Development 135, 2383-2390 (2008) doi:10.1242/dev.023275

Association of trxG and PcG proteins with the *bxd* maintenance element depends on transcriptional activity

Svetlana Petruk[1], Sheryl T. Smith[2], Yurii Sedkov[1] and Alexander Mazo[1,*]

Polycomb group (PcG) and trithorax group (trxG) proteins act in an epigenetic fashion to maintain active and repressive states of expression of the Hox and other target genes by altering their chromatin structure. Genetically, mutations in trxG and PcG genes can antagonize each other's function, whereas mutations of genes within each group have synergistic effects. Here, we show in *Drosophila* that multiple trxG and PcG proteins act through the same or juxtaposed sequences in the maintenance element (ME) of the homeotic gene *Ultrabithorax*. Surprisingly, trxG or PcG proteins, but not both, associate in vivo in any one cell in a salivary gland with the ME of an activated or repressed *Ultrabithorax* transgene, respectively. Among several trxG and PcG proteins, only Ash1 and Asx require Trithorax in order to bind to their target genes. Together, our data argue that at the single-cell level, association of repressors and activators correlates with gene silencing and activation, respectively. There is, however, no overall synergism or antagonism between and within the trxG and PcG proteins and, instead, only subsets of trxG proteins act synergistically.

KEY WORDS: Trithorax, Polycomb, Epigenetic regulation, Maintenance elements, Homeotic genes

INTRODUCTION

Two large gene families, the trithorax group (trxG) and the Polycomb group (PcG), are required to maintain the appropriate state of Hox gene expression throughout development (reviewed by Grimaud et al., 2006). The traditional view is that trxG proteins are required to maintain the active state of Hox gene expression, whereas PcG proteins repress their expression. Combining mutations of members of the same group enhances their mutant phenotype, whereas combining mutations from each group results in mutual suppression. Despite this general trend, some trxG genes can act as both activators and repressors (Busturia et al., 2001; Hodgson et al., 2001; Horard et al., 2000). Mutations in several PcG genes can enhance the phenotypes of trxG mutants, suggesting that a subset of PcG genes are required to activate as well as to suppress Hox expression (Gildea et al., 2000; LaJeunesse and Shearn, 1996; Milne et al., 1999; Sinclair et al., 1992). One such example is the PcG gene *Additional sex combs* (*Asx*), which is required for both activation and repression of different aspects of Hox expression in *Drosophila* embryos (Milne et al., 1999). Thus, there are some trxG and PcG proteins that have dual roles and, depending on a particular context, may act as activators or repressors. It has been suggested that these proteins should be called enhancers of trithorax and Polycomb (ETPs) (Gildea et al., 2000).

Genetic experiments have suggested that PcG/trxG proteins might alter the chromatin structure of their target genes (Grimaud et al., 2006). Consistent with these genetically derived concepts, accumulating data suggest that trxG and PcG proteins regulate transcription of their target genes by altering their chromatin structure. They are found in multiprotein complexes that either modify histones within nucleosomes, or remodel chromatin, or are components of the general transcriptional machinery. At present, we know the composition and enzymatic activities of several trxG proteins and their complexes in *Drosophila*. The Trithorax (Trx)-containing complex TAC1 possesses both histone acetyltransferase (HAT) and histone H3 lysine 4 (H3-K4) methyltransferase (HMTase) activities (Petruk et al., 2001; Smith et al., 2004). Another trxG protein, Ash1, is also an HMTase. Ash1 was previously shown to methylate a number of residues in histones H3 and H4 [H3-K4, H3-K9 and H4-K20) (Beisel et al., 2002; Byrd and Shearn, 2003)], but recent analysis suggests that it methylates exclusively H3-K36 (Tanaka et al., 2007). A trxG BRM complex is closely related to the well-known yeast SWI/SNF ATP-dependent chromatin remodeling complex (Papoulas et al., 1998). Several components of the BRM complex, including Brahma (Brm), Moira (Mor) and Osa, are encoded by trxG genes (Collins and Treisman, 2000; Crosby et al., 1999; Papoulas et al., 1998). Although the trxG protein Kismet (Kis) is similar to the SWI/SNF family of ATP-dependent remodeling factors, it is a general factor at some stages of transcriptional elongation (Daubresse et al., 1999; Srinivasan et al., 2005). Several other molecularly characterized trxG proteins are thought to be general transcription factors. For example, Skuld (Skd) and Kohtalo (Kto) encode homologs of TRAP240 and TRAP230, two subunits of the *Drosophila* Mediator complex (Janody et al., 2003). Two purified PcG complexes, PRC1 and PRC2, are also involved in chromatin alterations. PRC1 ubiquitylates histone H2A at lysine 119 (Wang, H. et al., 2004) and counteracts the chromatin remodeling activity of the SWI/SNF complex (Francis et al., 2001). The PRC2, or E(z)-Esc, complex may have several HMTase activities due to the SET domain of Enhancer of Zeste [E(z)], the major one being methylation of H3-K27 (Cao et al., 2002; Czermin et al., 2002; Kuzmichev et al., 2002; Muller et al., 2002).

The targets of trxG and PcG proteins are not limited to the Hox complexes. Both traditional cytological mapping of binding sites on salivary gland polytene chromosomes (reviewed by Brock and van Lohuizen, 2001) and recent genome-wide chromatin immunoprecipitation (ChIP) assays (Negre et al., 2006; Schwartz et al., 2006) demonstrate that these proteins are associated, often jointly, with a very large number of genes. The maintenance of gene expression by these groups of proteins is mediated by trxG and PcG response elements (TREs and PREs, respectively). These elements have been most extensively studied in the regulatory regions of the

[1]Department of Biochemistry and Molecular Biology, Thomas Jefferson University, Philadelphia, PA 19107, USA. [2]Department of Biology, Arcadia University, Glenside, PA 19038, USA.

*Author for correspondence (e-mail: Mazo@mail.jci.tju.edu)

Accepted 16 May 2008

Bithorax complex (BX-C). There are multiple TREs and PREs in the 300 kb BX-C region, and these elements tend to localize in close proximity to one another in regions termed maintenance elements (MEs) (Chan et al., 1994; Chang et al., 1995; Chiang et al., 1995; Fritsch et al., 1999; Hagstrom et al., 1996; Orlando et al., 1998; Simon et al., 1993; Strutt et al., 1997; Tillib et al., 1999). The best-studied ME is localized in the *bxd* regulatory region of *Ubx* ~25 kb upstream of the *Ubx* promoter. A number of PREs and Trx-regulated TREs have been mapped to juxtaposed DNA sequences in a 3 kb *bxd* ME (Tillib et al., 1999). This organization suggests that these proteins could interact in complex ways at the ME.

Despite advances in studies of the PcG and trxG proteins, there is little understanding of whether they act in concert at a ME. Even in the *bxd* ME, mapping data for trxG proteins is limited to Trx and Ash1 (Beisel et al., 2002; Papp and Muller, 2006; Petruk et al., 2007; Tillib et al., 1999). It seems likely that many trxG proteins besides Trx should act there, because Trx interacts directly with several trxG proteins. For example, Trx interacts directly with Snr1 (Rozenblatt-Rosen et al., 1998), a component of the BRM complex (Papoulas et al., 1998). Similarly, Trx and Ash1 can interact at the protein level (Rozovskaia et al., 1999). They are associated with the same regions of *Ubx* in vivo (Petruk et al., 2007; Petruk et al., 2006) and share most of their target genes (Rozovskaia et al., 1999). There is also very little data on whether trxG proteins are dependent on each other for binding to their target genes. The only exception is the finding that binding of Trx to its target genes is strongly affected in *ash1* mutants (Kuzin et al., 1994). Another important uninvestigated issue is how trxG functioning relates to that of the PcG and ETP proteins, such as Asx. The answers to these questions might reveal whether different complexes have similar functions in different places, act in the same place with different functions, or have different functions in different places.

In this work, we show that many trxG genes are required for functioning of the *bxd* ME. Genetic experiments show that the response elements for the ETP gene *Asx* and the trxG gene *ash1* either coincide with or are juxtaposed to the response element of *trx*. Consistent with this, binding of Ash1 and Asx to all their target genes is completely dependent on Trx, suggesting that they function cooperatively. Surprisingly, although the response elements of *trx* and *brm* are also juxtaposed, the BRM complex does not require Trx for its association with target genes. At the single-cell level, binding of Trx and components of the two major PcG complexes, PRC1 and PRC2, to the *bxd*-ME-containing transgene in vivo is mutually exclusive. Binding of PRC1 and PRC2 to their target genes is independent of Trx. Thus, our results indicate that although multiple trxG and PcG proteins are required for functioning of the *bxd* ME, many may function independently. Importantly, association of activators and repressors with the *bxd* ME correlates with the transcriptional status of the gene.

MATERIALS AND METHODS

Drosophila stocks

The following trxG alleles were used: $ash1^{B1}$, brm^{2}, brm^{5}, skd^{1}, skd^{2}, kto^{3}, kis^{1}, kis^{2}, dev^{1}, dev^{2}, mor^{2}, osa^{1}, urd^{1}, *Df(urd)*, sls^{1}, *vtd* (from J. Kennison, NIH, Bethesda, MD); $ash1^{11}$, $ash1^{2}$, $ash1^{22}$, $ash2^{2}$, $ash2^{18}$ (from A. Shearn, John Hopkins University, Baltimore, MD); Asx^{3} (from H. Brock, University of British Columbia, Vancouver, Canada), kto^{1}, osa^{2}, mor^{1} (from the *Drosophila* Stock Center, Bloomington, IN).

Genetic analysis

The strategy to determine *bxd* regulatory sequences that are responsive to trxG mutations is described in the legend to Table 1 and by Tillib et al. (Tillib et al., 1999). Construction of the *bxd* transgenes has been described previously (Tillib et al., 1999). Induction of the *trx* RNAi line has been described previously (Petruk et al., 2006).

Immunostaining of polytene chromosomes

Polytene chromosomes from third instar larvae were prepared and immunostained as described previously (Tillib et al., 1999). The following antibodies were used: Trx N1 (rat, dilution 1:20) (Tillib et al., 1999); Ash1 (rabbit, 1:150) (Rozovskaia et al., 1999); E(z) (rabbit, 1:25; from R. Jones, Southern Methodist University, Dallas, TX); Ph (rabbit, 1:120), Pc (rabbit, 1:100) and Asx (sheep, 1:30) from H. Brock; Osa (mouse, 1:15), Kis (rabbit, 1:50) and Brm (rabbit, 1:75) from J. Tamkun (University of California, Santa Cruz, CA).

RESULTS

To examine whether trxG genes other than *trx* also have response elements in the *bxd* ME, we determined the effect of trxG mutations on the expression of *white* in the adult eye under the control of the *bxd* ME in a transgene. Mutations in genes required for activation should reduce expression of *white*. We have shown previously that tests of *trx* function at the endogenous *bxd* ME in embryos give essentially the same results as those monitoring *white* expression in the eye, establishing the validity of this assay for the tests used here (Tillib et al., 1999). The results of these tests were entirely reproducible with two wild-type N transgenic lines (Fig. 1B). With the exception of *sls*, for which only one allele is available, we tested several alleles of most of the trxG genes examined. We also tested one *Asx* allele, to gain information with regard to this unusual ETP gene. As shown in Fig. 1A,B and Table 1, expression of the *white* marker gene is reduced in heterozygous mutants of nine newly

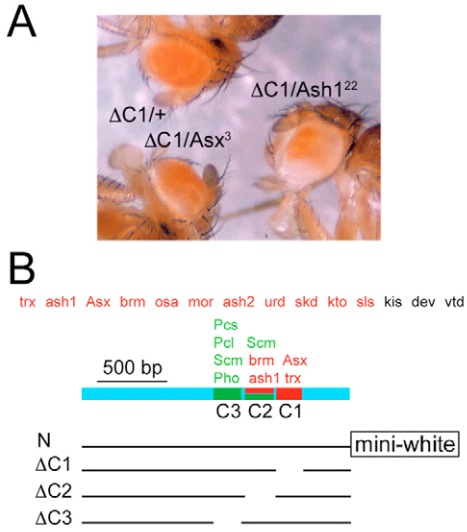

Fig. 1. Multiple trxG genes are essential for functioning of the *bxd* TRE/PRE maintenance element. (**A**) The effects of $ash1^{22}$ and Asx^{3} on the eye color of ΔC1 transgenic flies. (**B**) Map of trxG and PcG response elements in the *bxd* region of *Ubx* based on the results of the genetic *white* tests shown in Tables 1 and 2. Data from Table 1: genes that interact or do not interact with the *bxd* N constructs are shown in red and black, respectively, above the map. Data from Table 2: a scheme of the ΔC constructs used in these experiments is shown at the bottom. The B TRE has been deleted in the constructs because of redundancy with the C TRE (Tillib et al., 1999). Mapped Trx, Asx, Ash1 and Brm response elements are in red. Previously mapped PcG response elements are in green (Tillib et al., 1999).

Table 1. Responses of N construct transgenes to interactions with trxG alleles

trxG allele	N1	N2	trxG allele	N1	N2
ash1^{B1}	+	+	*Df(urd)*	+	+
ash1^{11}	+	+	*urd^{1}*	+	+
ash1^{12}	+	+	*skd^{1}*	+	+
ash1^{22}	+	+	*skd^{2}*	+	+
Asx^{3}	+	+	*kto^{1}*	+	+
ash2^{2}	+	+	*kto^{2}*	+	+
ash2^{18}	+	+	*sls^{1}*	+	+
trx^{B11}	+	+	*kis^{1}*	–	–
brm^{2}	+	+	*kis^{2}*	–	–
brm^{5}	+	+	*dev^{1}*	–	–
osa^{1}	+	+	*dev^{2}*	–	–
osa^{2}	+	+	*vtd^{3}*	–	–
mor^{1}	+	+	*vtd^{5}*	–	–
mor^{2}	+	+			

trxG alleles were crossed to two transgenic lines carrying independent insertions of the two N constructs containing the 3 kb *bxd* region [Fig. 1 and see Tillib et al. (Tillib et al., 1999)]. Comparisons of eye color were made between progeny carrying either N construct alone and those carrying N construct in the background of a given trxG allele. The decrease in eye color expression caused by a decreased dosage of wild-type trxG protein was designated (+), and no change in expression was designated (–).

tested trxG genes: *brm*, *osa*, *mor*, *ash1*, *ash2*, *sls*, *urd*, *skd* and *kto*. Three trxG alleles did not show genetic interaction with the *bxd* ME: *kis*, *dev* (*btl* – FlyBase) and *vtd*.

Interestingly, the *bxd*-ME-interacting alleles include *ash1* and three other genes that interact with Trx. They encode components of the BRM complex: *brm*, *osa* and *mor*. Ash1 and Snr1 (a component of BRM) directly interact with Trx (Rozovskaia et al., 1999; Rozenblatt-Rosen et al., 1998), and Ash1 has also been shown to genetically and physically interact with the TAC1 component dCBP (Nejire – FlyBase) (Bantignies et al., 2000). It is notable that the *Asx^{3}* mutation also caused a decrease in expression of the *white* gene (Fig. 1A), suggesting that despite the ETP nature of this gene, it behaves like a trxG gene in this assay. This is a potentially important finding because Asx interacts directly with Trx (J. Hodgson, personal communication). Together, these results suggest that these proteins might interact directly with Trx at their target genes. Since this also assumes that their elements would be located in close proximity to each other, we chose *ash1*, *Asx* and *brm* alleles for more detailed analysis.

Fine mapping of the *ash1*, *Asx* and *brm* response elements within the central C module of the *bxd* element was performed as described previously (Tillib et al., 1999), using multiple transgenic fly lines with constructs in which the C1, C2 or C3 sub-elements of the *bxd* ME were deleted (Fig. 1A,B). Table 2 shows that *ash1* and *brm* response elements reside within the C2 element, which is juxtaposed to the C1 *trx* response element. About half of the ΔC3 lines did not respond to the *ash1* mutations, suggesting that the response element for *ash1* detected in C2 might extend into the C3 element. Interestingly, the response elements for *Asx* and *trx* coincide in the C1 DNA element (Fig. 1B, Table 2). Note that the *Asx* analysis includes fewer transgenic lines, especially C2 lines, as this analysis was performed at later stages of this work when some of the original lines had been lost. Therefore, we cannot exclude the possibility that the *Asx* response element might also extend into the C2 region.

These results suggest a very complex organization of the *bxd* ME, in which trxG and PcG proteins occupy either the same or juxtaposed response elements. This raises the question of how the functioning of this element is mediated by multiple trxG and PcG proteins. This might occur by alternative binding of PcG versus trxG proteins, or by the preferential action of one of the two groups of simultaneously bound proteins. This can be best addressed by examining the association of these proteins in a single cell, where transcription of the *Ubx* transgene is either on or off. It is well established that many ME-containing transgenes have a variegated phenotype in the eye, i.e. that the *white* reporter gene in these transgenes is expressed in only a subset of cells in the eye. Consistent with this, we found that the *white* reporter gene in our construct also shows differential expression in the salivary glands of

Table 2. Determination of *ash1*, *brm* and *Asx* response elements in the *bxd* region of *Ubx*

			trxG alleles					
TRE/PREs*	Δ region C module	Transgenic line	*trx^{B11}*	*Asx^{3}*	*ash1^{22}*	*ash1^{12}*	*brm^{2}*	*brm^{5}*
C1 TRE: trx	ΔC1	1-11-42	–	–	+	+	+	+
	ΔC1	1-11-51	–	–	+	+	+	+
	ΔC1	1-11-17	–	–	+	+	+	+
	ΔC1	1-11-61	–	–	+	+	+	+
	ΔC1	1-11-11	–	NT	+	+	+	+
	ΔC1	1-11-10	–	NT	+	+	+	+
C2 PRE: Scm	ΔC2	14-7-42	+	+	–	–	–	–
	ΔC2	14-7-8	+	NT	–	–	–	–
	ΔC2	14-7-3	+	NT	–	–	–	–
	ΔC2	14-7-6	+	NT	–	–	–	–
	ΔC2	14-7-7	+	NT	+	–	–	–
C3 PRE: Psc, Pcl, Scm, pho	ΔC3	2-3-18	+	+	+	+	+	+
	ΔC3	2-3-13	+	+	+	–	+	+
	ΔC3	2-22-02	+	+	+	+	+	+
	ΔC3	2-3-84	+	NT	–	–	+	+
	ΔC3	2-22-85	+	NT	–	+	+	+

The effects of trxG alleles *trx^{B11}*, *ash1^{12}*, *ash1^{22}*, *brm^{2}*, *brm^{5}* and the ETP allele *Asx^{3}* on expression of N and ΔC transgenes containing deletions of the TRE or PRE (see Fig. 1). Adult flies from each line were crossed to each of the tested alleles and the eye color of the progeny was compared to the parent line. A decrease in eye color expression in the progeny was designated (+), and no change in eye color expression designated (–) as suggestive of a deleted response element for a given trxG or ETP protein. The transgenes containing the deleted C1 and C2 elements showed no further change in expression in the *trx* and *Asx* or *ash1* and *brm* mutants, respectively, suggesting that response elements for Trx and Asx reside in the C1 element, and that response elements for Ash1 and Brm reside in the C2 element. The response element for Ash1 may extend into the C3 element.
NT, not tested.
*As previously identified (Tillib et al., 1999).

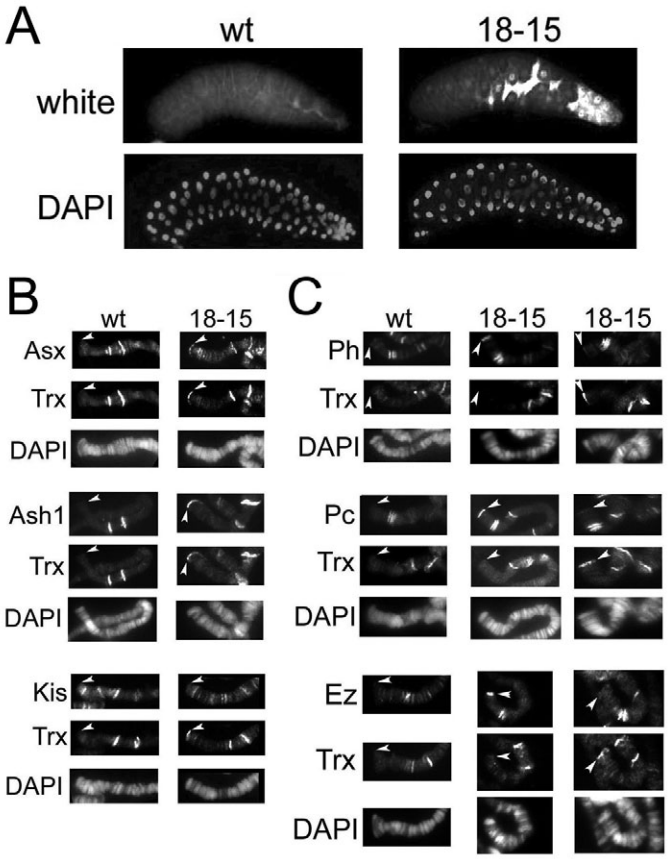

Fig. 2. In any one cell in a *Drosophila* salivary gland, trxG and PcG proteins alternatively associate with the *bxd* ME. (**A**) Variegating phenotypes of the *white* reporter gene of the 18-15 transgenic line in the salivary gland. DAPI staining of the same glands is shown in the lower panels. (**B**) Binding of trxG proteins Trx, Asx, Ash1 and Kis to chromosome 3 of the salivary gland polytene chromosomes of the wild type and 18-15 transgenic line. The site of insertion of the N construct is at the very tip of chromosome 3 and is indicated by arrowheads. Trx, Asx and Ash1, but not Kis, bind simultaneously to the site of insertion of the N construct in ~50% of nuclei from the salivary glands prepared from the same larvae. (**C**) PcG proteins Ph, Pc and E(z) bind to the site of insertion of the N construct in those nuclei where Trx protein is not associated with this site.

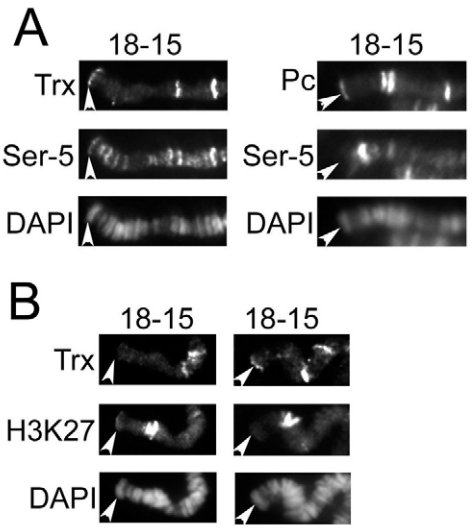

Fig. 3. Association of trxG or PcG proteins with the *bxd* ME in individual cells correlates with an activated or repressed *white* reporter gene, respectively. (**A**) Pol II phoshorylated at Ser5 is associated with the site of insertion of the N construct (arrowheads) in the same *Drosophila* salivary gland cells as Trx (left), but not Pc (right). (**B**) H3-meK27 is not associated with the site of insertion of the N construct in the same cells as Trx.

third instar larvae (Fig. 2A). Thus, tests for direct binding of trxG and PcG proteins to the *bxd* ME transgene in salivary glands provide a unique opportunity to address the above questions in individual cells in vivo.

We examined the physical association of several trxG and PcG proteins with the site of insertion of the 18-15 transgene that carries the wild-type N construct shown in Fig. 1B (Tillib et al., 1999). We found that Ash1 and Trx are always associated together at the site of insertion of the N transgene (Fig. 2B). Remarkably, the Asx protein was also found exclusively at the Trx-associated chromosomal sites (Fig. 2B). Together with localization of the *Asx* and *trx* response elements in the same region of the *bxd* ME, and the fact that expression of the *white* transgene is decreased in Asx^3 mutants (Fig. 1A, Table 2), these results suggest that at least in the larvae and the adults, this ETP protein is functioning exclusively as a trxG protein at the *bxd* ME in transgenes. The above results are also consistent with direct physical interactions between Trx and Asx (J. Hodgson, personal communication). Kis was not found at the site of insertion of this transgene (Fig. 2B), consistent with the results of the genetic *white* tests, which suggested that there is no *kis* response element in the *bxd* ME (Fig. 1B, Table 1). Brm and Mor could not be examined in these experiments because they are associated in wild-type animals with the same region where the transgene is inserted, at the tip of chromosome 3.

Two PcG proteins, Pc and Ph (Polyhomeotic), that have response elements in the *bxd* ME (Fig. 1B) (Tillib et al., 1999), associate with the N transgene in vivo (Fig. 2C). Similarly, E(z) protein is also associated with the *bxd* ME, which is consistent with previous ChIP analysis of larval imaginal discs (Cao et al., 2002; Papp and Muller, 2006). However, in sharp contrast with the above results, we found that Trx and each of the three tested PcG proteins were not simultaneously associated with the insertion site of the N transgene. Roughly one half of the nuclei from the same salivary gland contained Trx but not PcG proteins bound to the transgene, or vice versa (Fig. 2C).

The alternative association of several trxG activators and several PcG repressors with the N transgene in different subsets of chromosomes from the same gland indicates that N transgenes may be either activated or repressed, respectively, in different cells in salivary glands. This is in line with the variegated expression of the *white* transgene observed in salivary glands (Fig. 2A). To test this directly, we examined whether RNA polymerase II (Pol II) is associated with the transgene insertion site. Fig. 3A shows that an activated form of Pol II that is phosphorylated at Ser5 is co-localized with Trx but not with Pc at the alternative binding sites, suggesting that Trx binding correlates with activation of this transgene. By contrast, strong binding of Trx and H3-meK27 are mutually exclusive (Fig. 3B; co-localization of H3-meK27 and PcG proteins cannot be tested because these antibodies were raised in rabbits). Since H3-meK27 is a PRC2-generated mark of repressed transcription (Cao et al., 2002; Czermin et al., 2002; Kuzmichev et

al., 2002; Muller et al., 2002), this result indicates that all tested components of the PRC1 and PRC2 PcG complexes are associated with the repressed transgene.

The trxG and PcG genes interact genetically. Combining mutations of different trxG (or PcG) genes enhances their mutant phenotype, whereas combining mutations of trxG with PcG genes suppresses their mutant phenotypes. The finding of response elements for multiple, interacting trxG and PcG proteins in close proximity to each other (Fig. 1B), and the demonstration of mutually exclusive binding of trxG and PcG proteins to the activated and repressed ME (Figs 2, 3), suggest potential competition and synergism between the trxG and PcG proteins and between different trxG proteins, respectively. However, with the exception of two reports demonstrating hierarchical binding of several PcG proteins to the *bxd* ME in larval imaginal discs (Wang, L. et al., 2004), and that binding of Trx is strongly affected in *ash1* mutant larvae (Kuzin et al., 1994), there are no data with regard to the interdependencies between and within these two antagonistic groups of proteins for association with their common target genes. To address this, we asked whether elimination of the Trx protein by expressing a *trx* RNAi construct affected in vivo binding of trxG and PcG proteins to their binding sites on the salivary gland polytene chromosomes. Expression of *trx* RNAi was achieved using the Gal4-UAS system, as described previously (Petruk et al., 2006), and results in the efficient removal of the Trx protein from all of its binding sites on polytene chromosomes (Fig. 4A). The structure of the polytene chromosomes and binding of the unrelated protein, Ecdysone receptor (EcR), were not affected when *trx* RNAi was expressed (Fig. 4A) (Petruk et al., 2006).

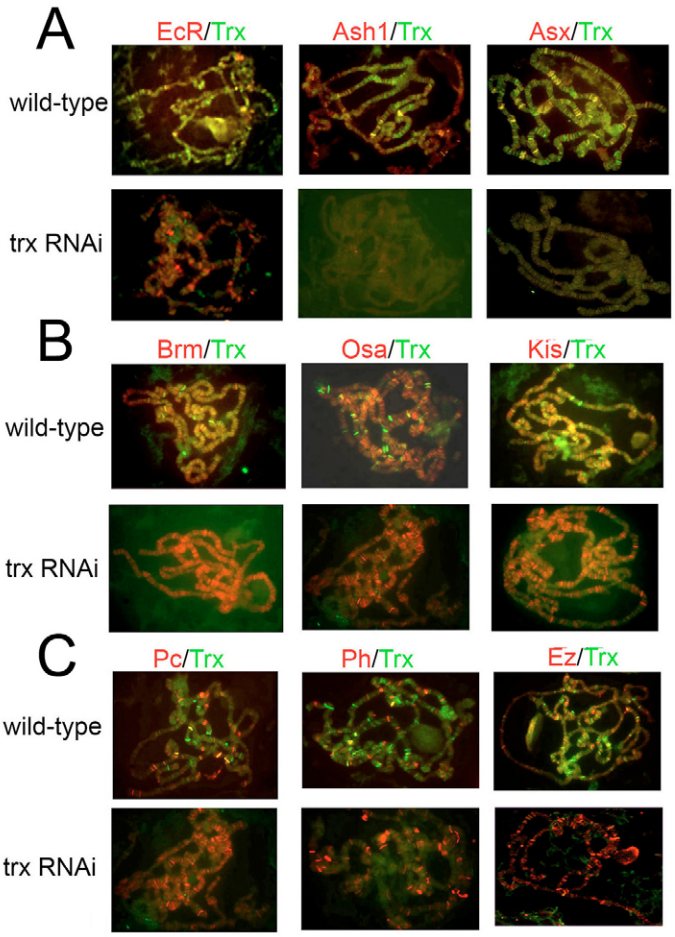

Fig. 4. Effect of *trx* RNAi on association of trxG and PcG proteins with their target genes. Salivary gland polytene chromosomes were prepared from wild-type third instar *Drosophila* larvae and from the line expressing *trx* RNAi as described (Petruk et al., 2006). The overall structure of these chromosomes is indistinguishable (Petruk et al., 2006). (**A**) Trx is essential for association of Ash1 and Asx. Binding of Trx (green), Ash1 (red, column 2) and Asx (red, column 3) is completely abolished in the *trx* RNAi line, whereas binding of the control protein, Ecdysone receptor (EcR, green) is unaffected (column 1). (**B**) Trx is not essential for association of the trxG BRM complex and Kis. Components of the BRM complex, Brm and Osa, and trxG protein Kis (red) bind to their sites on polytene chromosomes in a line expressing *trx* RNAi. Trx binding is in green. (**C**) Association of the PcG complexes PRC1 and PRC2 is not affected by induction of *trx* RNAi. The intensities and the number of bands for the components of PRC1, Pc and Ph, and the component of the PRC2, E(z) (all in red), are not significantly affected in the *trx* RNAi line.

Strikingly, binding of Ash1 was completely abrogated following induction of *trx* RNAi (Fig. 4A). This suggests that Trx is essential either for the recruitment or for stable association of Ash1 with all of its binding elements in the genome. These results are consistent with direct interaction of these proteins, and with the results that show that Trx and Ash1 are associated jointly at the juxtaposed response elements of the *bxd* ME (Rozovskaia et al., 1999) (Figs 1, 2 and Table 2). This is also consistent with the finding that Trx and Ash1 bind in vivo to essentially the same regions of *Ubx* (Petruk et al., 2007; Petruk et al., 2006). Since binding of Trx is also strongly affected in the *ash1* mutant larvae (Kuzin et al., 1994), this suggests that this dependency is reciprocal. Taken together, these results suggest that Trx and Ash1 are two interacting, mutually dependent trxG proteins. It is, however, important to note that these proteins do not appear to be components of the same protein complex.

In similar experiments, we tested the effect of *trx* mutation on binding to polytene chromosomes of the ETP protein Asx. *trx* mutation resulted in a complete loss of Asx binding to polytene chromosomes (Fig. 4A). These results are consistent with those above showing that response elements of *trx* and *Asx* reside in the same small C1 DNA element of the *bxd* ME (Fig. 1A,B, Table 2), as well as with the fact that Trx and Asx proteins interact directly (J. Hodgson, personal communication). The results indicate that, like Trx and Ash1, Trx and Asx are intimately related in their functioning at *Ubx* and other common target genes. Together, our results suggest that these three proteins, Trx, Ash1 and Asx, might be involved in direct interactions on the *bxd* ME.

By contrast, we were not able to detect any significant differences in association of Kis, Brm and Osa with salivary gland polytene chromosomes in the *trx* RNAi line (Fig. 4B). This is consistent with the absence of Kis response elements in the *bxd* ME (Fig. 1B, Table 1), and suggests that Kis might not be directly involved in the functioning of the epigenetic MEs, at least in the salivary glands. However, the results for Brm and Osa, as components of the BRM complex that are associated in close proximity to Trx on the *bxd* ME (Fig. 1B, Table 2) and can genetically and physically interact with TAC1, are very surprising. They imply that this complex functions completely independently of TAC1. They also suggest that there is no overall cooperativity in the association of trxG proteins with the *bxd* ME, and that only a subset of trxG proteins is recruited to this element synergistically.

We did not find significant differences in the association of the components of the two major PcG complexes, PRC1 (Pc and Ph) and PRC2 [E(z)], with their sites on polytene chromosomes in the

trx RNAi line (Fig. 4C). Since binding of Ash1 and Asx is strongly affected (Fig. 4A), these results also imply that PcG proteins function independently of Ash1 and Asx. We did not detect any increase in the number or intensity of the Pc and Ph polytene bands in the *trx* mutant larvae, suggesting that removing trxG proteins from their binding sites does not necessarily lead to enhanced binding of the PcG proteins. It is therefore likely that there is no continuous direct competition between these two groups of opposing regulators for binding to their neighboring response elements.

DISCUSSION

Despite much interest, there is little understanding of how the epigenetic TRE/PRE-containing MEs function. One key unresolved issue pertains to the organization of these complex transcription regulatory elements with regard to the response elements/binding sites of particular trxG and PcG proteins. Response elements for several PcG proteins were mapped in the *bxd* ME previously (Tillib et al., 1999), and some PcG proteins were detected at this DNA element in ChIP assays (Cao et al., 2002; Papp and Muller, 2006). However, information about the association of trxG proteins in the *bxd* ME is very limited. We previously mapped several Trx-dependent TREs in the *bxd* ME (Tillib et al., 1999). In addition, we and others have detected Trx and Ash1 proteins at the *bxd* ME in ChIP assays (Papp and Muller, 2006; Petruk et al., 2007; Petruk et al., 2006). Given the apparent functional heterogeneity of the trxG proteins, it is revealing that besides Trx, many other trxG genes are essential for functioning of the *bxd* ME. Two of the interacting genes, *skd* and *kto*, encode components of the *Drosophila* Mediator complex (Janody et al., 2003), so it is possible that their role in the functioning of the *bxd* ME relates to the transcription of some of the non-coding RNAs that are known to be transcribed through this element [Petruk et al. (Petruk et al., 2006) and references therein]. Ash2 is a component of several purified MLL (a human homolog of Trx) protein complexes (Dou et al., 2005; Nakamura et al., 2002; Yokoyama et al., 2004). The identification of an *ash2* response element in the *bxd* ME suggests that a second putative Trx-containing MLL-like complex might reside at the *bxd* ME. The genes *urd* and *sls* have only been minimally characterized, mainly as suppressors of *Pc* phenotypes. Therefore, it is premature to speculate about their function at this element, although they clearly interact there in some capacity.

Identification of multiple TREs and PREs within the same ME raises an important question with regard to potential interdependency or competition in the association of proteins from the same and different protein families. To address this, we focused on the fine mapping of response elements for several major trxG genes that are essential for functioning of the *bxd* ME: *ash1*, the *brm* component of the BRM chromatin remodeling complex, and the ETP gene *Asx*. These proteins or components of their protein complexes (i.e. Snr1, a component of BRM) can physically associate with Trx (Rozenblatt-Rosen et al., 1998; Rozovskaia et al., 1999) (J. Hodgson, personal communication). Thus, finding their response elements either in DNA fragments that are juxtaposed to (*brm* and *ash1*) or the same as (*Asx*) the previously mapped *trx* response element is consistent with direct interactions of these proteins with Trx. It should be noted, however, that all these proteins are components of protein complexes other than the Trx complex TAC1 (Papoulas et al., 1998; Petruk et al., 2001). Nevertheless, this suggests that there might be interdependency in recruitment and/or association of these protein complexes at the *bxd* ME. However, our results indicate that this suggestion is only partially true. Binding of the components of the BRM complex and of another trxG protein, Kis, were not affected by elimination of Trx. However, the association of Ash1 and Asx at all their sites on the salivary gland polytene chromosomes is completely dependent on the presence of Trx. Previous results of the reciprocal experiments indicated that binding of Trx is strongly decreased in *ash1* mutant animals (Kuzin et al., 1994). This suggests that Trx, Ash1 and Asx represent a special, and at least partially interdependent, set of trxG proteins. This also suggests, in contrast to the previously mentioned genetic studies, that not all trxG proteins are mutually dependent in their functioning.

Close proximity or even overlap between some TREs and PREs in the *bxd* ME suggests the existence of potential competitive relationships with regard to the binding of these functionally opposing groups of proteins. Furthermore, some ChIP assays indicate that some trxG and PcG proteins can bind to the *bxd* ME of both the activated and silenced gene (Papp and Muller, 2006), suggesting a potential interaction of these proteins on DNA. We tested this by asking whether binding of the components of two major PcG complexes, PRC1 and PRC2, is affected by elimination of Trx. We did not detect any significant change in the number or intensity of immunostained bands for all tested PcG proteins on the polytene chromosomes of *trx* mutant larvae. This suggests that not only is the association of PcG proteins independent of Trx, but also that Trx is not essential for preventing binding of the PcG proteins to their response elements. This is an important conclusion because some genetic studies have proposed that the main function of Trx and Ash1 is to prevent silencing by the PcG proteins (Klymenko and Muller, 2004).

An important issue in understanding the molecular mechanism of trxG/PcG functioning is to correlate their association at MEs with the state of expression of their target genes. Although most of the existing data were obtained in cultured cells, two studies addressed this issue in *Drosophila* larval tissues. ChIP analysis in larval imaginal discs suggests that some trxG and PcG proteins are associated with the *bxd* ME irrespective of the status of gene expression (Papp and Muller, 2006). However, the results of another study suggest alternative association of Trx and Pc at the site of the endogenous BX-C on polytene chromosomes from both fat body and salivary glands, where BX-C is correspondingly activated or repressed (Marchetti et al., 2003). Ideally, to resolve this issue it is essential to investigate the association of PcG and trxG proteins with the ME in the same tissue at the single-cell level and at a gene of defined expression status. We established such a test system in which the *bxd*-ME-containing transgene is either activated or repressed in cells within the same salivary gland. Direct visualization of the association of different proteins to the site of insertion of this transgene clearly indicates that major trxG and PcG proteins bind to the *bxd* ME in an alternative fashion. Importantly, using markers for activated and repressed transcription, we were able to correlate binding of trxG and PcG proteins in a single cell with either the activated or repressed *bxd* transgene, respectively. The differences between our results and those of Papp and Mueller (Papp and Muller, 2006) might be explained by technical differences and by the fact that trxG and PcG proteins may behave differently in different tissues and/or in polyploid versus diploid cells. It is important to note that although our analysis is limited to studies of a transgene, the detected alternative association of Trx and Pc on the *bxd* ME transgene correlates well with the results obtained at the endogenous BX-C on polytene chromosomes (Marchetti et al., 2003). We conclude, therefore, that on a cell-by-cell basis, binding

of trxG and PcG proteins is strictly dependent on the status of gene expression, in that they bind alternatively to the epigenetic regulatory elements of either activated or repressed target genes, respectively.

In summary, this is the first work on the fine mapping of multiple TREs at any target gene. This is also the first assessment of mutual dependencies within the trxG group of activators and between the trxG and PcG of antagonistic proteins. It provides a glance of the enormously complex regulatory element that binds proteins with opposite transcriptional regulatory activities. The main conclusions of this study are that two major trxG proteins, Trx and Ash1, and the ETP protein Asx, constitute a specific subgroup of interacting proteins that depend on each other in their functioning at the *bxd* ME and throughout the genome. Although multiple trxG proteins are essential for epigenetic functioning of the *bxd* ME, their association with this element and other binding sites in the genome might not necessarily require Trx and associated proteins, as exemplified by the components of the BRM complex and Kis. The components of the major PcG complexes, PRC1 and PRC2, also associate with target genes independently of Trx, Ash1 and Asx. Another important conclusion of this work is that trxG and PcG proteins are associated with the *bxd* ME only at activated and repressed genes, respectively. It will be important to determine whether the choice between the establishment of trxG-mediated activation or PcG-mediated repression occurs only at very specific early stages of development, or whether it can also occur at later developmental stages.

We thank H. Brock, J. Hodgson and J. Jaynes for critical comments on the manuscript, J. Tamkun, R. Jones and H. Brock for antibodies and J. Kennison, A. Shearn and H. Brock for mutant stocks. This work was supported by grants NIH 1R01GM075141 and March of Dimes 6-FY06-346 to A.M.

References

Bantignies, F., Goodman, R. H. and Smolik, S. M. (2000). Functional interaction between the coactivator Drosophila CREB-binding protein and ASH1, a member of the trithorax group of chromatin modifiers. *Mol. Cell. Biol.* **20**, 9317-9330.

Beisel, C., Imhof, A., Greene, J., Kremmer, E. and Sauer, F. (2002). Histone methylation by the Drosophila epigenetic transcriptional regulator Ash1. *Nature* **419**, 857-862.

Brock, H. W. and van Lohuizen, M. (2001). The Polycomb group-no longer an exclusive club? *Curr. Opin. Genet. Dev.* **11**, 175-181.

Busturia, A., Lloyd, A., Bejarano, F., Zavortink, M., Xin, H. and Sakonju, S. (2001). The MCP silencer of the Drosophila Abd-B gene requires both Pleiohomeotic and GAGA factor for the maintenance of repression. *Development* **128**, 2163-2173.

Byrd, K. N. and Shearn, A. (2003). ASH1, a Drosophila trithorax group protein, is required for methylation of lysine 4 residues on histone H3. *Proc. Natl. Acad. Sci. USA* **100**, 11535-11540.

Cao, R., Wang, L., Wang, H., Xia, L., Erdjument-Bromage, H., Tempst, P., Jones, R. S. and Zhang, Y. (2002). Role of histone H3 lysine 27 methylation in Polycomb-group silencing. *Science* **298**, 1039-1043.

Chan, C. S., Rastelli, L. and Pirrotta, V. (1994). A Polycomb response element in the Ubx gene that determines an epigenetically inherited state of repression. *EMBO J.* **13**, 2553-2564.

Chang, Y. L., King, B. O., O'Connor, M., Mazo, A. and Huang, D. H. (1995). Functional reconstruction of trans regulation of the Ultrabithorax promoter by the products of two antagonistic genes, trithorax and Polycomb. *Mol. Cell. Biol.* **15**, 6601-6612.

Chiang, A., O'Connor, M. B., Paro, R., Simon, J. and Bender, W. (1995). Discrete Polycomb-binding sites in each parasegmental domain of the bithorax complex. *Development* **121**, 1681-1689.

Collins, R. T. and Treisman, J. E. (2000). Osa-containing Brahma chromatin remodeling complexes are required for the repression of wingless target genes. *Genes Dev.* **14**, 3140-3152.

Crosby, M. A., Miller, C., Alon, T., Watson, K. L., Verrijzer, C. P., Goldman-Levi, R. and Zak, N. B. (1999). The trithorax group gene moira encodes a brahma-associated putative chromatin-remodeling factor in Drosophila melanogaster. *Mol. Cell. Biol.* **19**, 1159-1170.

Czermin, B., Melfi, R., McCabe, D., Seitz, V., Imhof, A. and Pirrotta, V. (2002). Drosophila enhancer of Zeste/ESC complexes have a histone H3 methyltransferase activity that marks chromosomal Polycomb sites. *Cell* **111**, 185-196.

Daubresse, G., Deuring, R., Moore, L., Papoulas, O., Zakrajsek, I., Waldrip, W. R., Scott, M. P., Kennison, J. A. and Tamkun, J. W. (1999). The Drosophila kismet gene is related to chromatin-remodeling factors and is required for both segmentation and segment identity. *Development* **126**, 1175-1187.

Dou, Y., Milne, T. A., Tackett, A. J., Smith, E. R., Fukuda, A., Wysocka, J., Allis, C. D., Chait, B. T., Hess, J. L. and Roeder, R. G. (2005). Physical association and coordinate function of the H3 K4 methyltransferase MLL1 and the H4 K16 acetyltransferase MOF. *Cell* **121**, 873-885.

Francis, N. J., Saurin, A. J., Shao, Z. and Kingston, R. E. (2001). Reconstitution of a functional core polycomb repressive complex. *Mol. Cell* **8**, 545-556.

Fritsch, C., Brown, J. L., Kassis, J. A. and Muller, J. (1999). The DNA-binding polycomb group protein pleiohomeotic mediates silencing of a drosophila homeotic gene. *Development* **126**, 3905-3913.

Gildea, J. J., Lopez, R. and Shearn, A. (2000). A screen for new trithorax group genes identified little imaginal discs, the Drosophila melanogaster homologue of human retinoblastoma binding protein 2. *Genetics* **156**, 645-663.

Grimaud, C., Negre, N. and Cavalli, G. (2006). From genetics to epigenetics: the tale of Polycomb group and trithorax group genes. *Chromosome Res.* **14**, 363-375.

Hagstrom, K., Muller, M. and Schedl, P. (1996). Fab-7 functions as a chromatin domain boundary to ensure proper segment specification by the Drosophila bithorax complex. *Genes Dev.* **10**, 3202-3215.

Hodgson, J. W., Argiropoulos, B. and Brock, H. W. (2001). Site-specific recognition of a 70-base-pair element containing d(GA)(n) repeats mediates bithoraxoid polycomb group response element-dependent silencing. *Mol. Cell. Biol.* **21**, 4528-4543.

Horard, B., Tatout, C., Poux, S. and Pirrotta, V. (2000). Structure of a polycomb response element and in vitro binding of polycomb group complexes containing GAGA factor. *Mol. Cell. Biol.* **20**, 3187-3197.

Janody, F., Martirosyan, Z., Benlali, A. and Treisman, J. E. (2003). Two subunits of the Drosophila mediator complex act together to control cell affinity. *Development* **130**, 3691-3701.

Klymenko, T. and Muller, J. (2004). The histone methyltransferases Trithorax and Ash1 prevent transcriptional silencing by Polycomb group proteins. *EMBO Rep.* **5**, 373-377.

Kuzin, B., Tillib, S., Sedkov, Y., Mizrokhi, L. and Mazo, A. (1994). The Drosophila trithorax gene encodes a chromosomal protein and directly regulates the region-specific homeotic gene fork head. *Genes Dev.* **8**, 2478-2490.

Kuzmichev, A., Nishioka, K., Erdjument-Bromage, H., Tempst, P. and Reinberg, D. (2002). Histone methyltransferase activity associated with a human multiprotein complex containing the Enhancer of Zeste protein. *Genes Dev.* **16**, 2893-2905.

LaJeunesse, D. and Shearn, A. (1996). E(z): a polycomb group gene or a trithorax group gene? *Development* **122**, 2189-2197.

Marchetti, M., Fanti, L., Berloco, M. and Pimpinelli, S. (2003). Differential expression of the Drosophila BX-C in polytene chromosomes in cells of larval fat bodies: a cytological approach to identifying in vivo targets of the homeotic Ubx, Abd-A and Abd-B proteins. *Development* **130**, 3683-3689.

Milne, T. A., Sinclair, D. A. and Brock, H. W. (1999). The Additional sex combs gene of Drosophila is required for activation and repression of homeotic loci, and interacts specifically with Polycomb and super sex combs. *Mol. Gen. Genet.* **261**, 753-761.

Muller, J., Hart, C. M., Francis, N. J., Vargas, M. L., Sengupta, A., Wild, B., Miller, E. L., O'Connor, M. B., Kingston, R. E. and Simon, J. A. (2002). Histone methyltransferase activity of a Drosophila Polycomb group repressor complex. *Cell* **111**, 197-208.

Nakamura, T., Mori, T., Tada, S., Krajewski, W., Rozovskaia, T., Wassell, R., Dubois, G., Mazo, A., Croce, C. M. and Canaani, E. (2002). ALL-1 is a histone methyltransferase that assembles a supercomplex of proteins involved in transcriptional regulation. *Mol. Cell* **10**, 1119-1128.

Negre, N., Hennetin, J., Sun, L. V., Lavrov, S., Bellis, M., White, K. P. and Cavalli, G. (2006). Chromosomal distribution of PcG proteins during Drosophila development. *PLoS Biol.* **4**, e170.

Orlando, V., Jane, E. P., Chinwalla, V., Harte, P. J. and Paro, R. (1998). Binding of trithorax and Polycomb proteins to the bithorax complex: dynamic changes during early Drosophila embryogenesis. *EMBO J.* **17**, 5141-5150.

Papoulas, O., Beek, S. J., Moseley, S. L., McCallum, C. M., Sarte, M., Shearn, A. and Tamkun, J. W. (1998). The Drosophila trithorax group proteins BRM, ASH1 and ASH2 are subunits of distinct protein complexes. *Development* **125**, 3955-3966.

Papp, B. and Muller, J. (2006). Histone trimethylation and the maintenance of transcriptional ON and OFF states by trxG and PcG proteins. *Genes Dev.* **20**, 2041-2054.

Petruk, S., Sedkov, Y., Smith, S., Tillib, S., Kraevski, V., Nakamura, T., Canaani, E., Croce, C. M. and Mazo, A. (2001). Trithorax and dCBP acting in a complex to maintain expression of a homeotic gene. *Science* **294**, 1331-1334.

Petruk, S., Sedkov, Y., Riley, K. M., Hodgson, J., Schweisguth, F., Hirose, S., Jaynes, J. B., Brock, H. W. and Mazo, A. (2006). Transcription of bxd noncoding RNAs promoted by trithorax represses Ubx in cis by transcriptional interference. *Cell* **127**, 1209-1221.

Petruk, S., Sedkov, Y., Brock, H. W. and Mazo, A. (2007). A model for initiation of mosaic HOX gene expression patterns by non-coding RNAs in early embryos. *RNA Biol.* **4**, 1-6.

Rozenblatt-Rosen, O., Rozovskaia, T., Burakov, D., Sedkov, Y., Tillib, S., Blechman, J., Nakamura, T., Croce, C. M., Mazo, A. and Canaani, E. (1998). The C-terminal SET domains of ALL-1 and TRITHORAX interact with the INI1 and SNR1 proteins, components of the SWI/SNF complex. *Proc. Natl. Acad. Sci. USA* **95**, 4152-4157.

Rozovskaia, T., Tillib, S., Smith, S., Sedkov, Y., Rozenblatt-Rosen, O., Petruk, S., Yano, T., Nakamura, T., Ben-Simchon, L., Gildea, J. et al. (1999). Trithorax and ASH1 interact directly and associate with the trithorax group-responsive bxd region of the Ultrabithorax promoter. *Mol. Cell. Biol.* **19**, 6441-6447.

Schwartz, Y. B., Kahn, T. G., Nix, D. A., Li, X. Y., Bourgon, R., Biggin, M. and Pirrotta, V. (2006). Genome-wide analysis of Polycomb targets in Drosophila melanogaster. *Nat. Genet.* **38**, 700-705.

Simon, J., Chiang, A., Bender, W., Shimell, M. J. and O'Connor, M. (1993). Elements of the Drosophila bithorax complex that mediate repression by Polycomb group products. *Dev. Biol.* **158**, 131-144.

Sinclair, D. A., Campbell, R. B., Nicholls, F., Slade, E. and Brock, H. W. (1992). Genetic analysis of the additional sex combs locus of Drosophila melanogaster. *Genetics* **130**, 817-825.

Smith, S. T., Petruk, S., Sedkov, Y., Cho, E., Tillib, S., Canaani, E. and Mazo, A. (2004). Modulation of heat shock gene expression by the TAC1 chromatin-modifying complex. *Nat. Cell Biol.* **6**, 162-167.

Srinivasan, S., Armstrong, J. A., Deuring, R., Dahlsveen, I. K., McNeill, H. and Tamkun, J. W. (2005). The Drosophila trithorax group protein Kismet facilitates an early step in transcriptional elongation by RNA Polymerase II. *Development* **132**, 1623-1635.

Strutt, H., Cavalli, G. and Paro, R. (1997). Co-localization of Polycomb protein and GAGA factor on regulatory elements responsible for the maintenance of homeotic gene expression. *EMBO J.* **16**, 3621-3632.

Tanaka, Y., Katagiri, Z., Kawahashi, K., Kioussis, D. and Kitajima, S. (2007). Trithorax-group protein ASH1 methylates histone H3 lysine 36. *Gene* **397**, 161-168.

Tillib, S., Petruk, S., Sedkov, Y., Kuzin, A., Fujioka, M., Goto, T. and Mazo, A. (1999). Trithorax- and Polycomb-group response elements within an Ultrabithorax transcription maintenance unit consist of closely situated but separable sequences. *Mol. Cell. Biol.* **19**, 5189-5202.

Wang, H., Wang, L., Erdjument-Bromage, H., Vidal, M., Tempst, P., Jones, R. S. and Zhang, Y. (2004). Role of histone H2A ubiquitination in Polycomb silencing. *Nature* **431**, 873-878.

Wang, L., Brown, J. L., Cao, R., Zhang, Y., Kassis, J. A. and Jones, R. S. (2004). Hierarchical recruitment of polycomb group silencing complexes. *Mol. Cell* **14**, 637-646.

Yokoyama, A., Wang, Z., Wysocka, J., Sanyal, M., Aufiero, D. J., Kitabayashi, I., Herr, W. and Cleary, M. L. (2004). Leukemia proto-oncoprotein MLL forms a SET1-like histone methyltransferase complex with menin to regulate Hox gene expression. *Mol. Cell. Biol.* **24**, 5639-5649.