

Spps, a *Drosophila* Sp1/KLF family member, binds to PREs and is required for PRE activity late in development

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SUMMARY

The Polycomb group of proteins (PcG) is important for transcriptional repression and silencing in all higher eukaryotes. In *Drosophila*, PcG proteins are recruited to the DNA by Polycomb-group response elements (PREs), regulatory sequences whose activity depends on the binding of many different sequence-specific DNA-binding proteins. We previously showed that a binding site for the Sp1/KLF family of zinc-finger proteins is required for PRE activity. Here, we report that the Sp1/KLF family member Spps binds specifically to *Ubx* and *engrailed* PREs, and that Spps binds to polytene chromosomes in a pattern virtually identical to that of the PcG protein, Psc. A deletion of the *Spps* gene causes lethality late in development and a loss in pairing-sensitive silencing, an activity associated with PREs. Finally, the *Spps* mutation enhances the phenotype of *pho* mutants. We suggest that Spps may work with, or in parallel to, Pho to recruit PcG protein complexes to PREs.

KEY WORDS: Sp1/Klf family members, Gene expression, Polycomb group genes

INTRODUCTION

Polycomb group genes (PcG) are an evolutionarily conserved group of proteins that were originally identified as repressors of homeotic genes in *Drosophila*. PcG gene products act as multi-protein complexes associating with chromatin through DNA elements called Polycomb group response elements (PREs), to repressively modify histones (for reviews, see Schwartz and Pirrotta, 2008; Müller and Verrijzer, 2009). Initially, two major PcG complexes were biochemically isolated, Polycomb repressive complexes 1 and 2 (PRC1 and PRC2). PRC1 core is made up of the PcG proteins Polycomb (Pc), Polyhomeotic (Ph), Posterior sex combs (Psc) and dRing/Sex combs extra (Sce) (Shao et al., 1999; Francis et al., 2001). The Pc subunit has a chromodomain that binds to H3K27me3 and dRing has H2A mono-ubiquitin ligase activity (Lagarou et al., 2008). PRC2 comprises Enhancer of Zeste [E(z)], extra sex combs (Esc) or Esc-like, Suppressor of zeste 12 [Su(z)12] and Nurf 55 (Cao et al., 2002; Czermin et al., 2002; Kuzmichev et al., 2002; Müller et al., 2002), and mediates histone H3K27 tri-methylation via the methyl-transferase activity of E(z). More recently, additional PcG-protein complexes have been isolated. These include dRAF and Pcl-PRC2. dRaf contains PSC and dRing in a complex with dKDM2, a histone demethylase (Lagarou et al., 2008). Pcl-PRC2 is required to generate high levels of H3K27 tri-methylation at PcG target sites (Nekrasov et al., 2007). In addition, Pcl may be important for recruitment of PcG protein complexes in larvae (Savla et al., 2008). The identification of PcG protein complexes is an area of intense investigation and it is likely that additional complexes will be identified. Given that not all PcG mutants have the same phenotype (Soto et al., 1995), it is likely that not all PcG targets are regulated by the same constellation of proteins.

Pleiohomeotic (Pho) is a PcG member that has specific DNA-binding activity and is thought to be important in recruiting PcG complexes to PREs (Brown et al., 1998; Fritsch et al., 1999). Pho has been shown to bind E(z), Esc and Pc in vitro, and thus could recruit PRC1 and PRC2 through direct protein-protein interactions (Mohd-Sarip et al., 2002; Mohd-Sarip et al., 2005; Wang et al., 2004). Pho is not a stable component of either PRC1 or PRC2 but it is found in a complex (PhoRC) with the PcG protein, dSfmbt (Klymenko et al., 2006). dSfmbt selectively binds to mono- or dimethylated H3K9 or H4K20, and may contribute to PcG repression by interacting with methylated histones in the chromatin flanking the PRE. dSfmbt can also physically and genetically interact with Sex combs on middle leg (Scm), a related MBT-repeat protein that can interact with Ph (Grimm et al., 2009) and associates substoichiometrically with PRC1 (Saurin et al., 2001; Peterson et al., 2004). Thus, in principle, PhoRC could recruit PRC1 to the DNA through an interaction with Scm. Although there are a lot of models, the reality is that very little is known about how PcG protein complexes become recruited to PREs.

Functional studies show that Pho-binding sites are a major determinant of PREs. Consistent with this, genome-wide ChIP-on-ChIP studies show that Pho is present at most or all PREs (Oktaba et al., 2008; Kwong et al., 2008; Schuettengruber et al., 2009). However, Pho-binding sites alone are not sufficient for PRE activity. PREs are made up of binding sites for many different proteins. Other proteins suggested to be important for PRE activity include GAGA factor (GAF), Pipsqueak, Dsp1, Zeste and Grainyhead/NTF1 (for a review, see Müller and Kassis, 2006). It is unclear what exactly these factors do at PREs; the genome-wide ChIP-on-ChIP data suggest that GAF, Dsp1 and Zeste binding does not closely correlate with DNA fragments bound by other PcG proteins, although some overlap is present. To date, Pho is the DNA-binding protein that tracks most closely with other PcG proteins (Schuettengruber et al., 2009; Nègre et al., 2006; Kwong et al., 2008; Oktaba et al., 2008).

Our interest has been to understand how PcG complexes are recruited to PREs, what constitutes a PRE and, in particular, how PREs work in the context of regulation of the *engrailed* gene. To this

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end, we have focused our analysis on an 181 bp PRE (–576 to –395 bp) upstream of the *engrailed* gene. Our approach has been to identify DNA-binding sites within this PRE that are important for PRE function and subsequently identify the proteins that bind to them. Importantly, this approach should allow us to identify proteins with redundant activities that may be missed by screening for mutations that cause PcG phenotypes. We previously reported that a Sp1/KLF binding site is required for the activity of the 181-bp *engrailed* PRE and that Sp1/KLF binding sites are present in all known PREs (Brown et al., 2005). Here, we describe the identification of a protein that binds this site: Spps. In polytene chromosomes, Spps is bound to the same chromosome bands as the PcG protein Psc. Furthermore, Spps is bound to the *en* and *Ubx* PREs. Finally, mutation of *Spps* suppresses pairing-sensitive silencing, an activity associated with PREs, and enhances the phenotype of *pho* mutants. These data show that Spps is important for PRE activity. We suggest it may play a role in recruiting PcG proteins to PREs.

MATERIALS AND METHODS

Antibodies

Rabbit polyclonal antibodies were raised against a gel purified HIS-Tag fusion protein that expresses amino acids 267 to 968 of Spps. Squashes and immunofluorescent staining of polytene chromosomes were performed as described previously (Brown et al., 2003) with the following changes. Primary antibodies were used at: α Pho, 1:200; α Psc, 1:25; α Spps, 1:200; α -mouse Alexafluor 488 (1:400) and α -rabbit Alexafluor 555 (1:500) were used as secondary antibodies.

Chromatin immunoprecipitation

The ChIP in S2 cells was performed using methods and *Ubx* primer sets described previously (Wang et al., 2004). The primer sequences for PCR over the *en* region are available on request. For ChIP from larval tissues, imaginal disks, brains and some cuticle were harvested from 40 larvae (2 \times 20 larvae) for each ChIP experiment. The 3rd instar larvae were dissected in PBS and then stored on ice in S2 culture media (GIBCO). The media was removed and the disks were fixed in 2% Ultrapur formaldehyde in 50 mM HEPES (pH 7.6), 100 mM NaCl, 0.1 mM EDTA, 0.5 mM EGTA with protease inhibitors for 15 minutes at room temperature. The disks were washed for 5 minutes with stop solution (1 \times PBS, 0.01% Triton X-100, 125 mM glycine) followed by two 5 minute washes in 50 mM Tris-HCl (pH 8.0), 10 mM EDTA, 0.5 mM EGTA, 0.25% Triton X-100. At this stage, the disks could be frozen at –80°C [in 10 mM Tris-HCl (pH 8), 1 mM EDTA, 0.5 mM EGTA] or used immediately by replacing the wash buffer with 300 μ l reaction buffer [50 mM Tris-HCl (pH 7.4), 1 mM EDTA]. The disks were homogenized then sonicated with a Biorupter sonicator according to the manufacturers' instructions. The debris was spun out, the 2 \times 300 μ l samples were pooled and an input control sample was removed. The remaining volume was divided into three equal aliquots and ChIP was carried out using the Millipore ChIP kit following the protocol supplied by the manufacturer. Pho and Spps antibodies were used at 1:200 dilutions. qPCR was performed on a Roche 480 Lightcycler with the 480 SYBR Master mix using standard protocols.

Oligos for larval qPCR

Oligos used were as follows: En PRE, GCTTATGAAAAGTGTCTGTG and GGGGCTTGTAGGCAGCAAT; En gene control, CGCCTA-AGGTGAGATTCAGTT and GGCGGTGTCATATTTTGGT; PRE_D, CGAAATGCTACTGCTCTCTA and GCGTAGTCTTATCTGTACT; *Ubx* non PRE, CCAGCATAAAACCGAAAGGA and CGCCAAACATTCA-GAGGATAG.

Targeted knockout of Spps

We used the ends out recombination strategy of Gong and Golic (Gong and Golic, 2003) to replace the Spps genomic region with GFP under the control of the Armadillo promoter (ARM). Flanking DNA (2 kb) from either side of the *Spps*-coding region (*CG13609* and *cav*) was amplified by

PCR and cloned into the zero blunt TOPO vector (Invitrogen). *CG13609* was flanked with *EcoRI* and *KpnI* sites and *cav* was flanked with *SpeI* sites. The two flanking sequences and intervening ARM/GFP was subcloned into pW30 as a *NotI-KpnI* fragment (Gong et al., 2005). The exact nature of the *Spps*¹ targeted knockout mutation was confirmed by PCR analysis and sequencing.

Generation of germline clones and other genetic crosses

To generate germline clones, *w*; *P{neoFRT}82B Spps¹/TM6B*, *Tb¹* virgin females were crossed to *yw hsFLP*; *P{neoFRT}82B P{ovoD1-18}3R/TM3*, *Sb¹* males. After 1 day, parents were transferred to a new vial and progeny were aged for 1 day and then heat-shocked at 37°C for 1 hour on 2 consecutive days (Chou and Perrimon, 1996). Virgin females of the genotype *yw hsFLP*; *P{neoFRT}82B Spps¹/P{neoFRT}82B P{ovoD1-18}3R* were crossed to *w*; *Spps¹/TM6B*, *Tb¹* males. Progeny were collected for embryo staining, polytene chromosome squashes and to assess the lethal phase.

To assess the genetic interaction of *pho* and *Spps*, a *w*; *Spps¹/TM6B*, *Tb¹*; *pho¹/ci^D* stock was made and progeny were analyzed. *pho¹* homozygotes normally die as pharate adults with a PcG phenotype (sex combs on the second and third legs visible through the pupal cases in pharate adults). *Spps¹/TM6B*, *Tb¹*; *pho¹/pho¹* pharate adults with a PcG phenotype were among the progeny of *w*; *Spps¹/TM6B*, *Tb¹*; *pho¹/ci^D*. However, we noticed that all *Spps¹/Spps¹* pharate adults lack the PcG phenotype. This suggested that either *Spps¹/Spps¹*; *pho¹/pho¹* do not survive to the pharate stage, or that *Spps* suppresses the *pho* phenotype. In order to determine which was the case, we tested 40 *Spps¹/Spps¹* pharate adults for the presence of the homozygous *pho¹* mutation by PCR (Brown et al., 1998). No homozygous *pho¹* mutants were detected, showing that *Spps¹/Spps¹*; *pho¹/pho¹* animals do not survive to the pharate adult stage.

To test whether the pharate adult lethal phase of *Spps¹* homozygotes was due to the mutation of *Spps* and not another mutation on the chromosomes, *Spps¹/TM6B*, *Tb¹* flies were crossed to two different deficiency stocks, *Df(3R)crb87-4 st e/TM6C* and *w¹¹¹⁸*; *Df(3R)Exel6198*, *P{XP-U}Exel6198/TM6B*, *Tb¹*. In both cases, *Spps¹/Df* flies died as pharate adults with no obvious transformations. To test whether the suppression of mini-white silencing also mapped to the *Spps* mutation, *w*; *PRE-mw-1/PRE-mw-1*; *Spps¹/TM3*, *Ser* virgins were crossed to *w*; *PRE-mw-1/+*; *Df(3R)crb87-4 st e/+* males. Like *PRE-mw-1/+* and *PRE-mw-1/PRE-mw-1*; *Spps¹/Spps¹* pharate adults, *PRE-mw-1/+* and *PRE-mw-1/PRE-mw-1*; *Spps¹/Df(3R)crb87-4 st e* pharate adults had red eyes, showing that the suppression of mini-white silencing was due to the *Spps¹* mutation and not to another mutation on the chromosome.

To express Spps under the control of enGAL4, we used line *P{Mae-UAS.6.11}CG5669[DP01353]*, an EP-line (Staudt et al., 2005) with the EP-element inserted just upstream of the *Spps* transcription start site, and crossed it to enGAL4.

RESULTS

We have previously shown that a Sp1/KLF-binding site is required for activity of a 181 bp PRE from the *Drosophila en* gene in two assays: pairing-sensitive silencing and maintenance of repression of a reporter gene in embryos (Americo et al., 2002; Brown et al., 2005). There are nine members of the Sp1/KLF family in *Drosophila*. Of the nine members, six of the proteins showed strong in vitro binding to the Sp1 site in the 181 bp PRE (Brown et al., 2005). These included: Bteb2, D-Sp1, btd, CG5669, CG12029 and CG9895. Of these genes, D-Sp1 and btd are involved in the development of the head, mechanosensory bristles and ventral imaginal disks (Schock et al., 1999; Wimmer et al., 1996; Estella et al., 2003), and it is unlikely that they play a role in Polycomb recruitment in general. Bteb2 and CG12029 are not expressed ubiquitously, a property of most PcG genes. We have not been able to detect the expression of CG9895 in embryos. Thus, we have concentrated our efforts on CG5669. Because of the phenotype of the CG5669 mutant (see below), we

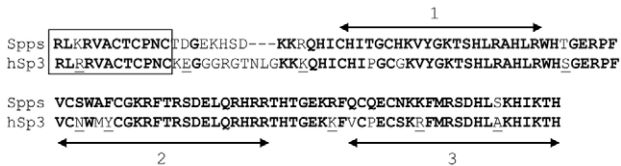


Fig. 1. Amino acid sequence comparison between *Drosophila* Spps and human Sp3. The boxed amino acids are the btd box, a domain present in Sp-subfamily members (Suske et al., 2005). The double-headed arrows indicate the extent of the three zinc-finger domains. Amino acids in bold are conserved between the two proteins. Underlined amino acids have similar properties.

have named this gene *Spps* (*Sp1-like factor for Pairing Sensitive-silencing*). *Spps* protein localization and mutant phenotype suggest it is important for PRE activity.

Of the over 20 mammalian Sp1/KLF family members, *Spps* shares the greatest sequence similarity to Sp1, Sp3 and Sp4. The homology spans the zinc-finger domains and a small region N-terminal to the zinc fingers that matches the consensus for a buttonhead (btd) box, a conserved region of unknown function also present in the btd protein. Over a 106 amino acid stretch of *Spps* there is 79%, 81% and 83% identity with the human Sp1, Sp3 and Sp4 factors, respectively. The sequence comparison of *Spps* with human Sp3 is shown in Fig. 1. The sequence similarity of *Spps* to the human factors is greater than the similarity to any of the other *Drosophila* Sp1/KLF family members.

Spps RNA is maternally deposited and ubiquitously expressed throughout embryogenesis (data not shown), an expression pattern that is typical of most PcG proteins. We made an antibody against the bacterially expressed C-terminal two-thirds of the *Spps* protein and affinity purified it for use in the studies described here. *Spps* is a nuclear protein, expressed ubiquitously throughout embryonic and larval development (Fig. 2 and data not shown). In Fig. 2A, the *Spps* is seen in all nuclei of the anterior half of a stage 13 embryo. The nuclear localization of *Spps* may be more easily seen in Fig. 2B. Here, *Spps* expression is under the control of an enGAL4 driver, causing its accumulation in a striped pattern and in particular cells of the nervous system. This confirms that our antibody is able to detect the *Spps* protein. In addition, we wanted to know whether our antibody was specific for the *Spps* protein, especially as the antigen we used contains the C-terminus, which includes the zinc-finger region that contains sequences similar to eight other Sp1/KLF family members. Importantly, in maternal-zygotic *Spps* mutants, that contain a deletion of the *Spps*-coding region, we detected no nuclear staining with the affinity-purified *Spps* antibody (Fig. 2C,D) (generation of this mutant is described below). Thus, our antibody is specific for *Spps*.

Does *Spps* associate with Polycomb targets?

We analyzed the pattern of binding of *Spps* to the polytene chromosomes of *Drosophila* in comparison with the binding pattern of the PcG protein Psc. It is striking that the patterns of binding of Psc and *Spps* are virtually identical, both in location and in intensity (Fig. 3A). This suggests that *Spps* functions at the same targets as Psc. This complete correlation in localization and intensity of polytene bands with Psc is somewhat different from that seen with polytene chromosomes double labeled with antibodies against Pho and Psc (Brown et al., 2003) (Fig. 3B). Although most of the bands of Pho and Psc are coincident, there are often differences in the intensity of staining relative to other bands in the spread. Some examples of this are highlighted in the last panel in Fig. 3B. In

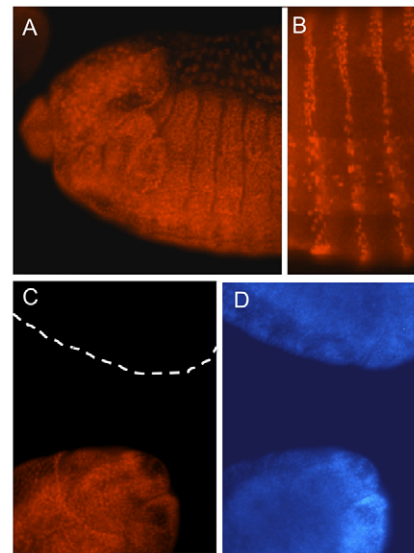


Fig. 2. *Spps* is a ubiquitous nuclear protein. (A) Anterior half of a stage 13 embryo (anterior leftwards, dorsal upwards), showing ubiquitous staining of *Spps* antigen. (B) Ventral view of abdominal segments of a stage 16 embryo expressing *Spps* via an enGal4 driver that causes *Spps* to be expressed in stripes and in specific cells of the CNS. This shows that the *Spps* antibody can detect *Spps*. (C,D) Embryos derived from *Spps* germline clones, stained with *Spps* antibody (C) and DAPI (D). The lower embryo received a wild-type *Spps* gene from the father, whereas the upper embryo received a mutant *Spps*1 chromosome from the father and thus has no *Spps* antigen. The absence of *Spps* staining in the upper embryo (broken line in C) shows the specificity of the *Spps* antibody for *Spps*. Both embryos are stage 15. The anterior end of the lower embryo and the lateral posterior end of the upper embryo are shown.

addition, there are polytene bands that have only Pho or Psc bound. Thus, *Spps* shows an even more specific association with Psc than does Pho, a protein known to be important for PRE activity.

Sp1/KLF-binding sites are present in all known PREs; therefore, we investigated whether we could show a specific association of *Spps* with PREs by chromatin immunoprecipitation in S2 cells and in larvae. First, we looked at the *en* 181 bp PRE and the 2 kb upstream of this that also exhibits PRE activity (see Fig. 4) (Kassis, 1994). The pattern of CHIP for *Spps* corresponds to the pattern of CHIP that we see with Pho, Ph and Pc at this PRE. In addition, we tested whether *Spps* binds to another well studied PRE, PRE_D from *Ubx* (Fritsch et al., 1999). *Ubx* PRE_D also contains a consensus-binding site for Sp1/KLF factors (Brown et al., 2005). As in the *en* PRE, there is an excellent correspondence between *Spps*, Pho, Ph and Pc binding to *Ubx* PRE_D. We also used CHIP followed by qPCR on larval samples and found that, like Pho, *Spps* binds to the *en* and *Ubx* PREs in larvae (Fig. 4E,F). *Spps* is therefore a very good candidate for a DNA-binding protein involved in the PcG response.

Generation of a *Spps* mutant

We generated a deletion of the *Spps*-coding region using the end-out recombination system developed by Gong and Golic (Gong and Golic, 2003). We replaced endogenous *Spps* with GFP under the control of the Armadillo promoter. The nature of the mutation was confirmed by PCR analysis, showing that the Arm-GFP is inserted at the expected location and that *Spps* is absent (data not shown).

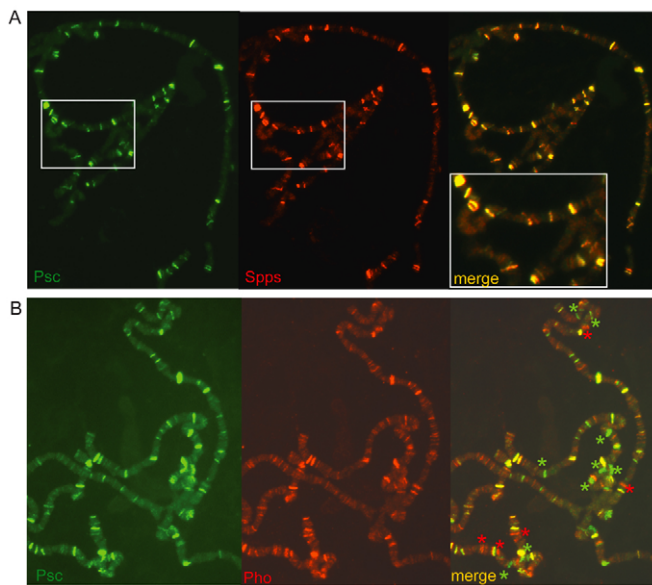


Fig. 3. Immunostaining of polytene chromosomes. (A) Green and red panels show the binding of antibodies against Psc and Spps respectively. The third panel shows the merged images. Virtually all of the bands are coincident. Boxed regions are magnified in the merged figure. (B) Green and red panels show the binding of antibodies against Psc and PHO proteins respectively. The third panel shows the merged images. The green asterisks represent some of the positions where Psc is bound at a higher incidence than PHO based on the comparable intensities of other bands nearby. The red asterisks represent positions where PHO is bound at a higher incidence than Psc when compared with the intensities of nearby bands.

The genes on either side of *Spps* were sequenced in the mutant to confirm that the open reading frame of these two genes (*CG13609* and *caravaggio*, *cav*) were still intact.

Mutation of *Spps* interferes with PRE function

Most homozygous *Spps*¹ mutants die as pharate adults, though some hatch and die shortly thereafter. Flies that are *Spps*¹ heterozygous with a deficiency that deletes *Spps* die at a similar stage. Aside from some wing defects (most flies do not open the wings at all), there is no other morphological defect, i.e. they do not show the homeotic phenotypes generally seen in PcG mutants. *Spps* has a large maternal component. In order to assess the role of the maternal contribution of *Spps*, we made germline clones. Surprisingly, the phenotype of maternal-zygotic mutants was exactly the same as that of the zygotic mutants, death at the pharate adult stage with no homeotic transformations. Consistent with this, in polytene chromosomes from *Spps* maternal-zygotic mutant larvae, we see no Spps antigen, but Psc is still bound to the DNA (data not shown). Furthermore, *Ubx* is not misexpressed in imaginal disks of *Spps* mutants (data not shown). This leads us to one of two conclusions. Either *Spps* plays no role in embryogenesis or larval development and instead plays an exclusively pupal/adult role or there is another protein that has some functional redundancy with *Spps*. Partial functional redundancy is seen with a number of members of the PcG genes [*pho* and *pho*-like (Brown et al., 2003), *Esc* and *esc*-like (Ohno et al., 2008; Kurzhais et al., 2008; Wang et al., 2006), *Psc* and *Su(z)2* (Lo et al., 2009)] and with the *Drosophila* [*Btd* and *Sp1* (Wimmer et al., 1996; Estella et al., 2003)] and mammalian Sp1/KLF factors [Sp1 and Sp3 (Li et al., 2004)]. Because the Sp1/KLF-binding site present in the *en* 181-bp

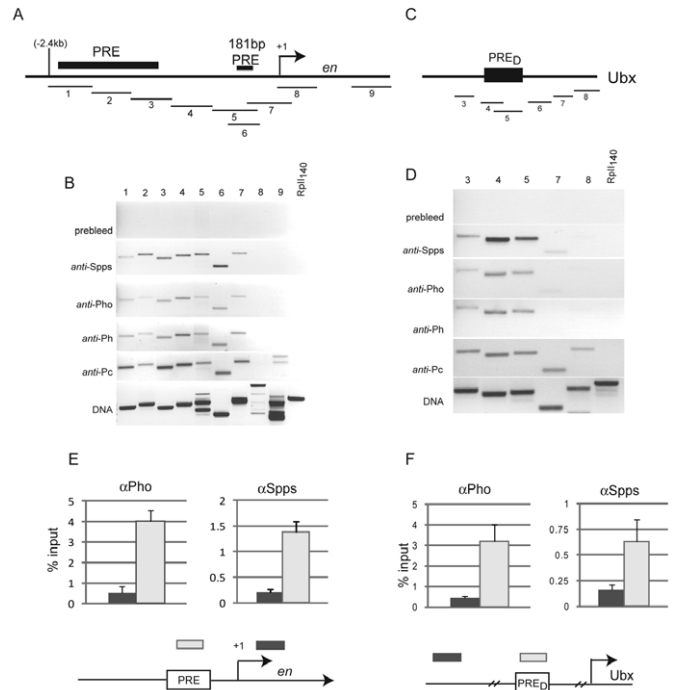


Fig. 4. *Spps* colocalizes with Pho, Ph and Pc on *en* and *Ubx* PREs in S2 cells and larvae. (A) Schematic of the *en* transcription start and the upstream 2.4 kb containing the identified PREs and the fragments amplified in the PCR after ChIP. The diagram is not to scale. (B) PCR of fragments after ChIP with antibody shown on the left. The numbers above the lanes indicate the fragment that was amplified in the PCR as shown in A. Rpl1140 primers were included as a negative control, as PcG proteins do not bind to this DNA. A DNA control and a sample incubated with pre-bleed from the rabbit used to generate the *Spps* antibody are also shown. (C) Schematic of *Ubx* DNA fragments used to analyze ChIP products over PRE_D. (D) PCR of fragments after ChIP with antibody shown on the left and the same controls as in B. (E, F) qPCR showing that Pho and *Spps* bind to the *en* and *Ubx* PREs in larvae.

PRE is important for PRE activity in an embryonic reporter assay (Brown et al., 2005), we believe that there must be a protein that acts through this site in embryos and provides an activity redundant to that of *Spps*. Currently, we do not know what the redundant protein is. It could be another member of the Sp1/KLF family, or possibly a different protein able to bind the same sequence.

Spps is necessary for PRE-mediated mini-white silencing

When PREs are cloned in reporter constructs with the eye color marker gene *mini-white*, they commonly silence its expression. This silencing activity is often stronger in flies that are homozygous for the reporter construct and is known as pairing-sensitive silencing (PSS). The Sp1/KLF-binding site in the 181-bp *en* PRE is required for PSS (Americo et al., 2002). In flies homozygous for the *Spps*¹ mutation, PRE-mediated *mini-white* silencing is suppressed, both in flies heterozygous for the PRE reporter gene, and in those homozygous for the insert. Fig. 5 shows the eyes of flies homozygous for a reporter construct with the 181-bp *en* PRE and *mini-white* at two different chromosomal insertion sites, in a fly with one wild-type copy of *Spps* and in a *Spps*¹ homozygous mutant. In flies with one wild-type copy of *Spps*, the PRE suppresses *mini-white* expression, and the eyes are white. By contrast, in *Spps*¹ homozygous mutants the eye color is red in one

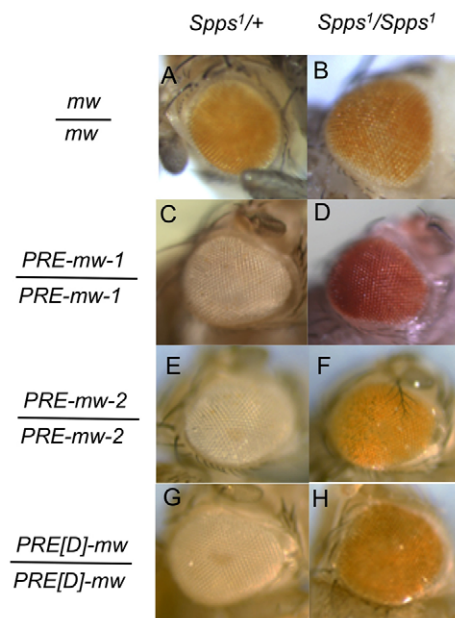


Fig. 5. Pairing-sensitive silencing is alleviated in *Spps*¹ homozygotes. (A,B) Eye from a fly homozygous for a mini-*white* construct without a PRE in either a heterozygous or homozygous *Spps*¹ background, showing that *Spps*¹ does not alter the eye color in the absence of a PRE. (C,D) Eye from a fly homozygous for a PRE-mini-*white* construct in either a heterozygous or homozygous *Spps*¹ background, showing that *Spps* alleviates pairing-sensitive silencing. This PRE-mini-*white* construct contains *en*-181 bp PRE in *pCaSpeR* [insert 8-10C on chromosome 2R (Kassis, 1994)]. (E,F) Eyes from flies with the insertion of another *en*-181 bp PRE *pCaSpeR* construct (4-8-3-1B, inserted on chromosome 2L, unpublished line). (G,H) Eyes from a line of flies with an insertion of a construct that has *Ubx*-*PRE_D* cloned in *pCaSpeR* (line K2-1, inserted on the X chromosome).

line, and orange in the other, showing that loss of *Spps* reduces or eliminates PSS in these flies. *Spps* also suppresses mini-*white* silencing mediated by *Ubx* *PRE_D* (Fig. 5G,H). Importantly, a mutation in *Spps* does not change the eye color of a line containing a mini-*white* reporter without a PRE (Fig. 5A,B). Thus, a mutation in *Spps* was able to abrogate the mini-*white* silencing activity of both the 181 bp *en* PRE and *Ubx* *PRE_D*, suggesting a general role in PRE-mediated silencing late in development.

Spps enhances the *pho* mutant phenotype

In order to further investigate the role of *Spps* in PcG-mediated repression, we looked for genetic interactions between *Spps*¹ and mutations in other PcG genes. We first looked at whether a mutation in *Spps* affected the extra sex comb phenotypes seen in flies heterozygous for *Psc*¹. We did not see any enhancement of the extra sex comb phenotype in *Spps*¹/*Spps*¹; *Psc*¹/+. Similarly, *Spps* does not enhance the extra sex comb phenotype seen in *Pc*⁴ heterozygotes. We next examined whether there was a genetic interaction between *pho* and *Spps*. There were two reasons for testing this. First, both *Pho* and *Spps* are PRE DNA-binding proteins; thus, they may work together or in parallel pathways to recruit PcG protein complexes to the DNA. Second, YY1 and Sp1, mammalian proteins that contain zinc-finger regions highly similar to *Pho* and *Spps*, are known to interact through their zinc-finger domains (Lee et al., 1993). Therefore, we examined the phenotype of *Spps*¹; *pho*¹ double mutants. Although both *pho*¹ and *Spps*¹ mutants die as pharate adults,

*Spps*¹; *pho*¹ double mutants die earlier in development, as third instar larvae or often before. Although theoretically one of every three *Spps*¹ homozygous third instar larvae should be a *pho*¹ homozygote in a *Spps*¹/*TM6b*; *pho*¹/*CiD* stock, the frequency was much less, about one in 15 (4/62) and double mutant larvae were often small. We examined the expression pattern of *Ubx* and *En* in *pho*¹ and *Spps*¹ single mutants, and in *Spps*¹; *pho*¹ double mutants in imaginal disks. As expected from the lack of homeotic phenotype, we did not see any mis-expression of *Ubx* or *En* in *Spps*¹ mutant larvae. The phenotype of *Ubx* and *En* expression in *pho*¹; *Spps*¹ double mutants was extremely variable and sometimes the disks were misshapen and it was difficult to discern which disk was which. Perhaps because the larvae are sick, in some double mutants there was a lot of mis-expression, in others there was much less (data not shown). Nevertheless, our data show that *Spps*; *pho* mutants die at a much earlier stage than either single mutant, and it is reasonable to propose that they may act either together or in parallel pathways to recruit PcG protein complexes to DNA.

DISCUSSION

Spps, a member of the Sp1/KLF family of proteins binds polytene chromosomes in a pattern comparable with the PcG protein *Psc* and, as shown by chromatin immunoprecipitation, is bound to both the *en* and *Ubx* PREs in S2 cells and in larvae. Furthermore, a mutation in *Spps* abrogates PRE activity in a mini-*white* assay, and enhances the phenotypes seen in a *pho* mutant. We suggest that *Spps* acts either with or in parallel to *Pho* to recruit PcG protein complexes to the DNA. This result is particularly interesting in lieu of the recent report that the PcG protein *Scm* is recruited to the DNA independently of *Pho* (Wang et al., 2010). Those authors speculate that *Scm* is in a complex with another PRE-DNA-binding protein, and show that, like *Pho*, *Scm* plays a role in recruitment of PRC1 and PRC2 to the PRE. It will be interesting to explore whether *Spps* or another Sp1/KLF family member recruits *Scm* to the DNA.

The mammalian homologues of *Pho* and *Spps*, YY1 and Sp1 are extremely versatile proteins. Their activities can be changed from repressor to activator or vice versa depending on the cellular and binding site context (for reviews, see Gordon et al., 2006; Lomber and Urrutia, 2005; Li et al., 2004). The activity of both these proteins is sensitive to the influence of many different co-repressors and co-activators. Both factors have been shown to bend DNA (Natesan and Gilman, 1993; Sjøttem et al., 1997). Finally, YY1 and Sp1 have also been shown to interact directly by a number of groups (Lee et al., 1993; Li et al., 2008). We find it very intriguing that such proteins bind to the PREs of *Drosophila* genes. Given that PREs may mediate the action of both the Polycomb and Trithorax group proteins, DNA-binding/recruitment proteins with such versatility and adaptability could be one way to facilitate the change from repression to activation. In fact, there is a report that *Pho* and *Phol*, in addition to their association with PREs, are bound to regions of chromatin with active histone modifications (Schuettengruber et al., 2009). Finally, a single *Pho*-binding site in a PRE in the *even-skipped* gene has been shown to be important for both activation and repression, dependent on the context (Fujioka et al., 2008). It will be interesting to explore whether *Spps* also has a dual role in gene regulation.

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Competing interests statement

The authors declare no competing financial interests.

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