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A Genetic Screen Identifies Novel Polycomb Group Genes in Drosophila

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ABSTRACT

Polycomb group (PcG) genes encode evolutionarily conserved transcriptional repressors that are required for the long-term silencing of particular developmental control genes in animals and plants. PcG genes were first identified in Drosophila as regulators that keep HOX genes inactive in cells where these genes must remain silent during development. Here, we report the results of a genetic screen aimed at isolating novel PcG mutants in Drosophila. In an EMS mutagenesis, we isolated 82 mutants that show *Polycomb*-like phenotypes in clones in the adult epidermis and misexpression of the HOX gene *Ubx* in clones in the imaginal wing disc. Analysis of these mutants revealed that we isolated multiple new alleles in most of the already- known PcG genes. In addition, we isolated multiple mutant alleles in each of ten different genes that previously had not been known to function in PcG repression. We show that the newly identified PcG gene *calypso* is required for the long-term repression of multiple HOX genes in embryos and larvae. In addition, our studies reveal that the Kto/Med12 and Skd/Med13 subunits of the Med12·Med13·Cdk8·CycC repressor subcomplex of Mediator are needed for repression of the HOX gene *Ubx*. The results of the mutant screen reported here suggest that the majority of nonredundant Drosophila genes with strong classic PcG phenotypes have been identified.

POLYCOMB (*Pc*) and a number of other Drosophila genes were originally identified because of specific mutant phenotypes that suggested that the products of these genes are needed for repression of multiple HOX genes (Lewis 1978; Struhl 1981; Duncan 1982; Ingham 1984). Subsequent molecular studies showed that different HOX genes are indeed misexpressed in *Pc* and in *extra sex combs* (*esc*) mutant embryos (Struhl and Akam 1985; White and Wilcox 1985; Beachy *et al.* 1985; Wedeen *et al.* 1986). Because mutations in several different Drosophila genes caused the same phenotype as mutations in *Pc*, this set of HOX gene repressors was named the "Polycomb group" (Duncan 1982; Jürgens 1985). Subsequent studies revealed that PcG genes encode a conserved set of transcriptional repressors that are required for the long-term repression of a variety of developmental control genes in both animals and plants. In particular, PcG repressors have been implicated in processes ranging from X-chromosome inactivation and maintenance of stem cell pluripotency in mammals to the control of seed development and flowering time in plants (reviewed in Calonje and Sung 2006; Sparmann and van Lohuizen 2006; Schwartz and Pirrotta 2007).

To date, 17 different loci in Drosophila are classified as PcG genes because of HOX misexpression phenotypes. Specifically, mutations in *Pc*, *esc*, *Sex combs on midleg* (*Scm*), *Sex combs extra/Ring* (*Sce/Ring*), *Posterior sex combs* (*Psc*), *Enhancer of zeste* [*E(z)*], *Suppressor of zeste 12* (*Su(z)12*), *Polycomb-like* (*Pcl*), *super sex combs* (*sxc*), *Additional sex combs* (*Asx*), *pleiohomeotic* (*pho*), and *dSfmbt* have all been shown to cause misexpression of HOX genes in Drosophila embryos or larvae (Beachy *et al.* 1985; Ingham 1985; Struhl and Akam 1985; Struhl and White 1985; Wedeen *et al.* 1986; Jones and Gelbart 1990; McKeon and Brock 1991; Pattatucci and Kaufmann 1991; Simon *et al.* 1992; Fritsch *et al.* 1999; Beuchle *et al.* 2001; Birve *et al.* 2001; Klymenko *et al.* 2006). It is important to note that in the case of some PcG genes, the Drosophila genome contains two loci that encode closely related proteins that function in a redundant fashion. These are the gene pairs *polyhomeotic-proximal* (*ph-p*) and *polyhomeotic-distal* (*ph-d*), *Psc* and *Suppressor of zeste 2* [*Su(z)2*], *pho* and *pho-like* (*phol*), *esc* and *esc-like* (*escl*). In the case of *esc*, *ph-p*, *ph-d*, or *phol*, single mutants are even viable and they show only very mild or no misexpression of HOX genes but, importantly, in double mutant animals that lack the function of both gene pair members, the PcG phenotype is usually much more severe than in either single mutant (Dura *et al.* 1987; Brown *et al.* 2003; Wang *et al.* 2006).

Biochemical studies on PcG proteins revealed that they exist in distinct multiprotein complexes that contain two or more different PcG proteins. To date, three different PcG protein complexes have been purified

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from Drosophila: PhoRC, PRC1, and PRC2. PhoRC contains Pho and dSfmbt (Klymenko *et al.* 2006); PRC1 contains Psc, Pc, Ph, Sce/Ring, and Scm (Shao *et al.* 1999), and PRC2 contains Esc, E(z), and Su(z)12 (Czermin *et al.* 2002; Müller *et al.* 2002). These PcG protein complexes possess specific enzymatic and/or chromatin-binding activities by which they modify and interact with the chromatin of HOX and other target genes (reviewed in Müller and Kassis 2006;; Schuettengruber *et al.* 2007; Schwartz and Pirrotta 2007). Nevertheless, there are also PcG genes, *e.g.*, *Asx* or *Su(z)2*, whose products are not known to be components of any of these complexes. These proteins may exist as individual molecules in the cell, but it is also possible that they are part of other protein complexes that contain additional, as yet unidentified PcG proteins.

Of the Drosophila PcG genes listed above, some genes (*e.g.*, *Pc*) were identified because heterozygous adults show subtle homeotic phenotypes (*e.g.*, Lewis 1947). A number of PcG genes were identified in the classic screens for embryonic lethal mutations that cause patterning defects in the embryonic epidermis (Nüsslein-Volhard and Wieschaus 1980; Jürgens 1985). However, only some PcG loci could be identified by this approach because all known PcG genes are expressed in the female germ line and maternally deposited wild-type protein often rescues homozygous mutant embryos to a considerable extent. For example, homozygous *E(z)*, *Su(z)12*, or *dSfmbt* mutants survive into the larval stages, and *sxc* homozygotes even reach the pharate adult stage (Ingham 1984; Jones and Gelbart 1990; Birve *et al.* 2001; Klymenko *et al.* 2006). More than 20 years ago, Jürgens (1985) attempted to estimate the total number of PcG genes in Drosophila using the following assay: embryos that are double homozygous for mutations in two different PcG genes typically show strongly enhanced homeotic transformations compared to the single mutants; the phenotype of such embryos is often similar to the null phenotype of the corresponding single mutants lacking both maternal and zygotic gene function. Jürgens used this striking property and generated embryos that were double homozygous for a particular PcG mutation and large chromosomal deficiencies (Jürgens 1985). From these tests, Jürgens estimated that the total number of PcG genes in the Drosophila genome would be in the range of 30–40 genes (Jürgens 1985). Although this number is frequently cited (*e.g.*, Landecker *et al.* 1994; Yamamoto *et al.* 1997), only 4 new PcG genes have been identified over the past 2 decades. Among those, mutations in *Su(z)12* (Birve *et al.* 2001) and *dSfmbt* (Klymenko *et al.* 2006) show strong classic PcG phenotypes, whereas *cramped* mutants show mild homeotic transformations in pharate adults (Yamamoto *et al.* 1997). *multi sex combs* (*mxc*), finally, encodes the La protein, which is essential for cell viability; however, mild homeotic phenotypes have been observed in hypomorphic *mxc* adult flies (Santamaría and Randsholt 1995; Saget *et al.* 1998). Together, these observations indicate that several PcG genes have remained unidentified, which prompted us to perform a genomewide systematic genetic screen for novel PcG mutants.

For this screen we made use of the observation that almost all PcG genes are required throughout embryonic and larval development to maintain repression of HOX genes. In particular, clonal analyses had shown that removal of PcG gene function in imaginal discs of developing larvae results in the misexpression of HOX genes (Beuchle *et al.* 2001). Moreover, if animals with such PcG mutant clones are allowed to develop into adults, HOX misexpression manifests itself in characteristic homeotic transformations in the differentiated epidermis (Struhl 1981; Duncan 1982; Ingham 1984; Busturia and Morata 1988). In this study, we used this property as the basis for a genetic screen in clones in the adult epidermis.

MATERIALS AND METHODS

Drosophila strains: The genotypes of the mutagenized males were as follows:

Chromosome 1: *y w hsp70-gfp* FRT19
Chromosome 2: *y w hsp70-flp*; FRT40 FRT42D P[y^+]
Chromosome 3: *y w*; FRT2A FRT82B.

The genotypes of the tester strains were the following:

Chromosome 1: *y w hsp70-gfp* FRT19; *vgBE-gal4 UAS-flp*
Chromosome 2: *y w hsp70-flp*; *vgBE-gal4 UAS-flp* FRT40 FRT42D
Chromosome 3: *y w hsp70-flp; vgBE-gal4 UAS-flp*; P[y^+] FRT2A FRT82B.

EMS mutagenesis and screen: Isogenic males of the genotypes described above were mutagenized with 25 mm ethyl methanesulphonate (EMS) and mated to females of the appropriate tester strains described above. The F_1 progeny was reared at 18°, and F_1 adults were screened for appearance of the PcG syndrome. F_1 candidate mutants were isolated and back-crossed to the tester strain. The F_2 progeny was again reared at 18°, and F_2 adults that showed the PcG syndrome were isolated to establish appropriately balanced stocks. The crossing scheme for the screen on chromosome 3 is depicted in Figure 1.

In the secondary screen for HOX gene misexpression, balanced mutants were crossed to the appropriate tester line and larval wing discs from the progeny were stained with antibody against Ubx to test for Ubx misexpression in clones. Positives in this rescreen were categorized as class I or class II mutants depending on the severity of the Ubx misexpression phenotype. Class I and II mutants were further examined by analyzing GFP-marked clones using the following strains:

Chromosome 2L: *y w hs-flp*; *hs-nGFP* FRT40
Chromosome 2R: *y w hs-flp*; FRT 42D *hs-nGFP*
Chromosome 3L: *y w hs-flp*; *hs-nGFP* FRT2A
Chromosome 3L: *y w*; *vgBE-Gal4 UAS-flp*; *hs-nGFP* FRT2A
Chromosome 3R: *y w hs-flp*; FRT82B *hs-nGFP.*

This also allowed us to map the mutation to the left or right arm of chromosomes 2 and 3, respectively.

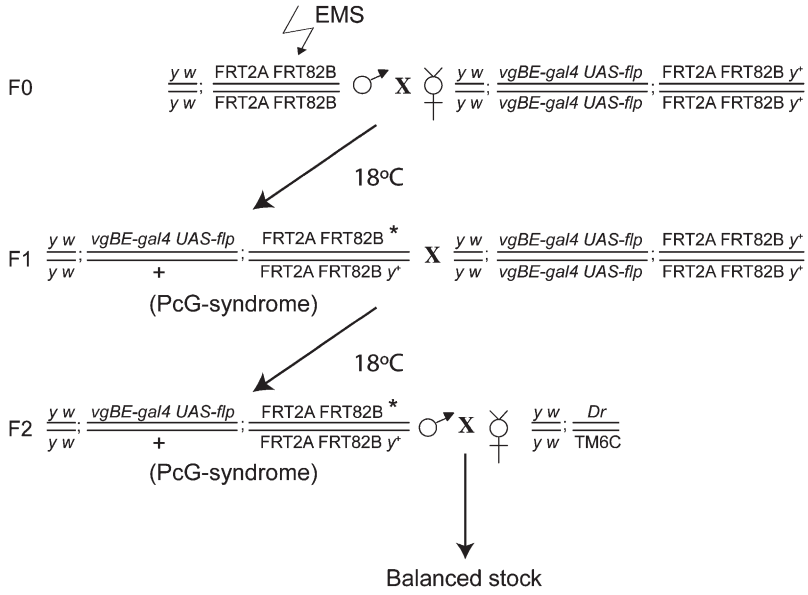

FIGURE 1.—Screening procedure for novel PcG mutants. The crossing scheme for the mutagenesis on chromosome 3 is shown as an example. EMS-fed F_0 males homozygous for *FRT2A FRT82B* were crossed to *vgBE-Gal4 UAS-flp; FRT2A FRT82B* y^+ "tester" females, and their F_1 progeny was screened for appearance of the PcG syndrome in the wing and thorax. Candidate F_1 mutants were retested using the same crossing procedure. The mutagenized chromosome was isolated from F_2 animals that showed the PcG syndrome, and balanced strains were established. EMS-induced mutation is represented with an asterisk (*). The same basic procedure with appropriate FRT chromosomes was used to isolate mutations on chromosomes 1 and 2 (see MATERIALS AND METHODS for detailed strain genotypes).

For analysis of kto^{T241} and skd^{T606} clones the *y w hs-flp; Ubi-GFP* FRT80 line was used.

Complementation analysis: After mapping of class I and class II mutants to individual chromosome arms, we tested whether the mutations were alleles of already known PcG genes. The following PcG mutations were used for this analysis: $Su(z)2^{1.b8}$, Psc^{e24}, Pcl^{D5}, Asx^3 (also known as Asx^{XT129}), $E(z)^{63}$, $Su(z)12^4$, Pc^{15} (also known as Pc^{XT109}), Scm^{D1}, and Sce^1. Class I and II mutants that were viable in *trans* with these mutations were then crossed to each other and complementation groups were established. In all crosses performed in this study, at least 100 F_1 animals were scored in the case of noncomplementation.

Mapping of *siren1* and *siren9*: Meiotic mapping with a *ru h th st cu sr e ca* chromosome revealed that $siren1^{32E16}$, $siren1^{33D19}$, and $siren9^{32A24}$ are located between the *th* marker at 72D1 and FRT2A at 79D–F. Subsequent complementation tests were carried out with the following deficiencies in the 72D–79D interval on 3L: W10, Cat, kto2, XS572, ri-79c, ri-XT1, Pc-101, Pc-2q, ED219, ED220, ED4606, ED223, ED224, ED225, ED4782, ED4786, ED4789, ED4799, ED228, ED229, ED4858, ED230, ED231, A13, A16, BSC20, XS705, 25-21, kto22, XS917, BSC2, and XS411.

Both $siren1^{32E16}$ and $siren1^{33D19}$ failed to complement ED228, ED229, XS572, XS705, 25-21, kto22, XS917, BSC2, and XS411, defining a chromosomal interval that contains the *CG32221*, *kto*, and *Paps* genes. Complementation tests showed that $siren1^{32E16}$, $siren1^{33D19}$, and $siren1^{33AC9}$ all fail to complement both kto^1 and kto^{12}. We therefore renamed all *siren1* alleles as kto^{32E16}, kto^{33D19}, kto^{33AC9}, kto^{144}, kto^{1251}, and kto^{33AC20}, respectively (see Table 1).

$siren9^{32A24}$ complemented each of the deficiencies listed above. *kto* and *skd* mutants show similar phenotypes (TREISMAN 2001; JANODY *et al.* 2003), *kto/siren1* and *siren9* mutants also show similar phenotypes, and since the *skd* locus was not uncovered by any of the deficiencies listed above, we tested whether *siren9* could be allelic to *skd*. We found that $siren9^{32A24}$, $siren9^{1855}$, and $siren9^{1052}$ all fail to complement both skd^2 and skd^3. We therefore renamed all *siren9* alleles as skd^{32A2}, skd^{1855}, and skd^{1052}, respectively (see Table 1).

Staining procedures and preparation of embryonic cuticles: Imaginal discs were stained as described (BEUCHLE *et al.* 2001) using antibodies against Ubx, Scr, or Kto/Med12. Cuticle preparations were done following standard protocols.

Screen for new *calypso* alleles: Males of the same isogenized line that was used for the screen on the second chromosome were mutagenized with 30 mM EMS and crossed to *y w*; *wg*/SM6b females. A total of 2500 males from the offspring bearing the mutagenized chromosome over SM6b were tested individually for viability of their mutagenized chromosome over $calypso^1$. One mutant was recovered that failed to complement $calypso^1$ and showed PcG syndrome and Ubx derepression after clone induction in the wing disc. This mutant was named $calypso^2$.

Generation of *calypso* mutant germ-line clones: *hs-flp slbo-LacZ/w*; FRT40 FRT42D y^+ $calypso^2$/FRT42D ovo^{D1} females were heat-shocked at third instar larval or pupal stage and subsequently crossed to *w*; FRT40 FRT42D y^+ $calypso^2$/CyO *Ubi-GFP* males. $calypso^2$ mutant embryos were distinguished by absence of GFP signal. *hs-flp slbo-LacZ*; FRT42D ovo^{D1}/CyO line was generated and kindly provided by Hsin-Ho Sung.

RESULTS AND DISCUSSION

An F_1 screen in clones in the adult epidermis identifies novel PcG mutants: We set up an F_1 screen based on the FLP–FRT system and screened for mutants that show PcG-like transformations in clones in the wing of adult flies. In particular, we generated F_1 adults that were heterozygous for a mutagenized genome but, due to FRT sites on one of the chromosomes, these animals contained clones of cells in the wing that were homozygous for a mutagenized chromosome arm. Induction of clones in the wing was done by expressing FLP recombinase from an *UAS-flp* transgene under the control of the *vgBE-Gal4* driver, as described by VEGH and BASLER (2003). Initial tests with this system, using previously characterized *Pc* and *Su(z)12* alleles, revealed that F_1 animals show a set of phenotypes that we call the PcG syndrome. The PcG syndrome is characterized by (1) a reduction of wing size and appearance of blisters and necrotic tissue in the wing blade, indicating partial transformation of wings into halteres (possibly due to misexpression of Ubx), (2) gaps in the triple row of bristles at the anterior wing margin, sometimes accompanied by

TABLE 1

Loci identified in screen

Locus	No. of alleles	Allele names	Class
2R			
Asx	6	22P4, 24E6, 26A3, 26D2, 27J6, 27K1	I
Pcl	3	22M21, 27O4, 27T7	I
calypso	2	1, 2[a]	I
Psc	3	24K4[b], 26E2, 27T5	II[b]
siren5	6[c]	21D2, 22N10, 22GE37, 23D10, 24B2, 26M3[c]	II
Single hits	2[d]	22F2, 22M2	II
3L			
E(z)	13	32A40, 32B20, 33D9, 33H25, 33M23, 33M30, 33Z14, 143, 328[e], 731[e], 914, 1025[e], 2434[e]	I
Pc	5	33A3, 33B10, 33E12, 33M4, 557	I
Su(z)12	6	33A8, 33C2, 33C4, 33P6, 974, 2036	I
siren1/kto	6	32E16, 33D19, 33AC9, 33AC20, 144, 1251	II
siren9/skd	3	32A24, 1052, 1855	II
siren2	2	31H4, 32B15	II
siren7	3[f]	31P7, 33T15, 33AC17[f]	II
siren8	3[f]	33U17, 33U18, 33AC17[f]	II
Single hits	4[d]	32C40, 33Q1, 33W1, 33AA12	II
3R			
Scm	1	32A23	I
Sce	1	33M2[g]	I
siren3	4	33C8, 33D22, 32E5, 33Z10	II
siren4	5[c]	33E6, 33M19, 33R1, 33W2[c], 33Z11	II
siren6	2	33U1, 33W16	II
circe	1	33D27	II[h]
Single hits	5[d]	33N15, 33R6, 33R11, 33AA14, 33AC1	II

The different loci for which mutants were recovered in the screen are grouped according to the chromosome arm where they are located. The number of alleles recovered for each locus is indicated, as well as whether Ubx misexpression in clones in the wing disc is strong (class I) or weak (class II).

[a] *calypso*^{2} was recovered from a screen for new *calypso* alleles.

[b] *Psc*^{24K4} shows Ubx misexpression corresponding to class II; the other two *Psc* alleles show Ubx misexpression intermediate between class I and class II.

[c] *siren5*^{26M3} and *siren4*^{33W2} do not show Ubx derepression in clones in the wing disc.

[d] "No. of alleles" in case of single hits indicates number of single hits.

[e] The *E(z)* alleles 328, 731, 1025, and 2434 have been previously described (Müller *et al.* 2002).

[f] The *33AC17* mutant fails to complement *siren7* and *siren8* mutants.

[g] *Sce*^{33M2} has already been described (Fritsch *et al.* 2003).

[h] *circe* mutant clones in wing disc show weak Ubx derepression. However, *circe* clones in haltere and third-leg disc show dramatic loss of Ubx expression.

transformation into posterior wing margin,possibly due to misexpression of Engrailed (Busturia and Morata 1988), (3) transformation of the macrochaete on the notum into bristles resembling those found on genitalia and analia (possibly due to misexpression of Abd-B), and, in extreme cases, (4) a longitudinal dorsal cleft splitting the dorsal notum. Representative examples of wings from animals with PcG syndrome are shown in Figure 2; the examples shown are all from mutants identified in the screen.

To screen for novel PcG mutants on the second and third chromosomes, we used FRT40 FRT42D and FRT2A FRT82B chromosomes, respectively. The use of these double FRT chromosomes allowed us to screen simultaneously for mutations on the left and on the right arm of these chromosomes (see Vegh and Basler 2003). In the case of the first chromosome, FRT19 was used. For chromosomes 2 and 3 we screened >200,000 F_1 animals each, and in the case of chromosome 1, 70,000 F_1 animals were screened. Candidate mutants showing the PcG syndrome were isolated and retested (Figure 1), to ensure that the mutation causing the phenotype was transmitted through the germ line. Mutants that scored positively in the retest were then isolated and stocks with appropriate balancer chromosomes were established (Figure 1). We thus isolated 124 mutants on chromosome 3, 99 mutants on chromosome 2, and 14 mutants on chromosome 1. In each case, homozygosity for the chromosome harboring the mutation caused lethality.

We then tested each of these candidate PcG mutants for misexpression of the HOX gene *Ubx*. Specifically, we

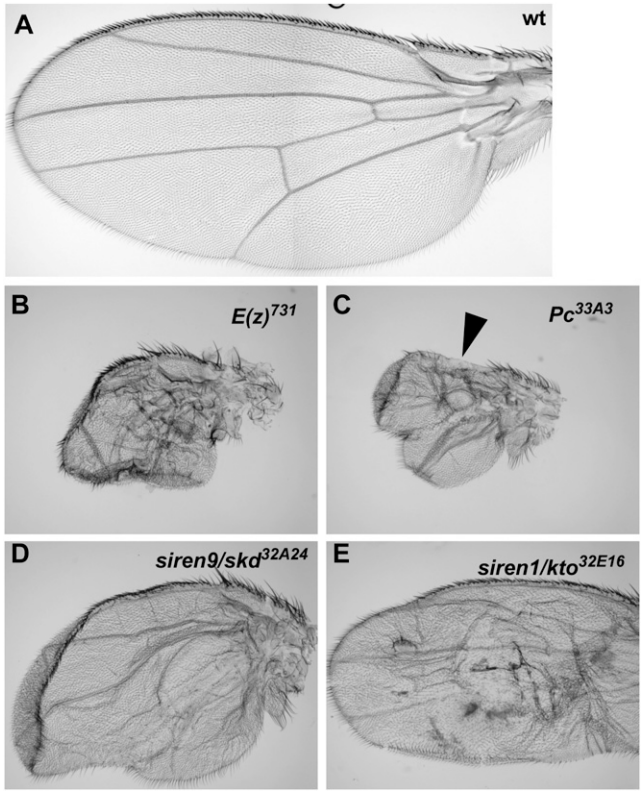

FIGURE 2.—PcG syndrome in wings of adult flies with clones of PcG mutant cells. (A) Wing of a wild-type adult. (B–E) Wings from F_2 adults isolated in the screen (see Figure 1). The animals were heterozygous for the indicated mutation and contained clones of homozygous mutant cells in the wing, induced by FLP recombinase expressed under the control of *vgBE-Gal4 UAS-flp*. Wings were photographed at the same magnification. Note the overall reduction of wing size, indicating partial transformation into haltere; blisters and necrotic tissue are observed in the wing blade, possibly reflecting sorting out of clone cells from the surrounding wild-type tissue. (C) Part of the anterior wing margin is transformed into posterior wing margin (arrowhead). Other phenotypes such as pattern duplications are visible as well, but these were not reliable indicators of PcG syndrome (see text). In the case of the two class I mutants $E(z)^{731}$ (B) and Pc^{33A3} (C), the PcG syndrome is more severe than in the class II mutants *siren9/skd*^{32A24} (D) and *siren1/kto*^{32E16} (E).

used the *vgBE-Gal4 UAS-flp* combination to induce clones of homozygous mutant cells in the wing imaginal disc and stained these discs with an antibody against the Ubx protein. Sixty-two mutants on chromosome 3, 20 mutants on chromosome 2, but none of the mutants on chromosome X showed misexpression of Ubx in the wing disc. Thus, a total of 82 mutants isolated in our screen showed a *bona fide* PcG phenotype.

The newly identified mutants can be grouped into two classes. Class I mutants show strong and widespread misexpression of *Ubx* in most clones in the pouch of the wing disc (Figure 3). Misexpression at high levels in the pouch and at lower levels in the notum and hinge region is observed in many of the classic PcG mutants (BEUCHLE *et al.* 2001). High-level misexpression of Ubx in the wing pouch likely reflects presence of (unidentified) transcription factors or activating signaling pathways that are localized in this area of the disc and promote transcriptional activation of *Ubx*. In contrast to class I mutants, class II mutants show misexpression of *Ubx* only in a small subset of clones in the pouch, and the levels of Ubx protein in these mutant clones are typically lower than in class I mutant clones (Figure 3). Interestingly, often only small clusters of cells within a clone show misexpression of *Ubx* (Figure 3). This suggests that repression is lost in a stochastic manner in the mutant cells within a clone and that the derepressed state is then maintained in a clonal fashion, similar to what is observed in the case of position-effect variegation (EBERT *et al.* 2006). The basis for this stochastic loss of repression is not known and will require further investigation. Taken together, these *Ubx* misexpression phenotypes correlated well with the severity of the PcG syndrome in clones in adult flies: class I mutants consistently showed a more extreme PcG syndrome than class II mutants. However, it should also be noted that the less severe PcG phenotype in class II mutants cannot simply be attributed to defects in clone growth; clones of class I and class II mutant cells show comparable growth rates (see Figure 3 for some examples).

We then performed complementation tests between the newly identified PcG mutants and mutants in known PcG loci. With the exception of *calypso*^{1} (see below), all class I mutants are novel alleles of previously described PcG genes (Table 1). Importantly, we identified multiple alleles for the majority of the known PcG loci (Table 1), an observation that strongly validates our screening strategy. In contrast to class I mutants, most class II mutants complemented mutations in the known PcG loci, indicating that these are mutations in novel genes that function in PcG repression (Table 1). Strikingly, crosses between the 46 different class II mutants revealed that the majority of them fall into nine different complementation groups (Table 1). We thus identified nine class II genes for which we had independently isolated two or more mutant alleles on the basis of their homeotic phenotype. We named these loci *siren1–9* (Table 1).

In the following we shall first briefly discuss some general aspects of the screen and shall then focus on the in-depth characterization of two class I and two class II mutants.

Identification of nonredundant PcG genes by screening for loss-of-function phenotypes: The use of the FLP–FRT system allowed us to screen for PcG mutants on all five major chromosome arms X, 2L, 2R, 3L, and 3R, provided that the mutated genes were located distally to the FRT cassette on these chromosomes. We thus isolated mutations in the known PcG loci *Pcl*, *Asx*, *Psc*, *Pc*, *Su(z)12*, *E(z)*, *Scm*, and *Sce* but also in previously uncharacterized genes. The inability to isolate alleles in other known PcG genes was either because of simple technical reasons or because of redundancy between

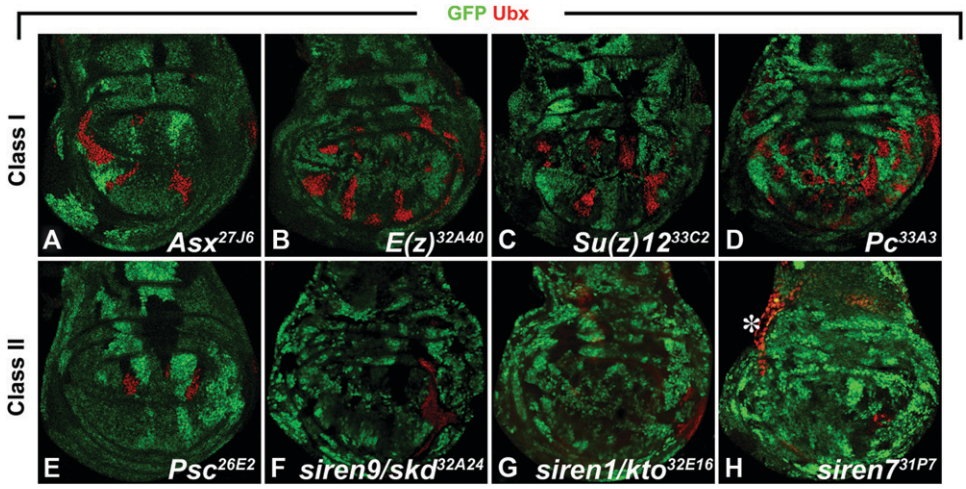

FIGURE 3.—Misexpression of the HOX gene Ubx in PcG mutant clones in the wing imaginal disc. Wing imaginal discs with clones of cells that are homozygous for the indicated mutant allele were stained with an antibody against the Ubx protein (red); clones of mutant cells are marked by the absence of GFP protein (green). (A and E) Clones were induced 96 hr before analysis by heat-shock-induced expression of FLP recombinase. (B–D and F–H) The clones were induced by FLP recombinase expressed from the *vgBE-Gal4 UAS-flp* driver; the clones were thus induced at different time points during larval development and, consequently, the clone size varies considerably. Ubx is normally not expressed in the wing imaginal disc, but it is strongly misexpressed in a large fraction of clones in class I mutants (A–D). In class II mutants (E–H), misexpression of Ubx is observed only in a few rare clones and the levels of Ubx signal in these clones is consistently lower than in class I mutant clones. The asterisk (*) in H marks the normal wild-type expression of Ubx in the wing trachea that was present in this preparation. All discs are oriented with the anterior compartment to the left.

two closely related PcG gene products. In particular, we did not isolate mutations in *pho* because the lack of suitable FRT sites on the fourth chromosome did not allow screening for mutants on this chromosome. Similarly, the *sxc* gene at 41C on the right arm of chromosome 2 (INGHAM 1984) is located between the centromere and the FRT cassette in 42D, and this precluded the isolation of *sxc* mutants because no mutant clones could be generated. In the case of the gene pairs *esc* and *esc-like*, *ph-p* and *ph-d*, *Psc* and *Su(z)2*, or *pho-like* and *pho*, we did not expect to isolate mutations because the two proteins of each of these gene pairs function in a redundant manner in imaginal discs (DURA *et al.* 1987; BEUCHLE *et al.* 2001; BROWN *et al.* 2003; WANG *et al.* 2006). Given that *Psc* null mutant clones show no misexpression of *Ubx* (BEUCHLE *et al.* 2001), it was therefore surprising that three class II mutants turned out to be alleles of *Psc* (Table 1). However, previous studies showed that certain mutant forms of Psc protein integrate into PRC1 *in vitro* but function in a dominant-negative fashion *in vivo* (KING *et al.* 2005). It is possible that our newly identified *Psc* alleles encode such aberrant forms of Psc protein that might, for example, compete with the redundantly acting Su(z)2 protein for complex formation. Finally, we note that we did not recover any mutant alleles of *dSfmbt*, even though *dSfmbt* null mutant clones in imaginal discs show widespread misexpression of HOX genes (KLYMENKO *et al.* 2006). However, when we tested *dSfmbt* mutants in our screen assay, *i.e.*, using the *vgBE-Gal4 UAS-flp* combination for clone induction, we found that the animals fail to hatch from the pupal case. It is therefore possible that we also failed to isolate mutations in uncharacterized PcG genes because of impaired survival.

Taken together, the screening strategy used here allowed us to isolate mutations in most of the non-redundant known PcG loci and, importantly, we also isolated mutations in 10 genes that were previously not known to be required for PcG-mediated repression.

***Calypso*, a novel Drosophila PcG gene:** One class I mutation on chromosome 2 complemented mutations in any of the known PcG genes on chromosome 2, suggesting that this mutation affects an uncharacterized PcG gene that is strictly required for repression of HOX genes. We named this gene *calypso* and the identified mutant allele *calypso*^{1} (Table 1). *calypso*^{1} mutant clones in imaginal discs show widespread misexpression of the HOX genes *Ubx* and *Sex combs reduced* (*Scr*) (Figure 4). To isolate additional *calypso* alleles, we performed an EMS mutagenesis in which we isolated *calypso*^{2} as a mutation that fails to complement the lethality of *calypso*^{1} (see MATERIALS AND METHODS). Clones of *calypso*^{2} mutant cells in imaginal discs show the same widespread misexpresssion of HOX genes as *calypso*^{1} mutant clones (Figure 4), providing further evidence that *calypso* is a *bona fide* PcG gene in Drosophila.

We next analyzed the requirement for *calypso* in PcG repression in embryos. As in the case of other PcG genes, maternally deposited wild-type Calypso products rescue *calypso* homozygous embryos into the larval stages, and such animals do not show any obvious phenotype in the cuticle or detectable misexpression of the HOX genes *Ubx* or *Abd-B* (data not shown). However, *calypso*^{2} homozygous embryos that are derived from *calypso*^{2} mutant germ cells die at the end of embryogenesis with homeotic transformations that are characteristic for PcG mutants (Figure 4). Specifically, in such *calypso* mutant animals, *i.e.*, lacking maternal and zygotic Calypso^{+} products, several abdominal segments are transformed into copies of the eighth abdominal segment (Figure 4). Together, these results clearly establish *calypso* as a novel PcG

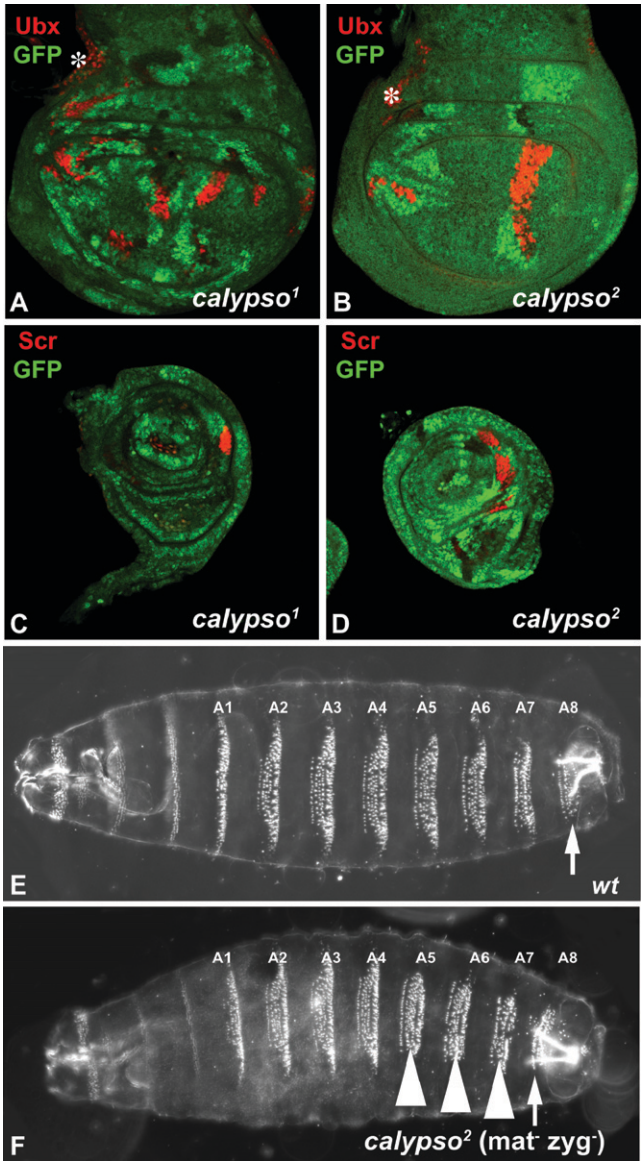

FIGURE 4.—The class I mutant *calypso* shows classic PcG phenotypes. Wing (A and B) and second leg discs (C and D) with *calypso*^{1} (A and C) or *calypso*^{2} (B and D) mutant clones were stained with antibodies against Ubx (A and B) or Scr (C and D) protein (red). In all cases, clones of *calypso* mutant cells are marked by the absence of GFP protein (green). Ubx is normally not expressed in the wing imaginal disc and Scr is normally not expressed in the second leg imaginal disc but strong misexpression of Ubx and Scr is detected in *calypso*^{1} and *calypso*^{2} mutant clones. An asterisk (*) in A and B marks normal Ubx expression in the trachea. (E and F) Ventral views of cuticles of a wild-type embryo (E) and a *calypso*^{2} homozygous embryo derived from a *calypso*^{2} germ-line clone (F). In the *calypso*^{2} mutant embryo, abdominal segments A5–A7 (arrowheads) are homeotically transformed, resembling the eighth abdominal segment (arrow) due to the lack of maternal and zygotic (mat^{-} zyg^{-}) wild-type Calypso protein.

gene that is required for repression of multiple HOX genes during both embryonic and larval development.

***Asx* is essential for PcG repression in larvae:** *Asx*^{3} (also called *Asx*^{XT129}) is considered to be a null mutation in *Asx* (SOTO *et al.* 1995). We previously reported that clones of *Asx*^{3} homozygous cells in imaginal discs show only very mild misexpression of *Ubx* and grow poorly (BEUCHLE *et al.* 2001). In the screen reported here, we identified six different *Asx* alleles as class I mutants that show severe misexpression of *Ubx* in clones of homozygous cells in the wing disc (Figure 3 and data not shown). None of these new *Asx* alleles show the growth defects observed in *Asx*^{3} mutant clones (Figure 3 and data not shown). One possible explanation for these differences could be that the chromosome carrying the *Asx*^{3} allele contains a second-site mutation that impedes cell growth/proliferation and that this phenotype masks the Polycomb phenotype in *Asx*^{3} mutant clones. Alternatively, *Asx* may have a function in cell growth or proliferation in addition to its role in PcG repression, and the *Asx* alleles that we isolated in our screen may encode proteins that have impaired PcG repressor activity but retain the function needed for cell growth or proliferation. However, the fact that we isolated six independent *Asx* alleles that cause a strong PcG phenotype with no proliferation defects strongly supports the first explanation. *Asx* thus appears to be a classic PcG gene that is required for the long-term silencing of HOX genes in embryos and larvae.

Kto/Med12 and Skd/Med13 are required for Ubx repression: To gain insight into the role of class II mutants in PcG repression, we mapped the *siren1* and *siren9* mutants by meiotic recombination, deficiency mapping, and complementation tests with candidate mutations. These experiments revealed that *siren1* is allelic to *kohtalo* (*kto*) and *siren9* is allelic to *skuld* (*skd*) (see MATERIALS AND METHODS). We therefore renamed the *siren1* and *siren9* alleles as *kto* and *skd* alleles, respectively (Table 1, Figure 3). The finding that we isolated mutations in *kto* and *skd* because of their *Ubx* misexpression phenotype (Figures 2 and 3) was surprising in several respects. *kto* and *skd* encode Med12 and Med13, both components of the very same protein complex, the Med12·Med13·Cdk8·CycC submodule of the Mediator complex (LEWIS and REINBERG 2003; BOURBON *et al.* 2004). The Med12·Med13·Cdk8·CycC subcomplex appears to function as a repressor module within Mediator; biochemical preparations of Mediator that lack it can stimulate transcription *in vitro*, whereas preparations including it cannot (BORGGREFE *et al.* 2002; TAATJES *et al.* 2002; BJÖRKLUND and GUSTAFSSON 2005). The repressive role of Med12 and Med13 is further supported by genetic studies in yeast, flies, and nematodes, where mutations in these Mediator components were identified in a variety of genetic screens for transcriptional repressors (CARLSON 1997; TREISMAN 2001; JANODY *et al.* 2004; YODA *et al.* 2005). Interestingly, clonal analyses in the eye/antenna disc showed that Kto/Med12 and Skd/Med13 are required for the repression or downregulation of *dachshund* (*dac*), *eyeless* (*ey*), and *decapentaplegic* (*dpp*) (TREISMAN 2001; JANODY *et al.* 2004; LONCLE *et al.* 2007).

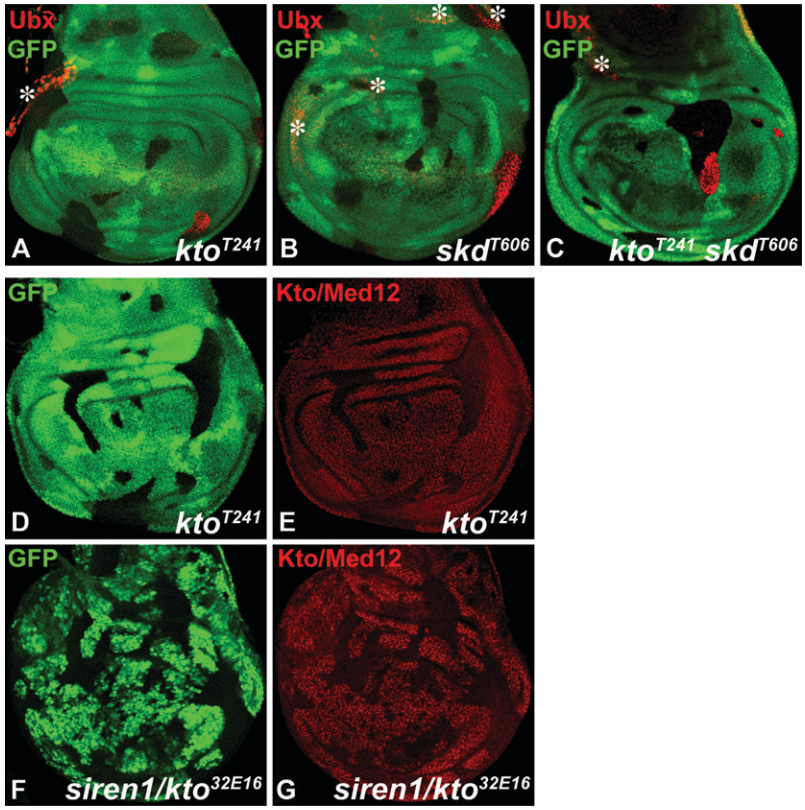

FIGURE 5.—Analysis of *kto* and *skd* mutant clones in the wing imaginal disc. (A–C) Wing discs with clones of cells homozygous for kto^{T241} (A), skd^{T606} (B), or double homozygous for kto^{T241} and skd^{T606} (C) were stained with antibodies against Ubx protein (red). In all cases, clones of mutant cells were marked by the absence of GFP protein (green). Note that in all cases Ubx is misexpressed only in a fraction of clone cells, but that this phenotype is comparable to that of $siren9/skd^{32A24}$ or $siren1/kto^{32E16}$ mutant clones shown in Figure 3, F and G, respectively. Asterisks (*) in A–C mark normal Ubx expression in the trachea and the peripordial membrane. (D–G) Wing discs with kto^{T241} (D and E) or $siren1/kto^{32E16}$ (F and G) mutant clones stained with antibodies against Kto/Med12 protein (red). Clones of mutant cells were marked by the absence of GFP protein (green). (D and E) Clones were induced 96 hr before analysis by heat-shock-induced expression of FLP recombinase. (F and G) The clones were induced by FLP recombinase expressed from the *vgBE-Gal4 UAS-flp* driver; the clones were thus induced at different time points during larval development and, consequently, the clone sizes vary considerably. Note the lack of Kto/Med12 antibody signal in both kto^{T241} and $siren1/kto^{32E16}$ mutant clones. Loss of the Kto/Med12 epitope is also observed in clones of other *siren1/kto* mutants (data not shown). All discs are oriented with the anterior compartment to the left.

This is intriguing because these three genes have been identified as target genes to which PcG proteins are bound in tissue culture cells (SCHWARTZ *et al.* 2006). Puzzlingly, mutations in *kto* and *skd* were, however, also identified as dominant suppressors of the extra sex combs phenotype that is observed in *Pc* heterozygotes and, because of this genetic interaction, these two genes were originally classified as trxG loci (KENNISON and TAMKUN 1988). The notion that mutations in *kto* and *skd* suppress traits of the PcG phenotype in a dominant genetic interaction assay but cause misexpression of certain PcG target genes in the recessive condition posed a conundrum. To address this issue, we performed a number of genetic tests. First, we asked whether Ubx misexpression in *kto* and *skd* mutant clones (Figure 3) was a specific feature of the *kto* and *skd* alleles that we had isolated in our screen or whether it was also observed in clones homozygous for kto^{T241} or skd^{T606}, two null alleles that were previously isolated in a different screen (TREISMAN 2001; JANODY *et al.* 2004). We found that kto^{T241} or skd^{T606} mutant clones in the wing imaginal disc also show misexpression of *Ubx* (Figure 5), demonstrating that this is a genuine *kto* and *skd* loss-of-function phenotype. Moreover, kto^{T241} skd^{T606} double-mutant clones show Ubx misexpression comparable to *kto* or *skd* single-mutant clones (Figure 5C), consistent with the observation that other phenotypes caused by the removal of either *kto* or *skd* are also not more severe in *kto skd* double mutants (JANODY *et al.* 2003). Second, we stained wing discs with clones homozygous for any of our six newly isolated *kto* alleles with antibodies against Kto/Med12. No Kto/Med12 epitope was detected in clones that were homozygyous for any of these alleles, indicating that none of the six alleles produces a stable full-length Kto/Med12 protein (Figure 5, F and G, and data not shown). No conclusive results were obtained with an antibody against Skd/Med13; this antibody gave uniform staining on wing discs with skd^{T606} mutant clones, even though the encoded Skd^{T606} protein is predicted to lack the corresponding epitope (TREISMAN 2001). Finally, we asked whether mutants lacking the CycC or Cdk8 subunits (LONCLE *et al.* 2007) of the Med12·Med13·Cdk8·CycC mediator submodule would also show PcG phenotypes. We were unable to detect misexpression of Ubx protein in imaginal disc clones that were homozygous for $CycC^{Y5}$ or $Cdk8^{K185}$, respectively (data not shown). This suggests that Kto/Med12 and Skd/Med13 do not mediate HOX gene repression through the kinase activity of Cdk8. Kto/Med12 and Skd/Med13 may thus have functions that are not linked to their association with CycC and Cdk8, an idea that is supported by the recent finding that several other phenotypes common to *kto* and *skd* mutants are not shared by *CycC* and *Cdk8* mutants (LONCLE *et al.* 2007).

Taken together, the loss-of-function phenotypes of *kto* and *skd* mutants show that the Kto/Med12 and Skd/Med13 proteins are required for repression of *Ubx* in imaginal discs. Furthermore, the deregulation of the putative PcG target genes *dac*, *ey*, and *dpp* (TREISMAN 2001; JANODY *et al.* 2004) suggests that the Kto/Med12

and Skd/Med13 proteins may be required for repression of multiple PcG target genes. Interestingly, we have, however, not been able to detect misexpression of the HOX genes *Scr*, *abd-A*, or *Abd-B* in either *kto* or *skd* mutant clones in leg, haltere, wing, or eye-antennal discs (data not shown). This raises the intriguing possibility that Kto/Med12 and Skd/Med13 proteins may be specifically required for repression of only a subset of PcG target genes. Recent studies that analyzed genomewide binding of PcG proteins by chromatin-immunoprecipitation assays identified a large number of putative PcG target genes in the Drosophila genome (Negre *et al.* 2006; Schwartz *et al.* 2006; Tolhuis *et al.* 2006). Systematic analyses of these target genes in different PcG mutants will allow addressing the question to what extent the different PcG protein complexes are required at each of these target genes.

Concluding remarks: In this study, we report the results of a genomewide genetic screen for mutations that cause loss of PcG repression in Drosophila. The screening strategy resulted in the isolation of mutant alleles in most of the known nonredundant PcG loci and identified a number of genes that were previously not known to play a role in Polycomb repression. Among these, *calypso* stands out as a novel PcG gene that is critically required for repression of HOX genes. The observation that *calypso* is the only new PcG gene with a strong phenotype that we identified, and the fact that we isolated multiple alleles in most of the nonredundant PcG genes, suggest that the majority of PcG genes with strong phenotypes in Drosophila have been identified. Nevertheless, we also isolated multiple alleles for nine different "class II" genes that show milder PcG phenotypes and less widespread misexpression of HOX genes. It is possible that class II genes show milder homeotic phenotypes because their products are not core components of the PcG system or because they are required for repression only at a subset of PcG target genes. However, as in the case of *Psc* and several other PcG genes, it is also possible that class II mutants show milder phenotypes because their products act redundantly with proteins encoded by other (*e.g.*, class II) genes.

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