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Non-genic transcription at the *Drosophila* bithorax complex – functional activity of the dark matter of the genome

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ABSTRACT *Drosophila melanogaster* is a powerful model system for the study of gene regulation due to its short generation time, high fertility and the availability of various genetic tools to manipulate the genome. Investigation into the regulation of homeotic genes and their role in embryonic patterning during development was pioneered in *Drosophila*. Recently, the molecular mechanisms responsible for regulating gene expression in the bithorax complex have been the focus of active study. Many of these studies have pointed to the importance of *cis*-regulatory modules, genetic sequences that direct the temporal and spatial patterns of gene expression over large genomic distances. Additional components of the regulatory code have emerged beyond the primary DNA sequence. In particular, non-genic transcription is an important mechanism for controlling gene expression either through direct transcriptional mechanisms that mediate dynamic epigenetic control of the chromatin environment or through functional activity of the RNA products.

KEY WORDS: *cis-regulation, RNA, transcription, chromatin, bithorax*

Overview of gene regulation in the bithorax complex

Homeotic (Hox) genes are a major player in determining the pattern of development in metazoan organisms. Hox genes encode transcription factors that modulate the expression of downstream genes in order to form specific developmental structures along the main anterior-posterior body axis of the developing embryo (Lemons and McGinnis, 2006). In *Drosophila*, segment identity along the anterior-posterior axis of the embryo is regulated by two Hox gene complexes: the Antennapedia complex (ANT-C), which determines the identity of the anterior segments, and the bithorax complex (BX-C), which determines the identity of the posterior thoracic and abdominal segments (Karch *et al*., 1985, Lewis, 1978, Lewis *et al*., 2003).

The BX-C has been studied extensively for over fifty years and details from this research have contributed immensely to our general understanding of gene regulation during embryonic development. The *Drosophila* BX-C is 330 kb in length, yet contains only three protein-coding Hox genes: *Ultrabithorax* (*Ubx*), *abdominal-A* (*abd-A*) and *Abdominal-B* (*Abd-B*) (Fig. 1) (Martin *et al*., 1995, Sanchez-Herrero *et al*., 1985). The BX-C contains an extensive array of *cis*-regulatory modules (CRMs) that control the function of these three genes (Fig. 1) (Akbari *et al*., 2006, Maeda and Karch, 2006). The CRMs include functionally diverse elements such as enhancers, insulators, anti-insulators and silencers that act synergistically to regulate the expression of the neighboring Hox genes (Akbari *et al*., 2006, Maeda and Karch, 2006). The functional activity of these CRMs in the intergenic *infraabdominal* (*iab)*, *abx/bx* and *bxd/pbx* regulatory regions are required to determine the specific identities of thoracic and abdominal segments in the embryo (Akbari *et al*., 2006, Maeda and Karch, 2006). Each *iab* region is thought to contain at least one IAB enhancer (Fig. 1), capable of upregulating gene expression by recruiting transcription factors that interact with the basal transcription machinery at the Hox target promoters (Busturia and Bienz, 1993, Muller and Bienz, 1992, Qian *et al*., 1991). These promoter-enhancer interactions result in characteristic spatial domains of Hox gene expression along the anterior-posterior axis of the embryo (Akbari *et al*., 2006, Maeda and Karch, 2006).

In concert with the other CRMs from the *iab* regions, the IAB enhancers direct distinct temporal and spatial patterns of Hox gene expression in the developing embryo. Mutations in the CRMs can cause homeotic transformations, which are pheno-

Abbreviations used in this paper: Abd, abdominal; BX-C, bithorax complex; CRM, cis-regulatory module; PcG, polycomb group protein; PRE, polycomb response element.

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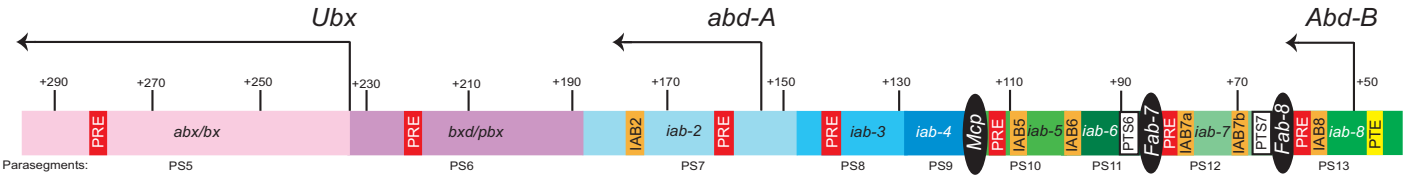

Fig. 1. Molecular organization of the bithorax complex (BX-C). *The* Ubx, abd-A *and* Abd-B *transcripts are indicated by leftward arrows. The* abx/bx, bxd/pbx *and* iab *(2-8) regulatory regions are indicated. Each region is thought to contain at least one characterized enhancer (orange rectangles). The* cis*-regulatory interaction between each region and their target promoters are specified by color.* abx/bx *and* bxd/pbx *interact with* Ubx *(violet).* iab-2, iab-3, *and* iab-4 *interact with* abd-A *(blue).* iab-5, iab-6, iab-7, *and* iab-8 *interact with* Abd-B *(green). The positions of the characterized Fab-7, Fab-8, and Mcp insulators (black ellipses), promoter targeting sequence (PTS) modules (white rectangles), promoter tethering element (PTE) (yellow rectangle) and polycomb response elements (PREs) (red rectangles) are indicated. Numbers above line refer to kilobase positions in DNA sequence accession number: DM31961.*

typic transformations of one or more segments to the identity of a neighboring segment (Karch *et al.*, 1985, Lewis, 1954, Lewis, 1978). The differential expression patterns of Hox genes established by the CRMs are maintained into larval stages of development by the polycomb group (PcG) and trithorax group (TrxG) proteins (Beuchle *et al.*, 2001, Busturia and Bienz, 1993, Busturia and Morata, 1988, Muller and Bienz, 1991, Pirrotta, 1995, Simon *et al.*, 1990). The PcG and TrxG proteins bind to maintenance elements (MEs), polycomb response elements (PREs) and trithorax response elements (TREs) in the BX-C and are thought to repress or activate *iab* regions by modifying the structure of the local chromatin environment (Klymenko and Muller, 2004, Ringrose and Paro, 2007).

Recently, the process of non-genic transcription and the associated non-coding RNA (ncRNA) products have emerged as important players in the activation and maintenance of gene expression in the BX-C. Transcription through the intergenic *iab* regions is extensive and temporally precedes the activation of the Hox genes during development (Bae *et al.*, 2002, Bender and Fitzgerald, 2002, Drewell *et al.*, 2002). Here we review recent studies that have demonstrated that the ncRNA products resulting from *iab* transcription may fine tune the expression patterns of the Hox genes. Later in development, non-genic transcription is also thought to play a role in the regulation of PcG and TrxG protein binding to PREs and TREs and in the nuclear localization of these DNA-protein complexes to maintain Hox gene expression patterns throughout development. The complex molecular roles emerging for this non-protein coding transcription suggest that non-genic sequences may provide a surprisingly rich level of functional diversity in the dark matter of the genome.

Transcription is required to establish functional activation of *cis*-regulatory regions

The earliest studies of transcription in the BX-C discovered extensive transcription through the intergenic regions during early development (Hogness *et al.*, 1985, Lipshitz *et al.*, 1987, Sanchez-Herrero and Akam, 1989). Transcription across the intergenic regions in the BX-C is spatially restricted along the anterior-posterior axis of the embryo and is temporally initiated prior to the activation of the Hox genes (Bae *et al.*, 2002, Sanchez-Herrero and Akam, 1989). Therefore, it is thought that the generation of transcripts across these chromosomal regions may correspond with the sequential opening of the chromatin in each of the *iab* regions in order to enable initiation of Hox gene expression (see Fig. 2) (Cumberledge *et al.*, 1990, Lipshitz *et al.*, 1987, Sanchez-Herrero and Akam, 1989), for detailed reviews see (Akbari *et al.*, 2006, Lempradl and Ringrose, 2008). Our own studies using high resolution *in situ* hybridization (ISH) of intergenic transcription in the BX-C have contributed to the understanding that non-genic transcription may define the embryonic domains of activity for the *iab* regions and the CRMs contained within these regions (Bae *et al.*, 2002, Drewell *et al.*, 2002). Intergenic transcription early in embryonic development is observed not merely over identified CRMs but over entire *iab* regions (Bae *et al.*, 2002). Based on these observations we proposed that transcription is necessary for the functional activity of the *cis*-regulatory genomic regions in the BX-C and subsequent initiation of Hox gene expression (Fig. 2) (Akbari *et al.*, 2006). Intergenic transcription is thought to activate the expression of the Hox genes through the sequential model. In this model, the production of non-genic transcripts through the *iab* regions opens up the chromatin environment, subsequently allowing proteins to bind their recognition sites in the BX-C (Fig. 2B) (Cook, 2003). Thus, transcription factors may bind to sites in enhancers, such as those in IAB5 (Fig. 2B) and IAB2 (Casares and Sanchez-Herrero, 1995, Shimell *et al.*, 1994) (see (Maeda and Karch, 2006) for a review). The transcription factors bound to the enhancers may then interact with the *Abd-B* promoter to initiate active transcription of the *Abd-B* gene (Fig. 2D).

The enigmatic role of transcriptional interference

After initiation of Hox gene expression in the BX-C, non-genic transcription continues to be important for the dynamic regulation of Hox genes. One mechanism of control is transcriptional interference (TI), originally termed “promoter occlusion”, wherein the process of transcription of one regulatory sequence prevents subsequent transcriptional initiation, elongation or premature termination of a nearby target gene and therefore results in the repression of that target gene (Adyha and Gottesman, 1982, Mazo *et al.*, 2007).

At the BX-C, the *bxd* genomic region lies between the *Ubx* and *abd-A* transcriptional units (see Fig. 1), and contains a maintenance element (ME) which regulates *Ubx* (Chan *et al.*, 1994, Cumberledge *et al.*, 1990, Lipshitz *et al.*, 1987, Muller and Bienz, 1991, Tillib *et al.*, 1999). Several ncRNAs have been shown to be transcribed through the *bxd* ME (Cumberledge *et al.*, 1990,

Sanchez-Herrero and Akam, 1989). Evidence for sense-TI at this region comes from deletions in the promoters of the *bxd* ncRNAs which lead to ectopic expression of *Ubx* in the embryonic domains where the ncRNAs are normally expressed (Petruk *et al.*, 2006). Because injection of dsRNA to ablate *bxd* ncRNAs does not affect the *Ubx* expression pattern (Petruk *et al.*, 2006), the process of non-genic transcription itself is thought to have greater functional importance than the RNA products. However, the endogenous ncRNAs remain localized in the nucleus, which may potentially prevent the dsRNA from modulating the stability of the ncRNAs in this experiment. Previous studies reported that transcription of *bxd* ncRNAs upregulates expression of *Ubx* in wing imaginal discs and that *bxd* transcripts are capable of recruiting Ash1, an epigenetic activator capable of methylating lysine residues in histones (Sanchez-Elsner *et al.*, 2006). The fact that Ash1 and Ash1-directed histone methylation patterns were observed at the *bxd* MEs after *bxd* transcription, and that Ash1 was shown to be capable of binding *bxd* RNA transcripts, suggest that the *bxd* transcripts may in fact play important functional roles (Sanchez-Elsner *et al.*, 2006). The apparent discrepancies in the regulatory activities of the *bxd* ncRNAs discovered in these studies are discussed in detail in a recent review (Lempradl and Ringrose, 2008). The authors of the review note that it is important to make close observations of developmental timing in studies on these ncRNAs (Lempradl and Ringrose, 2008). Petruk *et al.* note that the silencing of *Ubx* by *bxd* ncRNAs is a transient phenomenon, perhaps only functioning during early development, as in the case of the proposed silencing of *abd-A* by *iab* ncRNAs (Petruk *et al.*, 2006).

In vitro studies by Callen *et al.* find that transcription through the *bxd* regulatory sequence does not directly prevent RNA polymerase II (Pol II) from binding to the promoter of the target gene, *Ubx*, but reduces the ability of the open form of Pol II to clear the target promoter (Callen *et al.*, 2004). Nonetheless, the precise mechanism for TI at the *Ubx* gene remains elusive. Does the presence of Pol II at the *bxd* regulatory region prevent recruitment of the transcription initiation complex to the *Ubx* target promoter? Alternatively, Pol II recruitment at the *bxd* regulatory region may prevent transcriptional elongation by dislodging the elongation complex at the target *Ubx* promoter (Mazo *et al.*, 2007).

Communication between *cis*-regulatory modules facilitates higher order chromatin structures in the bithorax complex

The first documented example of a dynamic, long-distance physical interaction between distal regulatory modules *in vivo* was provided by studies of the BX-C (Cleard *et al.*, 2006). Dam methylase identification was used to detect physical interactions between the Fab-7 insulator and *Abd-B* promoter in adult head tissues, where *Abd-B* is not expressed (Celniker and Drewell, 2007, Cleard *et al.*, 2006). Recently, we have identified further interactions between the *Abd-B* promoter and other CRMs in the BX-C. A 255 bp promoter tethering element (PTE) located 40 bp upstream of the *Abd-B* transcription start site specifically directs interactions between the IAB5 enhancer and the *Abd-B* promoter

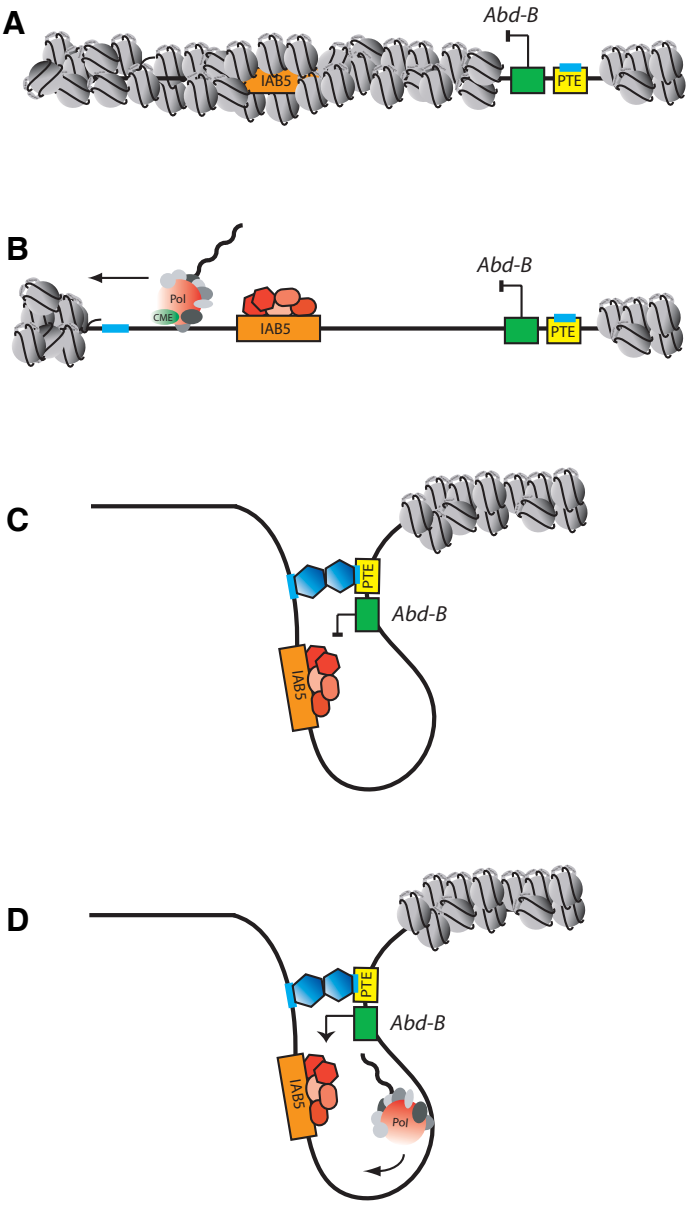

Fig. 2. Model for activation of *Abd-B* gene expression by sequential transcription and promoter-enhancer tethering. *A model for events occurring in the presumptive 5th abdominal segment of the developing* Drosophila *embryo, where the promoter tethering element (PTE) facilitates interactions between the IAB5 enhancer and the* Abd-B *promoter:* **(A)** *Prior to the activation of the Hox genes, the regulatory sequences 3′ of the* Abd-B *gene are in an inactive chromatin configuration.* **(B)** *Intergenic transcription early in embryonic development by RNA polymerase II (Pol) (red circle) and associated chromatin modifying enzymes (CME) (green circle) facilitates the opening of the chromatin at the regulatory regions in the BX-C, enabling transcription factors (red/peach polygons) to bind to enhancer modules, such as IAB5 (orange box).* **(C)** *Homo-dimeric interactions between a protein factor (blue hexagon) bound at conserved sequences (blue line) within the PTE (yellow box) and a region close to IAB5 facilitate promoter-enhancer interactions. The chromatin loop configuration formed by these specific interactions brings the IAB5 enhancer and associated transcription factors to the* Abd-B *promoter (green box).* **(D)** *The localized high concentration of transcription factors in the chromatin loop upregulates expression of* Abd-B *by interacting with the basal transcriptional machinery, including RNA polymerase II.*

in transgenic assays (Akbari *et al.*, 2008). Furthermore, these assays demonstrate that the PTE is capable of allowing enhancers to bypass intervening insulators to activate a distal promoter sequence (Akbari *et al.*, 2007). To account for this molecular activity we proposed that the PTE facilitates a chromatin loop in the BX-C which allows a stable interaction between the *Abd-B* promoter and enhancers from the 3' *iab* regulatory regions (Fig. 2C). This molecular bridge may be mediated by homo-dimerizing proteins that bind to conserved hexamer motifs within the PTE and close to the IAB enhancers in the BX-C (Fig. 2D) (Akbari *et al.*, 2007).

Recent *in vivo* analysis of the three-dimensional structure of chromatin in the *Drosophila* BX-C suggests several physical interactions between non-homologous CRMs that may mediate activated or repressed transcriptional states (Lanzuolo *et al.*, 2007). Chromatin Conformation Capture (3C) enables the chromatin arrangement of the BX-C to be deduced by quantitative PCR (qPCR) analysis of the frequency of crosslinking events. 3C on chromatin prepared from 5-8 hour embryos shows enriched physical interactions between the *abd-A* promoter and (1) all major genetic sequences that are known to bind PcG proteins in the BX-C (e.g. PREs and insulators), (2) the *Ubx* and *Abd-B* promoters, and (3) the 3' end of all the Hox genes in the BX-C (see Fig. 1) (Lanzuolo *et al.*, 2007). However, the *in vivo* function of these associations remains unclear, as these studies do not make it possible to distinguish between distinct spatial regulatory domains in the embryo which may possess different chromatin arrangements that may be dynamic in nature.

Visualization of the nuclear position of differentially transcribed BX-C loci relative to Polycomb bodies has recently become possible through the application of fluorescence *in situ* hybridization (FISH) and FISH-Immunostaining (FISH-I) technology. FISH and FISH-I on 5-8 hour embryos show that known PREs in the *bxd* and Fab-7 regions (see Fig. 1) co-localize with a large Polycomb body in 80% of nuclei from the anterior region of the embryo (Lanzuolo *et al.*, 2007). These nuclei are located in embryonic domains where the BX-C Hox genes are repressed. Conversely, in 80% of posterior nuclei, where the Hox genes are transcriptionally active, *bxd* co-localizes with a Polycomb body, but Fab-7 is mostly found far from this domain (Lanzuolo *et al.*, 2007). Because only 2D FISH was performed on the embryos, the co-localization data must be carefully interpreted. However, these data suggest that non-homologous CRMs may physically interact and associate with Polycomb bodies in order to facilitate alternative higher-order chromatin structures that result in transcriptionally repressed or active states. In light of these data, the formation of a chromatin environment that contains subnuclear silencing compartments may be a major mechanism through which regulation of Hox gene expression by CRMs occurs (Lanzuolo *et al.*, 2007).

In some strains of *Drosophila* S2 larval cells, *Ubx, abd-A,* and *Abd-B* are transcriptionally repressed, representing complete silencing of the BX-C complex (Breiling *et al.*, 2004). 3C on chromatin prepared from these S2 cells show that the *Ubx, abd-A,* and *Abd-B* promoters strongly interact with PREs. In contrast, in *Drosophila* S3 cells, *Abd-B* is transcribed, while *abd-A* and *Ubx* remain silenced (Breiling *et al.*, 2004). 3C on chromatin prepared from S3 cells shows a loss of Fab-7 interactions with most regions of the BX-C, including the Abd-Bγ and B/C promoters and maintenance of interactions with the less active *Abd-B* promoter A (Lanzuolo *et al.*, 2007). These data agree with the results from the Dam methylation reporter analysis in the BX-C (Cleard *et al.*, 2006), where lower levels of Dam methylation were found in the *Abd-B* promoter region of abdominal tissues where *Abd-B* is transcriptionally active, as compared to tissues of the adult head region where it is not transcribed (Celniker and Drewell, 2007).

Addition of double-stranded RNA (dsRNA) against *Polycomb* (*Pc*) results in loss of binding of PC bodies at PREs and subsequent derepression of the Hox genes in S2 cells (Lanzuolo *et al.*, 2007). 3C analysis after dsRNA knockdown of PC also confirms impairment of physical associations between distal PREs and the active *Abd-B*, *abd-A* and *Ubx* promoters (Lanzuolo *et al.*, 2007). These results suggest that disrupting interactions between these distal CRMs causes the derepression of the Hox genes. In addition, the dsRNA knockdown of PC results in weak transcriptional activity at the PREs (Lanzuolo *et al.*, 2007). The functional activity of these ectopic PRE transcripts is currently unclear. However, one possibility is that weak non-genic transcription through PREs may simply be a consequence of the impairment of physical associations between distal PREs and the promoters of Hox genes.

S2 or S3 cells, derived from unknown tissues in larval stages of development, may not have comparable three-dimensional chromatin structure to adult tissues. This limitation undermines the use of cell-based assays for the study of the chromatin environment and the physical interactions between CRMs at the BX-C during development. In addition, these studies highlight the overall difficulty in interpreting both 3C and 2D FISH experiments, in the one case due to the high frequency of random crosslinking in an uncondensed chromatin environment, and in the other due to the complications arising from distinct dynamic spatial regulatory domains within the embryo. In future studies it will be critical to find experimental techniques that enable the discrimination between important functional interactions and transient interactions. Examining these events in the distinct regulatory domains of the embryo will also be very important. Deciphering how the three-dimensional structure of the chromatin environment mediates alternative transcription states through physical associations between CRMs in the BX-C is a technically challenging task that may be best accomplished through the careful application of several parallel techniques. 3D FISH combined with Förster resonance energy transfer (FRET), a physical technique for determining molecular co-localization with high precision, could be utilized to yield a more complete and accurate picture of the protein-DNA interactions that mediate the dynamic three-dimensional structure of chromatin.

Functional role for RNAi pathway components in silencing at the bithorax complex?

Recent investigations have highlighted intriguing cases in which components of the RNAi machinery interact with polycomb group proteins (PcGs) at CRMs in the BX-C (Grimaud *et al.*, 2006). The RNAi pathway, triggered by the presence of double stranded RNA (dsRNA), generates short RNAs that can, through multiple mechanisms, directly repress homologous genes through gene silencing (Hammond, 2005). In the initiation step of the RNAi pathway, the long dsRNA is processed into small interfering

RNAs (siRNAs). In the effector step, the siRNAs are incorporated into the RNA-induced silencing complex (RISC) (Hammond *et al.*, 2000, Zamore *et al.*, 2000). MicroRNAs (miRNAs), stem-loop RNA structures which are formed from naturally occurring RNA transcripts, also extensively use the protein components of the RNAi pathway in order to effect silencing activity (Jackson and Standart, 2007, Kim, 2005). A recent study in the BX-C (Grimaud *et al.*, 2006) focuses on the specific roles of factors previously shown to be involved in these small RNA silencing mechanisms, including Dicer-2 (Dcr-2), an RNase III family member which processes dsRNA into siRNAs (Bernstein *et al.*, 2001, Lee *et al.*, 2004, Liu *et al.*, 2003, Pham *et al.*, 2004, Zamore *et al.*, 2000) and Argonaute1 (AGO1), which has a role in miRNA-mediated gene silencing (for review of argonaute proteins see (Hutvagner and Simard, 2008)). In contrast to the miRNAs, Piwi-associated RNAs (piRNAs), the latest addition to the small RNA world, are repeat-associated siRNAs processed independently of Dicer and Drosha that function to prevent the spread of active transposons in the germline (Aravin *et al.*, 2003, Hartig *et al.*, 2007). A functional role for the protein factors from the piRNA pathway at the BX-C has also been addressed (Grimaud *et al.*, 2006). In particular the requirement for PIWI, an argonaute family protein, which is involved in heterochromatin formation and piRNA processing (Brennecke *et al.*, 2007, Gunawardane *et al.*, 2007, Pal-Bhadra *et al.*, 2004, Saito *et al.*, 2006, Sarot *et al.*, 2004) and Aubergine (Aub), an argonaute family protein which is involved with piRNA processing and maintaining silenced states (Aravin *et al.*, 2004, Brennecke *et al.*, 2007, Carmell *et al.*, 2002, Gunawardane *et al.*, 2007, Harris and MacDonald, 2001, Vagin *et al.*, 2006) was investigated.

In order to understand the role of these RNAi components in maintaining long-range chromatin contacts, a transgenic copy of a 3.6 kb fragment containing the Fab-7 insulator and PRE was introduced on the X chromosome (Fab-X), close to the endogenous *scalloped* (*sd*) gene (Bantignies *et al.*, 2003, Grimaud *et al.*, 2006). Upon integration, the ectopic Fab-X sequence induced PcG-dependent silencing of the neighboring *sd* gene (Bantignies *et al.*, 2003, Grimaud *et al.*, 2006). In embryos carrying mutations affecting *dcr-2*, *ago1*, *piwi* or *aub*, there was no effect on physical interactions between Fab-7 and the Hox genes in the BX-C, or Fab-X and *sd* on the X chromosome (Grimaud *et al.*, 2006). However, in third instar larvae carrying the same RNAi component mutations, there was a loss of physical interactions from both the endogenous and ectopic Fab-7 CRMs. Therefore, it appears that while these RNAi components are not required during early embryonic development for the functional establishment of these interactions, they may possibly play a role in maintaining CRM interactions in later developmental stages (Fig. 3) (Grimaud *et al.*, 2006). These data suggest that the RNAi components may therefore be involved in maintenance of long distance Fab-7 repressive interactions with target sequences within the BX-C, such as the *Abd-B* promoter (Fig. 3D), as identified in parallel studies (Cleard *et al.*, 2006). However, it should be pointed out that none of the RNAi mutations result in segmental transformations which would be indicative of disruption of Hox gene expression (Grimaud *et al.*, 2006).

Investigation of the molecular mechanisms responsible for the long-range CRM interactions at the BX-C has also recently highlighted the role of the polycomb repressive complex 1 (PRC1). The

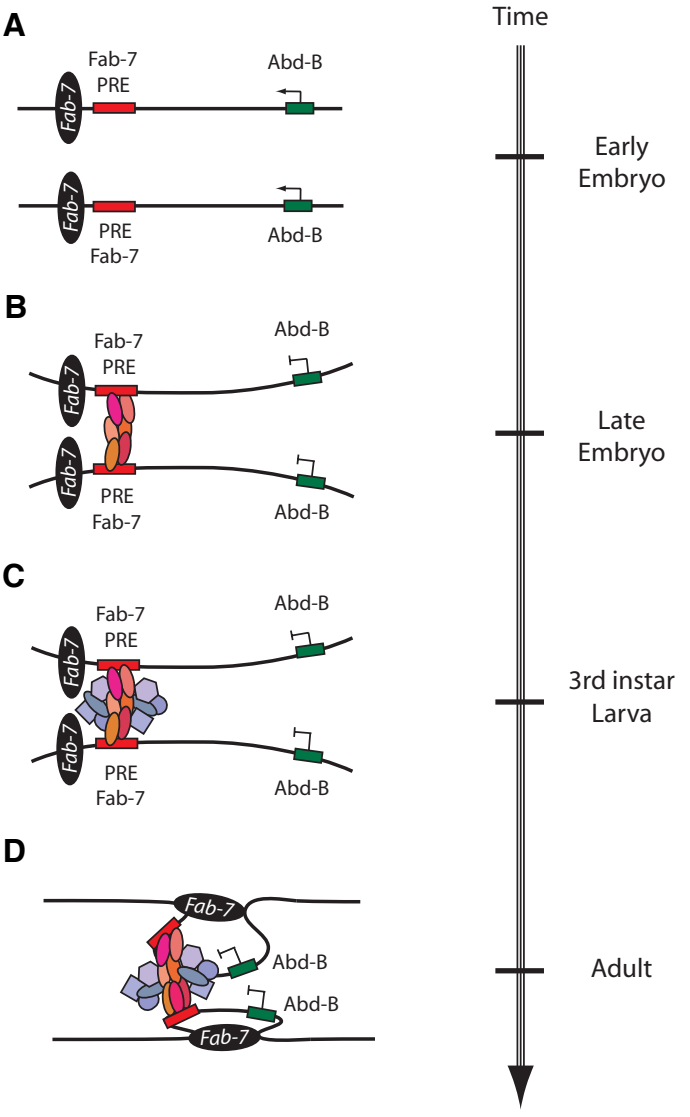

Fig. 3. Fab-7 *cis*-regulatory modules (CRMs) modulate long-distance repressive interactions at the *Abd-B* gene. **(A)** *The 3′ Fab-7 regulatory region that controls* Abd-B *expression is shown in an open chromatin formation early in embryonic development when* Abd-B *(green box) is transcribed. During this embryonic stage no PcG proteins are bound to the PRE (red box) located just upstream of Fab-7 boundary (black ellipse).* **(B)** *In late embryonic development, a repressive complex is formed as PcG proteins (red shaded ellipses) bind to the PRE and silence transcription of* Abd-B *(Zink and Paro, 1995), a process in which the RNAi machinery is not involved (Grimaud* et al., *2006). This repressive complex does not form in tissues in the posterior end of the embryo where* Abd-B *is expressed.* **(C)** *Upon reaching larval developmental stages in RNAi protein mutants, there is a loss of long range chromosome interactions of the Fab-7 PRE element (Grimaud* et al., *2006) This suggests that protein components of the RNAi machinery (blue shaded polygons), which co-localize with the PcG proteins, are necessary to maintain the repressed state of* Abd-B *(Grimaud* et al., *2006, Lei and Corces, 2006).* **(D)** *In adults, as a further step to maintain the repressed state of* Abd-B *in the head region of the fly,* Fab-7 *interacts with a region 1.6 kb 5′ of* Abd-B *which facilitates a chromatin loop configuration that brings* Abd-B *in closer vicinity with the repressive complex containing the PcG and RNAi proteins (Cleard* et al., *2006).*

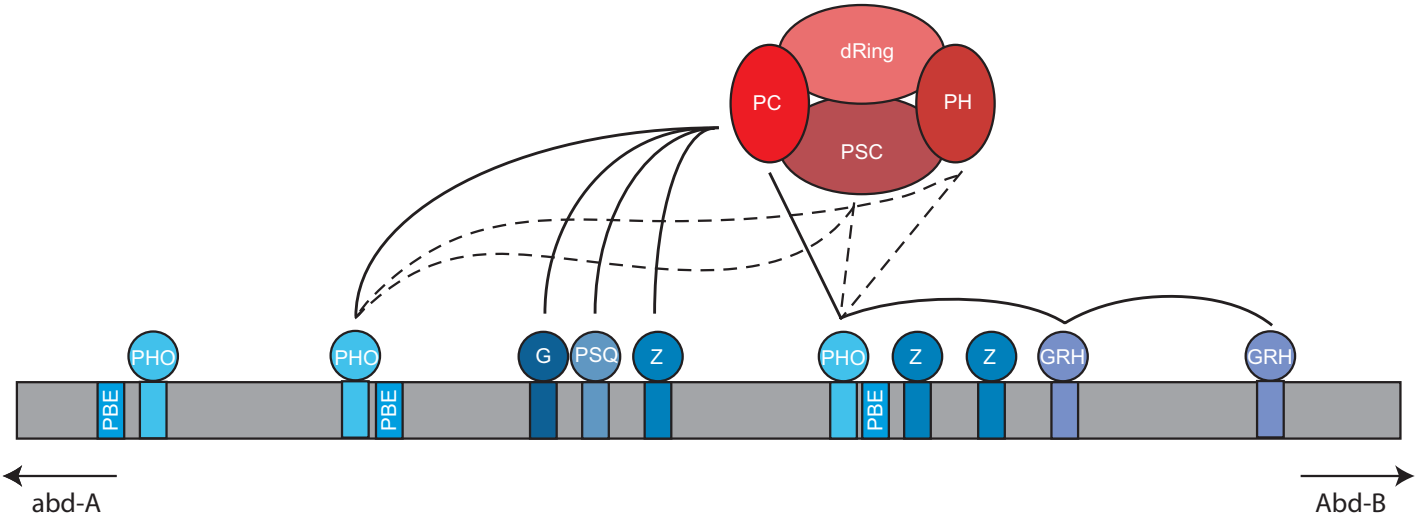

Fig. 4. Polycomb repressive complex 1 (PRC1) recruitment to Fab-7 polycomb response element (PRE). *An approximately 408 bp section from the Fab-7 minimal PRE of the bithorax complex is able to recruit the PRC1 complex (Blastyák* et al.*, 2006). The binding sites (blue boxes) for specific DNA binding factors; Pleiohomeotic (PHO), GAGA factor/Pipsqueak (G/PSQ), Zeste (Z) and Grainyhead (GRH) are spread throughout the Fab-7 DNA element. Components of the PRC1 core complex (PCC); polycomb (PC), polyhomeotic (PH), posterior sex combs (PSC) and dRING are indicated by red ellipses. Solid lines represent characterized interactions and cooperative binding to the DNA sites (see (Bantignies and Cavalli, 2006) for detailed description). Dashed lines represent proposed interactions (see detailed description in text). (Figure adapted from (Bantignies and Cavalli, 2006)).*

PRC1 core complex (PCC) is composed of four components: polycomb (PC), polyhomeotic (PH), posterior sex combs (PSC), and dRing, but PRC1 in its entirety is known to contain additional proteins (Shao *et al.*, 1999). Pleiohomeotic (PHO), a sequence-specific DNA-binding member of the PcG (Brown *et al.*, 1998), is essential for PRC1 to form a complex with target DNA (Brown *et al.*, 1998, Fritsch *et al.*, 1999). Double immunolabeling studies revealed co-localization of polyhomeotic (PH), a component of the polycomb repressive complex 1 (PRC1), with Dcr-2, PIWI, and AGO1 in larval nuclei, suggesting that the RNAi components are physically proximal to PcG nuclear bodies within a cell (Fig. 3) (Grimaud *et al.*, 2006). Moreover, FISH-I performed in larvae polytene chromosomes with mutations for *piwi, dcr-2*, and *ago1* revealed reduced long-distance interactions between PcG-dependent silenced regions in the BX-C and the ANT-C (Grimaud *et al.*, 2006). These studies suggest that the protein components of the RNAi machinery and PcG factors may play a combinatorial role in long range nuclear interactions at endogenous Hox target genes (Fig. 3), although correlation with the transcriptional activity of the Hox genes has yet to be functionally tested. However, a similar observation was recently made at the *gypsy* insulator (Lei and Corces, 2006). In flies carrying mutations for Piwi or AGO2, *gypsy* insulator function was diminished, suggesting that these two RNAi components may be critical for the function of different types of CRMs (Lei and Corces, 2006).

Alternate mechanism for the recruitment of polycomb group proteins to polycomb response elements in the bithorax complex

The current model for the molecular recruitment of PcG proteins to PREs is that the Polycomb (PC) homeodomain of PRC1 recognizes a specific epigenetic mark in chromatin (Fig. 4). The mark is thought to be due to the catalytic activity of polycomb repressive complex 2 (PRC2), which tri-methylates lysine 27 on histone H3 (H3K27me3) (Cao and Zhang, 2004, Czermin *et al.*, 2002, Kuzmichev *et al.*, 2002, Levine *et al.*, 2004, Muller *et al.*, 2002, Shao *et al.*, 1999, Wang *et al.*, 2004). Previous DNA crosslinking studies showed that PHO and PC bind strongly to DNA (Fig 4.) (Mohd-Sarip *et al.*, 2005). In contrast, Posterior Sex Combs (PSC) or PH bind DNA very weakly, although the authors could not distinguish between the binding affinity of these two proteins (Mohd-Sarip *et al.*, 2005). In the absence of other PCC subunits PC does not bind as effectively with PHO and DNA, as revealed by a reduced DNA footprint (Mohd-Sarip *et al.*, 2005). These data indicate that other factors in the PCC are cooperatively involved in the binding of DNA. FISH-I on polytene chromosomes from third-instar larvae carrying a *piwi* mutation revealed a loss of PC binding to Fab-X (Grimaud *et al.*, 2006). However, PH, another component of PRC1, still bound the Fab-X PRE in these mutants (Grimaud *et al.*, 2006), suggesting that PC and PH binding can be uncoupled and that the current model of recruitment is not universal (Fig. 4). These observations suggest that there may be multiple robust molecular mechanisms for the recruitment of the PRC1 complex to endogenous PREs, such as the Fab-7 PRE, in the BX-C (Fig 4). Additional PRC1 subunits, specifically PH or PSC, could account for alternative molecular methods of PcG protein recruitment (Fig. 4). Consequently, future studies should examine the specific roles of PSC and PH in PcG protein interactions with DNA.

Functional miRNAs in the bithorax complex regulate *Hox* gene expression

Recent evidence has also implicated the RNA products of non-genic transcription in the direct regulation of neighboring Hox genes. Aravin *et al.* characterized several microRNA (miRNA) clones from various stages of the developing *Drosophila* embryo (Aravin *et al.*, 2003). Specifically, they looked at two miRNAs

located in the *iab-4* region of the BX-C, which they referred to as mir-iab-4-5p and mir-iab-4-3p (see Fig. 5). These miRNAs are derived from the primary transcript, pri-mir-iab-4, previously referred to as *iab-4* (Cumberledge *et al.,* 1990), which results from transcription at the *iab-4* locus anti-sense relative to the Hox genes in the BX-C (Fig. 5). The orientation of the miRNAs as defined here is confirmed by sequencing results (Aravin *et al.*, 2003, Ruby *et al.*, 2007, Stark *et al.*, 2007). The pri-mir-iab-4 transcript is cleaved by Drosha (Lee *et al.*, 2003) to form pre-mir-iab-4, which is processed into a mature miRNA by Dicer (Hutvagner *et al.*, 2001). Generally, only one strand of the pre-miRNA becomes a functionally active mature miRNA (Aravin *et al.*, 2003). In the case of mir -iab-4-5p/-3p (which together form a single pre-miRNA) (Fig. 5), it appears to be mir -iab-4-5p which is functionally active (Bender, 2008, Ronshaugen *et al.*, 2005, Stark *et al.*, 2008, Tyler *et al.*, 2008).

A detailed study of mir-iab-4-5p showed that it is a direct repressor of *Ubx* (Ronshaugen *et al.*, 2005). Three more recent studies (Bender, 2008, Stark *et al.*, 2008, Tyler *et al.*, 2008) confirmed the role of mir-iab-4-5p as a repressor of *Ubx*, and revealed that transcription of the opposite DNA strand to mir-iab-4 (i.e. sense relative to the Hox genes in the BX-C) also produces a functionally active miRNA (Fig. 5), which is referred to as mir-

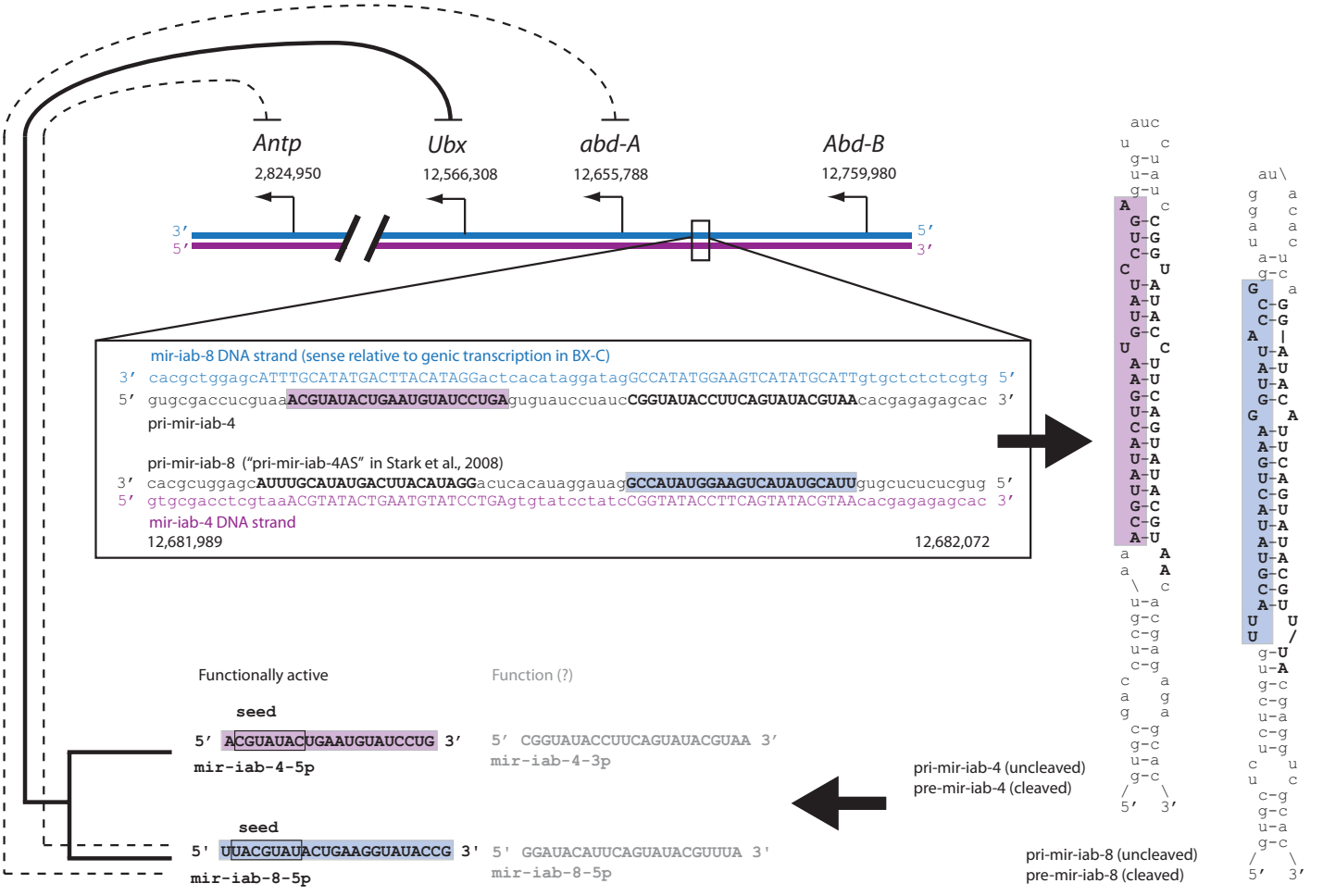

Fig. 5. Non-genic transcription produces two functionally distinct miRNAs at the *iab-4* locus in the bithorax complex (BX-C). *Transcription start sites of genes are indicated by leftward arrows. In* Drosophila melanogaster, *sense transcription of all the* Hox *genes, with the exception of* Deformed, *is toward the centrosome (not shown) on chromosome 3R. During embryonic development both sense (purple) and anti-sense (blue)* mir-iab-4 *transcripts, shown complementary to the DNA sequences in the central box, are synthesized at the* iab-4 *locus (solid box). Note that the sense direction of* mir-iab-4 *is opposite to that of the Hox genes in the BX-C. The primary transcripts are cleaved by Drosha to form the "hairpin" RNAs pre-mir-iab-4/-8, the 2-D structures of which are shown on the right. The hairpins are then processed by Dicer to form the mature, functionally active miRNAs: mir-iab-4-5p (shaded purple background) and mir-iab-8-5p (shaded blue background). The seed sequences within the miRNAs are indicated with solid boxes. The two functional miRNA products repress target genes in the ANT-C and BX-C (top). The thick line for* Ubx *indicates functional repression which is removed in flies homozygous for a deletion of mir-iab-4/-8 (Bender, 2008), and the dashed lines indicate putative targets as identified either by ectopic expression of mir-iab-4/-8 or by bioinformatic analysis (Stark* et al., *2008, Tyler* et al., *2008). It is likely that mir-iab-4 and not mir-iab-8 represses* Ubx in vivo, *based on the more anterior expression of mir-iab-4 (Bender, 2008, Stark* et al., *2008, Tyler* et al., *2008), although both mir-iab-4 and mir-iab-8 are shown to repress* Ubx *when ectopically expressed (Ronshaugen* et al., *2005, Stark* et al., *2008, Tyler* et al., *2008). It is not known if the alternate strands of the hairpins are processed into functionally active miRNAs. Genomic coordinates refer to the base-pair positions in the* Drosophila melanogaster *genome sequence on chromosome 3R.*

iab-8 by Bender (2008) and Tyler *et al.* (2008), respectively, and as mir-iab-4AS by Stark *et al.* (2008). The naming of mir-iab-8 refers to the suspected origin of the transcript at the *iab-8* locus (Bender, 2008). Although this origin has not been conclusively shown in our own studies (Bae *et al.*, 2002), we will nonetheless refer to the transcript as mir-iab-8 because it makes clear the direction of transcription. As with mir-iab-4, it is the 5' arm of pre-mir-iab-8 which appears to become the functionally active mature miRNA (Fig. 5) (Bender, 2008, Stark *et al.*, 2008, Tyler *et al.*, 2008). Ectopic expression of mir-iab-8-5p caused repression of both *Ubx* and *abd-A*, and this repression was stronger than that caused by ectopic expression of mir-iab-4-5p (Stark *et al.*, 2008, Tyler *et al.*, 2008). There is also some indirect evidence (based mainly on evolutionary conservation studies) that mir-iab-8-5p may be a repressor of *Antennapedia* (*Antp*) (Stark *et al.*, 2008, Tyler *et al.*, 2008). However, the only functional data comes from a homozygous deletion of both mir-iab-4/-8 (Bender, 2008), which indicates that one or both of these miRNAs repress *Ubx*. No effect on *abd-A, Abd-B*, or *Antp* expression was seen as a result of the deletion (Bender, 2008), contradicting evidence that ectopic expression of mir-iab-4 causes repression of *abd-A* (Stark *et al.*, 2008, Tyler *et al.*, 2008). One possibility is that the microRNAs are capable of repressing transcription of *abd-A* (and even *Antp*), but are not normally expressed in the correct spatial domain during development.

Thus, sense and anti-sense non-genic transcription at the *iab-4* chromosomal region may play functionally distinct roles. The primary miRNAs resulting from the sense and anti-sense transcripts adopt slightly different conformations and the resulting mature miRNAs have different seed sequences, which consequently recognize different target mRNA sequences (Stark *et al.*, 2008, Tyler *et al.*, 2008). Moreover, the spatial domains of sense and anti-sense transcription are temporally non-overlapping in the *iab-4* region in the developing embryo (Bae *et al.*, 2002, Drewell *et al.*, 2002, Stark *et al.*, 2008, Tyler *et al.*, 2008), further suggesting that transcription from the opposite strands of DNA in this region may play functionally distinct roles. In a discussion of *Drosophila* mir-307 and mammalian mir-338, Tyler *et al.* suggest that this duality of sense and anti-sense transcription may in fact be a common phenomenon, allowing for distinct functional miRNAs to be produced from a single locus (Tyler *et al.*, 2008). Although there are currently only a few known cases of anti-sense transcription of miRNAs, bioinformatic analysis reveals that a large fraction of miRNA loci are theoretically competent to produce both sense and anti-sense miRNAs (Tyler *et al.*, 2008). The entire mir-iab-4 sequence, as well as the known and putative target sequences for the functionally active miRNAs, shows extremely high sequence conservation in *Drosophila*, mosquito, bees and beetles (Bender, 2008, Stark *et al.*, 2008, Tyler *et al.*, 2008). The evolutionary conservation of this miRNA pathway indicates that this may be a significant functional component in regulating Hox gene expression at the BX-C.

Summary

The results from recent studies in the BX-C strongly indicate that non-genic transcription plays a crucial role in both the establishment and maintenance of the specific temporal and spatial patterns of Hox gene expression during development. Non-genic transcription is required for the selective activation of specific CRMs, most likely by either preventing or facilitating binding of transcription factors or by directly recruiting chromatin-modifying factors. Moreover, PcG proteins, protein components of the RNAi pathway and the basal transcriptional machinery act synergistically in the modification and maintenance of distinct chromatin structures, some of which regulate long distance interactions between CRMs that are capable of modulating Hox gene expression. Non-genic transcription also produces functional ncRNAs, including miRNAs, which can fine tune the expression levels of the neighboring Hox target genes. Thus, non-genic transcription is a critical component in the rich molecular toolbox responsible for the regulation of the Hox genes. Future detailed studies will enable us to more fully comprehend these distinct functional roles embedded in the dark matter of the genome.

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