

# The role of the histone H2A ubiquitinase Sce in Polycomb repression

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## SUMMARY

Polycomb group (PcG) proteins exist in multiprotein complexes that modify chromatin to repress transcription. *Drosophila* PcG proteins Sex combs extra (Sce; dRing) and Posterior sex combs (Psc) are core subunits of PRC1-type complexes. The Sce:Psc module acts as an E3 ligase for monoubiquitylation of histone H2A, an activity thought to be crucial for repression by PRC1-type complexes. Here, we created an Sce knockout allele and show that depletion of Sce results in loss of H2A monoubiquitylation in developing *Drosophila*. Genome-wide profiling identified a set of target genes co-bound by Sce and all other PRC1 subunits. Analyses in mutants lacking individual PRC1 subunits reveals that these target genes comprise two distinct classes. Class I genes are misexpressed in mutants lacking any of the PRC1 subunits. Class II genes are only misexpressed in animals lacking the Psc-Su(z)2 and Polyhomeotic (Ph) subunits but remain stably repressed in the absence of the Sce and Polycomb (Pc) subunits. Repression of class II target genes therefore does not require Sce and H2A monoubiquitylation but might rely on the ability of Psc-Su(z)2 and Ph to inhibit nucleosome remodeling or to compact chromatin. Similarly, Sce does not provide tumor suppressor activity in larval tissues under conditions in which Psc-Su(z)2, Ph and Pc show such activity. Sce and H2A monoubiquitylation are therefore only crucial for repression of a subset of genes and processes regulated by PRC1-type complexes. Sce synergizes with the Polycomb repressive deubiquitinase (PR-DUB) complex to repress transcription at class I genes, suggesting that H2A monoubiquitylation must be appropriately balanced for their transcriptional repression.

**KEY WORDS:** Polycomb repression, Sce (dRing), H2A monoubiquitylation, Calypso (Bap1), *Drosophila*

## INTRODUCTION

Polycomb group (PcG) genes encode regulatory proteins that control diverse developmental processes in animals and plants by repressing the transcription of developmental regulator genes. Genetic studies in *Drosophila* originally identified PcG proteins as repressors that are required for the long-term silencing of HOX genes in cells in which these genes have to remain inactive (Duncan, 1982; Jürgens, 1985; Lewis, 1978; Struhl, 1981). Eighteen different *Drosophila* proteins have been classified as PcG members on the basis that HOX gene silencing is lost in animals that lack these proteins. PcG proteins compose subunits of four principal protein assemblies: Polycomb repressive complex 1 (PRC1), Polycomb repressive complex 2 (PRC2), Polycomb repressive deubiquitinase (PR-DUB) and Pho repressive complex (PhoRC) (Czermin et al., 2002; Klymenko et al., 2006; Lagarou et al., 2008; Müller et al., 2002; Nekrasov et al., 2007; Scheuermann et al., 2010; Shao et al., 1999). Protein assemblies that are identical or similar to PRC1, PRC2 and PR-DUB have also been identified in mammals (Cao et al., 2002; Kuzmichev et al., 2002; Levine et al., 2002; Misaghi et al., 2009; Sarma et al., 2008; Sowa et al., 2009; Yu et al., 2010).

PcG protein complexes possess specific chromatin-modifying activities that are thought to be crucial for repression of target genes (reviewed by Müller and Verrijzer, 2009; Simon and Kingston, 2009). Specifically, PRC2 tri-methylates lysine 27 in histone H3 (H3-K27me3), and high levels of this modification at target genes correlates with their repression (Cao et al., 2008; Cao et al., 2002; Kahn et al., 2006; Kuzmichev et al., 2002; Nekrasov et al., 2007; Papp and Müller, 2006; Sarma et al., 2008; Schwartz et al., 2006; Schwartz et al., 2010). PRC1 inhibits nucleosome remodeling and transcription on chromatin templates and compacts chromatin in vitro (Francis et al., 2004; Francis et al., 2001; Levine et al., 2002; Shao et al., 1999). PRC1-like complexes that contain only a subset of PRC1 subunits, such as human PRC1L (hPRC1L) and the *Drosophila* dRing-associated factors (dRAF) complex, function as E3 ligases for the monoubiquitylation (ub) of a specific lysine in histone H2A: H2A-K119 in mammals and H2A-K118 in *Drosophila* (de Napoles et al., 2004; Lagarou et al., 2008; Wang, H. et al., 2004). The recently identified PR-DUB complex deubiquitylates H2A-K119ub1/H2A-K118ub1 in vitro, and *Drosophila* mutants lacking PR-DUB show a strong increase of bulk H2A-K118ub levels (Scheuermann et al., 2010). PhoRC does not possess any enzymatic activity but is the only PcG protein complex with sequence-specific DNA-binding activity (Klymenko et al., 2006). PhoRC has been implicated in the targeting of other PcG protein complexes to Polycomb response elements (PREs), possibly in conjunction with other DNA-binding proteins (Mohd-Sarip et al., 2005; Wang, L. et al., 2004; Brown and Kassis, 2010; Klymenko et al., 2006; Kwong et al., 2008; Mohd-Sarip et al., 2006; Oktaba et al., 2008; Schuettengruber and Cavalli, 2009) (reviewed by Müller and Kassis, 2006).

Genome-wide profiling of PcG proteins in *Drosophila* tissue culture cells and in developing animals revealed that PhoRC, PRC1, PRC2 and PR-DUB all co-occupy PREs of HOX and a

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large set of other developmental regulator genes (Kwong et al., 2008; Nègre et al., 2006; Oktaba et al., 2008; Scheuermann et al., 2010; Schuettengruber and Cavalli, 2009; Schwartz et al., 2006; Tolhuis et al., 2006). Nevertheless, for most of the non-HOX target genes it is still not known whether and in which cells the PcG machinery is required to repress their transcription and which activities of the different complexes are needed for this repression.

In this study, we investigated how the different chromatin-modifying activities of PRC1 contribute to the transcriptional repression of PRC1 target genes in *Drosophila*. In particular, we were interested in analyzing the role of H2A monoubiquitylation in repression. The core of *Drosophila* PRC1 comprises Sex combs extra (Sce; also known as dRing), Polycomb (Pc), the two highly related proteins Polyhomeotic-proximal (Ph-p) and Polyhomeotic-distal (Ph-d), and Posterior sex combs (Psc) or its paralog Suppressor of zeste 2 [Su(z)2] (Francis et al., 2001; Lo et al., 2009; Lo and Francis, 2010; Shao et al., 1999; Strübbe et al., 2011). The ability of PRC1 to inhibit nucleosome remodeling, to repress transcription on chromatin templates and to compact chromatin in vitro is primarily attributed to the Psc-Su(z)2 subunit (Francis et al., 2004; Francis et al., 2001; Lo and Francis, 2010). Psc interacts directly with Sce. In vitro, the Sce:Psc module and the corresponding RING1B (RNF2):BMI1 module of human PRC1 have E3 ligase activity to monoubiquitylate histone H2A in nucleosomes (Buchwald et al., 2006; Lagarou et al., 2008; Li et al., 2006). E3 ligase activity for monoubiquitylation of nucleosomal H2A has also been shown for the PRC1-related dRAF complex that comprises Sce, Psc and Kdm2 but lacks Ph and Pc (Lagarou et al., 2008). However, attempts to show an E3 ligase activity with reconstituted recombinant PRC1 in vitro have so far failed (Lagarou et al., 2008). In addition, a recent study in murine embryonic stem cells proposed that Ring1B compacts HOX gene chromatin and represses their transcription independently of H2A ubiquitylation (Eskeland et al., 2010). The question of whether H2A monoubiquitylation is central to the repression mechanism of PRC1 is therefore still unresolved.

Here, we find that genes that are bound by all four PRC1 core subunits fall into two broad categories with respect to their regulation by PRC1-type complexes. Class I genes require all four PRC1 subunits for repression, whereas repression of class II genes depends on Psc-Su(z)2 and Ph but not on Pc and Sce. Non-covalent chromatin modification by Psc-Su(z)2 and Ph thus appears to play a central role in the repression of all PRC1 target genes, whereas H2A monoubiquitylation by Sce only seems to be crucial for repression of a subset of these targets.

## MATERIALS AND METHODS

### Fly strains

The following *Drosophila* strains were used in this study:

*w ph<sup>504</sup> FRT101/FM7c*;  
*yw; FRT40A FRT42D Asx<sup>22P4</sup> y<sup>+</sup>/SM6b*;  
*yw; FRT40A FRT42D Asx<sup>22P4</sup> y<sup>+</sup>/CyO, twi::EGFP*;  
*w; FRT42D Su(z)2<sup>1.b8</sup>/SM6b*;  
*w; Pc<sup>XT109</sup> FRT2A/TM6B*;  
*w; FRT82B Sce<sup>1</sup>/TM6C*;  
*yw; FRT82B Sce<sup>33M2</sup>/TM6C*;  
*w; FRT82B cu sr Sce<sup>KO</sup>/TM6C*;  
*w; F82B cu sr Sce<sup>KO</sup>/TM3, Sb, Ser, twi::EGFP*;  
*w; FRT40A FRT42D Asx<sup>22P4</sup> y<sup>+</sup>; FRT82B cu sr Sce<sup>KO</sup>/SM5<sup>+</sup>TM6B*;  
*w hs-nGFP hs-flp FRT101*;  
*yw hs-flp; FRT42D hs-nGFP*;  
*yw hs-flp; hs-nGFP FRT2A*;  
*yw hs-flp; FRT82B hs-nGFP*;

*yw ey-flp; FRT42D cell lethal ubi-GFP.nls/CyO*;  
*yw; FRT40 FRT42D y<sup>+</sup>*;  
*yw; FRT42D Psc-Su(z)2<sup>P3C</sup>/CyO-CTG*;  
*yw; ey-GAL4 UAS-flp/CyO; cell lethal GMR-hid F2A/TM6B*; and  
*yw; ey-GAL4 UAS-flp/CyO; FRT82B cell lethal GMR-hid/TM6B*.

### Generation of the Sce<sup>KO</sup> deletion allele

The ends-out recombination strategy was used to disrupt *Sce* and replace its entire coding region with a *miniwhite* marker gene following the strategy described previously (Gong and Golic, 2003). In brief, 3.7 kb of *Sce* 3' flanking sequences (FlyBase 3R:23502707 to 23506380) and 3.5 kb of 5' flanking sequences (FlyBase 3R:23507729 to 23511264) were cloned into pw35 (Gong and Golic, 2003). In this construct, the entire coding region of *Sce* [FlyBase 3R:23506276 to 23507736 (on the minus strand)] is replaced by the *miniwhite* gene from pw35; only the C-terminal 14 codons of *Sce* are still present. Following the described strategy (Gong and Golic, 2003), we isolated several independent targeting events that all failed to complement the lethality of *Sce*<sup>1</sup>. One of these alleles, *Sce*<sup>KO</sup>, was selected for in-depth analysis by PCR using primers (5' to 3'): a-1, CAGTCGCGCTGACGCCCATGGAACCAACCC; a-2, CCGGAAAGACGACCTGCTGAATGCC; a-3, ACCTGCTCATCTCGAAGATCTATACCCC; b-1, TGATTCTCGGAAGAAAGTGAAGTGGG; b-2, ATCCCGGATGGCGATACTTGGATGCC; and b-3, TAGCCATCACCTTCCTCGGATGGCC (supplementary material Fig. S1).

### Knockdown of Sce by RNA interference (RNAi)

Males homozygous for the *UAS-anti-Sce hairpin RNA* transgene (VDRC transformant line 106328, construct ID 109179) were crossed to *P(GawB)T80/CyO-ubiGFP* females (Bloomington stock number 1878) to induce degradation of *Sce* mRNA in the offspring.

### Preparation of imaginal disc total protein extracts

Imaginal wing discs from third instar *Drosophila* larvae were dissected on ice and incubated in SDS gel loading buffer for 5 minutes at 95°C. The suspension was sonicated, debris was pelleted and the supernatant analyzed by SDS-PAGE.

### Acid-extraction of histones from *Drosophila* larval imaginal wing discs

Dissected imaginal wing discs from third instar larvae were homogenized and acid-extraction of histones was performed as described (Scheuermann et al., 2010).

### Preparation of embryonic cuticles and immunostaining of embryos and imaginal discs

Preparation of embryonic cuticles, staining of embryos and larval imaginal discs and clonal analysis were performed following standard protocols (Beuchle et al., 2001). Mutant eye-antennal discs were generated using the *eyeless-FLP/cell-lethal* system as described (Classen et al., 2009).

### Antibodies

Antibodies used in this study were Sce (Gorfinkel et al., 2004; Lagarou et al., 2008; Balicky et al., 2004), Psc (Poux et al., 2001), Ph (Oktaba et al., 2008), Calypso (Scheuermann et al., 2010), Asx (Scheuermann et al., 2010),  $\alpha$ -Tubulin (Sigma, T9026), H2A (Millipore, 07-146), H3K27me3 (Upstate, 07-449), Eve (Frasch et al., 1987), Dac (Mardon et al., 1994) (Developmental Studies Hybridoma Bank MR1A), Pros (Srinivasan et al., 1998), EIB (Weihe et al., 2004), Noc (Weihe et al., 2004), Doc2/3 (Reim et al., 2003), En (Developmental Studies Hybridoma Bank 4D9), Wg (Diaz-Benjumea and Cohen, 1995), Cad (Macdonald and Struhl, 1986), Ubx (White and Wilcox, 1984), Abd-B (Celniker et al., 1990) and Lamin (gift from D. Arndt-Jovin, Göttingen, Germany).

### Genome-wide profiling of Sce protein in *Drosophila* using Affymetrix whole-genome tiling arrays

Chromatin preparation from *Drosophila* larval brain and imaginal discs (wing, haltere and third leg) and chromatin immunoprecipitation (ChIP) were performed essentially as described (Oktaba et al., 2008). To determine Sce-bound regions, six ChIP assays were performed on independent batches of chromatin, three with each of two different antibodies raised

against full-length Sce protein (Balicky et al., 2004; Lagarou et al., 2008). The immunoprecipitated material was amplified by ligation-mediated PCR, fragmented, labeled and hybridized to Affymetrix whole-genome microarrays as described (Gambetta et al., 2009). The raw and processed microarray data have been deposited in the ArrayExpress database under accession number E-TABM-988. Data analysis was as described (Oktaba et al., 2008) with the following modifications.

#### Genome assembly and annotation

We used the third *Drosophila melanogaster* genome assembly (UCSC dm3 or FlyBase 5.x) and the gff export of FlyBase 5.23 for genome annotation.

#### Affymetrix probe remapping

We downloaded the Dm35b\_MR\_v02-3\_BDGPv4h.new.bpmap file from the CisGenome website (<http://www.biostat.jhsph.edu/~hji/cisgenome/>). This file contains the remapped location to genome version 5 (i.e. UCSC dm3) of all the original Affymetrix 25mer sequences, removing those probes that cannot uniquely map to the genome.

#### Detection of regions that are significantly bound by Sce

A quantile normalization (Bolstad et al., 2003) was applied to normalize together six ChIP hybridizations and three genomic DNA hybridizations. Significantly bound regions were identified using TileMap (Ji and Wong, 2005) with the hidden Markov model. A TileMap score where 60% of the previously identified 237 regions bound by PhoRC and Ph in larva (Gambetta et al., 2009; Oktaba et al., 2008) were recovered was used as threshold for considering significantly bound regions. Additionally, the top 5% regions below cut-off exhibiting overlap with either Pho-, Sfmt- or Ph-bound regions (Gambetta et al., 2009; Oktaba et al., 2008) were rescued and included in the final high-confidence Sce binding profile. We thus identified 624 regions (405 scoring above cut-off, plus 219 rescued) bound by Sce in brain and larval imaginal discs.

#### Venn diagram counts

For Venn diagram counts, two or more regions that overlap by at least one base were merged and defined as a 'common' region.

#### Sce-bound regions, target gene assignment and GO slim analysis

The relative distance of the midpoint of each Sce-bound region with respect to the closest transcription start site (TSS) was computed. Target genes were assigned based on TSS-proximal location. Assigned genes to each dataset were tested for enriched GO slim term annotations.

## RESULTS

### An Sce deletion mutant shows classic homeotic phenotypes

*Drosophila* Sce encodes a protein of 435 amino acids. Previous studies reported the isolation and characterization of two mutant Sce alleles (Breen and Duncan, 1986; Fritsch et al., 2003; Gaytán de Ayala Alonso et al., 2007; Gorfinkel et al., 2004). *Sce<sup>l</sup>* encodes a truncated Sce protein that contains the N-terminal 322 amino acids, whereas *Sce<sup>33M2</sup>* encodes a full-length Sce protein with an Arg65Cys mutation (Fritsch et al., 2003; Gorfinkel et al., 2004). To determine the Sce null mutant phenotype, we used a homologous recombination strategy (Gong and Golic, 2003) to generate *Sce<sup>KO</sup>*, a knockout allele that deletes the entire Sce coding region (supplementary material Fig. S1).

We analyzed Sce expression in protein extracts from *Sce<sup>KO</sup>*, *Sce<sup>33M2</sup>* or *Sce<sup>l</sup>* homozygous embryos that were derived from germ line clones and therefore lacked maternally deposited wild-type Sce protein. The extracts were probed with an antibody that had been raised against the N-terminal 274 amino acids of the Sce protein (Gorfinkel et al., 2004). We were unable to detect full-length Sce protein in extracts from any of these Sce mutants (Fig. 1A). The lack of detectable Sce protein in *Sce<sup>33M2</sup>* mutants suggests that the *Sce<sup>R65C</sup>* protein encoded by this allele is not stable. Staining of imaginal discs that contained clones of Sce mutant cells with anti-

Sce antibody corroborated this finding: Sce protein was undetectable not only in *Sce<sup>KO</sup>* but also in *Sce<sup>33M2</sup>* mutant clones (Fig. 1B). By contrast, the Sce antibody signal was undiminished in *Sce<sup>l</sup>* mutant clones (Fig. 1B), suggesting that the C-terminally truncated Sce protein encoded by this allele is expressed as a stable polypeptide. We were unable to detect the truncated *Sce<sup>l</sup>* protein by western blot analysis because of cross-reactivity of the anti-Sce antibody with an unknown epitope that migrates at the position of the predicted truncated *Sce<sup>l</sup>* protein in embryo extracts (data not shown). Given that *Sce<sup>KO</sup>* lacks the entire coding region, the phenotype of *Sce<sup>KO</sup>* mutants represents the null phenotype of Sce. *Sce<sup>KO</sup>* and *Sce<sup>33M2</sup>* mutant embryos derived from *Sce<sup>KO</sup>* and *Sce<sup>33M2</sup>* mutant germ cells, respectively, show comparably severe homeotic transformations (supplementary material Fig. S2). This suggests that *Sce<sup>33M2</sup>* is a strong loss-of-function or even a null mutation, consistent with the observation that *Sce<sup>33M2</sup>* mutants lack detectable Sce protein. Intriguingly, *Sce<sup>l</sup>* mutant embryos show more extreme homeotic transformations than *Sce<sup>KO</sup>* or *Sce<sup>33M2</sup>* mutants, and their phenotype resembles that of *Pc* mutants (supplementary material Fig. S2). The truncated *Sce<sup>l</sup>* protein lacks the C-terminal Pc-interaction domain (Gorfinkel et al., 2004; Schoorlemmer et al., 1997; Wang et al., 2010) but contains the N-terminal RING finger domain required for complex formation with Psc-Su(z)2 (Buchwald et al., 2006; Li et al., 2006). It is possible that *Sce<sup>l</sup>* mutants show a stronger PcG phenotype than *Sce<sup>KO</sup>* mutants because the *Sce<sup>l</sup>* protein sequesters Psc and/or Su(z)2 into non-productive protein assemblies and thereby interferes with their ability to repress target genes.

Taken together, these analyses show that the complete lack of Sce protein in embryos causes severe homeotic transformations that, nonetheless, are less extreme than the transformations observed in some of the other PcG mutants (see below). The truncated Sce protein expressed from the *Sce<sup>l</sup>* allele also lacks *Sce<sup>+</sup>* function but, in addition, acts in a dominant-negative fashion to exacerbate the loss of HOX gene silencing.

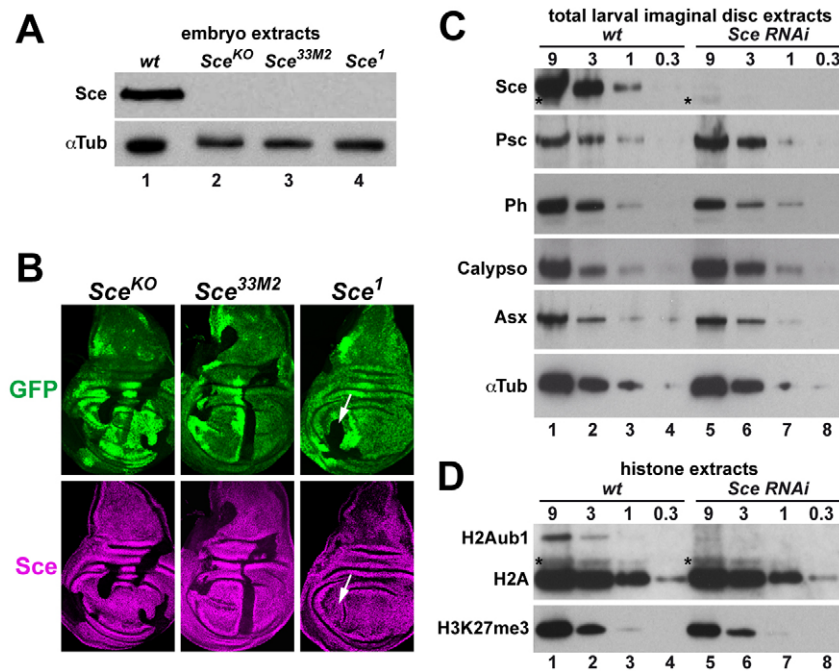
### Sce is the major E3 ligase for H2A monoubiquitylation in *Drosophila*

We next investigated how H2A-K118ub1 levels were affected by removal of the Sce protein. We used an RNAi strategy to knock down Sce protein in imaginal disc tissues. Sce protein levels in imaginal disc cells were reduced by more than 95% after Sce RNAi (Fig. 1C). By contrast, the levels of the PRC1 subunits Psc and Ph and of the PR-DUB subunits Calypso and Additional sex combs (Asx) were unaffected by the Sce RNAi treatment (Fig. 1C). We isolated bulk histones by acid extraction from imaginal disc tissue of wild-type and Sce RNAi-treated larvae and compared the levels of H2A-K118ub1 and H3-K27me3. Both modifications were readily detected in wild-type animals (Fig. 1D). In Sce RNAi-treated animals, H2A-K118ub1 levels were strongly reduced, whereas H3-K27me3 levels were comparable to those in wild-type animals (Fig. 1D). We conclude that Sce is responsible for the bulk of H2A monoubiquitylation in developing *Drosophila*. This is consistent with studies by Lagarou et al. (Lagarou et al., 2008) who reported that depletion of Sce by RNAi in *Drosophila* tissue culture cells results in a drastic reduction of H2A-K118ub1 levels.

### Mutants lacking PRC1 subunits show distinct morphological defects

*Sce* or *Pc* homozygous mutant embryos that are derived from females with a mutant germ line and therefore lack maternally deposited wild-type Sce or Pc protein, respectively, show





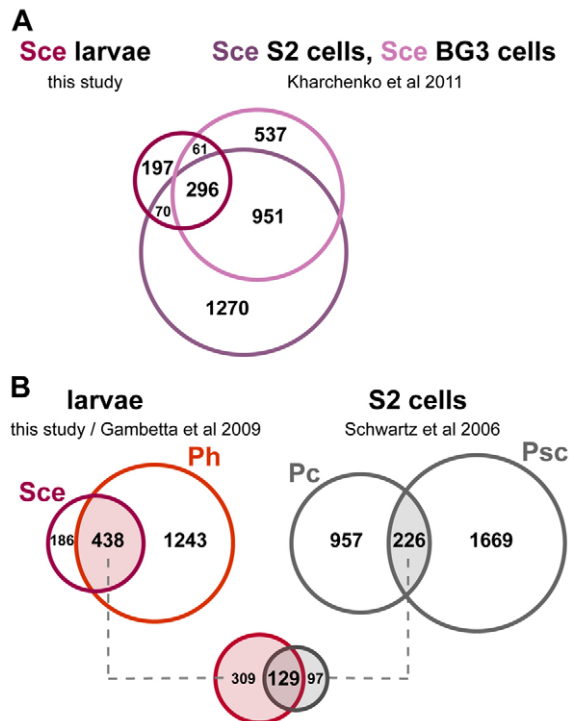
**Fig. 1. Analysis of Sce protein in *Sce* mutants and *Sce* requirement for H2A monoubiquitylation.** (A) Total extracts from wild-type (wt, lane 1), *Sce<sup>KO</sup>* (lane 2), *Sce<sup>33M2</sup>* (lane 3) and *Sce<sup>1</sup>* (lane 4) homozygous *Drosophila* embryos probed with antibodies against Sce and  $\alpha$ -Tubulin 1 ( $\alpha$ Tub) as loading control. The truncated *Sce<sup>1</sup>* protein is not shown (see text). (B) Wing imaginal discs from *Sce<sup>KO</sup>/GFP* (left), *Sce<sup>33M2</sup>/GFP* (middle) and *Sce<sup>1</sup>/GFP* (right) heterozygous third instar larvae with clones of cells that are homozygous for the indicated *Sce* mutation. Discs were stained with antibody against Sce protein, 96 hours after induction of clones. Clones of *Sce* homozygous cells are marked by absence of GFP (green). In *Sce<sup>KO</sup>* and *Sce<sup>33M2</sup>* homozygous cells, Sce protein is undetectable. In *Sce<sup>1</sup>* homozygous cells (arrows), the truncated *Sce<sup>1</sup>* protein is detected at levels that are comparable to those of the full-length wild-type Sce protein. (C) Western blots of serial dilutions (9:3:1:0.3) of total imaginal disc extracts from wild-type and *Sce RNAi*-treated third instar larvae, probed with antibodies against PRC1 and PR-DUB subunits and  $\alpha$ -Tubulin 1 ( $\alpha$ Tub). Sce signals in lanes 4 and 5 are comparable, suggesting that Sce levels are reduced more than 95% in *Sce RNAi*-treated larvae. Asterisks indicate a cross-reacting band. (D) Western blots of serial dilutions of histone extracts from imaginal discs of wild-type and *Sce RNAi*-treated third instar larvae, probed with antibodies against H2A and H3-K27me3. The H2A-K118ub1 band is identified as the monoubiquitylated form of H2A because it migrates at the same position as H2A-K118ub1 generated by monoubiquitylation of recombinant mononucleosomes with Ring1B-Bmi1 complex in vitro and at the same position as H2A-K118ub1 in extracts from *PR-DUB* mutant embryos in which the levels of H2A-K118ub1 are strongly increased (Scheuermann et al., 2010). Note that the H2A-K118ub1 signal in lane 5 is substantially weaker than in lane 2 and only slightly stronger than that in lane 3, suggesting that H2A-K118ub1 levels are reduced by about 80% in *Sce RNAi*-treated larvae. Asterisks indicate a cross-reacting band.

segmental transformations due to misexpression of HOX genes (supplementary material Fig. S2). By comparison, *ph<sup>0</sup>* embryos, which are mutant for both Ph-p and its paralog Ph-d, or embryos that are homozygous for a small deficiency that deletes both *Psc* and its paralog *Su(z)2* [i.e. *Psc-Su(z)2* mutants], show much more severe morphological defects, even if these embryos are derived from females with a heterozygous germ line and therefore contain the maternally deposited wild-type products of these genes (supplementary material Fig. S2) (see Dura et al., 1987; Feng et al., 2011; Martin and Adler, 1993; Smouse et al., 1988). *ph<sup>0</sup>* or *Psc-Su(z)2* mutant embryos that also lack maternally deposited Ph or Psc and *Su(z)2* proteins are highly abnormal already at the gastrulation stage, arrest development shortly afterwards and form no, or only very rudimentary, embryonic cuticle (supplementary material Fig. S2) (Smouse et al., 1988). Moreover, zygotic expression of wild-type Ph and *Psc-Su(z)2* proteins is unable to restore normal development to embryos that lack maternally deposited Ph and *Psc-Su(z)2* proteins, respectively (supplementary material Fig. S2) (Smouse et al., 1988). By contrast, zygotic expression of wild-type Sce or Pc protein rescues embryos that lack maternally deposited Sce or Pc proteins, respectively, into viable and fertile adults (not shown) (Breen and Duncan, 1986; Lawrence

et al., 1983). Taken together, comparison of the loss-of-function phenotypes of mutants lacking different PRC1 subunits shows that Ph, Psc and *Su(z)2* proteins are required for processes that appear to occur normally in the absence of Sce or Pc.

### Genome-wide binding maps of Sce, Ph, Psc and Pc identify a set of PRC1-bound genes

Ph, *Psc-Su(z)2*, Sce and Pc might regulate different sets of target genes and this could account for the differences in their mutant phenotypes. Here, we focused on target genes that are bound by all four PRC1 subunits and investigated how their expression is affected in the absence of individual subunits. Previous studies reported the genome-wide binding profiles of Psc, Pc and Sce in *Drosophila* tissue culture cells and that of Ph and Pc in *Drosophila* embryos and larval imaginal disc cells (Gambetta et al., 2009; Kharchenko et al., 2011; Kwong et al., 2008; Schuettengruber and Cavalli, 2009; Schwartz et al., 2006). To identify genes that are bound by Sce in developing *Drosophila*, we generated the genome-wide Sce protein-binding profile in imaginal disc and central nervous system (CNS) tissues from third instar larvae. We performed chromatin immunoprecipitation (ChIP) assays with two independent antisera that had been raised



**Fig. 2. Identification of cis-regulatory regions co-bound by Sce and other PRC1 subunits.** (A) Venn diagram showing the overlap of Sce-bound regions identified by genome-wide profiling in larval tissues and in *Drosophila* S2 and BG3 tissue culture cells. The overlap identifies 427 regions bound by Sce in both larval and tissue culture cells. (B) Venn diagram showing the overlap of Sce- and Ph-bound regions in larval tissues and the overlap of Pc- and Psc-bound regions in *Drosophila* S2 tissue culture cells. The overlap of 438 regions bound by both Sce and Ph and 226 regions bound by both Pc and Psc identifies 129 regions that are bound by all four PRC1 core subunits.

against the Sce protein and hybridized the immunoprecipitated material to high-density whole-genome tiling arrays. Only genomic regions significantly enriched by both anti-Sce antibodies were considered. Using a stringent cut-off, we thus obtained a high-confidence set of 624 genomic regions bound by the Sce protein in larval tissues (Fig. 2A,B; supplementary material Table S1). Comparison of this binding profile with the Sce profiles in two different tissue culture cell lines (Kharchenko et al., 2011) showed that 427 of the 624 regions bound by Sce in imaginal disc cells (68%) had also been identified as Sce target sites in tissue culture cells (Fig. 2A).

We then determined the overlap between Sce-bound regions and regions bound by other PRC1 subunits. For this analysis we used the genome-wide binding profiles of Ph in imaginal disc cells (Gambetta et al., 2009) and of Psc and Pc in tissue culture cells (Schwartz et al., 2006). The Ph profile from Gambetta et al. (Gambetta et al., 2009) permitted direct comparison with the Sce profile because both profiles had been generated in imaginal disc tissues dissected from developing third instar larvae. Similarly, the Psc and Pc datasets had both been generated in the same *Drosophila* S2 tissue culture cell line (Schwartz et al., 2006). Comparison of the Sce and Ph profiles in imaginal disc cells revealed that Ph is co-bound at 438 of the 624 Sce-bound regions (70%; Fig. 2B). Among these 438 regions bound by Sce and Ph, we identified 129 genomic locations that Schwartz et al. (Schwartz

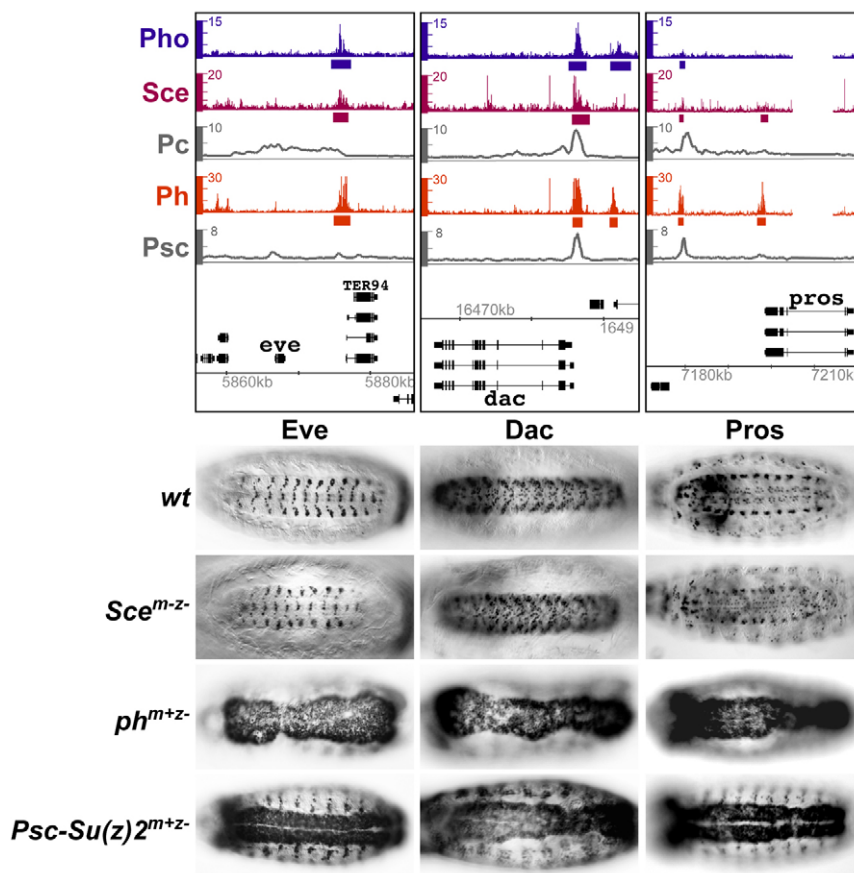
et al., 2006) had reported to be bound by both Psc and Pc in S2 cells (29%; Fig. 2B). These 129 regions thus represent genomic sites bound by Sce and Ph in imaginal disc cells and by Psc and Pc in tissue culture cells; they include PREs in HOX and other target genes that have been shown to be misexpressed in mutants lacking PRC1 subunits (see below). Because of the different source of material used for profiling (imaginal disc cells versus tissue culture cells), it is very likely that these 129 regions only represent a fraction of all regions that are bound by PRC1 in *Drosophila*. However, for simplicity, we shall here refer to the genes associated with these 129 regions as PRC1-bound genes.

### Repression of many PRC1-bound genes requires *ph* and *Psc-Su(z)2* but not *Sce* or *Pc*

We assessed how the expression of PRC1-bound genes is affected in animals that lack individual PRC1 subunits. For this analysis, we focused on target genes for which it was not known whether they are regulated by PRC1: *dachshund* (*dac*), *prospero* (*pros*), *elbow* (*elB*) and *no ocelli* (*noc*). We also included a few non-HOX target genes known to be regulated by the PcG system: *engrailed* (*en*), *caudal* (*cad*), *even-skipped* (*eve*) and *Dorsocross 2/3* (*Doc2/3*) (Beuchle et al., 2001; Busturia and Morata, 1988; Oktaba et al., 2008). Each of the analyzed genes is bound by all four PRC1 subunits (Figs 3, 4; supplementary material Fig. S3, Table S1).

In a first set of experiments, expression of the *eve*, *dac* and *pros* genes was analyzed in wild-type, *Sce*, *ph* and *Psc-Su(z)2* mutant embryos. In the case of *Sce*, the *Sce*<sup>KO</sup> homozygous embryos were derived from females with an *Sce*<sup>KO</sup> mutant germ line and therefore completely lacked Sce protein (*Sce*<sup>m-z</sup>). In the case of *ph*<sup>0</sup> and *Psc-Su(z)2*, the mutant embryos lacked zygotic *ph* and *Psc-Su(z)2* function but contained the maternally deposited wild-type products of these genes [*ph*<sup>m+z</sup> and *Psc-Su(z)2*<sup>m+z</sup>]. In wild-type embryos, *eve*, *dac* and *pros* are each expressed in a specific set of cells in the CNS of late stage embryos (Fig. 3). *eve* becomes misexpressed in most cells of the nervous system of *ph*<sup>0</sup> or *Psc-Su(z)2* mutant embryos (Fig. 3) (see Dura and Ingham, 1988; Oktaba et al., 2008). By contrast, *eve* expression remains confined in its normal pattern in embryos that lack Sce protein (Fig. 3). Similarly, we found that *dac* and *pros* are also widely misexpressed in the nervous system of *ph*<sup>0</sup> or *Psc-Su(z)2* mutant embryos, but their expression patterns in *Sce* mutant embryos are indistinguishable from those in wild-type embryos (Fig. 3). PcG regulation of *eve*, *dac* and *pros* genes thus differs from that of HOX genes in that their repression in embryos requires Ph, Psc and Su(z)2 proteins but not Sce.

We next analyzed expression of the PRC1-bound genes *elB*, *noc*, *Doc2/3*, *dac*, *en*, *eve* and *cad* in imaginal discs with clones of *Sce*<sup>KO</sup>, *Pc*, *ph*<sup>0</sup> or *Psc-Su(z)2* homozygous mutant cells. In wild-type animals, *elB*, *noc*, *Doc2/3*, *dac* and *en* are all expressed in characteristic patterns in the wing imaginal disc, whereas *eve* and *cad* are inactive in the wing disc (Fig. 4; supplementary material Fig. S3). Each of these genes becomes widely misexpressed in *ph*<sup>0</sup> or *Psc-Su(z)2* mutant clones in regions of the disc where these genes are normally not expressed (Fig. 4; supplementary material Fig. S3). By contrast, *Doc2/3*, *dac*, *eve* and *cad* all remained stably repressed in *Sce* or *Pc* mutant clones (Fig. 4; supplementary material Fig. S3). *en* also remained repressed in *Sce* or *Pc* mutant clones in most areas of the wing disc, but the gene was misexpressed in such clones in the presumptive dorsal wing hinge (supplementary material Fig. S3). Thus, among all the PRC1-bound genes analyzed here, only *elB* and *noc* were widely misexpressed in *Sce* or *Pc* mutant clones (Fig. 4).



**Fig. 3. Repression of *eve*, *dac* and *pros* in embryos requires *ph* and *Psc-Su(z)2* but not *Sce*.** (Top) ChIP profiles of *Sce*, *Pc*, *Ph* and *Psc* and, as reference, of *Pho*, the DNA-binding subunit of *PhoRC*, at the *eve*, *dac* and *pros* genes. PRC1 subunit profiles are from experiments shown in Fig. 2B and the *Pho* profile in imaginal disc tissues is the *Pho<sup>l</sup>* dataset from Oktaba et al. (Oktaba et al., 2008). Hybridization intensities for oligonucleotide probes are plotted as colored bars (*Pho*, *Sce*, *Ph*) or smoothed profiles (*Pc*, *Psc*) above the genomic map (release 5/UCSC dm3, kb coordinates) of *Drosophila*. In the *Pho*, *Sce* and *Ph* datasets, bars below plots mark regions that the analyses shown in Fig. 2 and in Oktaba et al. (Oktaba et al., 2008) identified as *Sce*-, *Ph*- or *Pho*-bound, respectively. (Below) Ventral views of 14- to 16-hour-old *Drosophila* embryos stained with antibodies against *Eve*, *Dac* or *Pros* protein.

One of the 129 PRC1-bound genes is *wingless* (*wg*) (Oktaba et al., 2008; Schwartz et al., 2006) (supplementary material Table S1). *wg* encodes a signaling molecule that acts as a positive regulator of *elB*, *noc*, *dac* and other genes involved in imaginal disc patterning (Lecuit and Cohen, 1997; Weihe et al., 2004). We have not been able to detect strong upregulation of Wg protein expression in *ph<sup>0</sup>*, *Psc-Su(z)2*, *Sce* or *Pc* mutant clones even though the Wg protein signal is often slightly increased in an erratic pattern in large clones of *ph<sup>0</sup>* or *Psc-Su(z)2* mutant cells (supplementary material Fig. S3). Misexpression of *elB* and *noc* in clones of *PcG* mutant cells (Fig. 4) is therefore unlikely to be simply an indirect consequence of the overexpression of Wg protein in the mutant clones. Finally, we note that we have not been able to detect misexpression of *nubbin*, *homothorax* or *vestigial* in either *ph<sup>0</sup>* or *Psc-Su(z)2* mutant clones in wing discs (data not shown). These three genes are all bound by PRC1 and are positively or negatively regulated by Wg in the wing imaginal disc. It is possible that they are repressed by PRC1 in other tissues or at other stages of development.

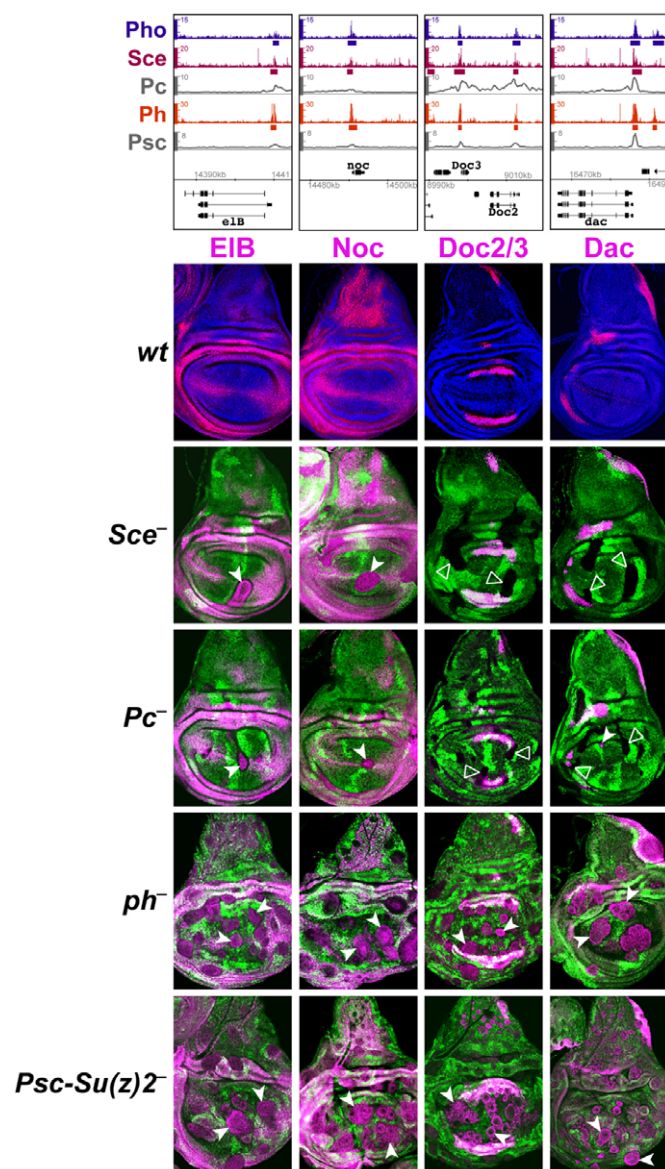
Taken together, these analyses suggest that several genes identified as PRC1 targets by genome-wide profiling are indeed directly repressed by PRC1 but with important differences with respect to the requirement for individual PRC1 subunits. PRC1 target genes can be grouped into two classes. Class I genes, such as *elB*, *noc*, *en* and the HOX genes, are derepressed in *ph*, *Psc-Su(z)2*, *Sce* and *Pc* mutants, whereas class II genes, such as *Doc2/3*, *dac*, *pros*, *eve* and *cad*, are only derepressed in *ph* and *Psc-Su(z)2* mutants but remain stably repressed in *Sce* and *Pc* mutants. Similar observations were made in *Sce* RNAi-treated imaginal discs (supplementary material Fig. S4). The global reduction of H2A-

K118ub1 levels in these discs (Fig. 1D) thus correlates with loss of repression of class I genes, whereas class II genes remain repressed.

### ***Psc-Su(z)2* and *ph* act as tumor suppressor genes but *Sce* does not**

We previously reported that clones of *Psc-Su(z)2* or *ph* mutant cells in imaginal wing discs show a tumor phenotype caused by hyperproliferation of the mutant cells, but that *Sce* or *Pc* mutant clones show normal cell proliferation (Beuchle et al., 2001; Oktaba et al., 2008). Recent studies reported that under conditions in which cell competition is removed, not only *Psc-Su(z)2* and *ph* but also *Pc* and *Sce* mutant cell clones show hyperproliferation and tumor formation (Classen et al., 2009). Interestingly, this hyperproliferation of *Pc* and *Sce* mutant cells was only observed in eye imaginal discs and not in wing discs (Classen et al., 2009). Because Classen et al. (Classen et al., 2009) had used the *Sce<sup>l</sup>* allele for their analyses, we re-examined the role of PRC1 components under the same assay conditions but with the *Sce<sup>KO</sup>* allele. Specifically, we used the *eyeless-FLP/cell-lethal* system to generate eye imaginal discs that consisted mainly of *Sce*, *Pc* or *Psc-Su(z)2* mutant cells (Fig. 5). *Psc-Su(z)2* and *Pc* mutant discs showed the tumor phenotype as previously reported (Classen et al., 2009) but *Sce<sup>KO</sup>* mutant discs were morphologically indistinguishable from wild-type discs (Fig. 5). Taken together, these analyses show that *Psc-Su(z)2*, *ph* and *Pc* have tumor suppressor activity to restrict cell proliferation in imaginal discs but that *Sce* does not show this activity. Tumor suppression by PRC1-type complexes therefore does not seem to require H2A monoubiquitylation.

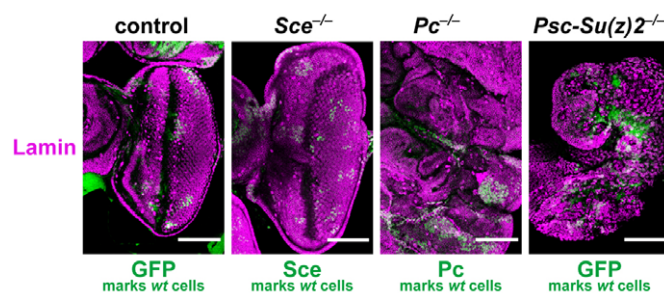




**Fig. 4. PRC1 subunits are differentially required for repression of *eIB*, *noc*, *Doc2/3* and *dac*.** (Top) ChIP profiles at the PRC1-bound genes *eIB*, *noc*, *Doc2/3* and *dac*, represented as in Fig. 3. (Below) Regulation of *eIB*, *noc*, *Doc2/3* and *dac* in wing imaginal discs with cell clones that lack individual PRC1 subunits. Row 1 shows wing discs from wild-type third instar larvae stained with antibodies against EIB, Noc, Doc2/3 and Dac proteins (magenta in each case) and co-stained with Hoechst (blue) to visualize nuclei. Rows 2 to 5 show wing discs with clones of cells lacking individual PRC1 subunits as indicated. Clones are marked by the absence of GFP and discs were analyzed 72 to 96 hours (rows 2 and 3) or 48 to 72 hours (rows 4 and 5) after clone induction. EIB, Noc, Doc2/3 and Dac proteins are all widely misexpressed in *Ph* or *Psc-Su(z)2* mutant clones (white arrowheads). EIB and Noc are also misexpressed in *Sce* and *Pc* mutant clones (white arrowheads) but the *Doc2/3* and *dac* genes remain stably repressed (open arrowheads).

### Sce and PR-DUB synergize to repress HOX genes

Finally, we investigated the interplay between H2A monoubiquitylation and deubiquitylation in the control of HOX gene repression. Recent studies identified PR-DUB as a major deubiquitinase for H2A-K118ub1 in *Drosophila* (Scheuermann et

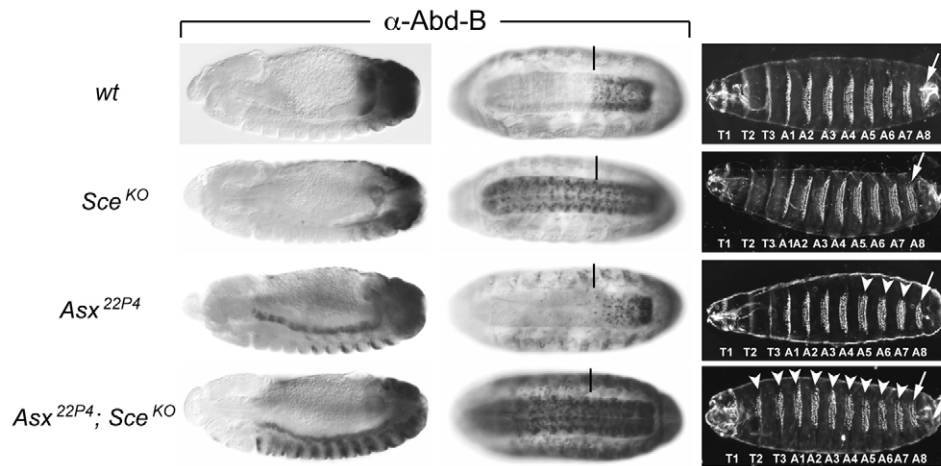


**Fig. 5. Sce lacks the tumor suppressor activity shown by other PRC1 subunits.** Eye imaginal discs consisting mainly of *Sce*<sup>-/-</sup>, *Pc*<sup>-/-</sup> or *Psc-Su(z)2*<sup>-/-</sup> mutant cells were generated with the *eyeless-FLP/cell-lethal* system. Discs were stained with antibody against Lamin to visualize all nuclei; wild-type cells were visualized by staining with antibodies against *Sce* or *Pc* or by the GFP marker protein. The *Sce*<sup>KO</sup>, *Pc*<sup>XT109</sup> and *Psc-Su(z)2*<sup>P3C</sup> alleles were used to permit direct comparison with the results reported by Classen et al. (Classen et al., 2009). Control shows clones of wild-type tissue (non-green) generated in the background of wild-type cells marked by GFP.

al., 2010). PR-DUB comprises the catalytic subunit Calypso (Bap1) and its essential co-factor Asx. Mutants lacking Calypso or Asx show misexpression of HOX genes and corresponding homeotic transformations (Gaytán de Ayala Alonso et al., 2007; Scheuermann et al., 2010), but they show no detectable misexpression of any of the other PcG target genes analyzed here (data not shown). We previously reported that *Asx*<sup>22P4</sup> *Sce*<sup>1</sup> double-homozygous embryos that were derived from heterozygous mothers show severe homeotic transformations that are comparable to the transformations observed in *Sce*<sup>1</sup> mutant embryos derived from females with an *Sce*<sup>1</sup> mutant germ line (Scheuermann et al., 2010). The same is true for *Asx*<sup>22P4</sup> *Sce*<sup>KO</sup> double-homozygous embryos; the homeotic transformations in such animals are more severe than those in *Asx*<sup>22P4</sup> or *Sce*<sup>KO</sup> single homozygotes and resemble the transformations observed in *Sce*<sup>KO</sup> homozygous embryos that lack maternally deposited *Sce* protein (compare the *Asx*<sup>22P4</sup> *Sce*<sup>KO</sup> embryo in Fig. 6 with the *Sce*<sup>KO</sup> embryo in supplementary material Fig. S2). As illustrated in Fig. 6, this is explained by the observation that repression of HOX genes in the epidermis of *Asx*<sup>22P4</sup> *Sce*<sup>KO</sup> double-mutant embryos is lost more rapidly than in *Asx*<sup>22P4</sup> or *Sce*<sup>KO</sup> single mutants and that the double-mutant embryos show much more widespread HOX gene misexpression in the epidermis of late-stage embryos compared with the single mutants. This suggests that the partial rescue of HOX gene repression by maternally deposited *Sce* protein in *Sce*<sup>KO</sup> homozygotes is abolished in the absence of PR-DUB. At present, we can only speculate how H2A deubiquitylation contributes to transcriptional repression (see Discussion), but the strong genetic synergy between PR-DUB and *Sce* in HOX gene silencing supports the idea that H2A ubiquitylation by *Sce* is important for this process.

### DISCUSSION

In this study, we analyzed how PRC1 regulates target genes in *Drosophila* to investigate how the distinct chromatin-modifying activities of this complex repress transcription in vivo. Because H2A monoubiquitylation is thought to be central to the repression mechanism of PRC1-type complexes, we focused on the role of *Sce*. The following main conclusions can be drawn from the work reported here. First, in the absence of *Sce*, bulk levels of H2A-



**Fig. 6. *Sce* and PR-DUB synergize to repress HOX genes.** Lateral views of stage 13 (left column) and ventral views of stage 16 (middle column) *Drosophila* embryos, stained with antibody against the HOX protein Abd-B. The right-hand column shows ventral views of embryonic cuticles. The genotypes are wild type (first row), *Sce*<sup>KO</sup> homozygous (second row), *Asx*<sup>22P4</sup> homozygous (third row), *Sce*<sup>KO</sup> and *Asx*<sup>22P4</sup> double homozygous (fourth row); in all cases the mutant embryos were derived from heterozygous females. In stage 16 embryos, a vertical bar marks the anterior margin of the normal Abd-B expression domain in parasegment (ps) 10 in the CNS. In embryonic cuticles, an arrow marks the denticle belt of the eighth abdominal segment. (Row 1) In wild-type embryos, Abd-B is expressed from ps 10–14, with lowest levels present in ps 10 and highest levels in ps 14. In the embryonic cuticle, every thoracic (T) and abdominal (A) segment contains a characteristic belt of denticles, specified by the level of the HOX protein expressed in that particular segment. (Row 2) In stage 13 *Sce*<sup>KO</sup> embryos, *Abd-B* is still repressed in most cells anterior to ps 10 and only a few Abd-B-positive cells are detected in more anterior regions. In stage 16 *Sce*<sup>KO</sup> embryos, *Abd-B* is expressed throughout the CNS but misexpression in the epidermis is low. The embryonic cuticle appears indistinguishable from that of wild type. (Row 3) In stage 13 *Asx*<sup>22P4</sup> embryos, *Abd-B* is misexpressed in the anterior visceral mesoderm and also in a subset of cells in the epidermis of abdominal segments. In stage 16 *Asx*<sup>22P4</sup> embryos, *Abd-B* is misexpressed in a subset of cells in the epidermis of every segment and in a few rare cells in the CNS. Within ps 10–14, there is a partial loss of Abd-B expression in the CNS but an increase in Abd-B levels in the epidermis (not visible here) and consequently A5–A7 in the cuticle are partially transformed into copies of A8 (white arrowheads). (Row 4) In stage 13 *Asx*<sup>22P4</sup> *Sce*<sup>KO</sup> double-mutant embryos, *Abd-B* misexpression in the epidermis is more widespread than in the single mutants and also extends throughout the thoracic and anterior abdominal segments. In stage 16 *Asx*<sup>22P4</sup> *Sce*<sup>KO</sup> embryos, *Abd-B* is strongly misexpressed throughout the epidermis and CNS and in more cells in every segment compared with the single mutants. Segments T2–A7 in the cuticle are extensively, but not completely, transformed into copies of A8 (white arrowheads).

K118ub1 are drastically reduced but the levels of the PRC1 subunits Psc and Ph are undiminished. *Sce* is therefore the major E3 ligase for H2A monoubiquitylation in developing *Drosophila* but is not required for the stability of other PRC1 subunits. Second, PRC1-bound genes fall into two classes. Class I target genes are misexpressed if any of the PRC1 subunits is removed. Class II target genes are misexpressed in the absence of Ph or Psc-Su(z)2 but remain stably repressed in the absence of *Sce* or Pc. At class II target genes, Ph and the Psc-Su(z)2 proteins work together to repress transcription by a mechanism that does not require *Sce* and Pc and is therefore independent of H2A monoubiquitylation. Third, removal of the Ph, Psc-Su(z)2 or Pc proteins results in imaginal disc tumors that are characterized by unrestricted cell proliferation. However, removal of *Sce* does not cause this phenotype, suggesting that this tumor suppressor activity by the PcG system does not require H2A monoubiquitylation. Finally, our analyses reveal that PRC1 subunits are essential for repressing the *elB*, *noc*, *dac* and *pros* genes outside of their normal expression domains in developing *Drosophila*. This expands the inventory of developmental regulator genes in *Drosophila* for which PcG repression has been demonstrated in a functional assay. Below we discuss the implications of the findings reported in this study.

#### Molecular role of *Sce* in PRC1 and dRAF

In the *Sce*<sup>33M2</sup> allele Arg65 is mutated to Cys, but this mutant *Sce* protein is undetectable and therefore does not appear to be stable in vivo (Fig. 1A,B). The crystal structure of the Ring1B-Bmi1

complex provides a molecular explanation for this observation: the Arg70 residue in Ring1B that corresponds to Arg65 in *Sce* is thought to be critical for interaction with Bmi1 (Buchwald et al., 2006; Li et al., 2006). A likely scenario therefore is that the *Sce*<sup>Arg65Cys</sup> protein in *Drosophila* is unstable and is degraded because it is unable to associate with Psc or its paralog Su(z)2. Interestingly, removal of *Sce* protein has no detectable effect on the levels of the Psc and Ph proteins (Fig. 1C). Psc is therefore stable in the absence of its binding partner *Sce*. This is in contrast to the situation in mice in which *Ring1B* mutant ES cells show a drastic reduction in the levels of the Ring1B partner protein Bmi1 and its paralog Mel18 (Pcgf62) and also a reduction in the levels of Mph2 (Phc2) and Mpc2 (Cbx4) (Leeb and Wutz, 2007). The interdependence between PRC1 subunits for protein stability is therefore different in mammals and *Drosophila*.

Reconstitution of the *Drosophila* PRC1 core complex in a baculovirus expression system suggests that *Sce* is important for complex stability (Francis et al., 2001). At present, we do not know whether the Psc, Ph and Pc proteins still form a complex in vivo in the absence of *Sce*. It is currently unknown whether Psc, Ph and Pc are still bound to all PRC1 target genes in the absence of *Sce*. However, the finding that class II genes remain repressed in the absence of *Sce*, even though their repression depends on Psc-Su(z)2 and Ph, argues against a crucial role of *Sce* in the targeting of these other PRC1 subunits to these genes. Interestingly, the repression of all class II target genes analyzed here always requires both the Ph and the Psc-Su(z)2 proteins. A possible explanation for



this observation is that Ph and Psc-Su(2) still form a PRC1 subcomplex in the absence of Sce and that this complex is fully functional to repress class II target genes. Alternatively, it is possible that Ph and Psc-Su(z)2 repress class II target genes as components of as yet uncharacterized complexes that are distinct from PRC1 and dRAF.

### Transcriptional repression by Psc-Su(z)2 and Ph

In vitro, Psc and Su(z)2 proteins compact nucleosome templates, inhibit nucleosome remodeling by SWI/SNF complexes and repress transcription on chromatin templates (Francis et al., 2004; Francis et al., 2001; Lo et al., 2009). The observation that repression of class II target genes requires Psc-Su(z)2 and Ph but not Pc and Sce supports the idea that the chromatin-modifying activities of Psc-Su(z)2 identified in vitro are the main mechanism by which PRC1 represses these genes. Previous structure/function analyses in *Drosophila* showed that the same domains of the Psc protein responsible for chromatin compaction and remodeling inhibition in vitro are crucial for HOX gene repression in vivo (King et al., 2005). Chromatin modification by Psc and Su(z)2 is therefore also crucial for repression of class I target genes. Regulation of the class I target gene *en* further illustrates this point. In some tissues (e.g. in the dorsal hinge region of the wing imaginal disc; supplementary material Fig. S3, white arrowhead) repression of *en* requires all PRC1 core subunits, but in other tissues (e.g. in the pouch of the wing imaginal disc; supplementary material Fig. S3, open arrowhead) *en* remains repressed in the absence of Sce and Pc, and only Psc-Su(z)2 and Ph seem to be crucial to keep the gene inactive. At present, the molecular mechanism of Ph is not well understood. In vitro, Ph protein has the capacity to inhibit chromatin remodeling and transcription but it does so less effectively than Psc (Francis et al., 2001; King et al., 2002). At the target genes analyzed here, Ph is required for transcriptional repression wherever Psc-Su(z)2 is required, suggesting that Ph and Psc-Su(z)2 act in concert in this repression. Nevertheless, it is possible that repression of other PRC1 target genes requires a different subset of PRC1 subunits, or that, as in the case of *en*, the subunit requirement for repression changes depending on the cell type.

### H2A ubiquitylation and deubiquitylation in transcriptional repression

In mammals, Ring1B and Ring1A are responsible for the bulk of H2A-K119 monoubiquitylation (de Napoles et al., 2004; Wang, H. et al., 2004). Similarly, Sce generates the bulk of H2A-K118 monoubiquitylation in *Drosophila*, both in tissue culture cells (Lagarou et al., 2008) and in the developing organism (this study). The requirement for Sce at class I target genes is consistent with the idea that H2A monoubiquitylation of their chromatin is part of the repression mechanism. Repression of a subset of class I genes, namely the HOX genes, also requires the H2A deubiquitinase PR-DUB (Gaytán de Ayala Alonso et al., 2007; Scheuermann et al., 2010). Moreover, PR-DUB and Sce strongly synergize to repress HOX genes. Specifically, the phenotype of *Sce PR-DUB* double mutants (Fig. 6) suggests that H2A monoubiquitylation becomes ineffective for HOX gene repression if PR-DUB is absent. However, embryos that lack PR-DUB alone show a 10-fold increase in the bulk levels of H2A-K118ub1 and we estimate that ~10% of all H2A molecules become monoubiquitylated in these animals [see figure 4A in Scheuermann et al. (Scheuermann et al., 2010)]. How could this conundrum be explained? One possibility is that H2A monoubiquitylation and deubiquitylation at HOX gene

chromatin need to be regulated in a precisely balanced manner. However, an alternative explanation considers H2A-K118ub1 levels in the context of ubiquitin homeostasis. In particular, the high H2A-K118ub1 levels in *PR-DUB* mutants suggest that Sce generates widespread H2A monoubiquitylation at most Sce-bound genes and possibly also elsewhere in the genome, but that in wild-type animals PR-DUB continuously deubiquitylates H2A-K118ub1 at these locations and thereby recycles ubiquitin. The observation that PR-DUB is widely co-bound with Sce, not only at HOX but also at many other class I and class II target genes, is consistent with this idea. It is tempting to speculate that the widespread H2A monoubiquitylation in *PR-DUB* mutants sequesters a substantial fraction of the pool of free ubiquitin. It is therefore possible that removal of PR-DUB effectively depletes the pool of free ubiquitin in the nucleus to an extent that H2A monoubiquitylation at HOX target genes becomes inefficient and, consequently, their repression can no longer be maintained. According to this model, the crucial function of PR-DUB would not be the deubiquitylation of H2A-K118ub1 at HOX genes but rather at class II target genes and elsewhere in the genome where Sce ‘wastefully’ monoubiquitylates H2A.

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### Competing interests statement

The authors declare no competing financial interests.

### Supplementary material

Supplementary material available online at <http://dev.biologists.org/lookup/suppl/doi:10.1242/dev.074450/-DC1>

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