otte, 1999). Biochemical fractionation of *Drosophila* nuclear extracts shows two major multimeric complexes. One, termed Polycomb Repressive Complex 1 (PRC1) has a size of about 2 MDa, contains the PcG products Polycomb (Pc), Polyhomeotic (Ph), Posterior sex combs (Psc), Sex combs on midleg (Scm) and *Drosophila* Ring, some components of the basal transcriptional machinery (TAFs) and other polypeptides (Shao et al., 1999; Saurin et al., 2001).

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Another complex, of about 600 kDa in size, does not contain any of the above proteins, but instead comprises the products of the *extra sex combs* (*esc*), *Enhancer of zeste* [*E*(*z*)] and *Suppressor of zeste 12* [*Su*(*Z*)12] genes (Muller et al., 2002; Ng et al., 2000). In contrast to the lack of enzymatic activities associated to the PRC1 complex, the so-called Esc–E(z) complex has histone deacetylase and histone methyltransferase activities (Czermin et al., 2002; Muller et al., 2002). A complexes-based function is consistent with the synergistic genetic interactions between any two PcG genes (Bel et al., 1998; Cheng et al., 1994; Jürgens, 1985). Additionally, the PcG products are chromosomal proteins that bind specific sites, visualized on salivary gland polytene chromosomes. Many of these binding sites are common for several PcG proteins (DeCamillis et al., 1992; Lonie et al., 1994; Martin and Adler, 1993; Rastelli et al., 1993; Sinclair et al., 1998; Zink and Paro, 1989). The large number of chromosomal sites that bind PcG proteins suggests that the homeotic complexes, BX-C and ANT-C, are only some of many target loci regulated by PcG (Maurange and Paro, 2002; Busturia and Morata, 1988; Moazed and O'Farrell, 1992; Pelegri and Lehmann, 1994).

Repression by PcG proteins occurs through Polycomb response elements (PRE), which are regulatory DNA sequences harbouring functional binding sites for PcG proteins. Until recently, PREs were identified in a few loci, including the homeotic genes of the BX-C and ANT-C complexes (Busturia and Bienz, 1993; Chiang et al., 1995; Gindhart and Kaufman, 1995; Orlando et al., 1998; Simon et al., 1993; Strutt and Paro, 1997; Strutt et al., 1997). Recently, computational methods have been used in *Drosophila* to predict PREs on a genome wide scale identifying about 170 of candidate PREs, which map to a variety of loci involved in development and cell proliferation (Ringrose et al., 2003). PREs have a modular structure and bind PcG complexes of different composition (Shimell et al., 2000; Strutt and Paro, 1997; Tillib et al., 1999). How these complexes are targeted to DNA is not known. PREs have DNA binding sites for proteins such as GAGA factor (Strutt et al., 1997), Zeste (Hur et al., 2002) and Pleiohomeotic (Pho), which is the only PcG product able to bind DNA (Brown et al., 1998; Fritsch et al., 1999). However, Pho is found only in PcG complexes at the earliest stages of *Drosophila* development (Poux et al., 2001). The molecular mechanism(s) by which the PcG repression function uses multimeric complexes is not known.

In a search for new mammalian PcG genes, we found *Ring*1/*Ring*1*A* and *Rnf*2/*Ring*1*B*, two mouse genes whose products interact both in vitro and in two hybrid assays with Pc, Psc and Ph homologs (Hemenway et al., 1998; Satijn and Otte, 1999; Satijn et al., 1997; Schoorlemmer et al., 1997). Ring1/Ring1A and Rnf2/Ring1B proteins are part of a PRC1 complex isolated from mammalian cells (Levine et al., 2002). The *Drosophila* PRC1 complex also contains the ortholog of vertebrate Ring1 proteins, which seems to play an essential role in the in vitro reconstitution of a PRC1 core complex together with Pc, Psc and Ph (Francis et al., 2001). In contrast to these components of the PRC1 core complex, initially identified by their mutant phenotypes, there is no genetic evidence for a role of Ring in *Drosophila*. In mice, null or hypomorphic mutations in the *Ring*1/*Ring*1*A* or *Rnf*2/*Ring*1*B* genes, respectively, show axial skeleton alterations consistent with a PcG function (del Mar Lorente et al., 2000; Suzuki et al., 2002).

Here, we have identified the product of the *Drosophila melanogaster Ring* gene (*Ring*) as *Sex combs extra* (*Sce*), one of the molecularly uncharacterized PcG mutants in *Drosophila*, which was defined by a single mutant allele Sce^{1} (Breen and Duncan, 1986) selected as a dominant enhancer of Miscadestral pigmentation (Mcp) (Lewis, 1978). We show that over-expression of *Ring*/*Sce* and also of the murine *Ring*1/*Ring*1*A* can rescue the extreme *Pc* embryonic phenotype derived from the lack of maternal and zygotic Sce^{1} (m^{-}, z^{-}), suggesting a functional conservation of the *Drosophila* and vertebrate proteins during evolution. In addition, we have found that *Ring*/*Sce* encodes a chromosomal protein that binds to more than 100 specific sites. Finally, we show that direct interactions between Ring/Sce and PcG proteins take place through the same domains as the interactions between their mammalian counterparts.

2. Results

2.1. Identification of Drosophila Ring proteins

We searched the EST databases of the BDGP with either murine *Ring*/*Ring*1*A* or *Rnf*2/*Ring*1*B* cDNAs and identified two overlapping cDNAs (LD3177 and LD6636), which were obtained from Research Genetics. The complete sequence of cDNA LD3177 was almost identical to a cDNA sequence termed *Ring* deposited in the databanks (CG5595).

The comparison between the fly and murine proteins revealed a high degree of conservation. Thus, the three domains (HD1, HD2 and HD3) identified in the murine (and human) proteins are also identified in the fly protein (Fig. 1A). These domains are separated, like in the mammalian proteins, by non-conserved sequences (Fig. 1B). Therefore, 78% of the 147 amino acids N-terminal domain (HD1), which contains a Ring finger, are identical between the fly and either of the murine Ring1 proteins. Conservation at the other two domains is lower: 53 and 60% identity with HD2 of Ring1/Ring1A and Rnf2/Ring1B, respectively, and 46% identity between *Drosophila* Ring HD3 and either HD3 of the murine Ring1 proteins. Curiously, the HD2 of *Drosophila* Ring is interrupted by a stretch of 11 amino acids.

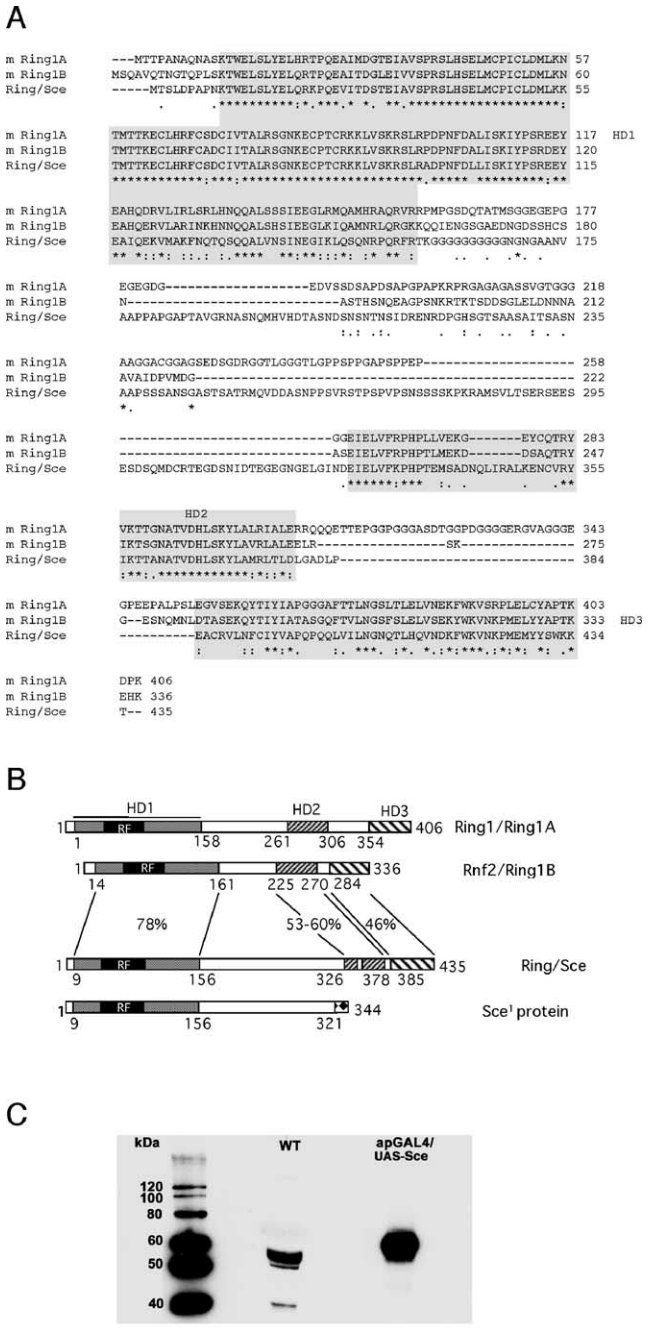

Fig. 1. Sequences and structural domains of Ring proteins. (A) Alignment of the deduced amino acid sequences encoded by the *Drosophila melanogaster Ring* (*Ring/Sce*) and *Mus musculus Ring*1/*Ring*1*A* and *Rnf*2/*Ring*1*B* cDNAs. The corresponding amino acid numbers are indicated. Shading indicates three regions of conserved sequences or homology domains (HD) HD1, HD2 and HD3. (*) indicates identical residues, (:) indicates strong homologies, (.) indicates weak homologies. (B) Schematic representation of Ring proteins. Shaded and stripped boxes represent the homology domains and open boxes represent non-conserved regions. The approximate percentage of sequence identity (similarity in parenthesis) is given. The predicted Ring mutant protein in *Sce*[1] mutants is also shown. RF, indicates the Ring finger domain. (C) Western blot showing the Ring/Sce protein from wild type imaginal discs (wt lane) and from imaginal discs over-expressing a Myc-tagged Ring/Sce fusion protein using the *apterous-GAL4* driver and revealed with the anti-Sce antibody.

By in situ hybridization to polytene chromosomes, *Drosophila Ring* was located at the end of the long arm of chromosome 3 in section 98A (data not shown). Interestingly, *Sce*, a non-molecularly characterized PcG gene, which was defined by a single mutant allele *Sce*[1], had been mapped by recombination to the 3–92 interval (Breen and Duncan, 1986). The proximity of such an interval to the cytological localization of the *Drosophila Ring* gene made us consider worth exploring a possible identity between the *Sce* and the *Drosophila Ring* gene.

We sequenced the region of genomic DNA from *Sce*[1] heterozygous embryos corresponding to the *Drosophila Ring* coding region. Comparing these sequences with the wild type, a deletion of 410 bp was found that it removes the codons for the C-terminal 113 amino acids, a small intron and 12 nucleotides of the 3′ untranslated region after the termination codon (data not shown). Therefore, the *Sce*[1] allele conceptually encodes a truncated Ring protein that is fused in frame to 23 novel amino acids at the C-terminal part of the protein. Fritsch et al. (2003) made an identical observation while this manuscript was in preparation. Herein, we will refer to *Drosophila Ring* as *Sce*.

2.2. Developmental analysis of Sce expression

To determine if *Sce* is developmentally regulated we examined the spatial distribution of *Sce* transcript and protein during development. In situ hybridization using a *Sce* cDNA probe showed a general expression in syncytial blastoderm embryos owed to the maternal component (Fig. 2A). This ubiquitous expression is maintained until stage 11 (Fig. 2B). However, by stage 13 of development *Sce* mRNA was restricted to the neuroectoderm (Fig. 2C) with no expression in the epidermis. Later on, at stage 15 of development, *Sce* transcripts were detected only in the central nervous system (Fig. 2D).

We also analysed expression pattern of the Sce protein using the anti-Sce antibody that we generated. In Western blots, this antibody recognized predominantly a unique band, corresponding to the mobility of a 58 kDa polypeptide (Fig. 1C). In embryos, we observed the same expression pattern of the Sce protein as the one detected by in situ hybridization (Fig. 2E–H). However, some differences can be observed. For example, note that at stage 14 of development, although no transcripts are detected in the epidermis, some Sce protein is still present in the epidermis of anterior segments. This observation suggests that *Sce* translation or stability might be spatially regulated.

We have also observed ubiquitous expression of *Sce* in all the imaginal discs detected by either in situ hybridization or antibody staining (data not shown). This expression in the imaginal discs is in agreement with the requirement of *Sce* function during all stages of larval development (data not shown and Beuchle et al., 2001).

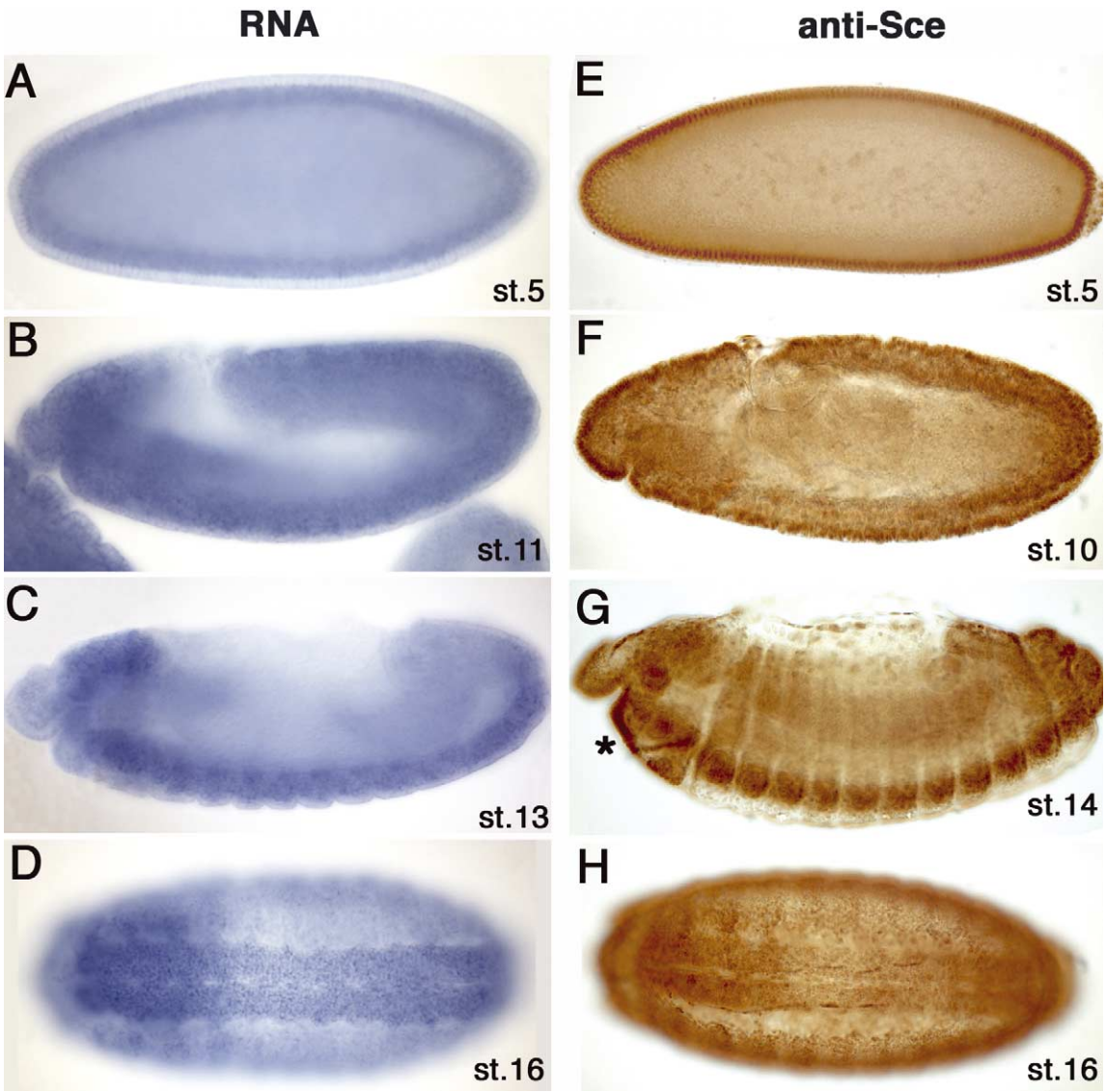

Fig. 2. Expression pattern of *Sce* during embryogenesis. (A–D) *Sce* mRNA expression detected by in situ hybridization. Note the ubiquitous expression of *Sce* mRNA in blastoderm and during the first stages of development (A,B). However, at stage 13 of development, *Sce* mRNA is restricted to neuroectoderm. (E–H) Sce protein detected by immunostaining with an anti-Sce antibody. The same expression pattern is observed except that at stage 14 of development (G) Sce protein is still detected in the epidermis of the anterior part of the body (*). Embryos are oriented anterior to the left and dorsal up.

2.3. Rescue of the Sce mutant embryos by ectopic expression of Drosophila Ring and mouse Ring1/Ring1A

Sce^1/Sce^1 embryos from $Sce^1/+$ mothers (m^+, z^- embryos) die as first instar larvae and show very weak posteriorly directed segmental transformation. In such larvae the ventral denticle belts of A7 develops with some A8 character (Breen and Duncan, 1986). Sce^1/Sce^1 embryos derived from Sce^1/Sce^1 germ-line mutant females crossed to Sce^1 males (m^-, z^- embryos) showed extreme posteriorly directed segmental transformation. All the thoracic and abdominal segments are transformed to A8 and head involution is blocked, as it was previously described (Breen and Duncan, 1986; Fig. 3B). Moreover, these mutants showed an anterior de-repression of the homeotic gene products such as *AbdB* (Fritsch et al., 2003 and data not shown). To test whether Sce^1 allele is a lack of function mutation, we looked for a deficiency that uncovered *Sce* locus. We tested all available deficiencies at 98A region where we found *Drosophila Ring* was located. *Df*(3*R*)*IR*16 (Shelton and Wasserman, 1993), whose breakpoints include 97F1–2; 98A on the cytological map, was lethal over Sce^1. In addition, homozygous Sce^1/Sce^1 germ-line mutant females crossed to Sce^1 males produced embryos that had identical phenotype than when crossed to *Df*(3*R*)*IR*16 males (data not shown). This result indicates that *Df*(3*R*)*IR*16 is a genuine deficiency for the *Sce* locus and suggest that Sce^1 is a null allele.

To verify that *Drosophila Ring* is *Sce*, we analysed the phenotype of Sce^1 (m^-, z^-) embryos when *Drosophila* Ring/Sce was over-expressed using *arm-GAL4* driver. We observed a complete rescue of the embryonic phenotype in

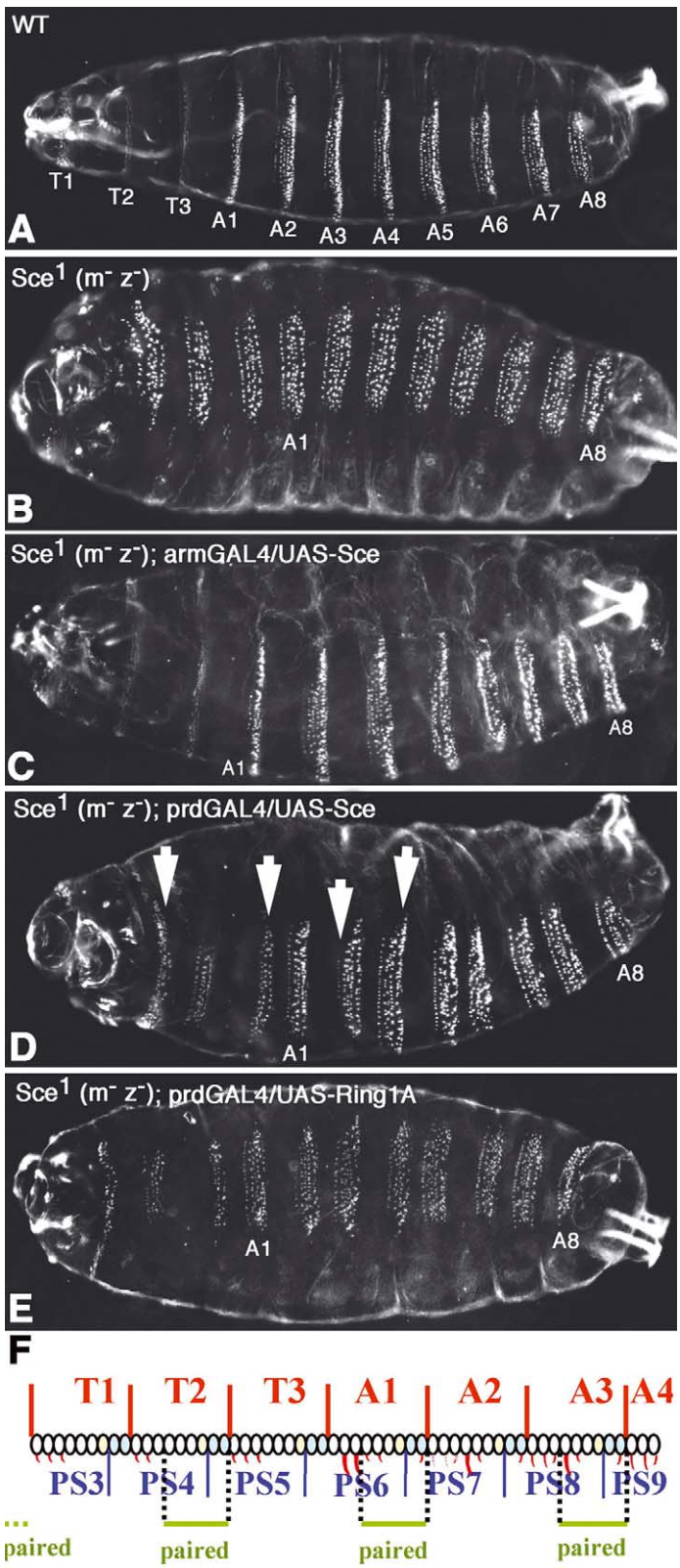

Fig. 3. Rescue of *Sce*[1] mutant phenotype by over-expression of *Drosophila Ring/Sce*. (A) Ventral cuticle structures of a wild type first instar larva. T1…A8 mark the corresponding segmental denticle belts. (B) Cuticle of *Sce*[1] (m^-, z^-) late embryo. (C) Ventral view of an *Sce*[1] (m^-, z^-) late embryo cuticle where *Ring* has been over-expressed with the *arm-GAL4* driver. (D) Ventral view of an *Sce*[1] (m^-, z^-) late embryo cuticle where *Sce* has been over-expressed in the *paired* domain. Rescued denticle belts are marked by arrows. (E) The same experiment than in (C), over-expressing murine *Ring1A* in the *paired* domain. (F) Schematic representation of a late embryo cuticle showing the expression domain of the *pairedGAL4* driver with respect to the denticle belts. *engrailed* expressing cells are light blue and *wingless* expressing cells are yellow.

such embryos, which were undistinguishable from wild type embryos (Fig. 3C). In the resulting embryonic population of the same experiment, there were also zygotic rescued embryos (m^-, z^+) that have almost wild type phenotype. To unequivocally distinguish the *Sce*^{1} embryos rescued by ectopic *Drosophila* Ring/Sce from the rest of the embryonic derived population, we expressed *UAS-Drosophila Ring/Sce* (*UAS-Sce*) ectopically using the *paired-Gal*4 line, which induces ectopic expression in alternate segments (Yoffe et al. (1995) and scheme in Fig. 3F). The areas of rescued cuticle in the *Sce*^{1} (m^-, z^- embryos); *prd-Gal*4/*UAS-Sce* embryos corresponded to those of *prd* expression domains (Fig. 3D). This rescue was visualized by the normalized T1 and T3 denticle belts and was also observed in anterior A2 and posterior A3 denticle belts (arrows, Fig. 3D). We then asked whether the murine Ring1/Ring1A protein would substitute for the fly Sce protein. As before, we expressed *Ring*/*Ring*1*A* in *Sce*^{1} (m^-, z^-) embryos using the lines *arm-Gal*4 and *prd-GAL*4 (Fig. 3E) as drivers, and we observed a rescue of the *Sce* phenotype similar to that seen with *Drosophila Ring/Sce*. Table 1 shows the number of *Sce*^{1} (m^-, z^-) and rescued embryos. Altogether these results further demonstrate that *Sce* locus encodes for the *Drosophila* ortholog of vertebrate *Ring*1/*Rnf*2 genes and that the function of the Ring proteins is conserved in mice and flies.

2.4. Immunolocalization of Sce on polytene chromosomes

PcG proteins are chromosomal proteins, which show binding to discrete euchromatic sites in polytene chromosomes. Many of these binding sites overlap among different PcG proteins. We have examined the distribution of Sce protein on salivary gland polytene chromosomes. We detected about 110 euchromatic sites of antibody staining in the polytene chromosomes (Fig. 4A). Table 2 lists these sites, indicates the differences in staining intensity, and compares them with the published binding sites for other PcG proteins as Pc, Ph, Pcl and Psc. Fifty-one of the 110 sites overlap with Pc/Ph/Pcl/Psc binding sites, 25 overlap with Pc/Ph/Pcl ones and 6 of them are common to the subset of unique Asx sites. Among the Sce sites are those of known targets of PcG genes, such as the ANT-C and BX-C clusters. Fig. 4B,C shows the Sce and Pc sites, respectively, on the segment of the third chromosome that contains the BX-C. Thus, the extensive co-localization of Sce and other PcG proteins at many chromosomal sites is in agreement with Sce being a functional partner of other PcG proteins in *Drosophila*.

Table 1
Rescue of the *Sce*^{1} (m^-, z^-) embryonic phenotype with *UAS-Sce* and *UAS-Ring*1*A*

	Sce^1 (m^-, z^-)	Rescued embryos
*arm-Gal*4; *UAS-Sce* (1)	140	55
*paired-Gal*4; *UAS-Sce* (2)	40	38
*paired-Gal*4; *UAS-Ring*1*A* (2)	22	17

(1) In this experiment, 3/4 of cuticles should be *Sce*^{1} and 1/4 of cuticles should be wild type, as the *arm-Gal*4 driver is in the X chormosome. We attribute the excess of wild type cuticles to the zygotic rescued embryos (m^+, z^-). (2) These experiments were done with GFP balancer chromosomes in order to avoid the zygotic rescued embryos (m^+, z^-). As *paired-GAL*4 was recombined to the *Sce*^{1} allele, equal number of *Sce*^{1} and rescued embryos is expected.

2.5. Interactions between Sce and PcG proteins

The PRC1 complex contains Psc, Pc, Ph, and Sce proteins (Shao et al., 1999; Saurin et al., 2001). Among these components of the PRC1 complex, it is known that Psc interacts directly with Ph and Pc (Kyba and Brock, 1998b) and that Psc and Ph interact homotypically (Kyba and Brock, 1998a; Peterson et al., 1997). Murine and human Ring1/Ring1A and Rnf2/Ring1B interact directly not only with the mammalian homologs of Pc, M33 and Pc2 (Satijn et al., 1997; Schoorlemmer et al., 1997), but also with orthologs of Psc such as Bmi1 (Satijn and Otte, 1999) and Mel18. In addition, Rnf2/Ring1B interacts with mPH2, a Ph homolog (Hemenway et al., 1998). To see whether the conservation of the patterns of pairwise interactions between *Drosophila* PcG protein and their mammalian counterparts also include Sce we studied its association with Pc, Psc and Ph using an in vitro protein binding assay (Fig. 5).

The complete Sce coding sequence (amino acids 1–435, Sce), and derivatives containing the domains HD1 [Sce amino acids 1–274, Sce(N)] or HD2 and HD3 [amino acids 274–435, Sce(C)] were fused to the glutathione S-transferase (GST) gene, and the resulting hybrid proteins were expressed in *Escherichia coli*. Fig. 5B shows that GST-Sce bound specifically Pc and Psc, but not Ph (Fig. 5B, lanes 1, 3–5, 7 and 8). Sce(C) but not Sce(N) bound Pc (Fig. 5B, lanes 9 and 13). This shows that Sce binding to Pc occurs through its HD2 and HD3 domains, as previously shown for mammalian Ring1 and Pc proteins. Moreover, the Pc variant lacking the conserved carboxyl domain (PcΔC) did not bind to Sce (Fig. 5B, lanes 6 and 10), a result consistent with previous findings in mammals showing that such domain is responsible for bind of Pc to Ring. However, binding to Psc occurred preferentially to Sce(N) (Fig. 5B, lanes 11 and 15), showing that the interaction between Sce and Psc involves the same domains as the interaction between mammalian Rings and Bmi1 proteins. Sce did not interact with the conserved domain of Ph (amino acids 1297–1576), which mediates homo and heterotypic interactions (Fig. 5B, lane 8). Although mouse Rinf2/Ring1B binds Ph (1297–1576) (data not shown) we cannot discard an interaction between Sce and regions in the rest of the Ph protein. These results indicate that of the interactions among mammalian Ring1/Rnf2 proteins and PcG proteins,

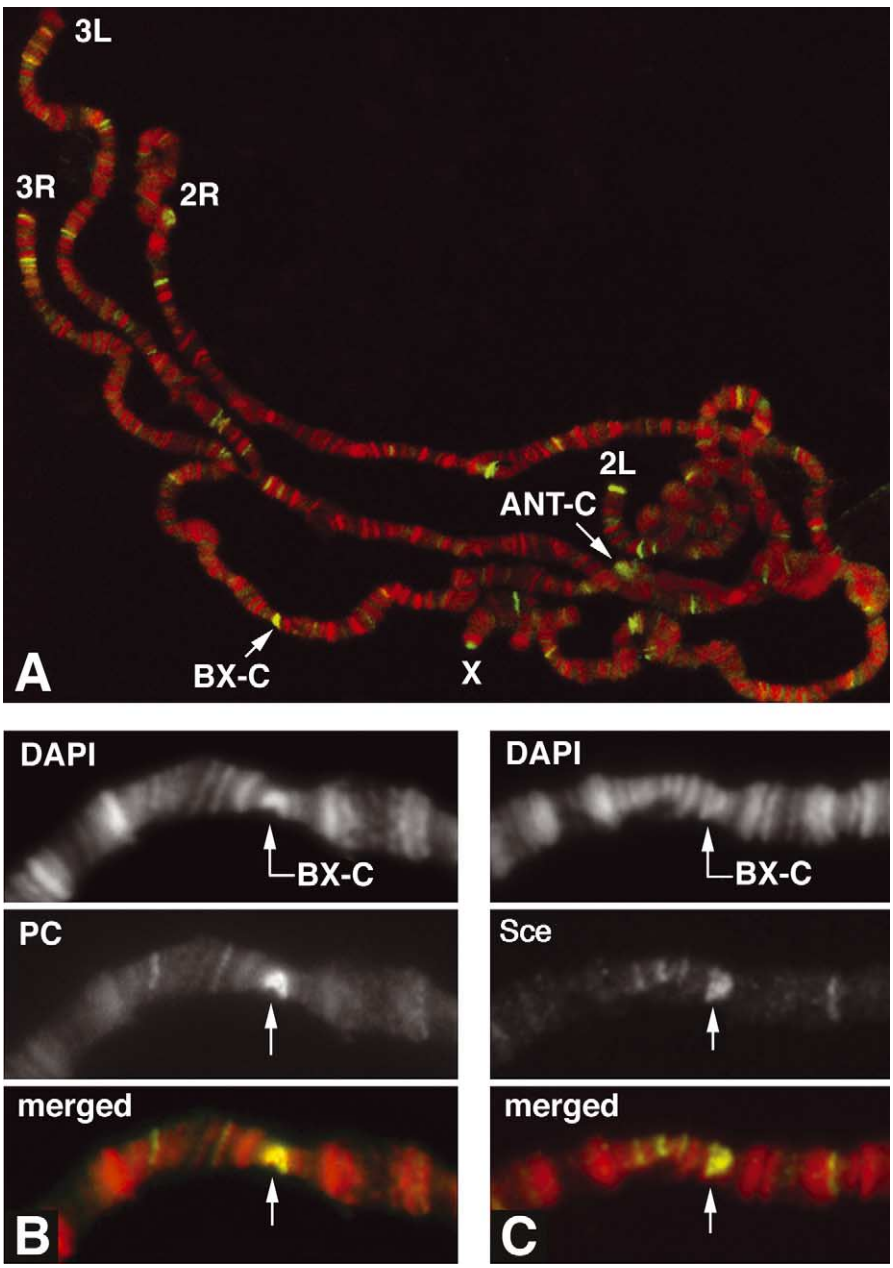

Fig. 4. Immunolocalization of Sce protein on salivary gland polytene chromosomes. (A) Merged image of a chromosomal spread, from wild type larva, stained with DAPI and with the anti-Sce antibody (DAPI in red; Sce in green). Arrows point at the ANT-C and BX-C complexes. (B and C) Enlarged sections of the third chromosome, stained with DAPI and with (B) anti-Pc or (C) anti-Sce antibodies. The immunopatterns clearly show that both the proteins are associated with the BX-C (arrows).

at least those between Ring and Pc and Psc are conserved in *Drosophila*.

3. Discussion

3.1. Sce encodes Drosophila Ring

Sce was identified as a gene required for regulation of BX-C genes. Sce^1 (m^-, z^-) embryos show posterior directed segmental transformations, and defects in head involution (Breen and Duncan, 1986; Fritsch et al., 2003; this work). Here, we have found that the Sce^1 allele bear a deletion that potentially results in a C-terminal truncated Ring protein. We also describe here *Df*(3*R*)*IR*16 (97F1–98A), a deficiency that uncovers the *Ring*/*Sce* locus. Embryos derived from Sce^1 mutant germ-line mothers crossed to *Df*(3*R*)*IR*16 males have a phenotype identical to Sce^1 (m^-, z^-) embryos. This strongly suggests that Sce^1 is a null mutant.

Table 2
Comparison of Sce with Pc/Ph/Pcl/Psc binding sites on polytene chromosomes

Chr. X	Sce	PcG proteins[a]	Chr. 2	Sce	PcG proteins[a]	Chr. 3	Sce	PcG proteins[a]
1A	+	Pc, Ph, Pcl, Psc	21AB	+	Pc, Ph, Pcl, Psc	61A	+	Pc, Ph, Pcl, Psc
2D	+	Pc, Ph, Pcl, Psc	21C	±	–	61C	+	Pc, Ph, Pcl
4C	+	Pc, Ph, Pcl, Psc	22A	+	Pc, Ph, Pcl, Psc	61D	±	Pc, Ph, Pcl
5A	+	Pc, Ph, Pcl, Psc	22B	+	Pc, Ph, Pcl, Psc	61E	+	–
5D	+	Pc, Ph, Pcl	22C	+	Pc, Ph, Pcl	61F	+	Pc, Ph, Pcl, Psc
7B	±	Pc, Ph, Pcl, Psc	22F–23A	−	Psc	62A	±	–
8A	+	Pc, Ph, Pcl, Psc	24A	+	Pc, Ph, Pcl, Psc	62F	−	–
8B	+	Pc, Ph, Pcl, Psc	24F	+	–	63E	+	Pc, Ph, Pcl, Psc
8E	+	–	25EF	+	Pc, Ph, Pcl, Psc	63F–64A	−	Pc, Ph, Pcl
8F–9A	+	Pc, Ph, Pcl, Psc	26F–27A	+	Pc, Ph, Pcl, Psc	64BC	−	Psc
12E	+	–	28A	+	Pc, Ph, Pcl	65CD	+	Pc, Ph, Pcl
13E	+	Pc, Ph, Pcl	29E	+	Pc, Ph, Pcl, Psc	66E	+	–
14B	+	Pc, Ph, Pcl, Psc	30B	+	Pc, Ph, Pcl	66EF	−	Pc, Ph, Pcl, Psc
16D	+	Pc, Ph, Pcl, Psc	30C	+	Pc, Ph, Pcl	66F	+	–
17A	−	Pc, Ph, Pcl	32EF	+	Pc, Ph, Pcl	67C	+	–
17E1,2	−	Pc, Ph, Pcl	33B	−	Pc, Ph, Pcl	67DE	+	Pc, Ph, Pcl, Psc
17F	−	Pc, Ph, Pcl	33F–34A	+	Pc, Ph, Pcl	68A	+	Pc, Ph, Pcl, Psc
18D	−	Psc	34C	+	Pc, Ph, Pcl	68B	±	–
19D	−	Pc, Ph, Pcl, Psc	34D	−	Pc, Ph, Pcl	69C	+	Pc, Ph, Pcl, Psc
			34F–35A	+	–	69D	+	Pc, Ph, Pcl, Psc
			35AB	+	Pc, Ph, Pcl, Psc	70AB	±	Pc, Ph, Pcl, Psc
			35D	+	–	70DE	+	Pc, Ph, Pcl, Psc
			36A	+	Pc, Ph, Pcl, Psc	70EF	+	–
			36B	−	Pc, Ph, Pcl	71F	+	Psc
			36C	+	–	72F	−	Psc
			36EF	+	–	74F	+	Psc
			37A	−	Psc	73F–74A	−	Psc
			37B	−	Pc, Ph, Pcl, Psc	75AB	+	–
						76C	−	Pc, Ph, Pcl, Psc
			37D	+	–	77E	+	Pc, Ph, Pcl, Psc
			38F	−	Pc, Ph, Pcl, Psc	78EF	+	Pc, Ph, Pcl, Psc
			39B	+	Pc, Ph, Pcl	79B	−	Pc, Ph, Pcl, Psc
			39EF	+	Pc, Ph, Pcl	82DE	+	Pc, Ph, Pcl, Psc
			41CD	+	Pc, Ph, Pcl	83C	−	Pc, Ph, Pcl
			42A	−	Psc	83D	+	–
			43BC	+	Pc, Ph, Pcl, Psc	84AB	+	Pc, Ph, Pcl, Psc
			43D	+	–	84D	+	Pc, Ph, Pcl, Psc
			44A	+	Pc, Ph, Pcl, Psc	84EF	−	Pc, Ph, Pcl, Psc
			44CD	+	–	84F	+	–
			45C	−	Pc, Ph, Pcl, Psc	85D	±	–
			46C	−	Pc, Ph, Pcl, Psc	85E	+	Pc, Ph, Pcl
			47AB	−	Psc	85EF	+	Pc, Ph, Pcl
			48A	+	Pc, Ph, Pcl, Psc	86C	+	Pc, Ph, Pcl, Psc
			49EF	+	Pc, Ph, Pcl, Psc	87B	+	Pc, Ph, Pcl, Psc
			50C	−	Psc	87BC	−	Pc, Ph, Pcl
			50D	+	–	87F–88A	+	Pc, Ph, Pcl, Psc
			51A	+	Pc, Ph, Pcl, Psc	88C	+	–
			51D	+	Pc, Ph, Pl	89B	+	Pc, Ph, Pcl, Psc
			55C	+	–	89C	+	Pc, Ph, Pcl, Psc
			55D	+	–	89E	+	Pc, Ph, Pcl, Psc
			56C	−	Pc, Ph, Pcl, Psc	90E	+	Pc, Ph, Pcl, Psc
			56D	+	–	93E	+	Pc, Ph, Pcl, Psc
			56E	+	–	94A	±	–
			57A	+	Pc, Ph, Pcl	94DE	+	Pc, Ph, Pcl, Psc
			57B	+	Pc, Ph, Pcl	95A	±	–
			57F	+	–	96A	+	–
			58CD	+	Pc, Ph, Pcl	96BC	+	Pc, Ph, Pcl, Psc
			59A	−	Pc, Ph, Pcl	96F–97A	+	Pc, Ph, Pcl
			59C	−	Pc, Ph, Pcl	97AB	+	–
			59F	+	PC, PH, PL, PSC	97DE	+	–
			60E	±	Pc, Ph, Pcl, Psc	98CD	−	Pc, Ph, Pcl
			60F	±	Pc, Ph, Pcl	99A	+	–

(*continued on next page*)

Table 2 (*continued*)

Chr. X	Sce	PcG proteins[a]	Chr. 2	Sce	PcG proteins[a]	Chr. 3	Sce	PcG proteins[a]
						99AD	−	Pc, Ph, Pcl, Psc
						99B	+	−
						99E	+	−
						100AB	+	Pc, Ph, Pcl, Psc
						100C	+	−

The identification of Sce chromosomal sites was been done after examination of 20 nuclei, and the intensities of the signals at the various sites is indicated by + (strong or moderate), ± (faint) and − (absence of signal).

[a] Localization of PcG proteins as reported (DeCamillis et al., 1992; Lonie et al., 1994; Martin and Adler, 1993; Rastelli et al., 1993; Sinclair et al., 1998; Zink and Paro, 1989).

Further evidence of the identity of *Drosophila Ring* and *Sce* is provided by rescue of the *Sce*^{1} embyonic phenotype when Ring protein is expressed from a transgene. Thus, in agreement with the presence of Ring in embryonic PcG complexes, our data support a PcG function for the Ring protein. Mice bearing null (*Ring*1/*Ring*1*A*) or hypomorphic (*Rnf*2/*Ring*1*B*) mutations had shown already an involvement of the *Ring* genes in the patterning of the antero-posterior axis (del Mar Lorente et al., 2000; Suzuki et al., 2002). However, in contrast to mutations in other vertebrate PcG genes, the alterations of the axial skeleton seen in the Ring mutant mice could not be associated clearly to a deregulation of *Hox* genes (del Mar Lorente et al., 2000). Therefore, the role of vertebrate Ring proteins as genuine PcG proteins is strengthened by our data showing a genetic evidence for a PcG function for Sce.

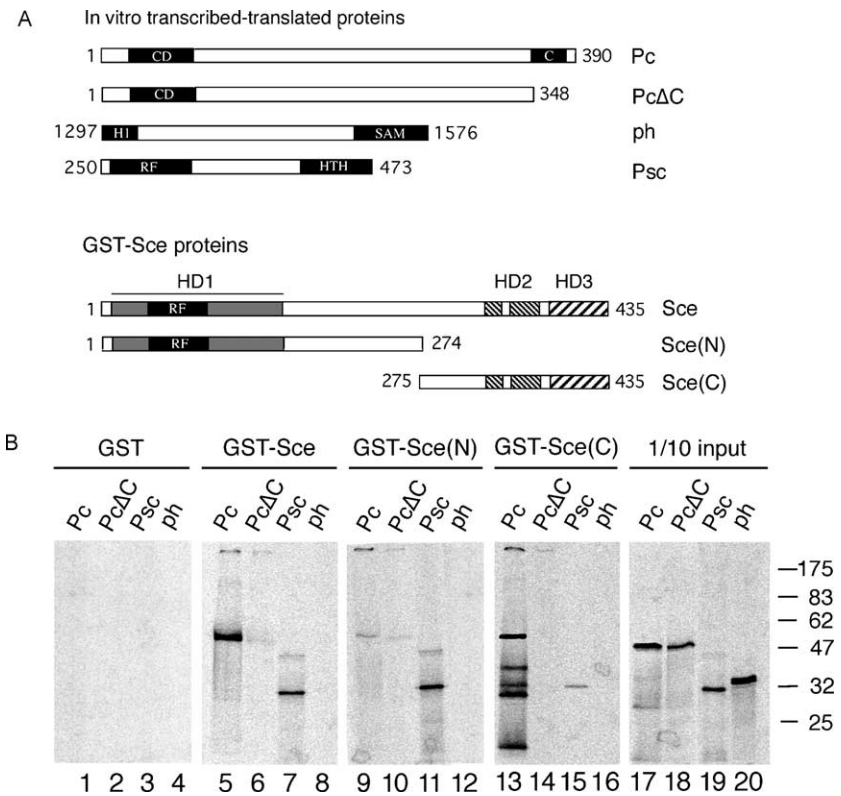

Fig. 5. Interactions between Sce and PcG proteins. (A) Diagram of the proteins used in the interaction assays. The conserved protein motifs in the in vitro transcribed–translated proteins are indicated by black boxes. These include: the chromodomain (CD) and carboxyl domains (C) of Pc; the homology region 1 (H1) and the self-association motif (SAM) of Ph; the ring finger (RF) and helix-turn-helix (HTH) motifs of Psc. The conserved motifs of Sce are indicated by shaded and stripped boxes, whereas the ring finger (RF) of the homology domain 1 (HD1) is indicated by a black box. Open boxes represent non-conserved regions. Amino acids are numbered in the various full length (Pc, Sce) and truncated [PcΔ, Ph, Psc, Sce(N) and Sce(C)] proteins. (B) Phosphorimager analysis of the indicated proteins bound to GST, GST-Sce and their derivatives after SDS-PAGE. Input represents 10% of the total [^{35}S]-labelled protein used in the interaction assay. Sizes of molecular weight (in kDa) are indicated on the right.

3.2. Functional and structural conservation of Ring proteins

Cross-species complementation experiments with *PcG* genes show contrasting results. Thus, *M*33, the mouse ortholog of *Drosophila Pc*, was shown to rescue the *Pc* mutant phenotype in early embryos (Muller et al., 1995). However, *eed*, the mouse ortholog of *Drosophila extra sex combs* (*esc*) is not only unable to rescue the embryonic lethality of *esc* embryos but show a dominant negative effect on the leg transformation phenotype of *esc* mutants (Wang et al., 2002). It has been suggested that the activity of *eed* in *Drosophila* cells is related to its inability to interact with E(z). Here, we show that mouse Ring1/Ring1A rescues the cuticle phenotype of *Sce* embryos, indicating that in early development, at least, the function of Ring is conserved between mice and flies. This might be due to the structural conservation of Ring proteins. The three domains conserved in Ring1/Ring1A and Rnf2/Ring1B are also present in Sce and constitute about 57% of this protein. Whereas the size and degree of conservation of the domains HD2 and HD3 are similar to other protein motifs identified in fly and vertebrate PcG proteins, domain HD1 is somewhat exceptional. This is a 147 amino acids domain of which 78% of them are identical in fly and vertebrate proteins, particularly in the RING finger motif. An indication of the relevance of the functionality of this region of Ring proteins is the Sce^{33M2} allele which shows a phenotype much milder than that Sce^{1} but that is due to a Ring protein with a single amino acid alteration in that region (Fritsch et al., 2003). The overall structural conservation between Ring proteins seems to dictate a conservation of interaction with other PcG proteins. In addition, we have shown that Sce interacts with Pc and Psc. In fact, the core of a PRC1 complex isolated from human cells is compositionally similar to that of flies and the biochemical activity of both complexes is similar (Levine et al., 2002).

Despite this conservation, it is possible that Sce serve diverse functions in late development. For example, expression of the mouse M33 protein in flies does not rescued the *Pc* adult phenotype (Muller et al., 1995). Our experiments have not addressed the activity of vertebrate Ring proteins at these later developmental stages and, therefore, whether vertebrate Ring proteins can fully substitute Sce needs to be approached experimentally.

3.3. Sce binding to polytene chromosomes

Previous genetic and biochemical evidence showed that PcG proteins act as protein complex(es) (Franke et al., 1992; Ng et al., 2000; Shao et al., 1999). Here, we have demonstrated that Sce interacts directly with Pc and Psc, but not with a Ph-fragment, which binds mouse Rnf2/Ring1B. In addition, our immunolocalization studies show that Sce binds to approximately 100 sites, which are in part shared by Pc, Psc, Ph, Pcl and Asx binding sites (DeCamillis et al., 1992; Lonie et al., 1994; Martin and Adler, 1993; Rastelli et al., 1993; Sinclair et al., 1998; Zink and Paro, 1989), including the ANT-C and BX-C complexes. These results are consistent with the presence of Sce in the PRC1 complex. However, almost a third of the sites that bind Sce do not bind any of the other PcG proteins. This contrasts with the observation that most Sce molecules in cell extracts are found complexed with PcG proteins in the PRC1 complex (Saurin et al., 2001). The discrepancy, however, may be related to the fact that the characterized PRC1 has been isolated from *Drosophila* embryos, whereas the Sce chromosomal sites correspond to binding sites in salivary glands from larvae. Psc, another component of the PRC1 complex, is also found in sites, which do not have Pc/Ph/Pcl (Martin and Adler, 1993). It is worth noting that, despite of the ability of Sce to interact with Psc, no Sce is found at these unique Psc sites. Nevertheless, some of these sites correspond with predicted PREs (Ringrose et al., 2003). Therefore, the partial overlapping patterns of Sce and other PcG binding sites suggest the existence of different Polycomb complexes in a tissue specific and developmentally controlled manner (Orlando et al., 1998; Soto et al., 1995; Strutt and Paro, 1997). An indication of complexes containing subsets of PcG proteins comes from studies in vertebrates where *Drosophila* Ring proteins are found together with other polypeptides but not Pc or Ph homologs (Ogawa et al., 2002).

An intriguing result of our studies on the chromosomal binding sites of Sce is that, in contrast to all PcG genes so far studied, the cytological localization of the *Sce* gene (98A in this study or 98B in www.flybase.org) is free of any PcG protein. The absence of PcG proteins at 98A, therefore, suggests that *Sce* is regulated somehow differently from other PcG loci.

In summary, in this study, we show that the PcG gene *Sce* encodes the *Drosophila* ortholog of mammalian Ring proteins. We also show that the product of the *Sce* gene binds to Pc and Psc and that it is a chromosomal protein associated to many sites in polytene chromosomes which also bind PcG proteins. Finally, we find that *Sce* is expressed and required throughout development and that the extreme *Pc* phenotype of *Sce* embryos is rescued by ectopic expression of *Drosophila* Ring/Sce and Ring1/Ring1A suggesting that the function of these proteins in conserved between flies and mammals, at least in the early stages of fly development.

4. Experimental procedures

4.1. Molecular cloning of genomic Drosophila Sce

Genomic DNA was isolated from wild type and Sce^{1} heterozygous larvae. Sequences spanning the coding region of *Drosophila Ring* were amplified using the following primers: 5′-GC CTC AGA ATT GGT GTG

AAA *ATG* AC-3′ (the conceptual starting codon is in italics) and 5′-TAG CGA GG ATT CCG AAA ACT CA-3′ which spans sequences 160 nucleotides 3′ to the conceptual stop codon. Wild type DNA produced a 1.4 kb PCR product, whereas *Sce*^{1}/+ DNA produced an additional 1.0 kb PCR product. PCR products were subcloned into pGEMTeasy plasmids for sequencing and further molecular manipulation.

4.2. Drosophila strains and phenotypic analysis

The wild type flies used were Oregon-R. The *Sce*^{1} strain (Breen and Duncan, 1986) was obtained from Duncan. Homozygous *Sce*^{1} embryos (between 16 and 24 h of development) were selected by using the *Kr-GFP*-tagged TM3 balancer chromosome (Casso et al., 2000). To generate maternal and zygotic *Sce*^{1} mutants (m^{-}, z^{-}), we induced germ-line clones in female flies with the following protocol: flip-out recombination for germ-line clones was induced in second instar larvae by a 30 min heat-shock at 37 °C in the progeny of *FRT*82*B Sce*^{1}/*TM6B* females crossed to *FLP*; *FRT82 ovo*^{D1}/*TM6B* males. The descendant females containing *Sce*^{1} germ-line clones were crossed to *Sce*^{1}/*TM6B* males. For the rescue experiments, female flies of the genotype *UAS-Sce/CyO*; *FRT*82*B Sce*^{1}/*TM6B* were crossed to *FLP*; *FRT82 ovo*^{D1}/*TM6B* males, and flip-out recombination for germ-line clones was induced as described above. From the F1, *FLP*/+; *UAS-Sce*/+; *FRT*82 *Sce*^{1}/*FRT*82 *ovo*^{D1} females were selected and crossed to *arm-GAL4*; *Sce*^{1}/*TM3*, *Kr-GFP* or *paired-GAL4*, *Sce*^{1}/*TM3*, *Kr-GFP* males. Non-GFP expressing embryos were selected for cuticle preparations 24 h after egg deposition. The same procedure was used to study the phenotypic rescue with *UAS-Ring*1*A*.

To study the *Sce*^{1} requirement during larvae development f^{-}, *FLP*122; *FRT*82*B* f^{+}/*TM6B* or *FLP*122; *FRT*82*B ubi-GFP*/*TM6B* were crossed with *FRT*82*B Sce*^{1}/*TM6B* females. Clones were generated by *FLP*-mediated mitotic recombination. Larvae of the corresponding genotypes were incubated at 37 °C for 1 h at 24–48 h after egg laying (AEL), or for 45 min at 48–72 h AEL.

4.3. Plasmids

The *Drosophila Sce* cDNA and its truncated variants were obtained by manipulation of *Drosophila* LD3177 clone purchased from Research Genetics. Recombinant proteins were isolated as GST fusions produced from pGEX4-T1 plasmids or as Maltose-binding protein (MBP) fusion produced from a pMalc2 plasmid. A *Drosophila* cDNA encoding both a full length PC protein and a truncated version lacking amino acids 349–390 were obtained by PCR from a *Drosophila Pc* cDNA obtained from Paro and Hogness (1991) and subcloned into pCITE-4a. Truncated Ph and Psc cDNAs were also subcloned in pCITE-4a as restriction fragments from plasmids phHD and pPSCHD, respectively, obtained from Kyba and Brock (1998b). The *Sce* and *Ring*1*A* transgenes to be expressed under the Gal4 control were obtained by sub-cloning the corresponding Myc-tagged full-length cDNAs in to the pUAST vector (Brand and Perrimon, 1993). The integrity of PCR fragments was verified by sequencing. Detailed descriptions of these plasmids are available upon request.

4.4. Antibody production and western blot analysis

Antibodies against *Drosophila* Sce were obtained using a GST-Sce (amino acids 1–274) protein produced in *E. coli* BL21 (DE3) and purified as described (Schoorlemmer et al., 1997).

To isolate monospecific antibodies an affinity column was prepared by coupling purified MBP-Sce (amino acids 1–274) to CNBr-activated Sepharose (Sigma Chemical Co.). The specificity of the antibodies was demonstrated by the detection of bands of the expected molecular mass on Western blots of *Drosophila* embryo extracts and of transfected mammalian tissue culture cells. Also, depletion experiments showed loss of immunoreactivity on Western blot after preincubation of the antibody with the immunogen.

Total extracts from *Drosophila* imaginal discs were prepared by homogenization in SDS-PAGE Laemmli's buffer, respectively. Western blot analysis was performed as described (Schoorlemmer et al., 1997), using horseradish peroxidase-conjugated goat anti-rabbit IgG antibodies (Bio-Rad) and a chemiluminescence kit (SuperSignal, Pierce). Extracts from imaginal discs over-expressing the Sce protein fused to a Myc tag were used as a control.

4.5. In situ hybridization and immunohistochemistry to Drosophila embryos and imaginal discs

A non-radioactive procedure described by Tautz and Pfeifle (1989) with some modifications, was used to detect *Sce* mRNA in embryos and imaginal discs. The *Drosophila* LD3177 cDNA, in pBluescript II SK, was used to prepare a riboprobe labelled in vitro with digoxigenin using a T7 polymerase and a digoxigenin labelling mix from Roche Molecular Biochemical. The RNA probe was cleavaged to an average 500 nt using a bicarbonate buffer. Embryos and imaginal discs were stained with anti-Sce antibody following standard protocols.

4.6. Fluorescence in situ hybridization and immunostaining of polytene chromosomes

Cytological preparations and fluorescence in situ hybridization (FISH) experiments were carried out as described in Pimpinelli et al. (2000). Probes were labelled using digoxigenin-11-dUTP and detected by rhodamine-conjugated antidigoxigenin (Roche). Immunofluorescence analyses of polytene chromosomes were performed according

to James et al. (1989). The anti-Sce antibodies were detected by fluorescein linked anti-rabbit Ig secondary antibody (Amersham). Digital images were obtained using a computer-controlled Nikon E 1000 epifluorescence microscope equipped with a cooled CCD camera (Coolsnap). The different fluorescent signals, detected using specific filters, were recorded separately as grey-scale images. Pseudo-colouring and merging of images were performed using Adobe Photoshop software.

4.7. In vitro transcription–translation and GST protein binding assay

Intact or truncated cDNAs were subcloned in the pCITE4-1 vector (Novagen). RNA was synthesized with 500 ng of supercoiled plasmids and translated in the presence of 40 μCi of [^{35}S] Met (10 mCi/ml, 800 Ci/mmol, New England Nuclear) using a rabbit reticulocyte lysate (Promega Co.). For the GST pull down assay 15 μl of a 1:1 suspension of GSH-agarose (Sigma Chemical Co.) and bacterial extracts prepared as described (Garcia et al., 1999) containing equivalent amounts of either GST alone or GST-Sce fusion proteins were mixed and rotated at 4 °C for 30 min. Agarose beads were washed three times with 0.02 M Hepes-KOH, pH 7.9, 0.1% NP-40, 0.15 M NaCl, 1 mM DTT and protease inhibitors (EDTA free Complete, Roche Molecular Biochemicals). The beads were then re-suspended in 200 μl of the same buffer, containing 1–4 μl of the in vitro translation mixtures and 1% bovine serum albumin. After rotation for 1 h at 4 °C, the beads were washed twice with 1 ml of buffer, transferred to fresh tubes and washed again. Bound proteins were eluted in 20 μl of loading buffer and separated in SDS-polyacrylamide gels. Dried gels were analysed using a Phosphorimager (Molecular Dynamics).

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