GENETICS

Polycomb-group recruitment to a *Drosophila* target gene is the default state that is inhibited by a transcriptional activator

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Polycomb-group (PcG) proteins are epigenetic regulators that maintain the transcriptional repression of target genes following their initial repression by transcription factors. PcG target genes are repressed in some cells, but active in others. Therefore, a mechanism must exist by which PcG proteins distinguish between the repressed and active states and only assemble repressive chromatin environments at target genes that are repressed. Here, we present experimental evidence that the repressed state of a *Drosophila* PcG target gene, *giant* (*gt*), is not identified by the presence of a repressor. Rather, de novo establishment of PcG-mediated silencing at *gt* is the default state that is prevented by the presence of an activator or coactivator, which may inhibit the catalytic activity of Polycomb-repressive complex 2 (PRC2).

INTRODUCTION

Polycomb-group (PcG) proteins play a critical role in metazoan development by maintaining the transcriptional silence of numerous genes, most of which encode developmental and/or cell cycle regulators (*1*). PcG proteins do not initiate transcriptional repression but maintain the repressed state following initial repression by gene-specific transcription factors (*2*–*4*). Recruitment of PcG proteins to targets in the *Drosophila* genome requires the presence of one or more Polycomb response elements (PREs) (*5*, *6*). It has been proposed that unmethylated CpG islands may serve a similar function in mammals (*7*). Following recruitment to repressed target genes, PcG proteins covalently modify histones and establish a repressive chromatin environment. The latter can involve local compaction of chromatin structure and/or inhibition of transcription initiation or elongation by RNA polymerase (RNAP) II (*8*, *9*).

PcG proteins largely function as components of protein complexes. There are three primary *Drosophila* PcG complexes: pleiohomeotic repressive complex (PhoRC), Polycomb repressive complex 1 (PRC1), and PRC2 (*5*). PhoRC includes the PRE binding protein Pho and Sfmbt. Canonical PRC1 includes Polycomb (Pc), Sex combs extra (Sce, aka dRing), Posterior sex combs (Psc), and polyhomeotic (Ph). A noncanonical form of *Drosophila* PRC1, dRAF, includes Sce, Psc, and Kdm2. Core subunits of PRC2 are Enhancer of zeste [E(z)], Extra sex combs (Esc), Suppressor of zeste 12 [Su(z)12], and Caf1-55. The catalytic subunit of PRC2, E(z), mono-, di-, and trimethylates histone H3 at lysine-27 (H3K27). A subset of PRC2 complexes includes Polycomb-like (Pcl). Pcl has been proposed to enhance PRC2 catalytic activity by stabilizing its association with chromatin substrates (*10*).

PRC2 deposition of H3K27me3 serves multiple functions. The Pc chromodomain specifically binds to H3K27me3, contributing to recruitment of canonical PRC1 (*11*–*13*). In addition, the aromatic cage of Esc binds to H3K27me3, stimulating PRC2 catalytic activity, thus providing a positive feedback effect on H3K27 methylation and contributing to propagation of the H3K27me3 mark (*14*, *15*).

PcG target genes must be expressed in specific subsets of cells and kept silent in others, yet PcG proteins are more ubiquitously expressed. This requires PcG proteins to distinguish between the active and repressed states of target genes and only assemble to form a repressive chromatin environment at genes that are transcriptionally inactive. Multiple models have been proposed for how PcG proteins initially distinguish between active and repressed target genes. Instructive models propose that PcG proteins may be recruited by physical interactions with repressors (and/or corepressors) or noncoding RNAs (ncRNAs) (*5*). The idea that repressors recruit PcG proteins is an obvious extension of the observation that PcG proteins maintain transcriptional repression following initiation of repression by transcription factors. However, only rare examples of this have been reported, including Runx1/CBFβ recruitment of mammalian PRC1 (*16*). Recruitment of PcG proteins by ncRNAs was first observed in mammalian X-inactivation but has been proposed to be a more general mechanism of recruitment (*17*). In contrast, responsive models suggest that the transcriptional state of a gene may determine whether PcG proteins are stably recruited (*5*). One version of these models suggests that RNA may compete with chromatin for PRC2 binding and that lack of local RNA synthesis may permit stable association of PRC2 with nucleosomes and deposition of H3K27me3 (*18*, *19*). Alternatively, it has been proposed that histone modifications associated with transcriptionally active genes, such as acetylation of lysine residues or methylation of certain residues, such as H3K4 and H3K36, which have been shown to inhibit the catalytic activity of PRC2, may locally inhibit H3K27me3 deposition (*5*, *20*).

We have experimentally tested these models using a previously described genetic system in which a PcG target gene, *giant* (*gt*), is ubiquitously repressed in *Drosophila* embryos (*21*). Here, we describe a modified version of this system in which *gt* is ubiquitously active and compare recruitment of PcG proteins and distribution of histone modifications under these disparate conditions. The respective roles of repressors and activators in identifying *gt* as active or repressed were tested by selectively depleting embryos of the *gt* repressor,

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Hunchback (Hb), and/or the activator, Caudal (Cad), and assaying the effects on PcG recruitment. In addition, by examining PcG recruitment to a transcriptionally inert *gt* transgene in embryos in which endogenous *gt* is active, we have tested the effect of local transcriptional activity on PcG recruitment. Our results support a responsive model in which PcG recruitment to a genomic target is the default state, but may be inhibited by the presence of an activator, and is not affected by local RNA synthesis.

RESULTS

Following fertilization of *Drosophila* eggs, nuclei undergo a series of rapid cycles of DNA replication and mitosis within a syncytium. After the ninth nuclear division (nc9), nuclei migrate to the periphery, forming the syncytial blastoderm. The durations of nuclear cycles gradually lengthen until the first prolonged interphase following nuclear cycle 13 (nc13) mitosis, during which cell membranes separate nuclei forming the cellular blastoderm in the second half of nc14 (nc14b). A small number of genes, including *gt*, begin to be transcribed during nc9 to nc13, but zygotic transcription of most genes does not begin until nc14 (*22*).

Transcriptional regulation of *gt* in syncytial and cellular blastoderm stage embryos is well characterized (*23*) and is controlled by four upstream enhancers (Fig. 1A) (*24*). In embryos produced by *bicoid* (*bcd*) *oskar* (*osk*) *torso-like* (*tsl*) females, the maternally expressed *gt* repressor Hb is ubiquitously expressed because of lack of *nanos* (*nos*) mRNA localization in the absence of Osk (*25*). The maternally expressed *gt* activator, Cad, also is ubiquitously expressed (due to the lack of Bcd), yet *gt* is initially repressed in all syncytial blastoderm nuclei by Hb (fig. S1B) (*21*). Bcd and the terminal system, which includes Tsl, are needed to activate zygotic *hb* transcription (*26*, *27*). Zygotic expression of *cad* also is activated by the terminal system but is repressed by maternal Hb (*28*, *29*). Therefore, as maternally expressed Hb and Cad are degraded [after nc13; (*27*, *30*)], they are not replaced by zygotically expressed Hb and Cad due to the *bcd osk tsl* maternal genotype (fig. S1B) (*21*). We have previously shown that both Hb and Cad bind to the *gt_(-3)* enhancer in these embryos, resulting in ubiquitous *gt* repression at the syncytial blastoderm stage and that the PcG is required to maintain *gt* repression when maternal Hb is degraded (*21*, *31*). Of the four *gt* enhancers, we will focus only on *gt_(-3)* and therefore will simply refer to it as "enhancer."

Testing the respective effects of Hb and Cad on PcG recruitment

To determine the respective roles of a repressor and an activator in recruiting or inhibiting recruitment of PcG proteins, we began by depleting embryos of Hb. Genetic crosses to generate RNA interference–mediated depletion of Hb in embryos produced by *bcd osk tsl* embryos were performed as diagrammed in fig. S1A. The *P(mat-tub-Gal4)* driver is expressed during oogenesis. Embryos derived from females that contain both *P(mat-tub-Gal4)* and *UAS-shRNA-hb* transgenes (*32*) exhibit markedly reduced levels of Hb protein. These embryos still ubiquitously express maternal Cad, resulting in *gt* expression throughout syncytial and cellular blastoderm stage embryos (fig. S1B). We will refer to these embryos as Hb-KD-*bcd osk tsl*. The relative levels of *gt* mRNA in *bcd osk tsl* and Hb-KD-*bcd osk tsl* embryos were confirmed by quantitative reverse transcription polymerase chain reaction (qRT-PCR) (Fig. 1D). No transcripts were detected from upstream regulatory regions when *gt* was transcriptionally active or repressed (fig. S1C).

At nc13, maternally expressed Hb is still abundant and repressing *gt* in embryos derived from *bcd osk tsl* females (*21*). Therefore, Hb depletion in Hb-KD-*bcd osk tsl* embryos was quantitatively assessed in chromatin immunoprecipitation (ChIP)–quantitative PCR (qPCR) assays of nc13 stage embryos. Hb signals were reduced approximately 80% at the enhancer (region 6) compared with their levels in *bcd osk tsl* embryos (Fig. 1B). Cad signals in this region were not affected by the reduced presence of Hb and were essentially the same in *bcd osk tsl* and Hb-KD-*bcd osk tsl* embryos. We note that our antigen affinity-purified anti-Cad antibody appears to also detect an unidentified protein localized at promoters, possibly a component of the transcription preinitiation complex (PIC). This interpretation is based, to a large extent, on positive ChIP signals at the promoter of the *miR-9A* gene (fig. S3), which is not regulated by Cad or the PcG. Therefore, the signals at regions 4 and 3 overrepresent the presence of Cad at and downstream of the *gt* promoter; we will primarily focus on the presence of Cad only at the enhancer (region 6). By nc14b, Hb and Cad signals were quite low at region 6 of both repressed and active *gt* (Fig. 1C), reflecting degradation of these maternally expressed proteins and consistent with expression levels observed in immunostained embryos (fig. S1B) (*27*, *30*).

Two *gt* regions, PRE1 and PRE2, previously have been shown to convey PcG-dependent maintenance of transcriptional repression to a heterologous enhancer and promoter within a reporter transgene and, therefore, are defined as PREs (*31*, *33*). PRE1, which contains two extended Pho consensus sites (*31*), includes the promoter region and transcription start site (TSS); PRE2 is approximately 6 kb upstream. Signals for Pho are positive at both PRE1 and PRE2 (regions 4 and 9, respectively) in nc14b embryos when *gt* is repressed (*bcd osk tsl*) (*21*). We chose nc14b embryos for these assays because that is the earliest stage at which components of all three major PcG complexes are stably recruited to both *gt* PREs (*21*). At transcriptionally active *gt* (Hb-KD-*bcd osk tsl* embryos), Pho signals at PRE1 were essentially unchanged from the repressed state (Fig. 2A). By nc14b, Pho is clearly positive at PRE2 of repressed *gt* (*21*). Pho also was positive at PRE2 of active *gt*, but, unlike its association with PRE1, Pho signals were reduced by about 50% in comparison to repressed *gt* (Fig. 2A). Also consistent with previous observations (*21*), under conditions of repression, core components of PRC1 and PRC2, Pc and E(z), respectively, and H3K27me3 were positive with peaks in the vicinities of the two *gt* PREs (Fig. 2, A and B). Likewise, signals for Pcl, an accessory component of PRC2, were highest at the PREs. At active *gt*, E(z) signals were only slightly above background at PRE1 and negative at PRE2 (Fig. 2A). Here and elsewhere, unless otherwise specified, "background" refers to signals equal to those at a negative control region, *PkaC1*, which have an enrichment value of 1 in these figures. Curiously, the signals for Pcl were almost twofold higher at PRE1 but reduced at PRE2 when *gt* was active (Fig. 2B). The discrepancy between Pcl and E(z) signals indicates that at least some of the Pcl at PRE1 likely is independent of PRC2. In light of observations that inclusion of a Pcl ortholog in mammalian PRC2 stabilizes the association of PRC2 with target chromatin, thereby increasing the ability of PRC2 to trimethylate H3K27 (*10*), it is possible that the presence of Pcl at PRE1 may stimulate the catalytic activity of low levels of PRC2, resulting in a small but positive peak of H3K27me3 in this region (Fig. 2A). Pc levels were correspondingly reduced to near-background levels across most of the *gt* region and

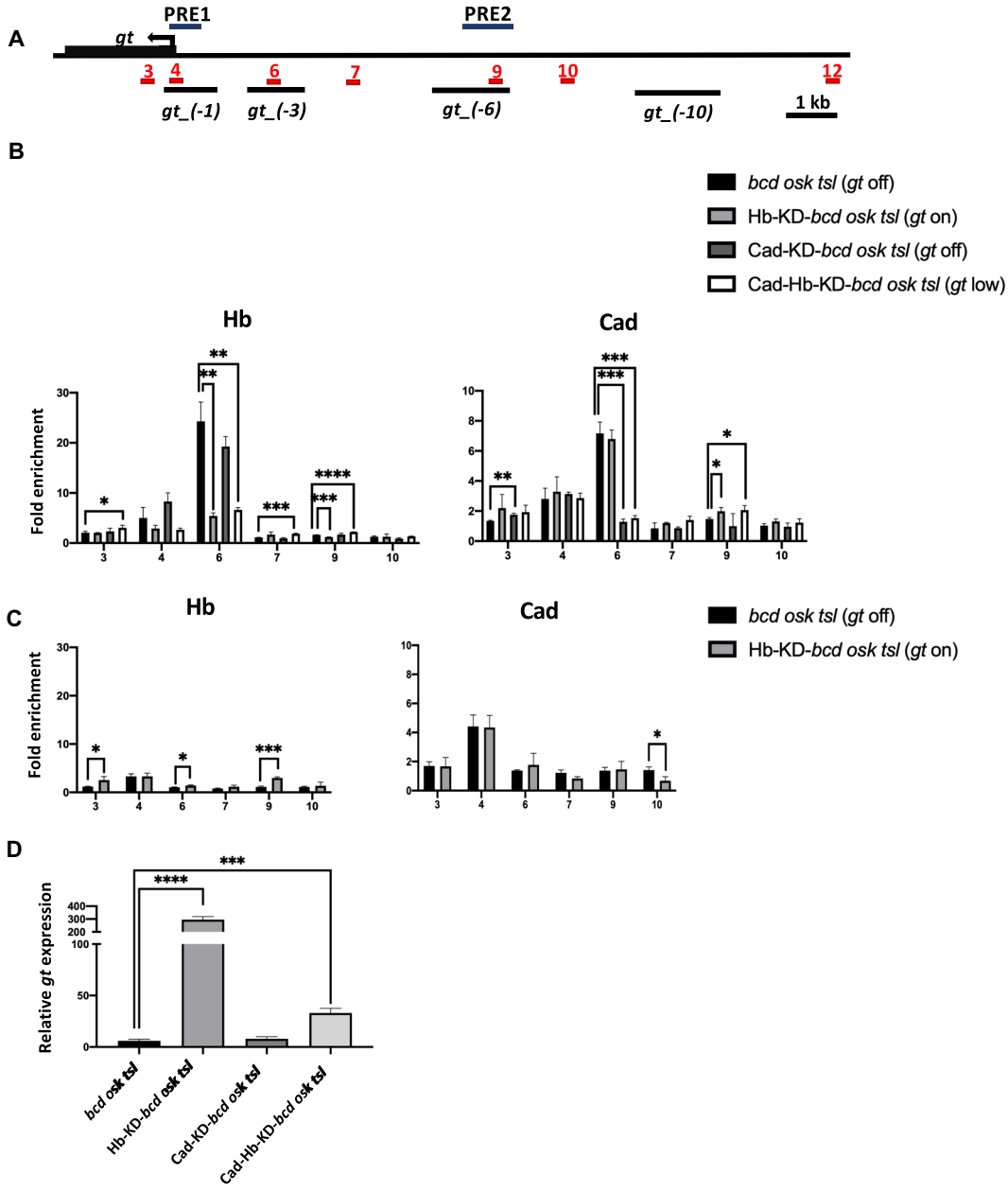

Fig. 1. Effects of RNAi-mediated depletion of Hb and/or Cad. (**A**) Map of the *gt* genomic region containing two PREs and four enhancers. Locations of PRE1 and PRE2 are indicated above. Below, numbers and positions of PCR amplified regions are shown in red; *gt* enhancers are shown in black. (**B** and **C**) ChIP-qPCR performed with anti-Hb and anti-Cad antibodies, as indicated, using (B) nc13 and (C) nc14b embryos. Maternal genotypes and the expression state of *gt* are indicated. ChIP signals are presented as fold enrichment relative to signals at *Pka-C1*, which have a value of 1. (**D**) qRT-PCR assays of *gt* mRNA expression in the indicated embryos. Values are shown relative to levels of *gt* in wild-type embryos, which has a value of 100. Signals from three biological replicas are presented. Error bars show the SD for three biological replicates. **P* < 0.05; ***P* < 0.01; ****P* < 0.001; *****P* < 0.0001.

only weakly positive in the vicinity of the H3K27me3 peak at PRE1 (Fig. 2B).

H3K27ac, a marker of transcriptionally active genes, was negative across much of repressed *gt* but moderately positive at the promoter/PRE1 (Fig. 2B). H3K27ac not only was clearly positive at this region when Hb was depleted but also was above background in the enhancer and an upstream region (Fig. 2B). This indicated that one effect of Hb is to inhibit acetylation of H3K27. RNAP II phosphorylated at serine 5 ($RNAPII_o^{ser5}$) was present at the repressed *gt* promoter (Fig. 2B), which is consistent with previous reports that RNAP II often is poised at promoters of PcG-repressed genes (*9*).

Thus, in the presence of Cad and near absence of Hb, PcG recruitment to PRE1 and PRE2 was differently affected. At PRE2, signals for PhoRC, PRC1, and PRC2 were all reduced by approximately 50% relative to their signals at repressed *gt*. Binding of Pho to PRE1 was unaffected by Hb depletion and transcriptional activation. However,

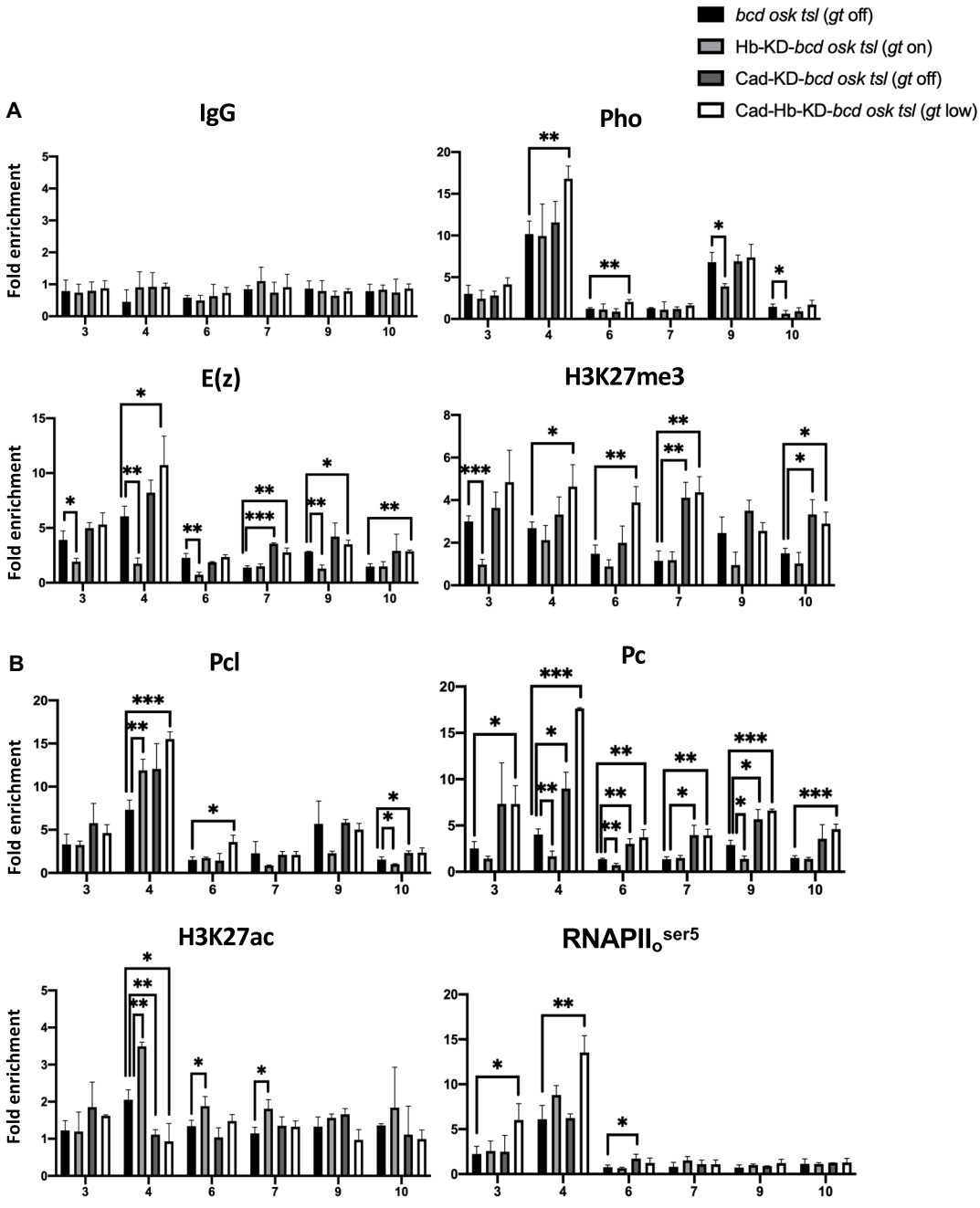

Fig. 2. Distribution of PcG proteins, histone modifications, and RNAP II at *gt* in the presence or absence of Hb and/or Cad. (**A** and **B**) ChIP-qPCR performed in nc14b embryos with the indicated antibodies. Genotypes of embryos are indicated in the key. (**A**) IgG, anti-Pho, anti-E(z), and anti-H3K27me3. (**B**) Anti-Pcl, anti-Pc, anti-H3K27ac, and anti-RNAPII$_o^{ser5}$. Maternal genotypes and the expression state of *gt* are indicated. Signals for histone modifications were normalized to total H3 signals that were obtained using an anti-H3 antibody. ChIP signals are presented as fold enrichment relative to signals at *Pka-C1*, as in Fig. 1, except for anti-H3K27ac and anti- RNAPII$_o^{ser5}$ signals, which are presented as fold enrichment relative to signals at *gt* region 12. Error bars show the SD for three biological replicates. **P* < 0.05; ***P* < 0.01; ****P* < 0.001.

signals for core components of PRC2 and PRC1 were strongly reduced, and H3K27ac signals were increased.

Two alternatives that are both consistent with these results were then considered. Either the repressor Hb may play a positive role in recruiting PcG proteins or the activator Cad may inhibit PcG recruitment. These two alternatives were tested by simultaneously depleting Hb and Cad in *bcd osk tsl* embryos. If the presence of Hb is needed for recruitment of PcG proteins, we predicted that the levels and distribution of PcG proteins at *gt* would resemble their profile at active *gt* in embryos depleted of Hb alone. Alternatively, if Cad inhibits PcG recruitment, we predicted that the levels and distribution of PcG proteins would more closely resemble the repressed state in *bcd osk tsl* embryos.

Neither Hb nor Cad was detectable in immunostained syncytial blastoderm embryos produced from the genetic cross diagrammed

in fig. S2A (fig. S2C). ChIP-qPCR assays of these nc13 embryos confirmed 73 and 78% reduction of Hb and Cad, respectively, from the enhancer region in comparison to their signals in *bcd osk tsl* embryos (Fig. 1B). Simultaneous depletion of both Cad and Hb resulted in low levels of *gt* transcription that were greater than observed in *bcd osk tsl* embryos (Fig. 1D), although Gt protein remained below detectable levels in immunostained embryos (fig. S2C). Low levels of *gt* transcription despite the reduced presence of an activator may be due to the presence of Zelda (Zld), which has been detected at the *gt* promoter and early enhancers during syncytial blastoderm stages (*34*). A TAGteam Zld binding site is located at −114 within the *gt* promoter. Zld facilitates DNA binding by transcription factors in pre-midblastula transition (pre-MBT) *Drosophila* embryos. In the absence of these transcription factors, Zld is able to cause low levels of target gene transcription, presumably by increasing the accessibility of promoters to basal transcription factors (*35*). Consistent with this interpretation, $RNAPII_o^{ser5}$ signals were positive at the *gt* promoter in these embryos (Fig. 2B).

To our surprise, signals for components of PhoRC, PRC1, PRC2, and H3K27me3 were significantly higher at PRE1 in Cad-Hb-KD-*bcd osk tsl* embryos (Fig. 2, A and B) compared with *bcd osk tsl* embryos (Fig. 2, A and B). Other than an increased signal in the enhancer, which was still quite low, Pho signals at other *gt* regions, including PRE2, were approximately equal to those in *bcd osk tsl* embryos. However, E(z) and Pc signals were increased across much of the *gt* regulatory region, including PRE2 (Fig. 2, A and B). H3K27me3 signals also increased in most upstream regions, with the exception of PRE2 (Fig. 2A). H3K27ac levels were close to background across most of the *gt* region, consistent with the reduced presence of the activator Cad. Particularly, H3K27ac signals were lower in comparison with *bcd osk tsl* at PRE1 (Fig. 2B). It is notable that PcG recruitment and H3K27me3 deposition occur despite the low levels of *gt* transcription that are above those in *bcd osk tsl* embryos (Fig. 1D), providing evidence that the act of transcription does not play a role in determining the active versus repressed state with respect to PcG recruitment at *gt*. The observation that PcG and H3K27me3 signals across much of the *gt* region in the reduced presence of Hb and Cad match or exceed their signals when both Hb and Cad are present and *gt* is repressed (*bcd osk tsl* embryos) contradicts a positive role for Hb in PcG recruitment and is consistent with an inhibitory effect of Cad.

This raised the question of what allows PcG protein recruitment when both Hb and Cad are present at *gt* in *bcd osk tsl* embryos. One possible explanation is that Hb only partially inhibits the inhibitory effect of Cad when both are present. To test this, embryos were generated in which Cad alone was depleted in a *bcd osk tsl* background (fig. S2B). As expected, *gt* transcripts were barely detectable in these embryos, and Gt was not detected in immunostained embryos (Fig. 1D and fig. S2C). Hb signals at the enhancer were equivalent to their signals in *bcd osk tsl* embryos (Fig. 1B). Signals for Pho at PRE1 and PRE2 were comparable to those in *bcd osk tsl* embryos (Fig. 2A), demonstrating that Cad does not inhibit binding by PhoRC. Although not statistically significant, signals for E(z), Pcl, and H3K27me3 were slightly elevated or at least unchanged at PRE1 and PRE2 (Fig. 2, A and B). Pc signals were significantly increased at both PREs and much of the *gt* regulatory region compared with their signals in *bcd osk tsl* embryos (Fig. 2B). Consistent with their dependence on Cad, H3K27ac signals at PRE1 were reduced.

These observations are consistent with Hb incompletely inhibiting the inhibitory effect of Cad on PcG recruitment in *bcd osk tsl* embryos. When both Hb and Cad are present, Hb prevails, but Cad still exerts a partial inhibition of PcG recruitment. By reducing the presence of Cad at *gt*, the balance may be shifted further toward absence of its inhibitory effect, permitting elevated levels of PcG recruitment. This is most clearly demonstrated by increased Pc signals when Cad was depleted (Fig. 2B). A hint at the mechanistic basis of this inhibition is revealed by the relatively higher Cad-dependent H3K27ac signals at PRE1 in Hb-KD-*bcd osk tsl* compared with *bcd osk tsl* embryos (Fig. 2C). By inhibiting acetylation of H3K27, Hb (or a corepressor it recruits) may enable PRC1 recruitment.

Testing the effect of transcription on PcG recruitment

To further test whether the transcriptional state of a gene identifies a target gene as active or repressed to PcG proteins, as has been proposed (*18*, *19*), we examined PcG recruitment to transgenes that are either transcriptionally active or transcriptionally inert. We have previously shown that a 10.8-kb *gt* fragment drives *lacZ* reporter gene expression in a *gt* pattern in embryos (from within the Pelican-*gt*-wt transgene; Fig. 3A) (*31*). To examine PcG recruitment to a transcriptionally inert *gt* regulatory region under conditions in which endogenous *gt* is active, we introduced clusters of base pair substitutions in the *gt* TATA box, Initiator region (Inr), and downstream promoter element (DPE) in this 10.8-kb fragment, which was then recombined into the same modified Pelican reporter vector that was used to generate Pelican-*gt*-wt (fig. S4A) (*31*). This construct, Pelican-*gt*-pm (promoter mutant), was integrated into the attP40 landing site, the same site into which the Pelican-*gt*-wt construct had been integrated (*31*). In situ hybridization and qRT-PCR confirmed that the Pelican-*gt*-pm transgene is not transcribed (figs. S3F and S4B).

Males containing either Pelican-*gt*-wt or Pelican-*gt*-pm were crossed to Hb-KD-*bcd osk tsl* females (fig. S4C). We then compared recruitment of Cad, Hb, PcG proteins, $RNAPII_o^{ser5}$, and deposition of histone covalent modifications to both Pelican-*gt*-wt and Pelican-*gt*-pm in embryos in which endogenous *gt* is transcriptionally activated by Cad. For each ChIP assay, signals from the transgene were compared with signals from endogenous *gt* in the same embryos. This is possible because the *gt* fragments within both transgenes contain mutations of PCR priming sites that permit transgene and endogenous *gt*-specific PCRs to be performed, but do not affect reporter gene expression (fig. S4B) (*31*).

At nc13, when signals for maternally expressed Cad and Hb at the endogenous *gt* enhancer (region 6) are highest when *gt* is repressed (*bcd osk tsl* embryos; Fig. 1B), their signals at this region of both transgenes were comparable to those at endogenous transcriptionally active *gt* (Fig. 3B). We note that Hb signals were relatively lower at the mutant promoter (region 4) of Pelican-*gt*-pm. Whether the weak Hb signals at the *gt* promoter are due to direct binding to promoter sites or to interactions with other promoter-associated proteins is not clear. We speculate that reduced signals of both Hb and Cad at the Pelican-*gt*-pm promoter may reflect the absence of PIC components and/or reduced access of this region when TFIID and other PIC components are not present. Elevated H3 signals at and downstream of the Pelican-*gt*-pm promoter are consistent with increased nucleosome density in this region (fig. S4D).

At nc14b, Pho was detected at PRE1 and PRE2 of Pelican-*gt*-wt at levels comparable to those at endogenous *gt* (Fig. 3C). Pho signals at PRE2 of Pelican-*gt*-pm were essentially the same as their signals at PRE2 of the active endogenous *gt*, which are approximately 50% of those at repressed *gt* (Figs. 2A and 3C). It was somewhat surprising

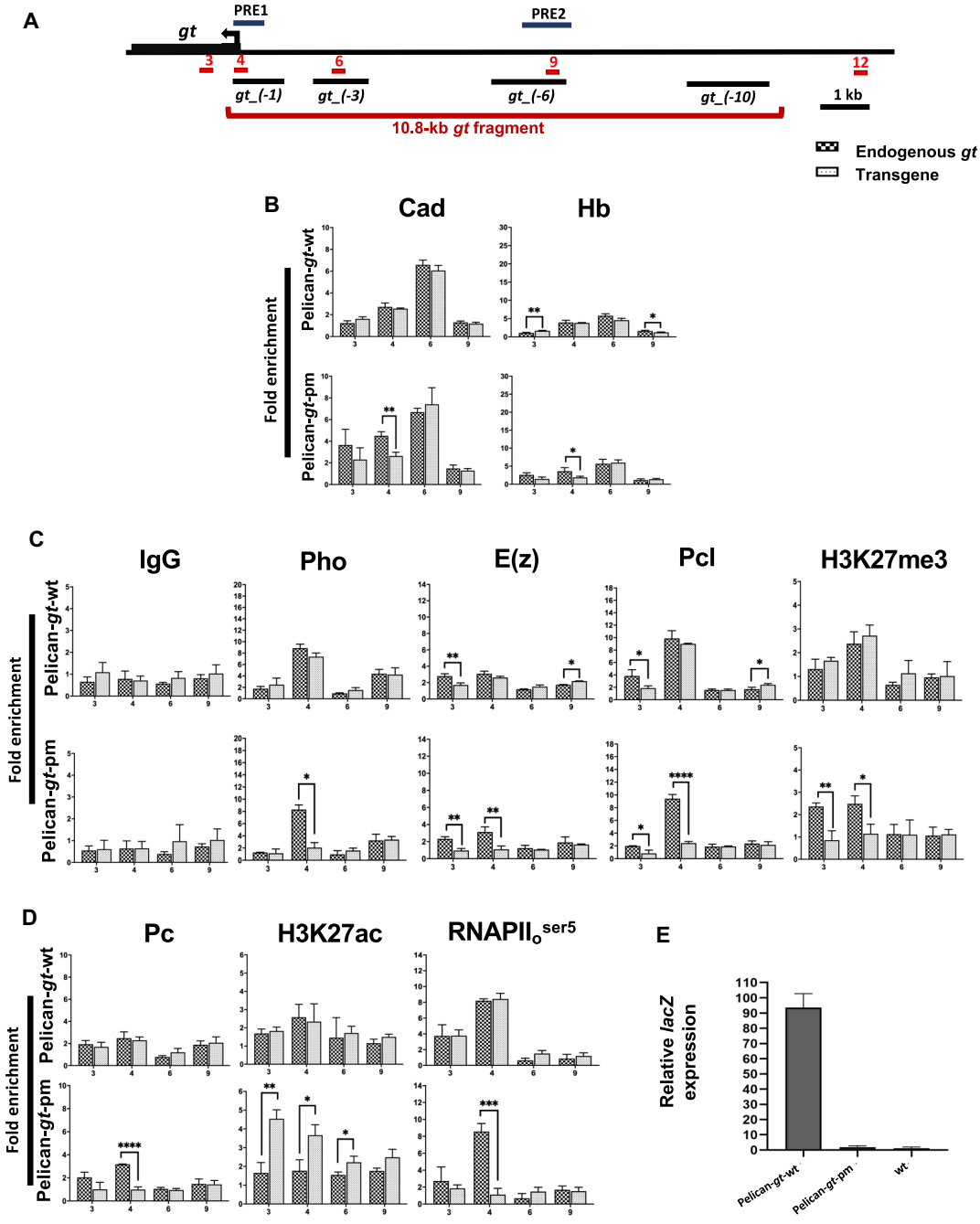

Fig. 3. PcG recruitment is not determined by the transcriptional state of a *gt* transgene. (**A**) Map of *gt* genomic region as in Fig. 1. The location of the genomic fragment in Pelican constructs is below. (**B** to **D**) ChIP-qPCR of (B) nc13 and (C and D) nc14b embryos from Hb-KD-*bcd osk tsl* females crossed to males that contained the transgene indicated on the left. Antibodies indicated above. ChIP signals from three biological replicates are presented as in Fig. 2. (**E**) qRT-PCR showing *lacZ* levels in nc13–nc14a Pelican-*gt*-pm, Pelican-*gt*-wt, and wild-type (wt; no transgene) embryos. *Y*-axis, qPCR signals relative to Pelican-*gt*-wt. Signals from three biological replicates are presented. **P* < 0.05; ***P* < 0.01; ****P* < 0.001; *****P* < 0.0001.

that Pho signals at PRE1 of Pelican-*gt*-pm were reduced in comparison to endogenous *gt* and Pelican-*gt*-wt. We have previously observed a correlation of chromatin accessibility and PhoRC binding with PREs (*21*). It may be that reduced accessibility due to lack of PIC assembly at the mutant promoter impedes PhoRC binding.

Consistent with dependence on PhoRC for recruitment of other PcG complexes to PRE1 (*31*), E(z), Pcl, H3K27me3, and Pc signals at region 4 also were reduced relative to endogenous *gt* (Fig. 3, C and D). For the question we are addressing, their signals at PRE2 closely matched those from active endogenous *gt* and do not resemble the elevated levels detected at PRE2 when *gt* is repressed (Figs. 2 and 3, C and D).

H3K27ac signals at Pelican-*gt*-pm also more closely resembled those at active endogenous *gt* and at Pelican-*gt*-wt, except that H3K27ac

was even more elevated at the promoter/PRE1, enhancer, and downstream of the TSS (region 3) of Pelican-*gt*-pm (Fig. 3E). We speculate that this may be due, in part, to the lack of histone H3 turnover in the absence of transcription. Consistent with the expected effects of the promoter mutations on TFIID binding and lack of transgene transcription, $RNAPII_o^{ser5}$ signals were negative at the promoter of Pelican-*gt*-pm (Fig. 3E).

We conclude that, despite its lack of transcription, Pelican-*gt*-pm is recognized as active by PcG proteins under conditions in which the endogenous *gt* gene is transcriptionally active. This observation is counter to a model for PcG recruitment in which lack of local RNA synthesis identifies PcG targets as repressed.

DISCUSSION

We previously have described de novo recruitment of PcG proteins and deposition of H3K27me3 at *gt* in embryos in which *gt* is ubiquitously repressed (*21*). Recruitment culminates by the second half of nc14 (nc14b) with high levels of PRC2-dependent H3K27me3 and the stable presence of PRC1 with peaks in the vicinities of PRE1 and PRE2. Here, we show that embryos in which the *gt* repressor, Hb, was depleted while the levels of the activator, Cad, were unchanged displayed ubiquitous transcription of *gt* in all nuclei.

Comparison of the recruitment of PcG proteins at transcriptionally active *gt* to their recruitment when the gene is repressed revealed that the transcriptional state of *gt* does not affect PRE1 binding by Pho despite the close proximity of the Pho binding sites to the *gt* promoter (at −73 and −304 from the TSS) (*31*). Rather, the difference is downstream of PhoRC binding. On the basis of the low E(z) and Pc signals at PRE1, it appears that some feature of active *gt* is incompatible with stable binding of PRC2, which likely then prevents stable recruitment of PRC1. The effect of Hb depletion and transcriptional activation on PcG recruitment to PRE2, which is independent of recruitment to PRE1 (*31*), is more consistent. Signals in this region for all tested PcG proteins were reduced by ~50%.

It has been proposed that RNA synthesis from an active gene may compete with chromatin for PRC2 binding, thus reducing local deposition of H3K27me3 and recruitment of canonical PRC1 (*18*, *19*). However, in embryos in which endogenous *gt* was transcriptionally active, PcG ChIP signals at the transcriptionally inert Pelican-*gt*-pm transgene more closely resembled their signals at active *gt* than to repressed *gt*. This was most clearly demonstrated by reduced PcG signals at PRE2. These results indicated that the presence or absence of transcription factors, or their effects on the local chromatin environment, and not production of RNA, is used by the PcG to distinguish between the active and repressed states of *gt*.

Direct recruitment of PRC1 by a repressor has been described (*16*). Therefore, it was feasible that Hb could play such a role at *gt*. However, by simultaneously depleting both Hb and Cad, we were able to rule out this possibility. In the virtual absence of both Hb and Cad, ChIP signals for all tested PcG proteins rose significantly at PRE1 and either did not decrease (Pho and Pcl) or increased [E(z) and Pc] at PRE2 and neighboring regions. This clearly demonstrated that Hb is not needed for PcG recruitment. Furthermore, *gt* was transcribed at low but detectable levels in these embryos. Significantly elevated PcG recruitment despite this transcription reinforces the conclusion that RNA synthesis from *gt* does not inhibit PRC2-dependent deposition of H3K27me3 and recruitment of other PcG proteins.

On the basis of these observations, we propose a model in which an activator, such as Cad, inhibits stable PRC2 recruitment, thereby reducing deposition of H3K27me3 and recruitment of PRC1 (Fig. 4). When both a repressor, for example, Hb, and Cad are present, Hb is able to partially inhibit the inhibitory effect of Cad, resulting in sufficient recruitment of PRC2 and PRC1 to maintain *gt* repression. In the absence of Cad, Hb is not needed for PcG recruitment. Its presence seems to impede Pho binding and subsequent recruitment of PRC2 and PRC1. We can only speculate as to why this is, but it may be that Hb recruits the NuRD corepressor complex to *gt*. Hb has been shown to directly interact with dMi-2, a component of the *Drosophila* NuRD complex, and dMi-2 contributes to PcG repression of Hox genes (*36*). The chromatin remodeling activity of NuRD complexes reduces access of activators to DNA target sites (*37*), which may similarly reduce accessibility of Pho binding sites within PRE1. We previously have described a correlation of chromatin accessibility and PRE binding by PhoRC (*21*).

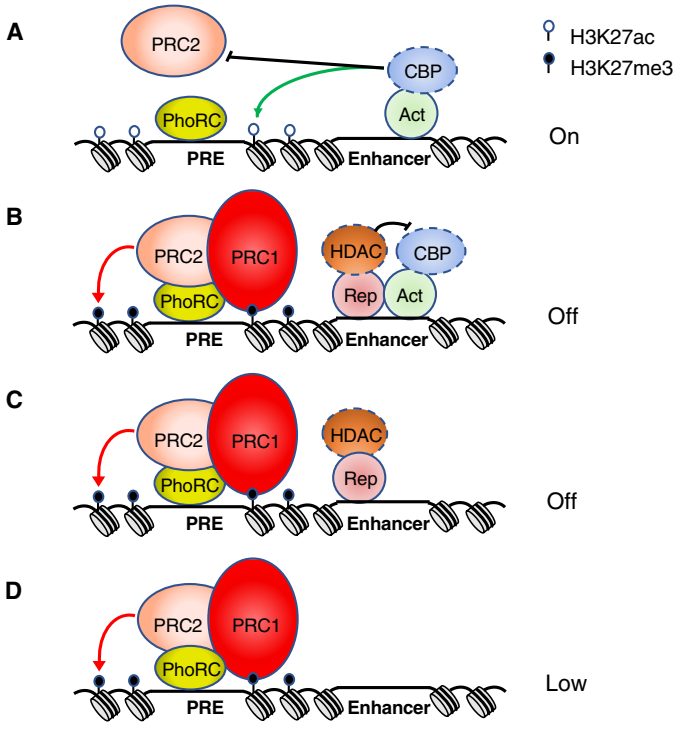

Fig. 4. Model for regulation of PcG recruitment. (**A**) In the presence of an activator (Act) (e.g., Cad) and absence of a repressor (Rep) (e.g., Hb), PhoRC binding is permitted, but stable PRC2 recruitment is inhibited. This may be due to recruitment of CBP, which acetylates H3K27, inhibiting H3K27me3 deposition and stable recruitment of PRC2 and PRC1. CBP may be directly recruited by the activator as a coactivator or as a component of an independent complex. (**B**) When both repressor and activator are present, the repressor sufficiently inhibits the inhibitory effect of the activator, permitting PRC2-dependent H3K27me3 deposition and stable recruitment of PRC2 and PRC1. This may be due to the deacetylase activity of an HDAC recruited by the repressor. (**C**) When the repressor is present, but the activator is absent, recruitment is similar to when the activator is also present. (**D**) In the absence of both activator and repressor, the default state is a stable recruitment of PcG complexes. The observed *gt* expression state in the presence or absence of an activator and/or repressor is indicated on the right. Solid outlines indicate the presence of proteins or complexes based on our observations. Dashed outlines indicate the presence of proteins based on previous reports.

It is likely that this antagonistic interaction between an activator and a repressor may be mediated by histone modifications. We observed a clear inverse correlation of H3K27ac signals and recruitment of PRC2 and PRC1. Consistent with a proposed inhibitory effect on recruitment of PRC2 and PRC1, acetylation of H3K27, which directly prevents H3K27 methylation, is not needed for transcriptional activation but rather to antagonize PcG repression in vivo (*38*, *39*).

Creb-binding protein (CBP) is a histone acetyl transferase (HAT) that is responsible for acetylating H3K27 in *Drosophila* (*40*). CBP is a coactivator of multiple transcription factors, including Cad, and also localizes to PREs, including *gt* PRE1 and PRE2 in wild-type embryos (*41*, *42*). In addition, it is a component of a Trithorax-group (TrxG) complex, TAC1, which also includes Trithorax (Trx) and dSbf1, that was isolated from *Drosophila* embryos (*43*). It is not known whether the presence of CBP at PREs is due to its recruitment as a coactivator of one or more transcription factors (e.g., Cad), which bind to enhancers in the vicinity of PREs, or as a component of TAC1 or similar TrxG complex. Consistent with the model represented in Fig. 4, CBP overexpression antagonizes PcG-mediated silencing (*40*). For the sake of simplifying the illustration of this model, we represent CBP as a Cad coactivator, but it should be emphasized that other means of recruitment are equally possible. H3K27me3 both facilitates recruitment of canonical PRC1 and stabilizes the presence of PRC2 due to H3K27me3 binding by its ESC subunit. Therefore, the presence of H3K27ac is predicted to inhibit stable binding by PRC2, consistent with our observations.

When both Hb and Cad are present, we propose that H3K27ac levels are sufficiently reduced to permit H3K27me3 deposition by PRC2. This is likely due to a corepressor with histone deacetylase activity that is recruited by Hb. In addition to chromatin remodeling activity, NuRD also includes the histone deacetylases HDAC1 or HDAC2 and has been shown to facilitate PRC2 binding to target loci in mouse embryonic stem cells (*44*). *Drosophila* HDAC1 has been shown to deacetylate H3K27 and function antagonistically to CBP (*40*).

In summary, our findings support a model in which PcG recruitment is the default state but which may be inhibited by the direct or indirect effects of an activator. The presence of a repressor is only required to counter the inhibitory effects of an activator. It is likely that this antagonistic interaction largely plays out through modulation of H3K27 acetylation, but further studies will be required to confirm the identities of the specific HAT and HDAC regulating H3K27ac at *gt*. Although this model may not apply to all PcG targets, H3K27 acetylation could play a central role in regulating recruitment at genomic sites where PRC2 deposition of H3K27me3 facilitates stable recruitment of PRC1.

MATERIALS AND METHODS

Construction of plasmids

The Pelican P element reporter vector (*45*) was modified for ϕC31 transgenesis and converted into a Gateway destination vector as previously described (*31*). Construction of Pelican-*gt*-wt, which contains a *gt* fragment from +360 to −10,421 from the *gt* TSS, was previously described (*31*). To generate a transcriptionally inert *gt* transgene, the TATA box, Inr, and DPE of this *gt* fragment within the pENTR1A vector were consecutively mutated using the Phusion II Site-Directed Mutagenesis Kit (Thermo Fisher Scientific), and primers are listed in table S1. The entire insert was sequenced to confirm that no unintended mutations were introduced, and the fragment was then recombined into the Pelican vector.

Drosophila stocks

The Pelican-*gt*-promoter mutant (Pelican-*gt*-pm) plasmid was injected into embryos (BestGene Inc.) and integrated into the attP40 landing site on the second chromosome. All fly stocks were obtained from the Bloomington *Drosophila* Stock Center: *maternal-tubulin-Gal4* driver (*mat-tub-Gal4;* no. 7062), *UAS-shRNA-hb* (no. 54478), and *UAS-shRNA-cad* (no. 57546). *bcd osk tsl* stocks were generated as previously described (*21*).

Immunostaining of embryos

Embryos were fixed and processed as previously described (*33*). Primary antibodies were used at the following dilutions: rabbit anti-Hb, 1:250; rabbit anti-Cad, 1:400 (*21*); guinea pig anti-Gt, 1:500 (*46*); and rabbit anti–β-galactosidase, 1:1500 (Cappel). Biotin-SP–conjugated goat anti-rabbit secondary antibodies (Jackson Immunoresearch) were diluted 1:10,000. Streptavidin–horseradish peroxidase (Jackson Immunoresearch) was diluted 1:5000. Images were obtained using a Zeiss Axiovert 200M microscope.

In situ hybridization of embryos

In situ hybridization of embryos was performed as previously described (*31*). Images were obtained using a Zeiss Axiovert 200M microscope.

Embryo processing and ChIP

Embryos were collected for 30 min and then aged at 25°C for 110 or 170 min to target nc13 and nc14b developmental stages. Embryos were then fixed and manually sorted as previously described (*21*). The following volumes of antibodies were used in ChIP experiments: Pc, 10 μl (*47*); Pho, 5 μl (*48*); E(z), 10 μl (*49*); Pcl, 10 μl (*50*); Hb, 5 μl; Cad, 5 μl (*21*); H3K27me3, 0.2 μl (Millipore #07-449); H3, 0.5 μl (Abcam #ab1791); mock, 0.5 μl (immunoglobulin G, Cell Signaling #2729); H3K27ac, 0.2 μl (Abcam #ab4729); and P-Rpb1 CTD (S5), 2 μl (Cell Signaling #D9N51). To approximately equalize the chromatin amount per ChIP using embryos at different developmental stages, we used a 2:1 ratio of nc13:nc14b as determined by total mass. Carefully staged embryos were then subjected to ChIP assays, and the enrichment values were calculated as previously described (*21*). Quanta Biosciences Perfecta SYBR green supermix was used for qPCR. All amplifications were performed in triplicate using Rotor Gene RG3000 thermocycler (Corbett Research). Sequences of PCR primers are listed in table S2.

Quantitative reverse transcription polymerase chain reaction

Embryos were collected for 1 hour and then aged at 25°C for 110 min to reach nc13 to nc14a. Embryos were then dechorinated and manually sorted. Total RNA was isolated, and complementary DNA was synthesized and analyzed by qPCR using gene-specific primers (table S3) as previously described (*31*).

Statistical analysis

For gene expression analysis, qRT-PCRs were performed in triplicate. To calculate the triplicate mean values, ΔΔCT quantification method with *rp49* transcript as normalization reference was used. All ChIP-qPCRs were run in triplicate. Rotor Gene 5 software was

used to determine Ct values, and sample concentrations were calculated using the ΔΔCT method (*51*) and normalized to an internal negative control region, *Pka-C1*, with the exception of anti-H3K27ac and anti-$RNAP_o^{Ser5}$, which were normalized to the *gt* 12 region. *Pka-C1* is in an H3K27me3-negative region in 0- to 4-hour embryos. The *gt* 12 region is located 12,077 base pairs (bp) upstream of the *gt* TSS and 1151 bp downstream of the 3′ end of the nearest flanking gene, *tko*, in a region that is not transcribed in 0- to 4-hour embryos and is not positive for H3K27ac (*52*). The *P* values for all statistical analyses were calculated using unpaired two-tailed Student's *t* tests. All graphs were plotted using GraphPad Prism 7.

SUPPLEMENTARY MATERIALS

Supplementary material for this article is available at http://advances.sciencemag.org/cgi/content/full/7/29/eabg1556/DC1

View/request a protocol for this paper from *Bio-protocol*.

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