Polycomb group-dependent *Cyclin A* repression in *Drosophila*

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Polycomb group (PcG) and trithorax group (trxG) proteins are well known for their role in the maintenance of silent and active expression states of homeotic genes. However, PcG proteins may also be required for the control of cellular proliferation in vertebrates. In *Drosophila*, PcG factors act by associating with specific DNA regions termed PcG response elements (PREs). Here, we have investigated whether *Drosophila* cell cycle genes are directly regulated by PcG proteins through PREs. We have isolated a PRE that regulates *Cyclin A* (*CycA*) expression. This sequence is bound by the Polycomb (PC) and Polyhomeotic (PH) proteins of the PcG, and also by GAGA factor (GAF), a trxG protein that is usually found associated with PREs. This sequence causes PcG- and trxG-dependent variegation of the mini-*white* reporter gene in transgenic flies. The combination of FISH with PC immunostaining in embryonic cells shows that the endogenous *CycA* gene colocalizes with PC at foci of high PC concentration named PcG bodies. Finally, loss of function of the *Pc* gene and overexpression of *Pc* and *ph* trigger up-regulation and down-regulation, respectively, of *CycA* expression in embryos. These results demonstrate that *CycA* is directly regulated by PcG proteins, linking them to cell cycle control in vivo.

[*Keywords:* Polycomb group; trithorax group; chromatin; Cyclin A; cell cycle; *Drosophila*]

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Polycomb group (PcG) and trithorax group (trxG) proteins are well-conserved, essential regulatory factors that maintain the silenced and activated states of developmental genes, respectively (Simon 1995; Pirrotta 1997). Much is known about the role of these proteins in maintaining homeotic gene expression patterns during metazoan development (Lund and van Lohuizen 2004b; Ringrose and Paro 2004). PcG genes encode two types of multimeric chromatin-binding protein complexes: the Polycomb repressive complex 1 (PRC1) and the PRC2/3 complexes. PRC1 contains Polycomb (PC), Polyhomeotic (PH), Posterior Sex Combs (PSC), and Sce/dRing (Shao et al. 1999). The PRC2 complex includes the proteins E(Z), Su(z)12, NURF-55, and ESC (Cao and Zhang 2004). To maintain silencing, PRC2/3 complexes can trimethylate Lys 27 of histone H3 (H3K27me3) and Lys 26 of histone H1 (H1K26me3) (Kuzmichev et al. 2004). The PRC1 complex can be recruited by H3K27me3 via binding of the chromodomain of the PC protein, and can then repress transcription by preventing ATP-dependent nucleosome remodeling by the Swi/Snf complex (Francis and Kingston 2001) as well as by establishing direct contacts with the transcriptional machinery (Levine et al. 2004).

In *Drosophila*, PcG-mediated repression occurs through *cis*-regulatory DNA sequences termed PREs (PcG response elements) (Zink et al. 1991; Simon et al. 1993). PREs share several tractable molecular and genetic properties: (1) PcG proteins bind directly to PREs (Zink and Paro 1995; Strutt and Paro 1997); (2) in vivo, PREs induce repression of the adjacent *white* reporter gene, producing a variegated phenotype (Fauvarque and Dura 1993); (3) this variegated phenotype is dependent on PcG and trxG proteins; and (4) PREs induce pairing-sensitive repression (PSR) (for review, see Kassis 2002).

Epigenetic regulation of gene expression is emerging as a key mechanism in the control of cellular proliferation. In vertebrates, PRC1 and PRC2/3 components have been implicated in the regulation of proliferation, acting as either potent activators or repressors of the cell cycle. Progression through the cell cycle depends primarily on the activity of the cyclin-dependent kinases (Cdks). Cdks, upon association with their respective cyclin partners, mediate the transitions between the different cell cycle phases. Although with increased complexity, cell

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cycle progression in mammalian cells follows the same general order of events as in *Drosophila* cells (Sherr 1995): Cdk4/Cdk6–Cyclin D complexes are critical for G1 progression, with the Cdk2/Cyclin E complex acting slightly later in G1. Progression through S phase requires the Cdk2/Cyclin A (CycA) complex, and the mitotic Cdk1/CycA and Cyclin B (CycB) complexes govern progression through mitosis. A specific class of proteins, the "cyclin-dependent inhibitors" or CKIs, also play a critical role in determining whether or not a cell divides. Other important cell cycle regulatory factors include the pRb and E2F proteins, which work together to coordinate the transcription of cell cycle genes and cell cycle progression (Cam and Dynlacht 2003). The activity of E2F transcription factors can be repressed by pRb and pRb-related proteins, which recruit several corepressor complexes to E2F-bound promoters of cell cycle genes (Dimova and Dyson 2005).

PcG complexes have previously been implicated in multiple levels of cell cycle control. For example, overexpression of *Bmi-1* (the mammalian homolog of *Psc*), a PRC1 component, causes lymphomas in transgenic mice (Lund and van Lohuizen 2004a). *Bmi-1* overexpression also correlates with a decrease in gene expression at the *ink4a/arf* tumor suppressor locus, which encodes the cell cycle inhibitors p16INK4a and p19ARF. p16 is a central component of the pRb growth suppression pathway, and significantly, in the absence of *Bmi1*, derepression of *p16* causes an active form of pRb to accumulate and produces proliferation defects.

PRC2 members may be involved in the control of cell cycle progression in collaboration with pRb. The pRb protein functions as a repressor of the cell cycle at the G0/G1-phase transition, at least in part by recruiting histone deacetylase (HDAC) activity (Brehm and Kouzarides 1999). When pRb binding to HDAC is disrupted, cells are committed to progressing through the cell cycle. pRb2 and the PcG protein Ezh2 have functionally opposite effects on the cell cycle, as *Ezh2* overexpression induces cellular proliferation (Sellers and Loda 2002), and inhibition of its expression causes cell cycle arrest (Varambally et al. 2002). This cell cycle arrest is accompanied by a reduction in *CycA* mRNA levels (Bracken et al. 2003). It was proposed that Ezh2-mediated regulation of *CycA* expression may be an indirect effect of competition between Ezh2 and pRB for HDAC recruitment (Tonini et al. 2004).

Unlike Bmi-1 and Ezh2, HPC2, a human homolog of PC, is a negative regulator of proliferation (Satijn et al. 1997). The molecular role of HPC2 is strongly debated. Studies on established tumor cell lines have suggested that HPC2 may cooperate with pRB to repress the *CycA* and *Cdk1* promoters during the G2 phase of the cell cycle (Dahiya et al. 2001), although it is unknown whether this also applies to normal cells that are not severely transformed.

In this work, we have analyzed whether PcG members are direct regulators of cell cycle genes. Following our initial discovery that *Pc* inactivation leads to a lengthening of G2 phase, we checked whether G2-specific cyclins are direct targets of PcG proteins. We identified a PRE in a region spanning the promoter and the first intron of the *Drosophila CycA* gene. *Drosophila* embryogenesis starts with 13 synchronous syncytial nuclear divisions, entirely under maternal control (Orr-Weaver 1994). Following S-phase 13, during which cellularization takes place, a G2 interphase is first introduced into the embryonic cycles (Edgar 1994). This G2 arrest marks the point at which transcription becomes dependent on the zygotic genome. During the subsequent gastrulation stage, the majority of the cells undergo three further mitotic cell divisions (cycles 14–16), in which S-phase entry is still immediately linked to the preceding mitosis.

We have found that overexpression of the *Pc* and the *polyhomeotic* (*ph*) genes in early embryos causes a down-regulation of endogenous zygotic *CycA* expression that is clearly visible in the 14th interphase. After the mitotic cycles 14–16, most cells arrest in the G1 phase of cycle 17 and remain quiescent until after hatching. We have observed that a loss of function mutation in *Pc* up-regulates *CycA* expression during this 17th interphase, when embryonic cells do not normally express *CycA*. Finally, we show that the *CycA* locus significantly colocalizes with the PC protein in the nucleus of diploid cells and that the colocalization rate increases gradually during embryonic development, reaching a maximum in cell cycle arrested cells. These results establish a direct link between PcG proteins and cell cycle control in vivo.

Results

PC depletion modifies the cell cycle profile in Drosophila *S2 cells*

To determine whether PcG complexes are involved in cell cycle control in *Drosophila*, we depleted cultured S2 cells for PC by RNA interference (RNAi) (Fig. 1A). As a negative control, we treated S2 cells with *neomycin* small interfering RNA (siRNA). While the control depletion had no effect on the cell cycle profile in flow cytometry, PC-depleted populations had a reproducibly decreased proportion of cells in G1 and S phase, with an accompanying increase in G2/M cells (Fig. 1B). This modification in the cell cycle profile occurred in the absence of any apparent growth arrest (data not shown), suggesting that the cells lacking *Pc* expression continued to divide. The doubling time of depleted cells did not differ significantly from that of wild-type cells. Similar results were obtained with *ph* RNAi (data not shown). We also observed that the *Pc* knockdown did not trigger apoptosis.

As *Pc* RNAi changes the normal cell cycle phase distribution in cultured cells, the PC protein could be directly or indirectly modulating the cell proliferation program of cycling *Drosophila* cells. In view of the apparent G2 lengthening observed after *Pc* knockdown, we hypothesized that PC may be directly acting on the *CycA*, *CycB*, or *CycB3* genes, which encode the *Drosophila* G2/M cyclins.

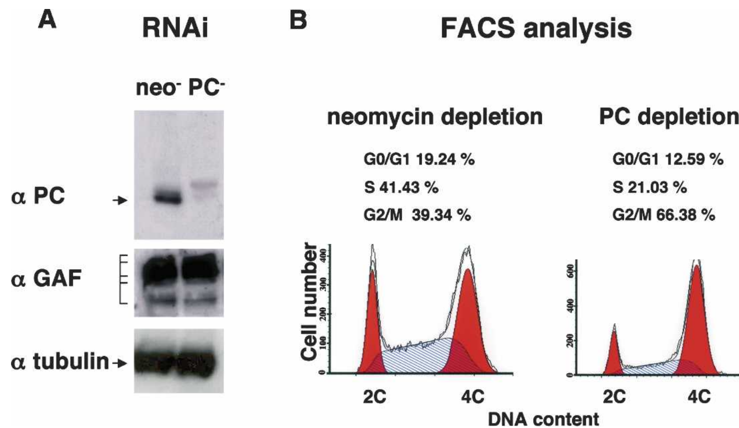

Figure 1. PC depletion by RNAi alters the cell cycle in proliferating S2 cells. (*A*) Incubation of S2 cells with double-stranded (ds) *Pc* RNA reduces PC expression but does not affect GAF or tubulin expression. As a control, *neomycin* (*neo*) dsRNA has no effect on PC expression. (*B*) Comparative FACS analysis of S2 cells after *neo* and *Pc* RNAi treatment showing altered cell cycle phasing after PC depletion. Histograms display DNA content (*X*-axis) and cell number (*Y*-axis). DNA content of neomycin and PC depleted cells is expressed in terms of G1, S, and G2 percentages.

PcG proteins associate with the CycA *gene*

To address this possibility, we mapped the binding of PC, PH, and GAF to the *CycB*, *CycB3*, and *CycA* genomic regions using chromatin immunoprecipitation (ChIP) (Orlando et al. 1997). These three factors have previously been found to be hallmarks of PREs (Strutt et al. 1997; Horard et al. 2000; Busturia et al. 2001). We first hybridized PC-, PH-, and GAF-immunoprecipitated materials from S2 cells to BACs containing the *CycB* and *CycB3* genomic regions (BACR04C10/BACR09K23 and BACCR33F18, respectively). In these experiments, we were unable to detect any enrichment for PC/PH at or close to the *CycB* or *CycB3* genes (data not shown). The same immunoprecipitated material was also hybridized to a genomic stretch of 153.4 kb encompassing the *CycA* gene (BAC48G03) (data not shown). In this case, we detected PC/PH/GAF binding in the region of the transcription unit. To fine-map the location of the binding sites, we subdivided the *CycA* gene region into 1-kb-sized PCR fragments (from 3.2 kb upstream of the *CycA* transcription start site according to transcript CG5940-RA to 2 kb downstream of the end of the gene) (see Fig. 2). Slot-blot hybridizations of immunoprecipitated and PCR-amplified material identified two main fragments in which PC/PH and GAF were strongly enriched (Fig. 2A). The first (fragment 5 in Fig. 2A) is located in a region extending from the promoter to the end of the first intron, from 294 base pairs (bp) upstream to 739 bp downstream of the transcription start site. The second binding site (fragment 6 in Fig. 2A) extends from the beginning of the first exon to the middle of the second exon, from 469 to 1446 bp downstream of the transcription start site. With both fragments, we observed a substantial overlap of PC, PH, and GAF binding. The same peak of enrichment for PC/PH/GAF binding was also observed using immunoprecipitated materials from embryos (data not shown). We next subdivided the two overlapping 1-kb target PCR fragments into eight subfragments of ~200 bp each. This allowed us to narrow down the element bound by the three proteins to a region spanning from 162 bp upstream to 962 bp downstream of the transcription start site in S2 cells (Fig. 2B) as well as in embryonic cells (Fig. 2C).

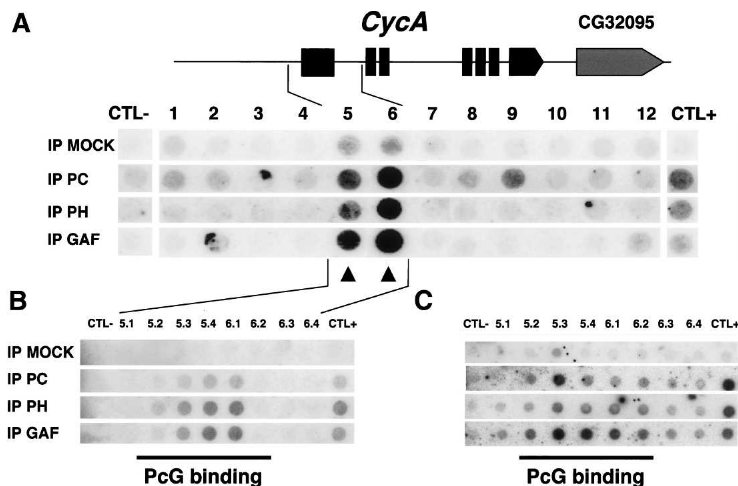

Figure 2. Identification of a target region for PC, PH, and GAF binding within the *CycA* gene. (*A*) Slot-blot hybridization. Chromatin from *Drosophila* S2 cells was either mock immunoprecipitated or immunoprecipitated with anti-PC, anti-PH, or anti-GAF antibodies. One-kilobase PCR fragments from the *CycA* genomic region were then blotted onto a nylon membrane, and the immunopurified DNA was radiolabeled and used as a probe for hybridization (arrows indicate the signals corresponding to the strongest enrichment as compared with mock). One bound fragment is located upstream of the transcription start site (fragment 5), and the second encompasses the first exon and spreads into the neighboring intron (fragment 6). As a negative control, a fragment of 203 bp in the mini-*white* gene was used. As a positive control, a central region of 206 bp within the *Fab-7* PRE was used. All oligonucleotide sequences are listed in the Supplemental Material. (*B*) The PRE products corresponding to fragments 5 and 6 were sub divided into 200-bp subfragments and subjected to hybridization using immunoprecipitated chromatin from S2 cells. PC/PH/GAF binding was detectable in four subfragments. (*C*) The same 200-bp subfragments were also hybridized using immunoprecipitated chromatin from stage 9–13 embryos (4–12 h after egg laying at 25°C).

Several sequence-specific DNA-binding proteins have emerged as likely candidates for PcG targeting factors. These include Pleiohomeotic (PHO) (Brown et al. 1998; Mihaly et al. 1998), GAF, Pipsqueak, Zeste, and the DSP1 protein (Dejardin et al. 2005). The identified 1124-bp *CycA* fragment contains several PHO, GAF/Pisqueak, Zeste, and DSP1 consensus motifs, similar to the composition of other well-characterized PREs. Together with the observed direct binding of PcG proteins to *CycA* in vivo, this finding suggested that PcG complexes may directly control *CycA* expression via a PRE.

A genomic fragment of the CycA *gene exhibits PcG- and trxG-dependent PRE activity*

Having shown that PcG proteins target the *CycA* gene in S2 cells and in embryos, we next studied whether the mapped PC/PH/GAF-binding site could function as a PRE in transgenic constructs. We linked the 1124-bp *CycA* fragment, extending from the promoter region to the first intron (according to transcript CG5940-RA from FlyBase, http://www.flybase.org) and containing the putative PRE (Fig. 2B), to the mini-*white* gene, which served as a reporter and transformation marker. Transgenic flies were produced using the pUZ vector (Lyko et al. 1997). We obtained 17 independent transgenic lines. Seven lines exhibited variegated expression of mini-*white* in the eyes (Fig. 3A), five of which showed variegation when heterozygous. Two of these lines also exhibited PSR, a phenomenon often associated with PREs (Kassis 2002).

To test whether silencing of the mini-*white* reporter gene by the 1124-bp *CycA* fragment depends on PcG genes, transgenic lines were placed in *Pc* and *ph* mutant backgrounds. Silencing was reduced in the *Pc* and *ph* mutant flies (Fig. 3A,B). In contrast, when the element was introduced into flies carrying a null mutation in the *brahma* (*brm*) trxG gene, mini-*white* silencing was strongly increased (Fig. 3C). The eye variegation phenotype observed in the transgenic lines is thus genetically linked to PcG and trxG activity. Taken together, these observations strongly suggest that the 1.1-kb fragment from *CycA* is a PRE that represses transcription in a PcG- and trxG-dependent manner in vivo. We should note, however, that the eye variegation was generally less pronounced than in homeotic PRE lines, suggesting that regulation of the 1124-bp *CycA* fragment might differ from that of the well-known homeotic PREs.

PC binds neither the endogenous CycA *locus nor a transgenic* CycA *PRE in polytene chromosomes*

We next analyzed whether PcG proteins are recruited at the endogenous *CycA* locus by combining immunostaining and DNA-FISH (immuno-FISH) on polytene chromosomes (Lavrov et al. 2004). Polytene chromosomes are derived from specialized salivary gland cells that undergo multiple rounds of replication without chromosome segregation. This cell cycle conversion is dependent on the elimination of mitotic Cdk1 activity and periodic activation of Cdk2/Cyclin E (Edgar and Orr-Weaver 2001). During these endoreplicative cell cycles, the expression of *CycA* is absent, making this tissue a priori ideal for the analysis of stable PcG association with *CycA*. Surprisingly, we found no recruitment of PC or PH at this locus (Fig. 4A). We then analyzed whether PcG proteins were recruited to the PRE-containing transgenes, since such transgenes have generally been shown to create ectopic PcG-binding sites at their insertion sites in polytene chromosomes (Zink and Paro 1995; Dejardin and Cavalli 2004). Again, no recruitment of PC could be found (Fig. 4B), except in one line (33.1) in which the *CycA* transgene integrated into a preexisting PcG-associated locus (data not shown). This phenomenon, termed "homing," has been previously described for PRE-containing transgenes (Fauvarque and Dura 1993).

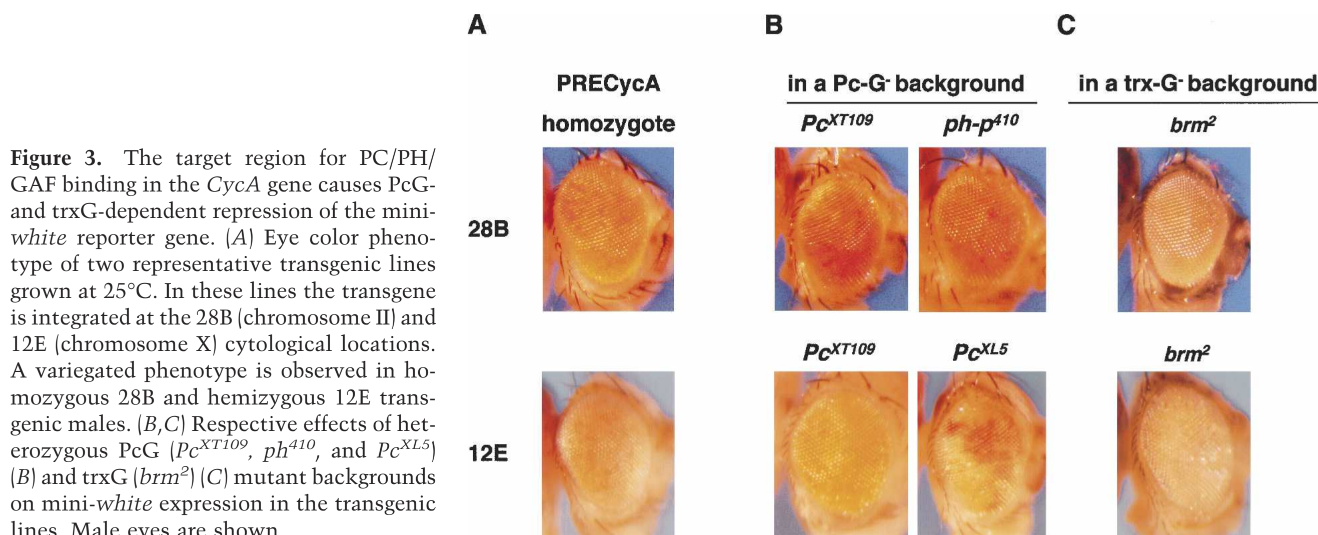

Figure 3. The target region for PC/PH/GAF binding in the *CycA* gene causes PcG- and trxG-dependent repression of the mini-*white* reporter gene. (*A*) Eye color phenotype of two representative transgenic lines grown at 25°C. In these lines the transgene is integrated at the 28B (chromosome II) and 12E (chromosome X) cytological locations. A variegated phenotype is observed in homozygous 28B and hemizygous 12E transgenic males. (*B*,*C*) Respective effects of heterozygous PcG (Pc^{XT109}, ph^{410}, and Pc^{XL5}) (*B*) and trxG (brm^2) (*C*) mutant backgrounds on mini-*white* expression in the transgenic lines. Male eyes are shown.

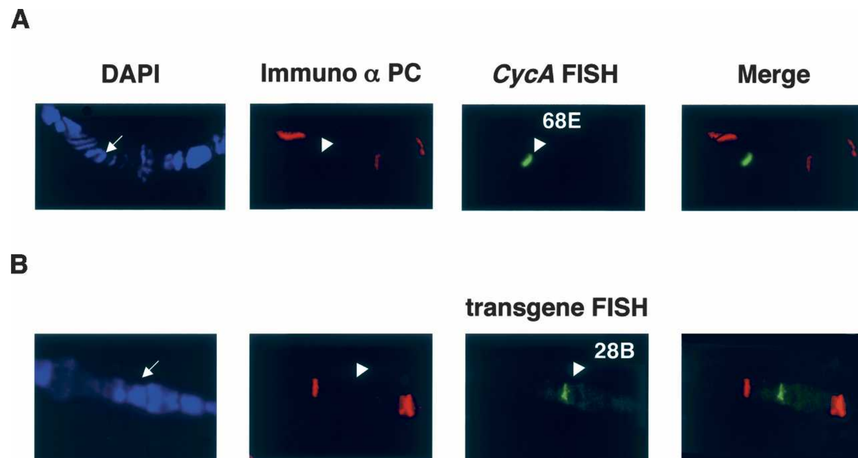

Figure 4. PC is neither recruited at the *CycA* locus nor at the integration site of the transgenic PRE. Polytene chromosome immuno-FISH experiments performed on wild-type (*A*) and the 28B transgenic line (*B*) using antibodies raised against the PC protein. For each experiment, DAPI staining, immunostaining, FISH, and a merge between immunostaining and FISH are shown. In DAPI panels, the transgene insertion position is indicated by an arrow. With immunostaining, the position of the transgene is indicated by arrowheads.

In conclusion, no PcG proteins were detected at either the endogenous *CycA* locus or at the insertion sites of PRE-containing transgenes. We therefore conclude that the repression of the *CycA* gene in endoreplicative cells might be controlled by a PcG-independent mechanism.

The CycA*, but not* CycB*, locus progressively recruits PcG complexes in embryonic nuclei*

In view of the fact that we detected PcG protein binding to *CycA* by ChIP, but failed to detect PcG bands at *CycA* PRE sites on polytene chromosomes, we tested whether the *CycA* locus colocalizes with PcG proteins in diploid embryonic cells. After three rounds of mitosis (14th–16th), cells stop dividing and enter a G1/0 phase for the first time (Edgar 1994); transcription of the genes encoding CycA, CycB, and CycB3 is then blocked. As CycA must be eliminated for the timely arrest of cell proliferation at interphase 17 in epidermal cells, we examined the nuclear localization of the *CycA* gene during embryonic development since cellularization (14th cycle/embryonic stage 5) until the terminal embryonic cell cycle arrest (stage 13). If PcG complexes were involved in the transcriptional repression of *CycA*, we would expect to see a substantial colocalization of *CycA* with PcG proteins.

Immunostaining of PC proteins in embryos showed a nuclear punctate pattern with foci of varying sizes, similar to the structures that have previously been called "PcG bodies" (Buchenau et al. 1998; Saurin et al. 1998). To analyze whether the *CycA* locus is colocalized with PcG bodies, we combined 3D-FISH with immunostaining of the PC protein (FISH-I) (see Materials and Method; Fig. 5; Supplementary Fig. S1). At the cellular blastoderm stage (stage 5), when nuclei enter interphase 14 metachronously, PC foci just emerge and are of small size (Fig. 5A). Quantification of PC and *CycA* colocalization can hardly be done at this early stage. From the gastrulation stage (stage 7) until the slow germ band elongation stage (stage 9), most of the cells pass through mitosis of the 14th cycle. In stage 9 embryos, PC bodies are well distinguished and we observed colocalization between *CycA* and PC bodies in 47% of the nuclei (Fig. 5A,C). However, these bodies were of small size. Later, at germ band retraction, when a large portion of cells asynchronously accomplish their two subsequent 15th and 16th mitoses (stage 11–12), we found that *CycA* and PC bodies colocalize in 56% of the nuclei (Fig. 5A,C). Remarkably, the size of *CycA*-colocalizing PcG bodies increased significantly in stage 11 compared with stage 9. Finally, at the end of germ band retraction (stage 13), the stage in which cells in most organ primordia begin to differentiate, the percentage of *CycA* and PC colocalization reached 76.5% (Fig. 5A,C). This is the same level as that seen between PcG bodies and the silenced *Abdominal-B* homeotic gene (*Abd-B*) (i.e., 80% in embryonic parasegments 4 and 5, where transcription of *Abd-B* is totally repressed by PcG complexes) (F. Bantignies, V. Roure, J. Bonnet, and G. Cavalli, unpubl.).

In contrast to the high colocalization rates observed with *CycA* and *Abd-B*, colocalization was reduced to an average of 19% of the nuclei when embryos were stained with a FISH probe directed against the *CycB* locus (Fig. 5B). This low-level colocalization may reflect random proximity of the locus to unrelated PcG bodies that are present in the cell nucleus, as we were unable to detect PC/PH binding to the *CycB* genomic region by ChIP. Consistent with a lack of recruitment of PcG proteins to *CycB*, *Abd-B* colocalizes with PC in 18% of the nuclei of parasegments 13 and 14, where *Abd-B* is strongly transcribed (F. Bantignies, V. Roure, J. Bonnet, and G. Cavalli, unpubl.). Remarkably, we note that the frequency of colocalization with PC bodies increases with the progression of embryonic development (Fig. 5C) at the *CycA*—but not the *CycB*—locus, concomitant with an increase in the average size of these PC bodies (Fig. 5A).

These results suggest that PcG proteins can induce silencing of the *CycA* gene in late embryos via a direct association with its PRE. However, the same analysis suggests that PcG proteins might also be present on the *CycA* locus in dividing embryonic cells, albeit less robustly bound.

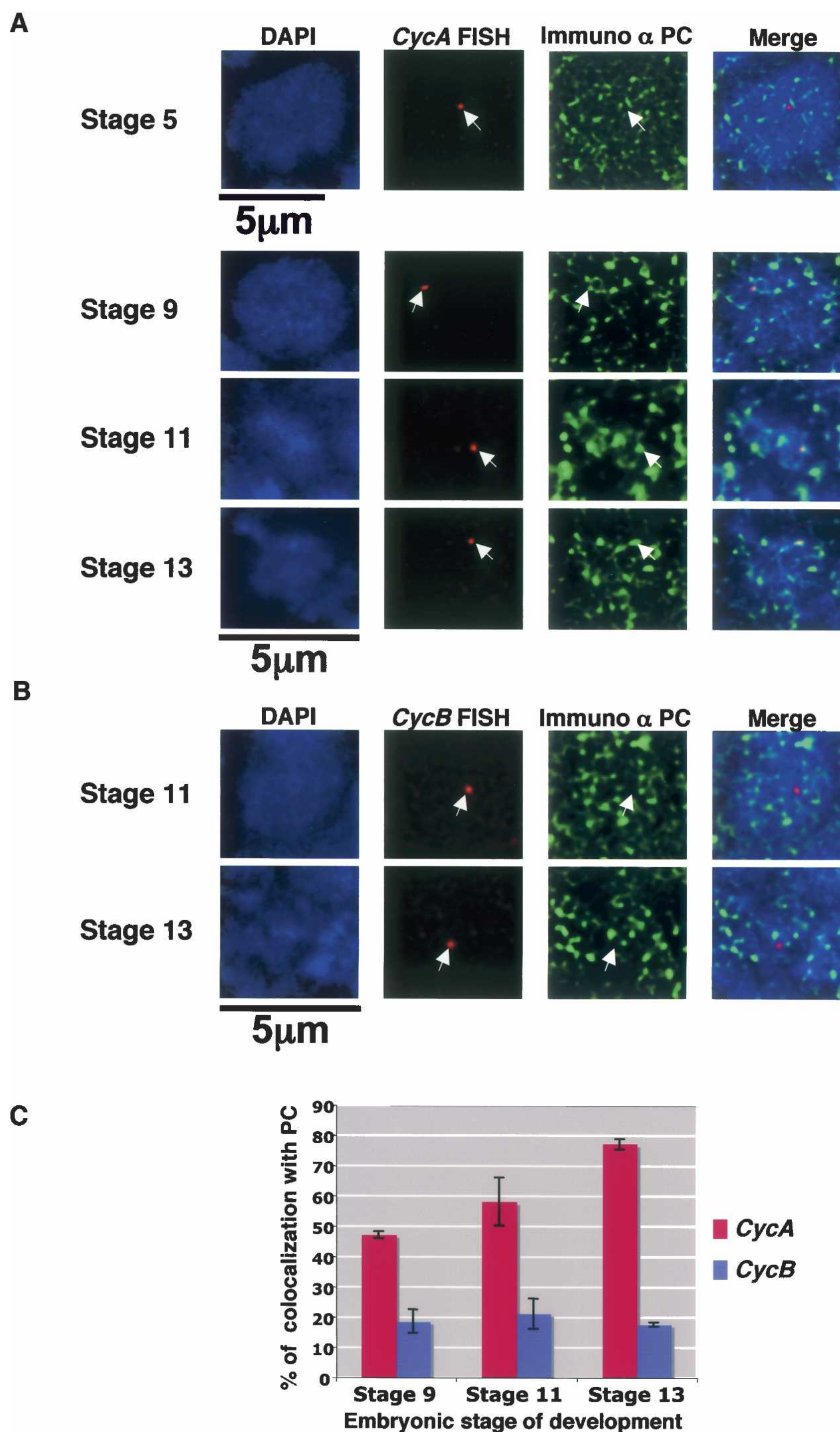

Figure 5. The *CycA*, but not *CycB*, locus colocalizes with PC foci in nondividing diploid embryonic nuclei. FISH-I in whole-mount embryos. Examples of merged images of DAPI labeling (blue), PC foci (in green) after deconvolution (Supplementary Fig. S1), and the FISH probe (red) are shown for the *CycA* (*A*) and *CycB* (*B*) loci. Single slices of individual nuclei show characteristic examples of data obtained with different nuclei. (*A*) Cellular blastoderm stage (stage 5), early germ band elongation stage (stage 9), late germ band elongation stage (stage 11), and germ band retraction stage (stage 13) nuclei are shown for *CycA*. Arrows indicate cases of colocalization between *CycA* and PcG foci. Note the progressively larger size of the PcG body colocalizing with *CycA* as development progresses from stage 9 to 13. (*B*) Characterictic nuclei at the germ band elongation stage (stage 11) and the germ band retraction stage (stage 13) show absence of colocalization between *CycB* and PC foci. (*C*) Quantification of the percentage of colocalization between the *CycA* and *CycB* loci and PcG foci during embryonic development was performed in at least 100 nuclei per embryo. Three embryos were analyzed for each experiment.

Overexpression of Pc *and* ph *down-regulates* CycA *expression in early embryos*

To test whether endogenous *CycA* gene expression is dependent on the levels of PC protein in vivo, we overexpressed *Pc* in actively dividing cells in early embryos. If PC can functionally silence the *CycA* gene, we would expect this treatment to cause a decrease in the levels of CycA. Before cellularization, the embryonic nuclear cycles 1–13 are driven almost exclusively by maternal products, and embryos contain an abundant supply of maternal *CycA* transcript. Zygotic transcription gradually increases during cycles 11–13, reaching a high level for the first time during interphase 14. Zygotic CycA supports the three subsequent divisions (mitoses 14–16), which are no longer synchronous and occur in spatially restricted domains of the embryo called mitotic domains (Foe 1989). Expression of the *CycA* gene is perfectly cor-

related with the mitotic domains, as CycA is degraded at each mitosis. CycA staining does not reflect the pattern of mitosis 14, however, as CycA accumulates uniformly in all cells of the embryo in interphase 14 (Fig. 6A).

We studied the effect of *Pc* overexpression on zygotic CycA levels at the 14th interphase. We used a *hsPc* transgenic line in which *Pc* expression is under the control of a *heat-shock* promoter (Fauvarque et al. 1995). As expected, before heat shock, CycA and PC are ubiquitous and generally coexpressed in gastrulation-staged embryos (stage 8–9) during interphase 14 (Fig. 6A). After induction of PC (which was homogeneous in some embryos while only partial in others, as illustrated in Fig. 6A) at the same stage, however, weak or no CycA staining was observed in regions of *Pc* overexpression, while CycA staining persisted outside of these regions. Interestingly, CycA staining was maintained in the nondividing amnioserosa cells even in the context of *Pc* overexpression (squares in Fig. 6A). It is known that amnioserosa cells stop dividing earlier than epidermal cells do, that they never enter mitosis 14, and that they are arrested in the G2 phase of cycle 14. In the amnioserosa cells, the maternally expressed CycA persists and is slowly degraded rather than disappearing abruptly as in other cells (Lehner and O'Farrell 1989). Our results thus suggest that *Pc* overexpression represses zygotic *CycA* in cycling cells but does not affect the maternal component of CycA deposited in the embryo. To confirm that PcG complexes were indeed being able to repress *CycA* during early embryonic development, we also overexpressed *ph* using a GAL4-responsive transgene *UASph* under the control of *engrailedGAL4* (*enGAL4*) and *pairedGAL4* (*prdGAL4*), two drivers that express GAL4 during embryonic development. In stage 8–9 embryos, PH and CycA are uniformly coexpressed (Fig. 6B). The *enGAL4*-directed expression of PH in the posterior part of each segment reduced CycA levels specifically in the GAL4 expressing domains, whereas CycA expression was maintained in the anterior part of segments, indicating a general repressive effect of PH on *CycA* (Fig. 6B). The pair-rule *prd* gene has a complex dynamic pattern of expression during embryonic development (Gutjahr et al. 1994). At gastrulation (stage 8), seven *prd*-expressing stripes split into anterior and posterior stripes and result in doubling of the number of stripes toward the end of gastrulation. *Prd* is then transiently expressed in a 14-stripe pattern, with a fainter anterior expression. Overexpression of *ph* driven by *prdGAL4* leads to CycA

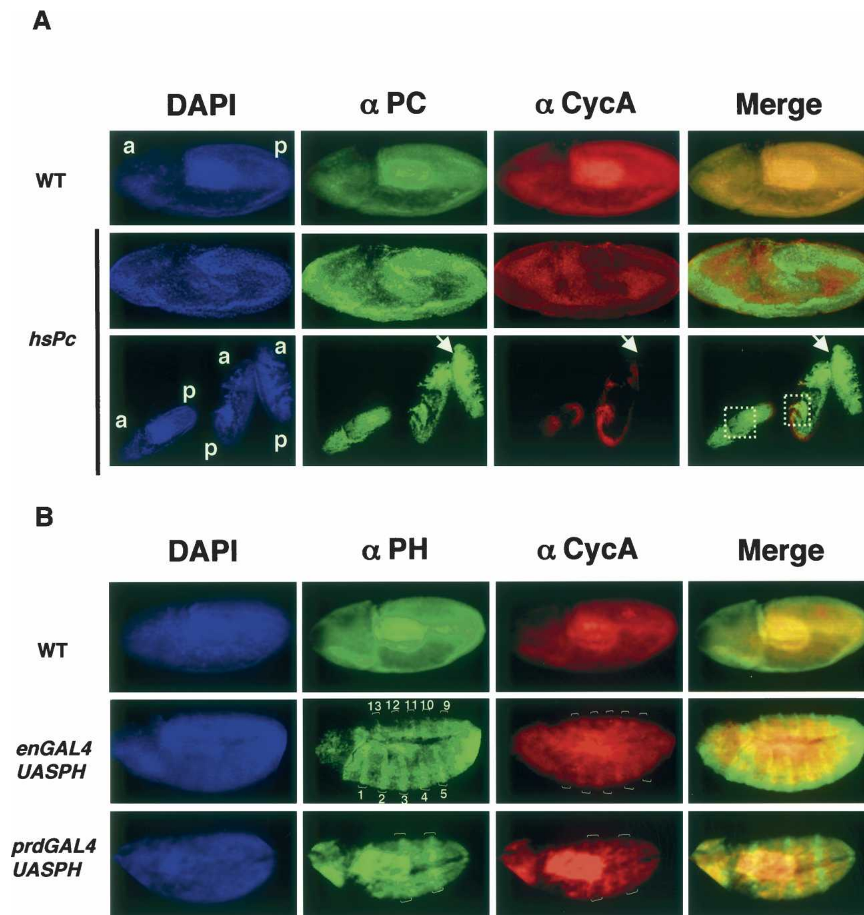

Figure 6. *CycA* expression is down-regulated following *Pc* and *ph* overexpression in mitotically active embryos. (*A*) PC and CycA expressions are ubiquitous in wild-type stage 8 embryos (WT) and coexpression is often observed (merge). After heat-shock treatment of transgenic *hsPc* embryos (carrying a *hsPc* transgene), *Pc* overexpression triggers the down-regulation of *CycA* expression (*hsPc*). The *middle* row shows a *hsPc* embryo with partial ectopic PC overexpression. *CycA* is repressed in the PC-overexpressing domain. (*Bottom* row) This down-regulation is observed throughout the body plan of the affected embryos during germ band elongation stages (8–9), except in cells of the amnioserosa. Dotted squares highlight the amnioserosa region. *CycA* expression persists outside of the PC overexpression domains. Anterior (a) and posterior (p) parts of the embryos are indicated. The arrows point to an embryo with a strong homogeneous PC overexpression, which silenced *CycA* throughout the embryonic body. (*B*) Analysis of CycA and PH expression in wild-type and PH overexpression backgrounds during early embryogenesis. PH and CycA are ubiquitous in wild-type stage 8/9 embryos (WT). In *enGAL4*/*UASph* embryos, PH is overexpressed in the posterior part of each segment. In these PH-overexpressing stripes, CycA staining is reduced. In *prdGAL4*/*UASph* embryos, PH is overexpressed by the *prd* driver, and CycA staining is reduced in PH-overexpressing regions (in red). Regions of *ph* overexpression are highlighted in the anti-PC-labeling panel by white marks, and reported in the CycA-labeling panel.

down-regulation in PH-expressing cells (Fig. 6B). We noted that *CycA* repression occurred also in stripes expressing low levels of *prdGAL4*, resulting in a regularly spaced pattern of *CycA* repression from the anterior to the posterior of the embryo. These results confirm the general ability of PcG complexes to repress *CycA* in all parts of early embryos containing actively dividing cells.

Loss of function of Pc *correlates with ectopic* CycA *expression in late embryos*

To confirm this *Pc*-dependent *CycA* repression, we next analyzed CycA levels in the absence of PC in nondividing late embryonic cells. In wild-type embryos, at germ band retraction (stage 10) the first cells enter mitosis 16th. At stages 11/12, most epidermal cells and cells of the nervous system have stopped dividing. At these late stages, epidermal cells are arrested in the G1 phase of cycle 17 (Edgar and O'Farrell 1990) and CycA staining is normally weak or absent (Fig. 7, upper panels). In Pc^{XL5}/Pc^{XL5} homozygous embryos, however, *CycA* was ectopically expressed in epidermal cells at stages 11, 12, and 13 (Fig. 7, lower panels). This result suggests that *CycA* is derepressed in a *Pc* loss-of-function mutant context. Together, the consequences of *Pc* overexpression and mutation demonstrate that PcG proteins act to control *CycA* gene expression during embryonic development. *Pc* homozygous mutant embryos cannot be identified before stage 11. We were thus unable to investigate a potential derepressive effect of PcG on *CycA* in cycling embryonic cells. However, *Pc*-RNAi treated S2 cells show a slight but consistent CycA up-regulation compared with control RNAi treated cells (Supplementary Fig. S2). This result suggests that PcG proteins might also modulate *CycA* transcription during the cell cycle of dividing cells.

Discussion

Given the well-described nature of homeotic gene silencing by PcG proteins (i.e., stable maintenance of repression throughout development), PcG genes would not appear at first glance to be obvious candidates for factors controlling the dynamic expression of cell cycle genes. Indeed, actively proliferating cells must reexpress their rate-limiting division components with each cell cycle. We have observed, however, that RNAi-mediated depletion of PC in cycling S2 cells modifies their cell cycle profile, although it does not affect the overall rate of cell proliferation. We have also identified the *Drosophila* *CycA* gene as a direct target in vivo for PC, PH, and GAF in cycling S2 and embryonic cells. In ChIP experiments, we precisely mapped the PcG-binding element in the *CycA* gene to a region spanning from the promoter to the first intron. This *CycA* region shares some but not all properties with homeotic PREs. First, the sequence is sufficient to silence the mini-*white* reporter gene in vivo, producing a characteristic eye variegation phenotype. Second, as expected for a PRE, mini-*white* silencing is genetically dependent on the activities of the PcG and trxG genes. Third, we demonstrated that the endogenous *CycA* gene is repressed in a *Pc*-dependent manner during embryonic development: In homozygous *Pc* mutants *CycA* expression is derepressed in late (stages 11/12/13) embryos. Finally, stable repression of *CycA* in normal

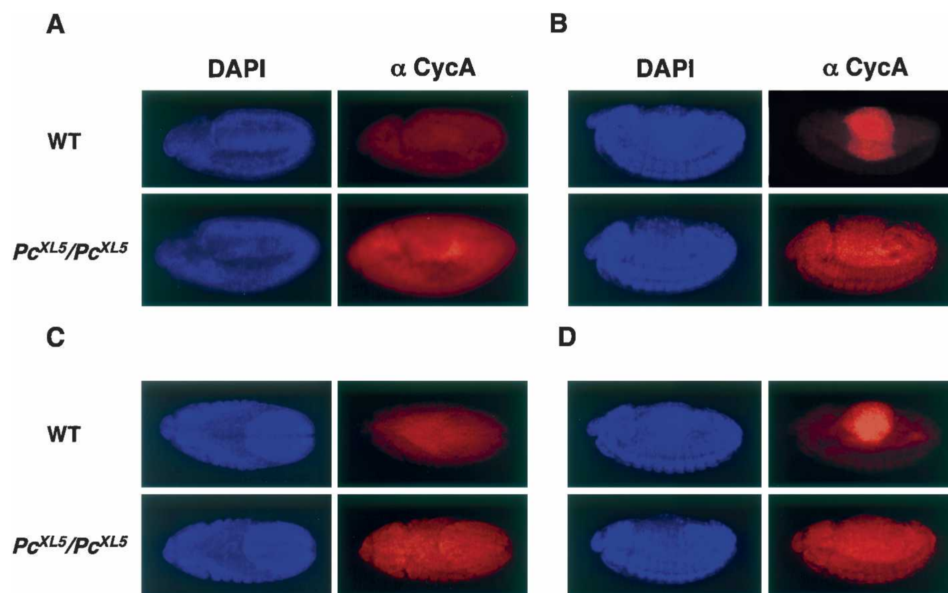

Figure 7. Loss of function of *Pc* triggers ectopic expression of the CycA protein in late embryos. (*A–D*, *top* panels) In wild-type (WT) embryos, CycA expression levels are very low in stage 11 (*A*), stage 12 (*B*,*C*), and stage 13 (*D*) embryos. (*A–D*, *bottom* panels) At the equivalent stages in Pc^{XL5}/Pc^{XL5} homozygous embryos, *CycA* is ectopically expressed in epidermal cells. (*A*) Stage 11 embryos, lateral view. (*B*) Stage 12 embryos, lateral view. (*C*) Stage 12 embryos, dorsal view. (*D*) Stage 13 embryos, lateral view.

embryos can be visualized as a colocalization between the *CycA* locus and PcG bodies that gradually increases, reaching a maximum at the time when cells totally stop dividing and begin to differentiate. Together, these results are consistent with PcG proteins playing a functional role in the stable repression of the *CycA* gene in vitro and in vivo.

In addition, *Pc* and *ph* overexpression in rapidly proliferating cells during early embryonic development caused a systematic decrease in the expression of the *CycA* gene. This suggests that PcG proteins may play a dual molecular role in the regulation of *CycA*, acting as stable silencing factors in mitotically quiescent cells and as modulators of promoter output in proliferating cells.

Role of PcG complexes in the control of CycA *expression during the cell cycle*

In our experiments, PcG members bound the *CycA* PRE in actively dividing S2 cells. This binding is most likely functionally relevant, since depletion of PC in S2 cells reproducibly modified the cell cycle division profile, correlating with increased CycA levels. We found an accumulation of cells in the G2/M phase of the cell cycle in PC-depleted cells in comparison to control cells. This accumulation is reminiscent of the phenotype observed in the *Drosophila dally* mutant, in which the cell division pattern is altered in the nervous system and G2/M progression is disrupted in specific sets of dividing cells in the larval brain and eye disc (Nakato et al. 1995). In this mutant, lamina precursor cells retain high levels of CycA for a prolonged period of time (Nakato et al. 2002). Although our experiments do not allow us to define exactly which step within the G2/M transition is abnormal, we propose that elevated levels of CycA, or an abnormally long persistence of CycA, might cause a delay in exit from mitosis. Accumulation of CycA has been previously shown to accelerate the G1/S transition (Sprenger et al. 1997). Consistent with this finding, in our experiments the population of S2 cells in G1 and S phases was largely decreased after PC depletion (Fig. 1).

The implication of PcG members in cell cycle control during active proliferation is surprising. Interestingly, Müller and colleagues removed individual PcG proteins from clones of proliferating cells in imaginal discs (Beuchle et al. 2001) and showed that *Psc-Su(z)2* and ph^{0} mutant clones are large and round (Beuchle et al. 2001), reminiscent of clones of mutations that cause disc tumors (Justice et al. 1995; Xu et al. 1995). While the exact nature of the defect was unknown, it could be rescued by resupplying Psc and Su(z)2 several hours after the induction of the clone. This suggests that the effects produced by altering PcG-mediated regulation of cell proliferation/growth might be reversible.

In our experiments, we found that *Pc* and *ph* overexpression in cycling embryonic cells can silence endogenous *CycA* expression. This result demonstrates that the effect of PcG proteins on the endogenous *CycA* PRE is dose-dependent in cycling cells, and suggests that *CycA* maintains an intrinsic capacity to be silenced despite being normally transcribed. In normal proliferating cells, induced transcription through the *CycA* locus, which would necessarily transverse the PRE, might be sufficient to counteract the PRE silencing activity of the *CycA* PRE. Indeed, it has recently been shown that intergenic transcription through a PRE counteracts silencing (Schmitt et al. 2005).

Dynamic repression versus stable silencing of the CycA *gene during development*

Our results suggest that the *CycA* PRE might present dual functional properties depending on whether cells are cycling or are arrested in the cell cycle. The *CycA* PRE might behave as a transcriptional attenuator element in cycling cells and as a stable silencer in a subset of mitotically quiescent cells. Recent data suggest the existence of functionally distinct PcG protein complexes that differ in composition as a function of developmental stage and cellular proliferation status (Lund and van Lohuizen 2004a; Voncken et al. 2005). It would thus be of great interest to biochemically characterize the composition of PcG complexes present at different phases of the cell cycle or during different developmental stages in *Drosophila*.

Although PcG proteins can repress *CycA* in mitotically arrested embryonic cells, this does not account for all aspects of stable *CycA* repression. For example, terminally differentiated cells of the salivary glands from third instar larvae do not express *CycA* (Edgar and Orr-Weaver 2001), but neither the endogenous gene nor the isolated PRE are able to attract PcG proteins in this tissue. This situation is similar to the *hh* gene, which is a known target of PcG proteins (Chanas and Maschat 2005). Another chromatin-silencing activity must therefore be responsible for this silencing. One possible candidate is the recently described dREAM complex (Korenjak et al. 2004), which contains the *Drosophila* E2F and RBF (pRb homolog) factors and binds to silent E2F-binding-site-containing genes during development, including in salivary glands. Whether or not this is the case, silencing of the *CycA* gene seems to be regulated in a complex manner that might change during different phases of the cell cycle and might depend on the developmental stage and the tissue under analysis.

In addition to *CycA* being regulated by PcG members, the converse might also be possible; i.e., PcG-binding and/or silencing activity might be regulated in a cell cycle-dependent manner. In a preliminary genetic analysis involving *trans*-heterozygous allelic combinations, we have found that the homeotic phenotypes of extra sex combs in the T2 and T3 thoracic legs in males and the pigmentation of the A4 tergite (*Mcp* phenotype) associated with mutations in the *Pc* and *ph* genes are enhanced when combined with a *CycA* mutation. This may suggest the existence of a feedback regulatory loop between *PcG* genes and *CycA*.

A global role for PcG proteins in the regulation of cell proliferation

From studies in vertebrates, it is clear that PcG proteins repress *p16ink4a* and *p19arf*, although a strict demonstration of direct repression is still missing. We do not know whether *plutonium*, the putative *Drosophila* homolog of *p16ink4a*, is silenced by PcG proteins. However, we have carried out a "ChIP-on-chip" analysis (Ren et al. 2000) of the binding profiles of PC, PH, and GAF proteins in a region covering 10% of the *Drosophila melanogaster* genome. This analysis led to the identification, among others, of several potential PcG target genes that play a role in the control of proliferation and growth (N. Nègre, J. Hennetin, L.V. Sun, S. Lavrov, M. Bellis, K.P. White, and G. Cavalli, unpubl.). These include the *escargot* (*esg*), *elbowB* (*elB*), and *no ocelli* (*noc*) genes, in addition to a p53-like factor encoded by *bifid*. Interestingly, *esg* and *elB*, as well as the known PcG target gene *hh*, have been coidentified as potential tumor suppressors in a protein overexpression screen (Tseng and Hariharan 2002). Finally, recent evidence suggests that *hh* regulates both proliferation and differentiation in the developing *Drosophila* retina (Thomas 2005).

Together with the role of PcG proteins in the regulation of *CycA*, this evidence suggests that PcG proteins may be globally involved in the coupling of cell proliferation with growth or differentiation during development in *Drosophila* and perhaps also in vertebrates. This intriguing possibility warrants future investigation.

Materials and methods

Antibodies

ChIP experiments were done with a rabbit affipure anti-PC antibody kindly provided by R. Paro (Zentrum fr Molekulare Biologie Heidelberg, University of Heidelberg, Heidelberg, Germany) (Zink and Paro 1989). The rabbit polyclonal anti-PH antibody has been previously described (Strutt and Paro 1997). The rabbit anti-GAF antibody was kindly provided by P. Becker (Adolf Butenandt Institute, University of Munich, Munich, Germany). For Western blot and immunostaining experiments, a polyclonal rabbit anti-PC antibody was used. This serum was raised against the C-terminal 199 amino acids of PC (same epitope as the affipure antibody) and recognizes the same sites in polytene chromosomes (Zink and Paro 1989). This anti-PC antibody was used at a dilution of 1:200. The anti-GAF antibody is already described (Melnikova et al. 2004). The A12 monoclonal anti-CycA antibody was generated by C. Lehner (Department of Genetics, University of Bayreuth, Bayreuth, Germany) and is available at the Developmental Studies Hybridoma Bank. For immuno-
staining, this antibody was used at a dilution of 1:5.

Fly stocks and handling

Flies were raised in standard corn meal yeast extract medium. The Oregon-R w^{1118} line was obtained from R. Paro. Transgenic lines, were obtained by injection of Canton-S w^{1118} embryos.

Mutant stocks used in this study had the following genotypes: Pc^{XT109}*, b, pr, cu, sbd/TM3Ser*, Pc^{XL5}*/TM3,Ser,Sb*, w^{1118}; ph^{410}*/FM7*, brm^{2}*e[s]ca[1]/TM3,Ser*. A stock carrying the *KrGFP-CyO* balancer chromosome was used for the selection of homozygous *Pc'* mutants. The *hsPc* line is described by Fauvarque et al. (1995). Eye pigment determination was done as described previously (Reuter and Wolff 1981). The *UASphL7* transgenic line was kindly provided by F. Maschat (Institute of Human Genetics, CNRS, Montpellier, France) and crossed with the *enGAL4* and *prdGAL4* (Brand and Perrimon 1993) drivers.

Immunostaining of embryos

Embryos were classically fixed (Mitchison and Sedat 1983) and stained with a monoclonal anti-CycA antibody diluted at 1:5 (A12 provided by Development Studies Hybridoma Bank [DSHB], University of Iowa) and polyclonal anti-PC antibodies at 1:250.

Heat-shock experiments

Embryos of the genotype w^{1118} or *hsPc* were collected for 1 h at room temperature on thin grape collection medium and aged 90 min after egg laying at 25°C (until early stage 4, cycle 10). They were then given a 30 min heat shock in a 37°C water bath and allowed to recover at 25°C until stage 6–9. The embryos were then fixed and stained for PC and CycA and viewed by fluorescent microscopy.

Transgenic constructs, P-element transformation, and fly work

The putative *CycA* PRE fragment was obtained by PCR using specific primers (S-14rev). Primer sequences are listed in the Supplemental Material. The PCR fragment was subcloned into the pGEM-T Easy Vector System (Promega), sequenced, and then excised from pGEM and cloned into the pUZ vector at the NotI restriction site. Insertion orientation and insert copy number were verified by PCR. The construct was then injected in embryos from w^{1118} Canton-S strain using a classical transgenesis protocol (Spradling 1986). Details of crosses used to obtain transgenic lines into trx^{E2}, brm^{2}, Pc^{XL5}, and ph^{410} mutant backgrounds are available upon request. Eyes were imaged with a Nikon DXM1200 digital camera mounted on a Nikon SMZ1000 binocular. For eye color comparison, all individuals were of the same age and were imaged in the same frame.

RNAi of gene expression in cultured Drosophila cells

For RNAi, we followed the protocol of the Dixon laboratory (Worby et al. 2001). Two oligonucleotides were designed for *Pc* exon 2 and the *neomycin* (*neo*) locus: *Pc* forward, 5′-GGCGTC GTGGAGTACCGTGTC-3′; *Pc*-rev, 5′-CGAAGACACCGGT CACCCCAC-3′; and *neo*-forward, 5′-GCGCGGCTATCGTG GCTGGCC-3′; *neo*-reverse, 5′-GCTAAGGCTTCGGGTTGG AAAG-3′. Each of these oligos incorporates a T7 RNA polymerase-binding site. The *Pc* and *neo* PCR products have a size of 725 and 708 bp. Double-stranded RNAs (dsRNAs) generated with the Ambion MEGAscript T7 kit were incubated with cultured S2 *Drosophila* cells. After 4 d of RNAi incubation, cell extracts were prepared using 2.5×10^5 cells and Western blots were performed and revealed using antibodies directed against the PC, GAF, and tubulin proteins. In parallel, 2×10^4 cells were analyzed by FACS.

In vivo formaldehyde cross-linking of Schneider cells and immunoprecipitation of cross-linked chromatin

ChIP in *Drosophila* S2 Schneider cells and 4- to 12-h-old embryos was carried out as previously described (Strutt et al. 1997;

Cavalli et al. 1999). Purified DNA (100 ng) from antibody immunoprecipitations or from control immunoprecipitations performed with an affipure rabbit HRP conjugate antibody (Mock IP; Promega) was used as a hybridization probe on Southern blots, as previously described (Orlando and Paro 1993; Orlando et al. 1997).

Immuno-FISH on polytene chromosomes

The detailed protocol for immuno-FISH was described previously (Lavrov et al. 2004). FISH probes (with the pUZ vector as a template) were labeled using the Bio-Nick nick-translation kit (Life Technologies) according to the manufacturer's instructions. Chromosomes were imaged with a DMRA2 Leica microscope coupled with a CoolSnap HQ CCD camera (Roper Scientific). Acquisitions were carried out using the Metaview software (Universal Imaging Corporation) and images were processed using Adobe Photoshop. For each fly line, 10–20 chromosomes from two or more preparations were analyzed.

Combination of FISH and immunostaining (FISH-I) on whole-mount embryos and image analysis

For the *CycA* and *CycB* loci, 10 overlapping genomic PCR fragments of 1 kb, covering 11.1 kb of the genomic region, were pooled for probe labeling. Probes were labeled by nick translation with digoxigenin-11-dUTP (Roche Diagnostics) according to the manufacturer's instructions. Detailed coordinates of fragments used to produce probes are available in the Supplemental Material. FISH on whole-mount embryos was performed as previously described (Bantignies et al. 2003). After post-hybridization washes, embryos were blocked in PBSTr (PBS, 0.3% Triton), 10% Normal Goat Serum (NGS) for 2 h at room temperature and incubated overnight at 4°C in PBSTr/10% NGS with a PC rabbit polyclonal antibody at a dilution of 1:250. After incubation with the anti-PC antibody, embryos were washed several times in PBSTr, blocked again in PBSTr/1% BSA/10% NGS for 1 h at room temperature, and incubated sequentially in blocking buffer with the secondary antibodies: first with anti-digoxygenin-Rhodamine (Roche Diagnostics) at a dilution of 1:45 for 1 h at room temperature, and then with the anti-rabbit Alexa 488 (Molecular Probes) at a dilution of 1:500, for 1 h at room temperature. DNA was counterstained with DAPI, and embryos were mounted in Prolong Antifade medium (Molecular Probes). Images were acquired with a cooled CCD camera (Micromax YHS 1300, Roper Scientific) mounted on a DMRXA Leica microscope, and with a 100× Plan/Apo Ojective (NA 1.4) mounted on a piezo electric (Roper Scientific). For each color channel, z-stacks of 6–7 µm were collected at 0.5-µm intervals along the *Z*-axis (i.e., 13–15 slices per stack) with Metamorph software (Universal Imaging Corporation). Three-dimensional (3D) stacks of raw images were reconstituted for each channel and color combined to give multichannel 3D stacks. For each locus, the statistical analysis was performed by analyzing 300 nuclei on three different multichannel 3D stacks. It is important to note that, at the developmental stage used in our analysis, the percentage of homologous pairing was high for both cyclin loci, and most nuclei had only one FISH spot. For figure display, single slices from z-stacks were deconvolved with the Huygens MLE single tif procedure (Scientific Volume Imaging) (Supplementary Fig. S1).

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