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RNAi Components Are Required for Nuclear Clustering of Polycomb Group Response Elements

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SUMMARY

Drosophila Polycomb group (PcG) proteins silence homeotic genes through binding to Polycomb group response elements (PREs). *Fab-7* is a PRE-containing regulatory element from the homeotic gene *Abdominal-B*. When present in multiple copies in the genome, *Fab-7* can induce long-distance gene contacts that enhance PcG-dependent silencing. We show here that components of the RNA interference (RNAi) machinery are involved in PcG-mediated silencing at *Fab-7* and in the production of small RNAs at transgenic *Fab-7* copies. In general, these mutations do not affect the recruitment of PcG components, but they are specifically required for the maintenance of long-range contacts between *Fab-7* copies. Dicer-2, PIWI, and Argonaute1, three RNAi components, frequently colocalize with PcG bodies, and their mutation significantly reduces the frequency of PcG-dependent chromosomal associations of endogenous homeotic genes. This suggests a novel role for the RNAi machinery in regulating the nuclear organization of PcG chromatin targets.

INTRODUCTION

Polycomb group (PcG) genes are highly conserved regulatory factors that are responsible for the maintenance of silent states of homeotic genes (Dejardin and Cavalli, 2005; Ringrose and Paro, 2004). PcG proteins form multimeric complexes that exert their respective functions by modifying chromatin structure. To date, two types of PcG complexes have been described. The first, called the E(Z)/ESC complex or the Polycomb repressive complex 2 (PRC2), contains the PcG proteins Enhancer of zeste (E(Z)), Extra sex combs (ESC), and SU(Z)12 and the histone binding protein NURF-55. E(Z) methylates lysine 27 of histone H3 (H3K27) (Cao and Zhang, 2004), creating an epigenetic mark that leads to the recruitment of the second type of PcG complex, named PRC1, via binding of the chromodomain of one of its components, the Polycomb (PC) protein. PC is a stoichiometric component of PRC1, together with Polyhomeotic (PH), Posterior sex combs, and dRing (Shao et al., 1999).

Drosophila PcG proteins regulate their target loci through binding to chromosomal regulatory elements named PREs (Chan et al., 1994; Fauvarque and Dura, 1993; Tillib et al., 1999). *Fab-7* is a well-characterized, PRE-containing element that is involved in the regulation of the homeotic gene *Abdominal-B* (*Abd-B*), which is located within the bithorax complex (BX-C) locus on the right arm of the third chromosome (Mihaly et al., 1997). When inserted into the fly genome, *Fab-7*-containing transgenes can ectopically recruit PcG proteins, leading to PcG-dependent silencing of reporter genes (Zink and Paro, 1995). Silencing is manifested as variegated expression phenotypes of the reporter genes. Another typical feature of PRE-mediated gene silencing is that repression is stronger when two copies of the transgene are present (the homozygous state) than when only one copy is present (the heterozygous state). The existence of this effect, called pairing-sensitive silencing (PSS), suggests that the proximity of two homologous PRE sequences reinforces their individual repressive functions (Kassis, 2002). PREs are also occasionally capable of enhancing silencing when multiple copies are present at independent chromosomal loci (the *trans*-heterozygous configuration) (Muller et al., 1999). In one such case that was analyzed in detail by 3D-FISH (three-dimensional fluorescence in situ hybridization), multiple copies of the *Fab-7* element were shown to be capable of inducing long-distance interactions within the nucleus, bringing independent chromosomal loci into close spatial proximity (Bantignies et al., 2003). These chromosomal interactions were stable throughout development and required both DNA sequence homology between the two interacting loci and wild-type (wt) function of PcG proteins.

The RNAi machinery has been implicated in a wide variety of biological processes (Hannon, 2002). One of these processes is the formation of heterochromatin (Fukagawa et al., 2004; Kanellopoulou et al., 2005; Lippman and Martienssen, 2004; Matzke and Birchler, 2005; Volpe et al., 2002). In *S. pombe*, this involves bidirectional transcription of RNA molecules from repetitive sequences and their cleavage into short interfering RNAs (siRNAs) of 21–23 nt by an RNase III enzyme called Dicer-1. siRNAs guide the RNA-induced initiation of transcriptional gene silencing (RITS) complex to homologous sequences in the nucleus (Noma et al., 2004; Verdel et al., 2004). Clr4, the homolog of the histone methyltransferase Su(Var) 3-9, is recruited along with the RITS complex to chromatin, where it methylates lysine 9 of histone H3 (H3K9). This epigenetic mark promotes the formation of heterochromatin by recruiting the heterochromatin protein Swi6, the homolog of HP1, via its chromodomain (Grewal and Rice, 2004). Consistent with these data, a redistribution of H3K9 methylation has been observed in *Drosophila* chromosomes in flies mutant for components of the RNAi machinery (Pal-Bhadra et al., 2004).

The RNAi machinery is also required for cosuppression, a phenomenon whereby the introduction of multiple transgenic copies of a gene phenocopies its loss of function instead of increasing its expression (Napoli et al., 1990; Pal-Bhadra et al., 2002). In *Drosophila*, cosuppression can act at either the transcriptional or posttranscriptional level and involves PcG proteins as well as the RNAi machinery (Pal-Bhadra et al., 1997, 1999, 2002).

The *Drosophila* RNAi machinery includes two Dicer proteins encoded by the *dicer-1* (*dcr-1*) and *dicer-2* (*dcr-2*) genes. Dcr-2 is specifically required to process double-stranded RNAs into siRNAs and mediates the assembly of siRNAs into the RNA-induced silencing complex (RISC). Dcr-1 is involved in the metabolism of siRNAs as well as the processing of pre-microRNAs into microRNAs (Lee et al., 2004). RNA silencing also involves several highly conserved genes coding for PAZ-domain proteins. *Argonaute1* (*AGO1*) and *Argonaute2* (*AGO2*) are involved in microRNA biogenesis and RNA interference (RNAi) (Okamura et al., 2004; Williams and Rubin, 2002). *piwi* is involved in cosuppression (Pal-Bhadra et al., 2002), silencing of retrotransposons (Kalmykova et al., 2005; Sarot et al., 2004), and heterochromatin formation (Pal-Bhadra et al., 2004). *aubergine* (*aub*) was first isolated based on its role in germline development (Harris and Macdonald, 2001) but is also responsible for maintaining the silenced state of an X-linked male fertility gene locus (*Stellate*) via RNAi (Aravin et al., 2004). The Aub protein is required for RNAi and RISC assembly in ovaries (Tomari et al., 2004). In addition, *homeless/spindle-E* (*hls*) is involved in silencing of *Stellate* and in heterochromatin formation (Aravin et al., 2004; Pal-Bhadra et al., 2004). Here, we tested whether RNAi components are involved in the PcG pathway. Our results show that the RNAi machinery affects the PcG response via a novel regulatory function in nuclear organization.

RESULTS

Effect of the RNAi Machinery on Silencing Mediated by Transgenic *Fab-7* Copies

We tested the role of a variety of RNAi components in a specific transgenic line called Fab-X (Bantignies et al., 2003). This line contains a construct carrying a 3.6 kb fragment from the *Fab-7* region, cloned upstream of a mini-*white* reporter and inserted into the X chromosome. In the Fab-X line, the presence of the *Fab-7* sequence is sufficient to induce PcG-dependent silencing, both of the mini-*white* eye-color reporter gene and of the endogenous *scalloped* (*sd*) gene, which is required for wing-blade morphogenesis and is located 18.4 kb downstream of *Fab-7*. These two repressed phenotypes are abolished in the presence of mutations in PcG genes and are not present in heterozygous females and hemizygous males, indicating that both mini-*white* and *sd* expression are subject to PSS (Bantignies et al., 2003).

The eye-color and wing phenotypes were used as a basis to analyze the effect of the RNAi machinery on PcG-dependent repression. Mutations in RNAi components were introduced into the Fab-X line and placed over a balancer chromosome containing a GFP marker. As the *AGO1* mutant alleles involve P element insertions containing the mini-*white* reporter gene, they could not be tested using the eye phenotype. A null mutation in *dcr-2* (*dcr-2*^{L811fsX}) decreased silencing of the mini-*white* reporter gene relative to the Fab-X line when in the homozygous state (Figure 1A). Likewise, two different mutant alleles of *piwi* (*piwi*^{1} and *piwi*^{2}) decreased mini-*white* silencing (Figure 1A), with the effect being more pronounced in *piwi*^{2} mutant flies. This effect was not restricted to the Fab-X line since it was also observed when *piwi*^{2} was recombined into another *Fab-7*-containing line (See Figure S1 in the Supplemental Data available with this article online). In contrast to the effects seen for *dcr-2* and *piwi* alleles, Fab-X females homozygous mutant for *hls*^{E1} or that carried the heteroallelic *hls*^{E1}/*hls*^{E616} combination silenced mini-*white* like wt Fab-X females (Figure 1A).

The *sd* phenotype was then analyzed in all mutant backgrounds at 28.5°C, a temperature inducing a strong wing phenotype in Fab-X. A preselection of non-GFP female larvae was carried out in order to selectively analyze homozygous or *trans*-heterozygous mutant adults. This analysis revealed that mutating any of the components of the RNAi machinery, except for *hls* and the heterozygous *dcr-1* mutation, leads to a strong decrease in the *sd* phenotype (Figures 1B and 1C). These data show that the RNAi machinery can affect PcG-mediated silencing. The fact that *hls* mutants had no effect suggests that this process might be mechanistically distinct from the role of RNAi components in heterochromatin formation.

The global level of PRC1 proteins as measured by Western blot did not decrease in RNAi mutants (Figure S2). This suggests that the loss of PcG-dependent silencing at the *sd* locus in the Fab-X line might not depend on a global loss of PcG proteins. Another possibility is that RNAi

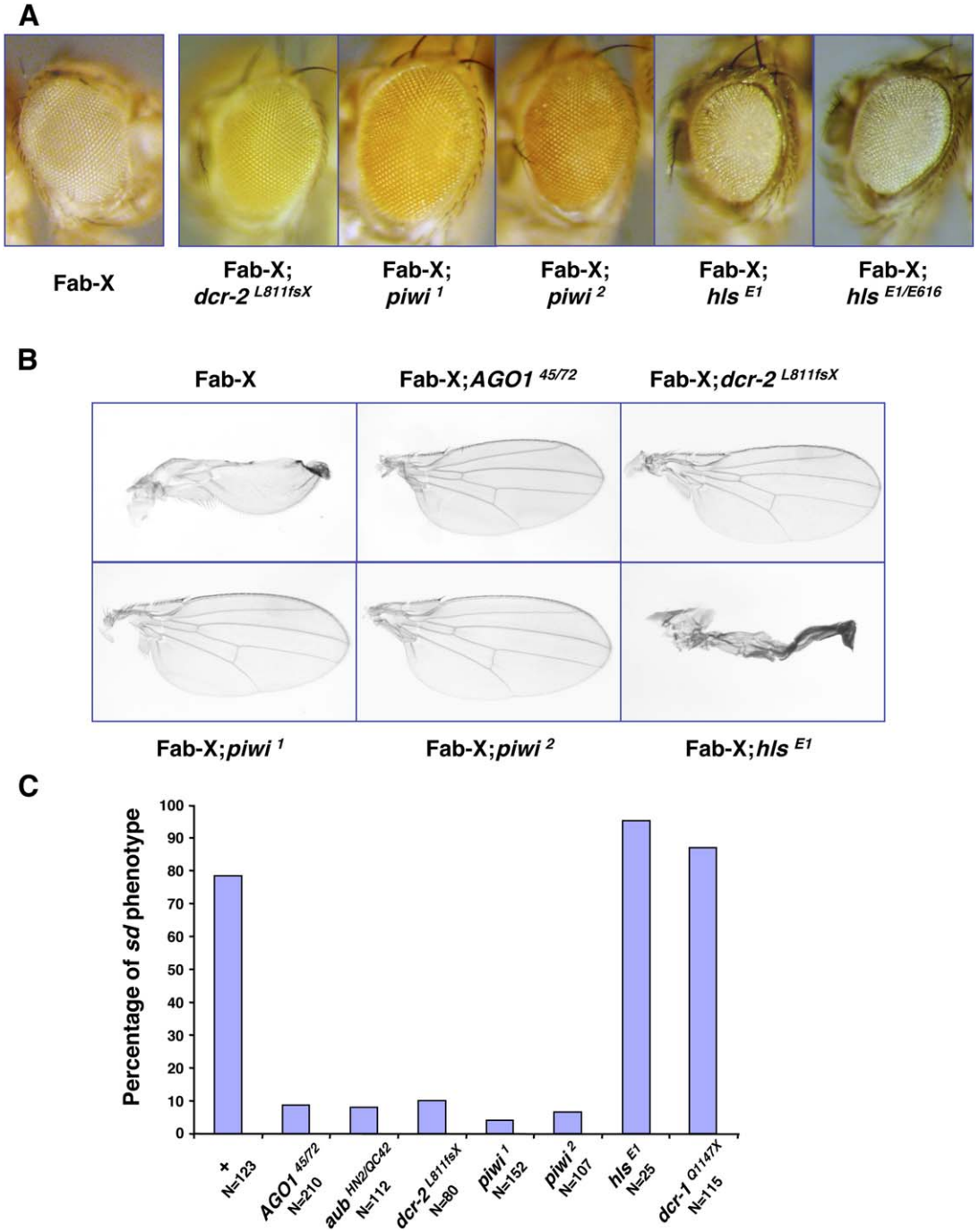

Figure 1. RNAi Components Affect *Fab-7*-Mediated Silencing

(A) Eye-color phenotype of female flies of different genotypes, indicated in each panel, grown at 25°C.

(B) Examples of *scalloped* (*sd*) wing phenotypes of female flies from different genotypes, grown at 28.5°C.

(C) Quantification of the percentage of *sd* wings in females for the different genotypes. The genotypes and the number of flies analyzed are indicated below each bar.

components might be required to generate small-RNA species that might recruit PcG silencing factors at the PRE. We did not detect significant levels of endogenous *Fab-7* transcripts by RT-PCR in adult wt flies. On the other hand, the presence of multiple copies of *Fab-7* in the Fab-X line induced the production of *Fab-7* transcripts in both sense and antisense orientations, and the amount of these transcripts was further increased in the presence

of mutations in *piwi* but not *hls* (Figure S3). Moreover, RNA species of 21–23 nt were detected in the *Fab-7* region of lines carrying a *Fab-7* transgene. The level of these small RNAs was increased in the additional presence of two endogenous *Fab-7* copies at the BX-C, while it was strongly reduced by mutations in *dcr-2* and *piwi* but not *hls* (Figure 2). This strong reduction is reminiscent of the effect of *dcr-2* mutation on the levels of siRNAs induced by a transgene producing a hairpin RNA (Lee et al., 2004) and of the effect of *piwi* mutations on siRNAs produced upon posttranscriptional silencing of multiple copies of an *Adh* transgene (Pal-Bhadra et al., 2002). Therefore, silencing at the transgenic *Fab-7* correlates with the RNAi-dependent production of small RNAs.

Effect of the RNAi Machinery on the Recruitment of PcG Proteins

In order to test the possibility that the RNAi machinery might be involved in the recruitment of PcG proteins to PREs, we combined FISH with immunostaining (FISH-I) on polytene chromosomes of third-instar female larvae. It has previously been shown that *Fab-7* recruits PcG proteins at transgene insertion sites (Cavalli and Paro, 1999; Dejardin et al., 2005; Zink and Paro, 1995). Consistent with this, we observed that two components of the PRC1 complex, Polycomb (PC) and Polyhomeotic (PH), were recruited to the insertion site of the *Fab-7* transgene in the Fab-X line (Figure 3A).

After selecting homozygous or *trans*-heterozygous larval females mutant for the different RNAi alleles, we performed FISH-I experiments with PH and PC. The *hls* and heterozygous *dcr-1* mutant backgrounds did not affect PH and PC binding at the transgene locus, as expected from the absence of effects on *Fab-7*-dependent silencing. The same result was observed with *dcr-2*, *aub*, and the *AGO1* mutant background (Figure 3A), even though these three genes are required for PcG-dependent *sd* silencing in the Fab-X line. However, a different result was obtained in the *piwi* mutant background, in which PC binding to the *Fab-7* transgene was abolished (Figure 3A). Surprisingly, this loss was specific for PC, as PH was not lost from the transgene (Figure 3A). This unexpected finding shows that the binding of PC and PH can be uncoupled and that PC is more sensitive than PH to the presence of the RNAi machinery.

As shown in Figure 3B, the loss of PC binding in *piwi* mutant backgrounds was restricted to the *Fab-7* transgenic insertion site, as overall PC binding at endogenous cytological positions was not visibly affected. Taken together, these data show that the RNAi machinery does not play a major role in recruiting PcG proteins to their endogenous chromatin targets, although PIWI can play a role in stabilizing PcG components at a PRE isolated from its natural genomic context.

Since PRC1 is thought to be recruited to chromatin by the PRC2 complex, we next analyzed whether mutations in *piwi* could also affect the recruitment of PRC2 member E(Z) to the *Fab-7* transgene. FISH-I experiments using an antibody against E(Z) showed that E(Z) is specifically recruited to the *Fab-7* transgene in the Fab-X line. This binding was completely lost in the *piwi*^{1} homozygous mutant background, while it was maintained in the presence of a mutation in *AGO1* (Figure 3C). Therefore, the destabilization of PC from the transgene in *piwi* mutant backgrounds might be due to a loss of PRC2. Again, E(Z) binding to endogenous sites was not visibly affected, suggesting that the tethering of PcG proteins by isolated PREs is less robust than is binding to endogenous sites.

Although our immunofluorescence data cannot exclude a minor decrease of PcG binding at its target sites, they clearly show that the RNAi machinery does not play an

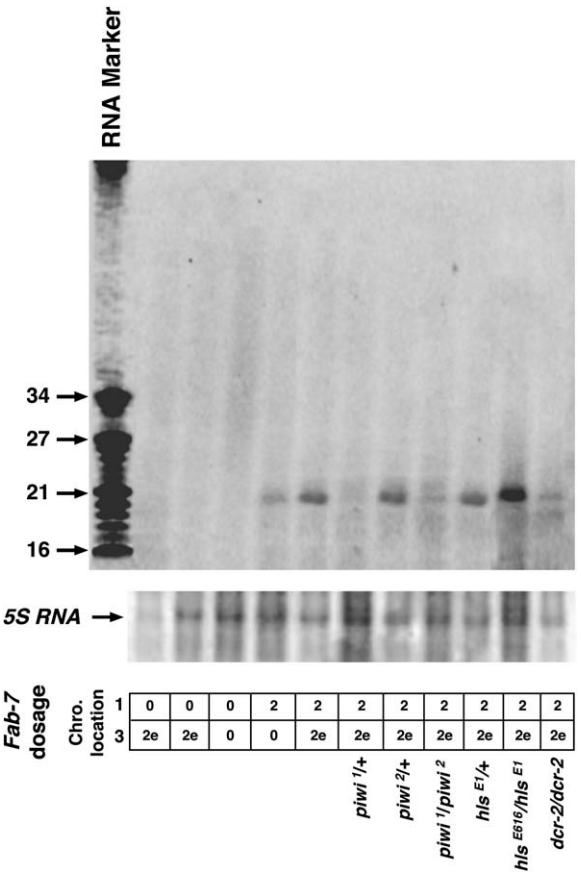

Figure 2. Transgenic *Fab-7* Copies Induce RNAi-Dependent Production of Small-RNA Species

Twenty-one to twenty-three nucleotide RNA species in the *Fab-7* region were detected in female adult flies by Northern blot using an RNA probe corresponding to the sense strand of the chromatin boundary portion of *Fab-7* (see also Figure S3). The number of transgenic or endogenous *Fab-7* copies from the indicated chromosome is given below the image. The first two lanes correspond to RNAs extracted from Canton S or w^{1118} females. The third lane corresponds to a w^{1118};*Fab-7*^{1} line carrying no *Fab-7*. The fourth lane corresponds to a Fab-X;*Fab-7*^{1} line carrying two transgenic but no endogenous *Fab-7* copies (Bantignies et al., 2003). The other lanes correspond to Fab-X carrying the indicated mutations. The 5S RNA from the same gel, stained by ethidium bromide, was used as a loading control.

essential role in PcG recruitment. This is consistent with the absence of homeotic phenotypes in every RNAi mutant that has been analyzed to date.

The RNAi Machinery Is Required for the Maintenance of Long-Range *Fab-7* Chromosomal Interactions

Since PcG repression is lost in RNAi mutants even though PcG proteins can still bind to the *sd* locus, we reasoned that RNAi components might regulate a different step in *Fab-7*-dependent gene silencing. We have previously demonstrated that long-distance chromosomal interactions between transgenic *Fab-7* sequences and the endogenous *Fab-7* within the BX-C can lead to strong silencing of the *sd* gene in the Fab-X line (Bantignies et al., 2003). As RNAi components are known to play a role in the nuclear organization of *S. pombe* chromosomes (Hall et al., 2003), we asked whether mutations in the RNAi machinery might affect the nuclear interaction of *Fab-7* sequences. To do this, we performed two-color FISH on whole-mount embryos carrying homozygous or *trans*-heterozygous RNAi mutations. These embryos were selected by the absence of GFP fluorescence during germband retraction, a stage at which PcG-mediated silencing has already initiated (Orlando et al., 1998).

In the control w^{1118} line, which does not contain *Fab-7* transgenic copies at the *sd* locus, the BX-C and *sd* loci interacted in 5% of the nuclei (Figure S4). This frequency may reflect random collisions of the two loci caused by chromosomal movements within the cell nucleus. In the Fab-X line, we observed a significant percentage of nuclei (25%) showing colocalization of the *sd* locus (which harbors the transgenic *Fab-7* copy) with the BX-C locus. RNAi mutant backgrounds for *AGO1*, *aub*, *piwi*, and *hls* did not affect the long-distance chromosomal interactions between *sd* and BX-C (Figure S4), suggesting that the RNAi machinery does not participate in the establishment of *Fab-7* contacts.

We then tested whether RNAi components are necessary for the maintenance of these contacts by performing two-color FISH experiments on wing imaginal discs of third-instar larvae. In the negative control line (w^{1118}), the percentage of colocalization was similar to that observed in embryos (Figure 4B), while in the Fab-X line, the colocalization of *sd* with the BX-C loci reached 38%. This frequency was not changed in the hls^{E1} homozygous mutant background. Strikingly, however, we observed a complete loss of *Fab-7* contacts in larval nuclei with all other RNAi mutant derivatives tested (Figures 4A and 4B). These data indicate that specific components of the RNAi machinery participate in PcG-dependent repression at the *sd* locus by contributing to the maintenance of long-distance *Fab-7* interactions during development. Importantly, a failure to maintain long-distance *Fab-7* contacts was also observed in mutants in which PcG proteins were still recruited to the *Fab-7* transgene. Thus, the role of RNAi factors in PcG protein recruitment can be uncoupled from their role in regulating long-range contacts between multiple *Fab-7* copies.

RNAi Components Colocalize with PcG Nuclear Bodies

We then analyzed the subcellular localization of RNAi components in larval nuclei by immunostaining (Figure 5A). PIWI is an exclusively nuclear protein, while AGO1, Dcr-2, and Hls stain in both the nucleus and the cytoplasm. All four proteins form discrete nuclear bodies, with approximately 13–15 foci for PIWI and Dcr-2, 10–12 foci for AGO1, and 5–6 foci for Hls. Remarkably, Hls foci were often localized close to the heterochromatin (as seen by intense DAPI staining), consistent with its genetic effect on heterochromatin silencing (Pal-Bhadra et al., 2004). Dcr-1 showed a predominantly cytoplasmic staining, with distinct protein accumulations close to the nuclear rim. In addition, there are 1–2 distinct Dcr-1 foci in a minority of the nuclei (Figure 5A).

PcG proteins also form nuclear bodies (Buchenau et al., 1998; Ficz et al., 2005; Saurin et al., 1998). In embryos, we detected 45–50 PC and PH bodies per nucleus, with apparent sizes ranging from 200 nm to 450 nm (data not shown). In larval tissues, we observed a clear decrease in the number of PC and PH bodies to approximately 30 for each of the proteins (Figure 5A, Figure 6A, and Figure 7A). This decrease is consistent with a recently published analysis of the subnuclear distribution of GFP-tagged forms of these two proteins (Ficz et al., 2005).

In double-immunolabeling experiments in larval imaginal discs, we observed 52% colocalization of PIWI foci with PH bodies. This colocalization was also seen for AGO1 (42% colocalization) and was even more pronounced for Dcr-2, reaching 62% (Figure 5B). In contrast, Hls colocalized only to 19.5%, consistent with the absence of a genetic effect of *hls* mutations in *Fab-7*-mediated silencing and in long-range contacts. Likewise, we found only 26% colocalization between Dcr-1 spots and PH. Since only 40% of the nuclei show Dcr-1 foci, this means that only about 10% of the total nuclei show colocalization between one Dcr-1 spot and PH, suggesting that Dcr-1 is not directly linked to PcG target genes. A statistical analysis of the levels of colocalization for Hls and the Dcr-1 proteins revealed a highly significant difference compared to the values observed for AGO1, PIWI, and Dcr-2. In summary, these data suggest that a distinct subset of nuclear RNAi components are physically located at, or close to, PcG nuclear bodies, although they are not integral stoichiometric components of PRC1.

Fab-7 Contacts and Targeting of *Fab-7* to PcG Bodies

We developed a combined 3D two-color FISH and immunostaining technique (FISH-I) that allows the detection of the relative localization of genes with PcG bodies, which are visualized using an anti-PC antibody. Analysis of the locations of known target genes of the PcG factors indicates that PcG bodies correspond to physical sites of gene silencing (F.B., V. Roure, J. Bonnet, and G.C., unpublished data). FISH-I experiments with *sd* and the BX-C were performed on imaginal discs of third-instar larvae, in which the

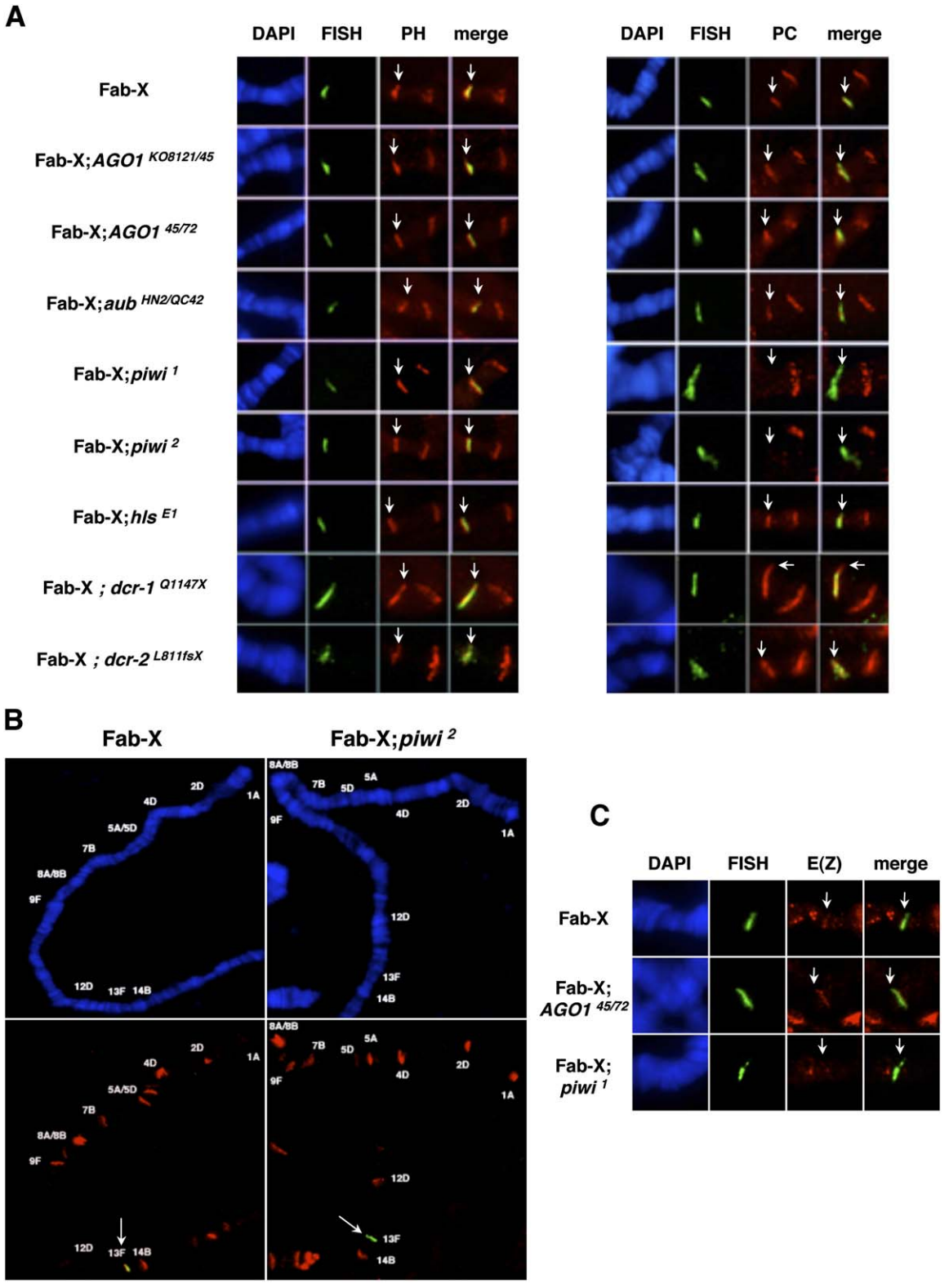

Figure 3. The Role of RNAi Components in PcG Recruitment

(A) FISH-I analysis with PH (left panel) and PC (right panel) antibodies on polytene chromosomes of third-instar female larvae of the Fab-X line and its RNAi mutant derivatives. DAPI labeling, the FISH signal of the transgenic insertion at the 13F *sd* locus, PH or PC, and the merge of the three channels are shown. Arrows indicate the position of the transgene.

BX-C is silenced by PcG proteins. We found that the BX-C was localized within PcG bodies in 89% of the nuclei in the w^{1118} control line and that the *sd* locus was localized within PcG bodies in 14% of the nuclei (Figure 6). As *sd* is not regulated by PcG proteins in the absence of *Fab-7* sequences, this 14% value likely corresponds to a coincidental proximity due to chromosomal movements.

We next analyzed the positioning of the *sd* and BX-C loci relative to PcG bodies in the Fab-X line, in either a wt background or the presence of mutations in RNAi genes. In the wt Fab-X line, the *Fab-7* transgene induced the localization of the *sd* locus within PcG bodies in 54% of the nuclei, while the BX-C was present within PcG bodies in 87% of the nuclei, similar to the w^{1118} control line (Figure 6). Colocalization with PcG bodies was not significantly perturbed in the presence of *dcr-2* and *AGO1* mutations, indicating that the loss of long-range *Fab-7* contacts is not a consequence of the loss of PcG protein targeting at PREs. On the other hand, a mutation in *piwi* reduced colocalization to 16.5% (Figure 6), consistent with a loss of PC protein from the transgene in polytene chromosomes. The percentage colocalization between the BX-C and PcG bodies remained unchanged in RNAi mutants. This result parallels the persistence of PC at endogenous sites in polytene chromosomes, suggesting that RNAi-independent pathways suffice to recruit PcG proteins to endogenous target genes.

Since *piwi* is required for *Fab-7* contacts, recruitment of E(Z) and PC to the *Fab-7* transgene in polytene chromosomes, and colocalization of the transgenic *Fab-7* with PC bodies and since the PIWI protein colocalizes with PH, we wanted to analyze whether PIWI associates with *Fab-7* transgenes by FISH-I. When we tried to do this, however, the FISH-I protocol caused a loss of PIWI nuclear bodies and an appearance of cytoplasmic staining, indicating that the protein might be displaced from the nucleus during the FISH treatment. Reversing the protocol (i.e., immunostaining, postfixation with formaldehyde, and then FISH) also induced a complete loss of PIWI nuclear staining. This was true also when the protocol was applied to polytene chromosomes. Furthermore, we did not see enrichment of PIWI at *Fab-7* in chromatin immunoprecipitation experiments, while PC was strongly enriched as expected (data not shown). This suggests that, although cytologically colocalized with PH, PIWI might only be loosely associated with chromatin. This would also be consistent with the previously reported predominantly nucleoplasmic localization of this protein (Cox et al., 2000).

RNAi Components Are Necessary for the Maintenance of Natural PcG-Dependent Long-Distance Gene Contacts

Polytene-chromosome staining shows around 120 binding sites for the PC protein (Paro and Zink, 1993), although we detected a much smaller number of PcG bodies in diploid nuclei, particularly in larval imaginal tissues. These numbers suggest that, in addition to contacts between homologous *Fab-7* copies, there may be a significant number of contacts among endogenous PcG target loci. Two candidate loci for establishing such contacts are the homeotic genes of the BX-C and the *Antennapedia* locus (*Antp*), which is located at a distance of 10 Mb from the BX-C. We thus performed FISH-I in two different imaginal-disc tissues: in antennal discs, where all these genes are repressed, and in wing discs, where the BX-C is silenced but *Antp* is active. We first analyzed the colocalization of the two loci in w^{1118} larvae. In antennal discs, the two loci were colocalized in about 23% of the nuclei. In all cases, this colocalization occurred within PcG bodies. In contrast, in wing discs, the colocalization between the two loci dropped to 7% (Figures 7A–7C). Therefore, the colocalization of endogenous PcG target genes in the three-dimensional nuclear space reflects their silencing (Figure 7 and F.B., V. Roure, J. Bonnet, and G.C., unpublished data).

In order to test whether RNAi components are required for long-range nuclear interactions among endogenous PcG target genes, we performed FISH-I experiments in mutant backgrounds for *dcr-2*, *AGO1*, and two different alleles of *piwi* ($piwi^1$, which was introduced into w^{1118} flies, and $piwi^2$, which was introduced into the Fab-X line). In each case, we observed a statistically significant decrease in the ability of BX-C and *Antp* to contact each other in antennal discs, with a colocalization of approximately 13% (Figures 7A and 7B). These results show that the effects of RNAi components are not restricted to transgene contacts but also extend to contacts between Hox genes silenced by PcG proteins. Remarkably, the BX-C and the *Antp* gene were still present within PcG bodies in antennal imaginal discs upon mutation of RNAi genes (Figure 7A and data not shown). This confirms that the roles of RNAi proteins in regulation of nuclear organization and in stabilizing PcG protein recruitment to chromatin are functionally separable.

DISCUSSION

This work shows that the RNAi machinery participates in specific nuclear PcG-dependent functions. While most RNAi components are not required for binding of PcG proteins to PREs, they are required to mediate long-range contacts between multiple copies of the *Fab-7* element. Moreover, Dcr-2, PIWI, and AGO1 colocalize with PH in the cell nucleus, and their effect correlates with the presence of small RNAs homologous to *Fab-7* sequences. Finally, in addition to their effects on transgenic *Fab-7* copies, mutations in these genes also reduce the frequency of long-distance contacts between endogenous PcG target

(B) FISH-I analysis of endogenous PC binding sites of the distal half of the X polytene chromosome in Fab-X and Fab-X;$piwi^2$, showing that the only difference is at the transgenic 13F *sd* locus, visualized by the FISH signal in green and pointed to by an arrow.
(C) FISH-I with an E(Z) antibody on Fab-X, Fab-X;$AGO1^{45/72}$, and Fab-X;$piwi^1$. Arrows indicate the position of the transgene.

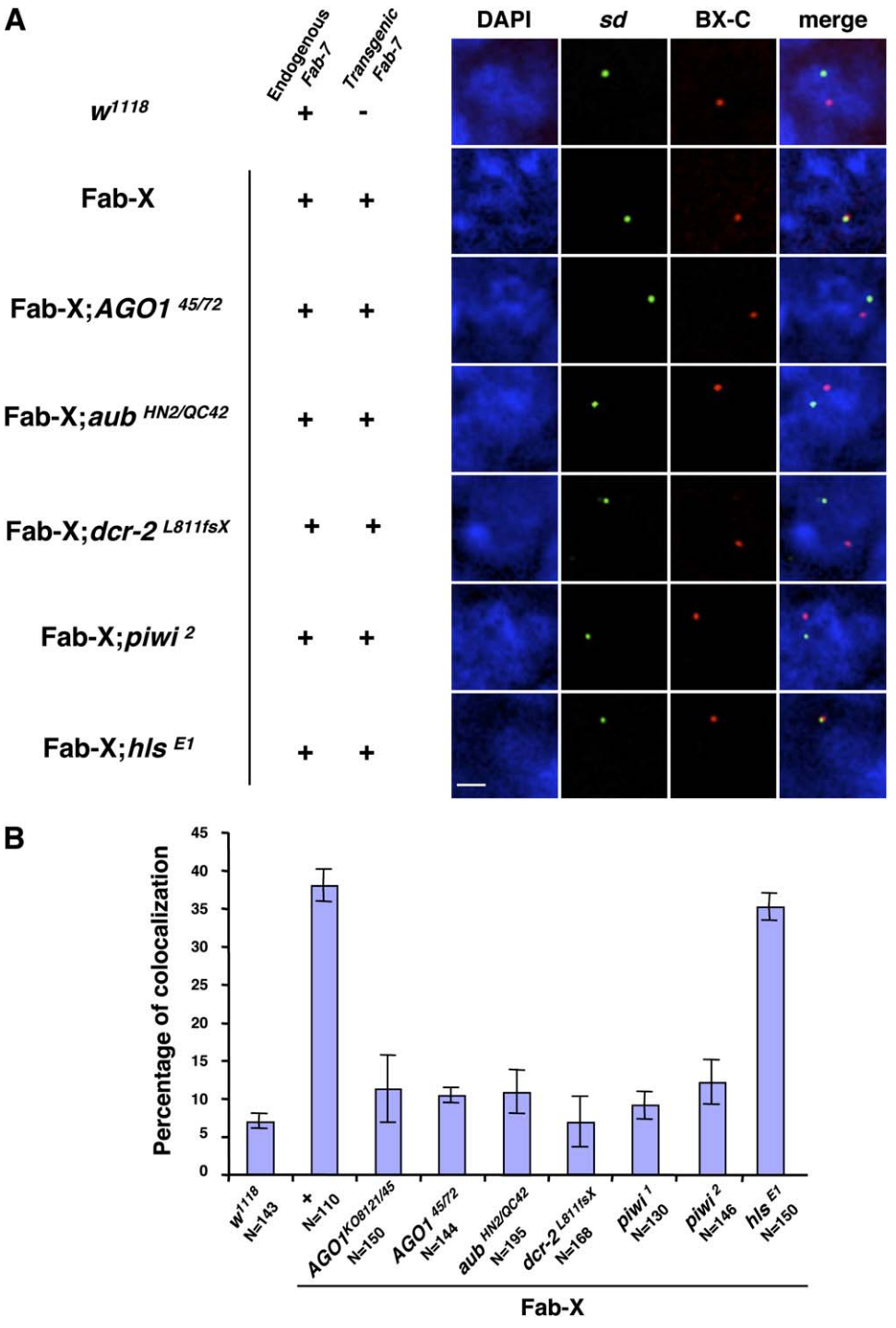

Figure 4. The Maintenance of Long-Distance *Fab-7* Chromosomal Interactions in Larvae Depends on the RNAi Machinery

(A) Characteristic examples of individual nuclei: DAPI staining, the *sd* locus, the BX-C locus, and the merge of the three channels are shown. Genotypes and the presence of endogenous and transgenic *Fab-7* copies are indicated for each line. Bar, 1 μm.

(B) Quantification of the percentage of colocalization between the *sd* and BX-C loci in third-instar larval wing imaginal discs. Genotypes and the total number of nuclei analyzed are indicated below each bar. Bars represent the standard deviation based on the analysis of 5 larval wing discs. Colocalization in *AGO1*, *aub*, *dcr-2*, and *piwi* was statistically different compared to Fab-X (all p values $< 2 \times 10^{-6}$) but not compared to the w^{1118} control (all p values > 0.18).

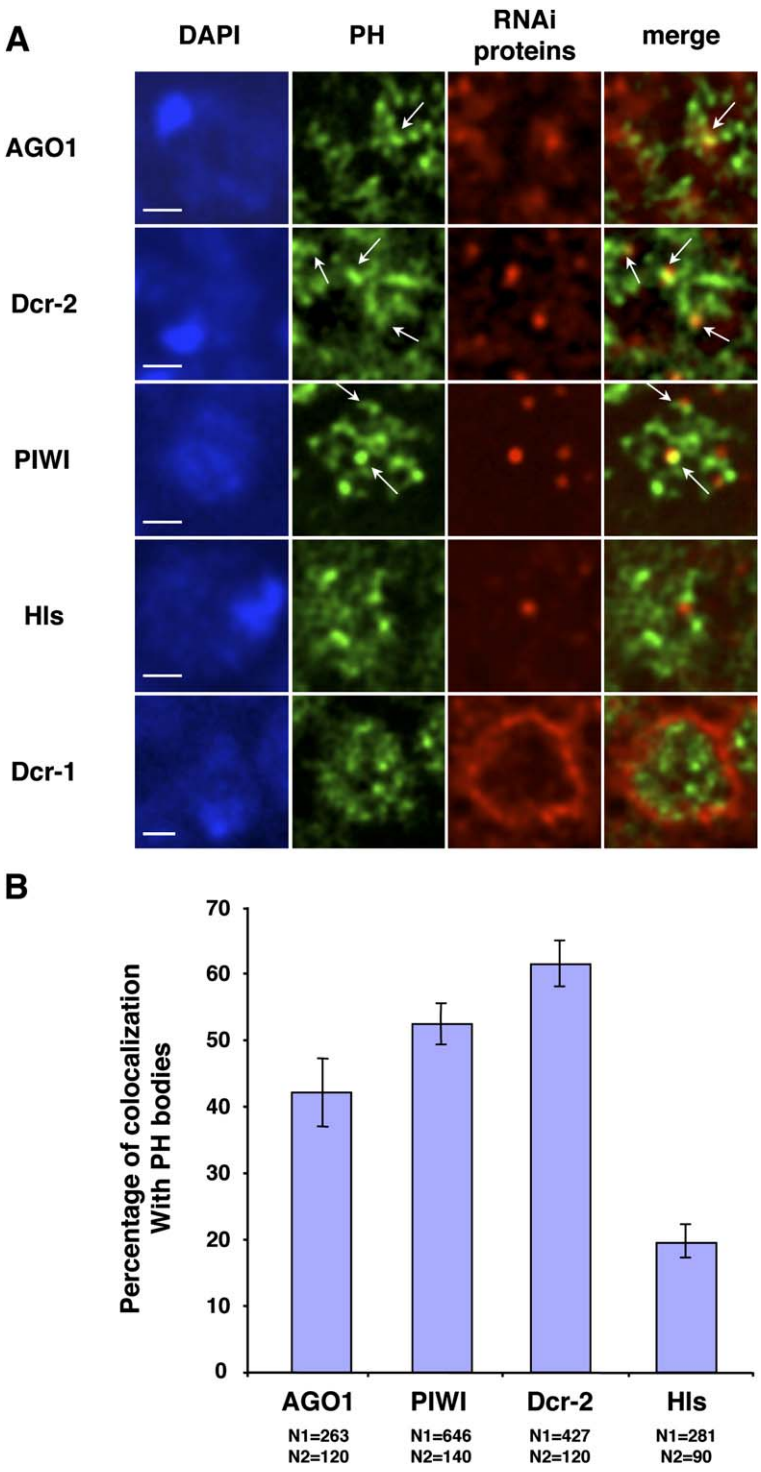

Figure 5. PcG and RNAi Components Co-localize in the Nucleus

(A) Representative examples of nuclei with double immunostaining using PH antibodies (in green) and antibodies against AGO1, Dcr-2, PIWI, Hls, and Dcr-1 (in red). Bar, 1 μm. Arrows point to co-localizing RNAi and PH nuclear bodies.

(B) Quantification of the percentage of colocalization between RNAi components and PH foci in w^{1118} larval imaginal discs. Bars represent standard deviation based on the analysis of 3 to 4 larval imaginal discs. N1 indicates the total number of RNAi foci analyzed and N2 the number of nuclei. The colocalization between PH and AGO1, Dcr-2, or PIWI was significantly higher than between PH and Hls (all p values < 2×10^{-8}).

genes. Taken together, these results reveal a novel and unexpected role for the RNAi machinery in the regulation of euchromatic genes in the nuclear space.

The effects caused by mutations in different RNAi components suggest the existence of distinct molecular roles for these proteins in the regulation of PcG function. First, the *hls* gene does not seem to play a major role in silencing at the *Fab-7* PRE or maintaining long-distance *Fab-7* contacts. Since Hls has been shown to play a central role in heterochromatin formation (Pal-Bhadra et al., 2004), there may be different subtypes of nuclear RNAi machineries for heterochromatin formation and for regulating PcG

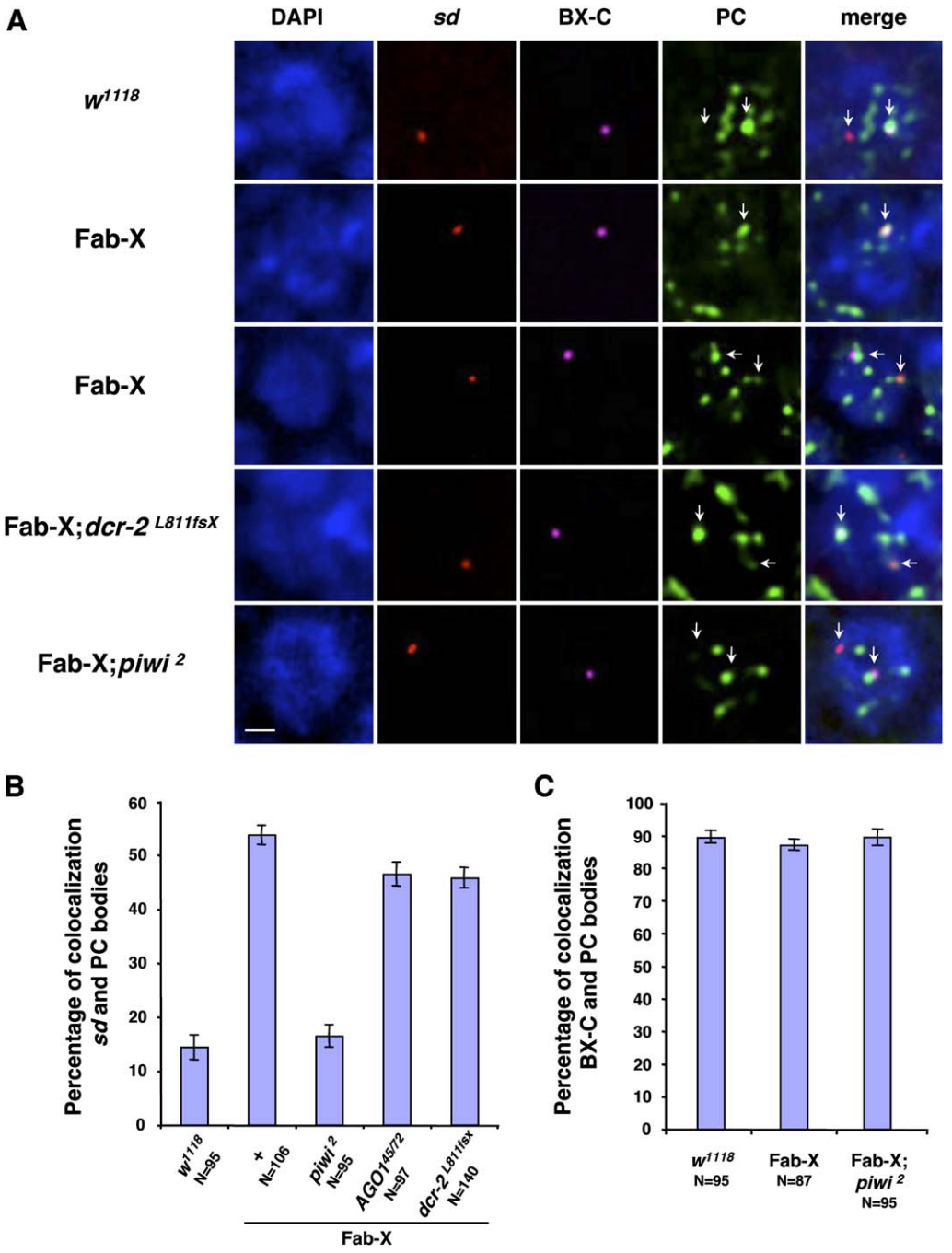

Figure 6. Loss of *Fab-7* Contacts and Localization of *Fab-7* in PcG Bodies

(A) Characteristic individual nuclei analyzed by FISH-I in larval wing imaginal discs: DAPI staining, the *sd* locus, the BX-C locus, the PC bodies, and the merge of the four channels are shown. Two specific examples are shown for the Fab-X line: one in which the two loci interact, and one in which they do not, although they are close to each other. Arrows point to the position of FISH signals. Bar, 1 μm.

(B) Quantification of the percentage of nuclei in which the *sd* locus is localized in a PC body. Colocalization in Fab-X;*piwi*^{2} was not significantly different from w^{1118} ($p = 0.54$), but it was different from Fab-X ($p = 5.92 \times 10^{-9}$). In contrast, the *AGO1* and *dcr-2* derivatives were not different from Fab-X (p values > 0.26). Bars represent the standard deviation based on the analysis of 2 to 3 larval wing discs.

(C) Quantification of the percentage of nuclei in which the BX-C locus is present in a PC body. Genotypes and the total number of nuclei analyzed are indicated below each bar. Bars represent the standard deviation based on the analysis of two larval wing discs.

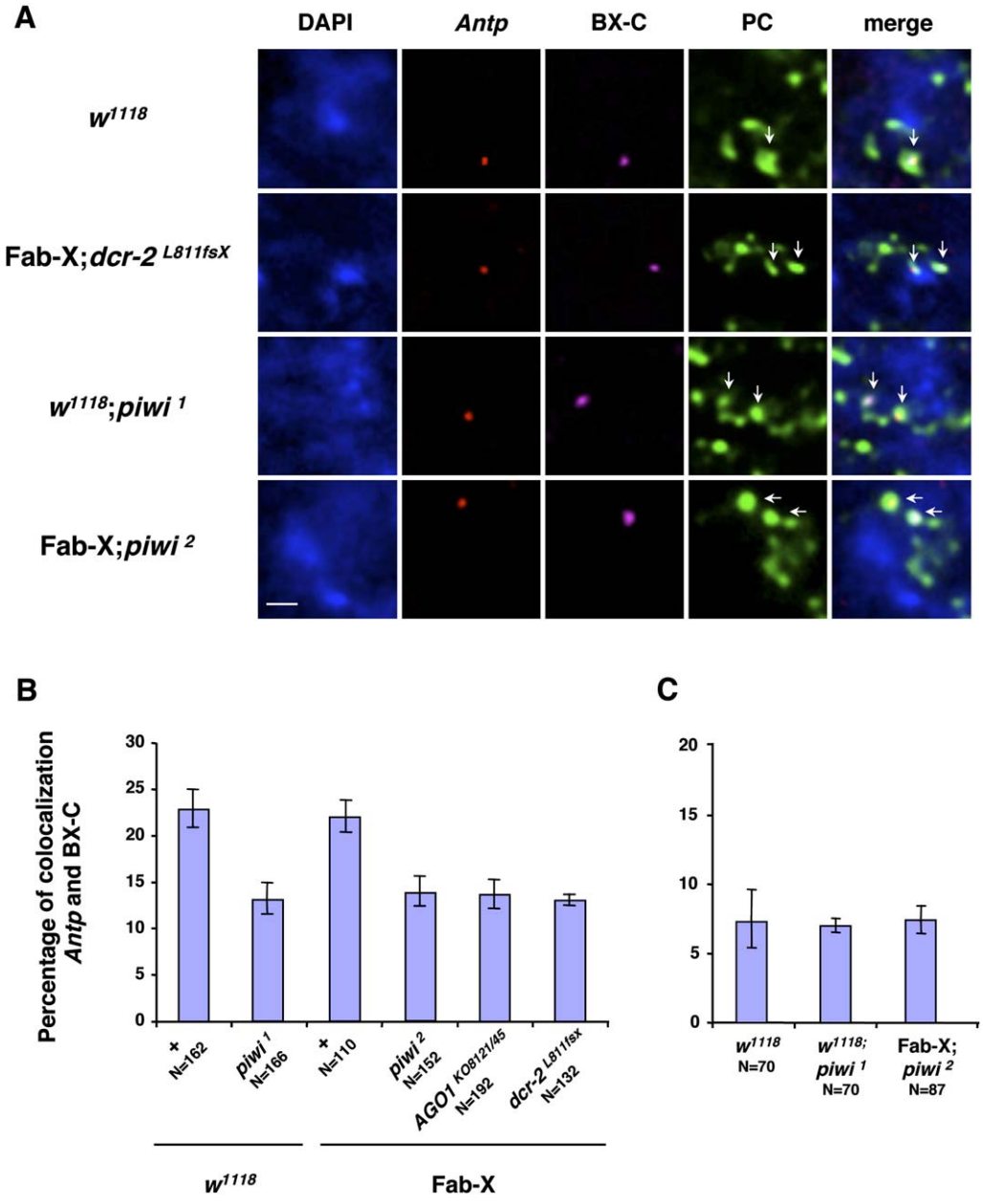

Figure 7. RNAi Mutations Disrupt Long-Distance Chromosomal Interactions between Endogenous PcG Target Loci

(A) Characteristic individual nuclei analyzed by FISH-I in larval antennal imaginal discs: DAPI staining, *Antp*, BX-C locus, the PC bodies, and the merge of the four channels are shown. Arrows point to the position of FISH signals. Bar, 1 μm.

(B) Quantification of the percentage of colocalization between *Antp* and the BX-C loci in antennal imaginal discs. Genotypes and the total number of nuclei analyzed are indicated below each bar. The standard deviation represents the analysis of 2 to 4 larval discs. The colocalization in *piwi*, *AGO1*, and *dcr-2* mutants was statistically different from the control lines (p values: $piwi^1$-w^{1118} = 0.034; $piwi^2$-w^{1118} = 0.024; *AGO1*-Fab-X = 0.075; *dcr-2*-Fab-X = 0.018).

(C) Same quantification as in (B), in wing imaginal discs.

function. We also found no effect when a mutation in the *dcr-1* gene was analyzed at the heterozygous state, but the elucidation of the function of *dcr-1* in PcG-mediated silencing awaits further analysis in a homozygous mutant background. A second class of RNAi components that participate in PcG-mediated repression contains *dcr-2*, *AGO1*, and the *aub* gene. Loss of any of these RNAi gene products affects PcG-dependent silencing at *Fab-7*, although it does not impact the binding of PcG proteins to *Fab-7*. Only mutations in *piwi* affected the binding of

PcG proteins to *Fab-7*, at least in polytene chromosomes, but even PIWI did not affect recruitment of PcG factors at endogenous genes. In *S. pombe*, both RNAi components as well as DNA binding proteins are involved in recruiting heterochromatin proteins to the mating-type region (Jia et al., 2004). At PREs, multiple DNA binding factors and chromatin-associated proteins are known to contribute to PcG protein recruitment, such as PHO, the GAGA factor, DSP1, and the CtBP proteins (Dejardin et al., 2005; Mohd-Sarip et al., 2005; Srinivasan and Atchison, 2004). Their combinatorial action might play a key role in the robust and specific chromatin tethering of PcG proteins, while, in contrast to the situation in *S. pombe*, the RNAi machinery might play a relatively minor role.

It is interesting to note that *piwi* mutations affected recruitment of E(Z) and PC to *Fab-7* in polytene chromosomes but had no effects on PH, another PRC1 component. In the current model for recruitment of PcG proteins to PREs, histone H3 methylation by the E(Z) protein recruits PRC1 via the chromodomain of PC. Our results indicate that multiple mechanisms might be used to anchor different PRC1 components to PREs and that the loss of PC does not necessarily lead to the disintegration of the entire PRC1 complex at PREs.

To date, all transcriptional gene-silencing phenomena that depend on the RNAi machinery involve the production of small-RNA molecules. RNAi components were also shown to affect telomere clustering in *S. pombe* (Hall et al., 2002, 2003), although binding of Swi6 and H3K9me to individual telomeres is not affected (Cam et al., 2005; Hall et al., 2003). The production of siRNAs is believed to be essential for the nuclear clustering of telomeres since cells carrying a catalytically dead RNA-dependent RNA polymerase (which abolishes siRNA production) are defective in telomere clustering (Sugiyama et al., 2005). Consistent with a role for small RNAs in mediating gene contacts, we found sense and antisense transcription of *Fab-7* as well as small-RNA species in *Fab-7* transgenic lines. Moreover, a mutant allele of *dcr-2* producing a truncated polypeptide lacking the RNase III domain, which is required for dsRNA processing, is defective in long-range interactions of PcG target sequences as well as in accumulation of *Fab-7* small RNAs. These data suggest that small-RNA species could be involved in these gene contacts. However, we were unable to detect small *Fab-7* RNA species in the wt situation, although RNAi mutants affect the contact of the endogenous *Fab-7* locus with the *Antp* gene. This might indicate that other RNA species produced in the endogenous Hox genes could contribute to gene clustering. However, the possibility remains that RNA-independent functions of RNAi proteins contribute to the maintenance of gene contacts, in particular in the case of endogenous PcG target genes.

Interestingly, none of the RNAi mutants tested are defective in the establishment of long-distance chromosomal interactions. *Fab-7* contacts are correctly established during embryogenesis but decay during later stages of development. This suggests that the RNAi machinery is not required to initiate contacts but rather to maintain them via the stabilization of gene clustering at specific nuclear bodies. This clustering could be important in cosuppression, where transgene silencing can occur at the transcriptional and posttranscriptional levels, both requiring the RNAi machinery (Pal-Bhadra et al., 2002). It is difficult, however, to understand how a relatively modest increase in transcript levels caused by an increase in the copy number of a gene could trigger a robust silencing of all copies. This is particularly puzzling considering that the transcript levels of endogenous single-copy genes can vary, e.g., during normal physiological gene regulatory processes, without triggering gene silencing. One explanation might be that cosuppressed genes are clustered in the cell nucleus. Indeed, clustering of multiple gene copies has been reported in plant cells (Abranches et al., 2000).

We propose that the RNAi machinery, perhaps in conjunction with PcG proteins, might stabilize this gene-clustering phenomenon. Specifically, the colocalization of multiple gene copies with components of the RNAi machinery might increase the local concentration of RNA species. Once this concentration overcomes a critical threshold, double-stranded RNAs might assemble and be cleaved in situ by the enzymatic activity of the RNAi machinery. RNA molecules might contribute to hold together loci containing PcG proteins that produce noncoding transcripts encompassing PREs. This gene clustering might involve contacts with components of the RNAi machinery as well as PcG proteins assembled in the same nuclear compartments.

One important question is, what is the role of the RNAi machinery in the regulation of endogenous PcG target genes? Our data indicate that RNAi components affect only a subset of these genes since the colocalization of PcG bodies with RNAi bodies is limited. Hox loci are characterized by extensive noncoding RNA transcription, and, recently, other PcG target genes have been shown to be associated to intergenic transcription (Bae et al., 2002; Schmitt et al., 2005). RNAi components might be targeted to this subset of PcG target genes, while other PcG target genes that are characterized by the absence of noncoding transcripts might be independent on RNAi factors.

The fact that no homeotic phenotypes are visible in RNAi mutant backgrounds suggests that the function of RNAi components can be rescued by other chromatin factors. Indeed, the decrease in the level of nuclear interaction between the homeotic complexes was incomplete in RNAi mutant backgrounds. Our data suggest that, while the RNAi machinery does not act in the establishment of PcG-dependent gene silencing, RNAi factors might help stabilize silencing during development by clustering PcG target genes at RNAi nuclear bodies. Thus, in addition to its role in defending the genome against viruses, transposons, and gene duplications, the RNAi machinery might participate in fine tuning the expression of PcG target genes through the regulation of nuclear organization. Finally, it must be noted that the developmental expression profile of the components of the RNAi machinery is highly

specific. The function of specific RNAi components is therefore likely to be highly variable in different cell types and as a function of time. It will be of great interest to explore this issue in the developmental context of the whole organism, in *Drosophila* as well as in other species.

EXPERIMENTAL PROCEDURES

Fly Stocks and Handling

Flies were raised in standard cornmeal yeast medium. Oregon R w^{1118} was used as a control line. The Fab-X line has been described previously (Bantignies et al., 2003). The $AGO1^{KO8121}$ allele has been described previously (Williams and Rubin, 2002). $AGO1^{45}$ and $AGO1^{72}$ alleles were generated by P element mobilization on the regulatory region using a P delta [2,3], ry [506]/ry [506] transposase stock and correspond to loss-of-function alleles of *AGO1*. In $AGO1^{72}$, the P element was relocated to −5 bp relative to the transcriptional start site. $AGO1^{45}$ is derived from $AGO1^{72}$, in which excision of the P element has retained one P foot and has deleted the neighboring sequence, including the transcriptional start site. Western blot analysis of the AGO1 protein shows that $AGO1^{72}$ has significantly reduced protein levels and that, in $AGO1^{45}$, there is no detectable AGO1 protein. $dcr\text{-}2^{L811fsX}$, $dcr\text{-}1^{Q1147X}$, aub^{HN2}, aub^{QC42}, $piwi^{1}$, $piwi^{2}$, hls^{E616}, and hls^{E1} were described previously (Aravin et al., 2004; Cox et al., 2000; Harris and Macdonald, 2001; Lee et al., 2004; Pal-Bhadra et al., 2004). RNAi stocks carrying the KrGFP-CyO or KrGFP-TM3 balancer chromosomes (from stocks BL#5194 and BL#5195 of the Bloomington *Drosophila* Stock Center) were used for selection of homozygous or *trans*-heterozygous mutants.

Analysis of Small RNAs

Small RNAs were extracted and probed as described earlier (Hamilton and Baulcombe, 1999). The small RNAs were run in 10% PAGE and electroblotted on nylon membrane. A pBSK plasmid containing the chromatin boundary portion of *Fab-7* (Mihaly et al., 1997), corresponding to coordinates 84,120 to 86,156 of the BX-C sequence (Martin et al., 1995), accession number U31961, was digested by the HindIII enzyme and transcribed with T7 polymerase in order to produce a transcript in the sense direction (the same as the orientation of the natural noncoding *Fab-7* RNA). This transcript was used for the preparation of a ^{32}P-labeled hydrolyzed probe, which was used to detect antisense small *Fab-7* RNAs. The membrane was hybridized at 65°C for 24 hr as described previously (Pal-Bhadra et al., 1997).

FISH-I on Polytene Chromosomes

Chromosome-staining procedures were adapted from a previously described protocol (Lavrov et al., 2004). The main difference is that FISH is done before immunostaining. A detailed protocol is available at http://www.epigenome-noe.net/researchtools/protocol.php?protid=4. A rabbit anti-PH antibody, provided by R. Paro, was used at a dilution of 1:350. A PC rabbit polyclonal antibody developed in our lab was used at a dilution of 1:300. This serum recognizes the same sites as those previously mapped on polytene chromosomes.

Two-Color FISH and FISH-I on Whole-Mount Tissues, Microscopy, and Image Analysis

Two-color FISH on whole-mount tissues was performed as previously described (Bantignies et al., 2003), and a detailed protocol is available at http://www.epigenome-noe.net/researchtools/protocol.php?protid=5. After FISH, immunostaining was performed as described in the Supplemental Data. Double immunostaining, microscopy, 3D image analysis, and statistical analysis of the data are described in the Supplemental Data.

Supplemental Data

Supplemental Data include Supplemental Experimental Procedures, Supplemental References, one table, and four figures and can be found with this article online at http://www.cell.com/cgi/content/full/124/5/957/DC1/.

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