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Transcriptional elongation of non-coding *bxd* RNAs promoted by the Trithorax TAC1 complex represses *Ubx* by a transcriptional interference mechanism

Svetlana Petruk[1], **Yurii Sedkov**[1], **Kristen M. Riley**[1], **Jacob Hodgson**[2], **Francois Schweisguth**[3], **Susumu Hirose**[4], **James B. Jaynes**[1], **Hugh W. Brock**[2], and **Alexander Mazo**[1,*]

1 *Department of Biochemistry, Thomas Jefferson University, Philadelphia, PA 19107, USA*

2 *Department of Zoology, University of British Columbia, 6270 University Boulevard, V6T 1Z4, Vancouver, BC, Canada*

3 *Ecole Normale Superieure, CNRS UMR 8542, Paris, France*

4 *Department of Developmental Genetics, National Institute of Genetics, and Graduate University for Advanced Studies, Mishima, Shizuoka-ken 411-8540, Japan*

Summary

Much of the genome is transcribed into long non-coding RNAs (ncRNAs). Previous data suggested that *bithoraxoid* (*bxd*) ncRNAs of the Drosophila bithorax complex prevent silencing of *Ultrabithorax* (*Ubx*), and recruit activating proteins of the trithorax group to their maintenance elements. We found that, surprisingly, *Ubx* and several *bxd* ncRNAs are expressed in non-overlapping patterns in both embryos and imaginal discs, suggesting that transcription of these ncRNAs is associated with repression, not activation, of *Ubx*. Our data rule out siRNA or miRNA-based mechanisms for repression by *bxd* ncRNAs. Rather, ncRNA transcription itself, acting in *cis*, represses *Ubx*. The Trithorax complex TAC1 binds the *Ubx* coding region in nuclei expressing *Ubx*, and the *bxd* region in nuclei not expressing *Ubx*. We propose that TAC1 promotes the mosaic pattern of *Ubx* expression by facilitating transcriptional elongation of *bxd* ncRNAs, which represses *Ubx* transcription.

Introduction

The Hox genes of the bithorax complex (BX-C) have spatially restricted expression patterns that vary within and between segments and tissues. Transcription factors encoded by segmentation genes (Carroll et al., 1988; Irish et al., 1989) establish the patterns of the Hox genes *Ubx*, *abd-A*, and *Abd-B* of the BX-C in embryos. After the segmentation proteins decay, Hox expression patterns are maintained epigenetically by proteins of the trithorax group (trxG) and the Polycomb group (PcG) (Grimaud et al., 2006b). PcG genes maintain the silent state, whereas trxG genes maintain the active state of Hox genes. PcG and trxG proteins act through partially overlapping sets of response elements known as maintenance elements (MEs, as in Figure 1A) (Hodgson et al., 2001; Pirrotta et al., 1995; Tillib et al., 1999).

* Correspondence: mazo@mail.tju.edu.

One of the most startling discoveries of the genomic era has been that much of the genome is transcribed into non-coding RNAs (ncRNAs) (Eddy, 2002; Gottesman, 2002; Mattick and Makunin, 2006). Recent attention has focused on small interfering RNAs (siRNAs) and micro-RNAs (miRNAs) that modulate gene activity by an antisense mechanism termed RNA interference (RNAi), which interferes with mRNA stability or translation (Carthew, 2006; Massirer and Pasquinelli, 2006; Sen and Blau, 2006). However, the most abundant and least characterized class of ncRNAs are long and have mostly unknown functions (Goodrich and McClure, 1991; Mattick and Makunin, 2006).

The intergenic regions of the Hox genes in *D. melanogaster* produce many long ncRNAs that may regulate Hox gene coding sequences. Increasing attention has been directed to the role of transcription of MEs in the regulation of BX-C genes. Several ncRNAs are transcribed through a well-studied ME in the *bxd* regulatory region that lies between the *Ubx* and *abd-A* transcription units (Cumberledge et al., 1990; Lipshitz et al., 1987; Sanchez-Herrero and Akam, 1989). The *bxd* ME regulates *Ubx* (Chan et al., 1994; Muller and Bienz, 1991; Simon et al., 1993) (Figure 1A). Transcription through *bxd* precedes activation of *Ubx* coding RNAs (hereafter referred to as "*Ubx* RNA", or simply as "*Ubx*"), suggesting that ncRNAs might regulate *Ubx* (Rank et al., 2002). Transcription patterns of ncRNAs appear similar to those of the neighboring Hox genes and are collinear with regulatory domains along the chromosome (Bae et al., 2002). A synthesis of genetic (Bender and Fitzgerald, 2002; Hogga and Karch, 2002; Rank et al., 2002) and transgenic studies (Schmitt et al., 2005) led to the idea that transcription of ncRNAs through MEs interferes with PcG-mediated silencing, perhaps by preventing recruitment of PcG proteins. A recent study suggests that transcription of MEs may recruit trxG proteins to maintenance elements (Sanchez-Elsner et al., 2006). If indeed transcription of MEs simultaneously prevents PcG binding and establishes trxG binding, this could be a key element of Hox regulation. Clearly, this model requires that intergenic *bxd* RNAs are expressed in the same cells as *Ubx*. However double-labeling of intergenic and coding RNAs at high resolution has not been performed, so this attractive model has not been rigorously tested.

The trxG protein complex TAC1 plays important roles in maintaining expression of homeotic genes throughout embryogenesis (Petruk et al., 2001). Recent attention has focused on the role of trxG proteins in histone modifications and in altering nucleosome positioning (Beisel et al., 2002; Smith et al., 2004). TAC1 contains three proteins, Trx, Sbf1 and dCBP, and thus acetylates histones and methylates histone H3 at Lys-4 (H3-K4), due to the enzymatic activities of dCBP and the SET domain of Trx, respectively (Petruk et al., 2001; Smith et al., 2004). How binding of Trx to MEs regulates expression of Hox genes is unclear. Because embryos have a mixture of cells expressing and not expressing each Hox gene, it has not been possible to determine precisely how trxG protein binding correlates with transcription of *Ubx* and *bxd* ncRNAs.

In this study, we show that *Ubx* is repressed by *bxd* transcription. The *bxd* ncRNAs do not act by siRNA or miRNA-based mechanisms, but repress *Ubx* in *cis* through a transcription-dependent mechanism. Alternative association of TAC1 with either *Ubx* or *bxd* correlates with their transcription. TAC1 appears to be part of an interdependent network of general elongation factors that associate with active genes. We suggest that a key role of TAC1 in establishing the mosaic pattern of *Ubx* expression is to promote elongation of *bxd* ncRNAs, which in turn represses expression of *Ubx*.

Results

Ubx and intergenic ncRNAs are expressed in different cells in embryos

We concentrated on *Ubx*, and the upstream *bxd* region because multiple ncRNAs are transcribed through the *bxd* region (Figure 1A). Two promoters of *bxd* ncRNAs (P1 and P2 in Figures 1A and 2C) are localized upstream of the *bxd* ME, and two others lie downstream (P3, P4). The *bxd* regulatory region contains several *trx*-responsive elements as separable regions within the ME (Tillib et al., 1999).

The development of a high-resolution, multiplex RNA *in situ* hybridization technique (Kosman et al. 2004) allows one to test the hypothesis that *bxd* ncRNAs are necessary for activation of *Ubx*. The model requires that *Ubx* and *bxd* ncRNAs be expressed in the same cells. We compared expression patterns of *Ubx* with those of *bxd* ncRNAs using 4 probes that are specific to several exons described previously (Lipshitz et al., 1987), as well as a probe that includes three transcripts (Sanchez-Elsner et al., 2006) ("*bxd*" in Figure 1A) described previously as giving a *Ubx*-like pattern (Rank et al., 2002). Expression of all tested *bxd* ncRNAs begins at syncytial blastoderm, and precedes that of *Ubx* (probes 1–7 and *bxd* in Figure 1B; *bxd* ncRNAs 1–8, 4 and 5: data not shown). At cellular blastoderm, the initial domain of *Ubx* expression is just anterior to the primary domains of ncRNA expression. During germband elongation, *Ubx* and *bxd* ncRNAs are expressed within the same restricted portion of the embryo, as reported previously. At all embryonic stages, no *bxd* ncRNA is expressed in cells that express *Ubx* (Figure 1B and data not shown). *Ubx* is expressed in the posterior, while *bxd* ncRNAs are expressed in the anterior, of each parasegment 6–13. This expression of *Ubx* and *bxd* ncRNAs in alternative sets of cells is confirmed by results described below using sorted nuclei.

The *bxd* ncRNA 1–7 shown in Figure 1B is expressed in a region that is more restricted dorsoventrally than that of *Ubx*. Overall, different *bxd* ncRNAs show significant overlap in their expression domains, which have the same anterior-posterior boundary. This overlap may be because these RNAs share alternatively spliced small exons. Strikingly, *bxd* ncRNAs are expressed in different germ layers (Figure 1C), suggesting that expression of individual *bxd* ncRNAs may be driven by different tissue-specific regulatory elements.

Elimination of intergenic transcripts leads to ectopic expression of *Ubx*

Our findings argue strongly that *bxd* ncRNAs cannot activate, but may instead repress, *Ubx*. To test this idea, we analyzed expression of *Ubx* in mutants that carry deletions in the transcription units of ncRNAs. The pbx^1 mutation that causes homeotic defects in adults, deletes promoter P1 and the first exon of two ncRNAs, 1–7 and 1–8, while the pbx^2 mutation deletes promoter regions P1, P2 and P4, and the 5'-exons of these and several other ncRNAs (Irvine et al., 1991) (Figures 1A and S2). Consistent with these molecular lesions, the corresponding *bxd* ncRNAs are not expressed in pbx^1 and pbx^2 mutant embryos (Figure 1D). Importantly, absence of *bxd* ncRNA is accompanied by clear ectopic expression of *Ubx* in the posterior region of the embryo where these *bxd* RNAs are expressed in wildtype embryos (Figure 1D), consistent with a role for *bxd* ncRNAs in *Ubx* repression. This ectopic expression of *Ubx* is very unlikely to be caused by deletion of an unmapped PcG response element, because mis-expression of *Ubx* in PcG maternal and zygotic homozygous mutant embryos is not yet detectable at this stage of embryogenesis (Soto et al., 1995; Struhl and Akam, 1985).

ncRNAs do not repress *Ubx* by RNAi-based mechanisms

Grimaud et al. (Grimaud et al., 2006a) report that mutations in genes required for RNA interference (RNAi) have no homeotic phenotypes, suggesting that RNAi-based mechanisms are not essential to the regulation of *Ubx*. To test directly whether *bxd* ncRNAs repress *Ubx* by RNAi, we prepared a mixture of dsRNAs that are specific to *bxd* RNA exons 1, 3 and 7,

and to other *bxd* RNAs mapped previously (Sanchez-Elsner et al., 2006) (grey bars in Figure 1A) and introduced these into embryos using two strategies. First, we injected dsRNA into adult females, where it is taken up by oocytes (Dzitoyeva et al., 2003), and second, we injected the dsRNAs into preblastoderm embryos. These dsRNAs diffuse through the oocyte or embryo, and will eliminate any RNAs containing homologous sequences by RNAi.

As shown in Figure 2A, introduction of dsRNA by either method leads to an almost complete elimination of the products of the corresponding ncRNAs. In all tested embryos, elimination of *bxd* ncRNAs had no effect on *Ubx* expression (Figure 2A). This shows that an RNAi-based degradation mechanism (i.e. siRNA) cannot be responsible for repression of *Ubx*. Note that because our injected dsRNA did not cause *Ubx* degradation, we have also eliminated the possibility that our probes contain sequences that are normally responsible for degradation of *Ubx*. We also carefully examined expression of a reporter transgene, that mimics the *Ubx* pattern, but does not contain the *Ubx* 3' UTR, a common target of miRNA-based translational repression (Figure 2C). There is no apparent ectopic expression of this reporter in regions of *bxd* transcription, arguing against miRNA-dependent translational repression (Figure 2B). These experiments together with the lack of homeotic phenotypes in RNAi mutant flies argue that ncRNAs do not act in *trans*, and suggest that repression is due to a *cis*-acting mechanism associated with *bxd* transcription per se.

Ubx expression is repressed by transcription from the promoters of ncRNAs

Transcription-based mechanisms of repression, namely promoter competition or transcriptional interference (Martens et al., 2004), offer an attractive possibility for *cis*-repression of *Ubx* by *bxd* ncRNAs. These *cis*-repression models predict that deletion of the promoter of an ncRNA will lead to expression of the repressed promoter in cells where these RNAs are normally transcribed. To test this, we used a *Ubx* transgene that closely mimics expression of endogenous *Ubx* in mid-to-late stage embryos (Tillib et al., 1999). This construct lacks promoters P1 and P4 which drive several ncRNAs, but contains promoters P2 (in the *bxd* region) and P5 (Figure 2C). The absence of these two promoters in the transgene leads to ectopic expression of the *GFP* reporter gene in the posterior region of blastoderm embryos (Figure 2D). During germband elongation, *GFP* is clearly expressed in some mesodermal regions where endogenous *Ubx* is not expressed, suggesting that the absence of transcription from the P1 and P4 promoters causes a loss of *cis*-repression of *Ubx* transcription in some mesodermal cells.

The *cis*-repression model of *bxd* ncRNA function predicts that the expanded domain of *GFP* expression from the transgene lacking P1 and P4 promoters should correspond to the domains of *bxd* ncRNAs normally transcribed from the endogenous promoters (see Figure 2E). As predicted, *GFP* expression significantly overlaps with that of the endogenous *bxd nc*RNAs 1–7 and 4, which are produced from the P1 and P4 promoters deleted in our transgene, in the posterior region of the blastoderm embryo (Figure 2F). Moreover, this overlap of *GFP* with ncRNAs in the mesodermal regions continues into germband extension (compare Figures. 2D and 2F, right panels). In contrast, endogenous *Ubx* does not overlap with any *bxd* ncRNA in embryos at either stage (Figures 1C and 2G). These results strongly corroborate a mechanism of *Ubx* repression by *bxd* transcription that acts in *cis*.

Ubx may be repressed by a transcriptional interference mechanism

Promoter competition occurs when nearby promoters, like those of *bxd* ncRNAs and *Ubx*, compete for rate-limiting transcription factors. The alternative *cis*-acting mechanism of transcriptional interference occurs when Pol II does not terminate at the 3'-exon of an upstream RNA, but proceeds through a promoter or enhancer, disrupting essential protein interactions

with these regulatory elements. The results above cannot distinguish between these two mechanisms.

To address this issue, we asked whether transcription of *bxd* ncRNAs proceeds to the vicinity of the *Ubx* promoter. First, we tested by RT-PCR for the presence of transcripts in the 8kb region between the *bxd* ncRNA 3'-exons 7 and 8 and the *Ubx* start site (Figure 3A). As Figure 3B shows, cDNA synthesized from the primer located just upstream of the *Ubx* promoter contains sequences both from the vicinity of the *Ubx* promoter and exons 7 and 8 of *bxd* ncRNAs, as well as from intervening sequences. This suggests that Pol II transcribes *bxd* ncRNAs and continues to the vicinity of the *Ubx* promoter. To test whether these read-through RNAs are expressed in the cells where *Ubx* is repressed, we used a probe to the upstream regulatory region of *Ubx* (P5 in Figure 3A) for *in situ* hybridization. Figure 3C shows that there is almost complete overlap of RNA from the vicinity of the *Ubx* promoter with the 3'-exon 7 of ncRNAs. Importantly, as for the *bxd* ncRNAs in general, synthesis of these ncRNAs precedes synthesis of *Ubx*, and occurs in cells not expressing *Ubx* (Figure 3D), confirming that these read-through RNA products correlate with *Ubx* repression.

ncRNAs are not required for activation of *Ubx*

Our data suggest that expression of *bxd* ncRNAs represses expression of *Ubx* in embryos, in contrast to a report that expression of *bxd* ncRNAs (Figure 1A) are required for activation of *Ubx* in larval imaginal discs and in S2 cells by specific recruitment of the trxG protein Ash1 (Sanchez-Elsner et al., 2006). Therefore we re-examined the proposed activating role of ncRNAs in larval discs.

Our RT-PCR and *in situ* hybridization results (Figures 4A,B and S3) show that *Ubx* is expressed at low levels in wing disks, in agreement with studies showing that *Ubx* is expressed in the peripodial membrane but not in the epithelium proper (Brower, 1987; Pallavi and Shashidhara, 2003), but in contrast to recent reports (Sanchez-Elsner et al., 2006). Importantly, *bxd* ncRNAs detected by exon 1, exon 5 and *bxd* probes are not expressed in any of the three tested larval discs at any significant levels (Figures 4A,B and S3), agreeing with previous data for exon 1 and exon 5 RNAs in larval imaginal discs (Lipshitz et al., 1987), but disagreeing with (Sanchez-Elsner et al., 2006).

The *Ubx-GFP* transgene used above (Figure 2C) ectopically expresses exon 5 and the three ncRNAs ("*bxd*" in Figure 1A) described by Sanchez-Elsner et al. (2006) in wing, 3rd leg, and haltere discs (Figure 4A,B). Sanchez-Elsner (2006) report that overexpression of each of these transcripts induces expression of endogenous *Ubx* in wing discs. We show by RT-PCR that expression of *bxd* RNA from the transgene does not lead to an increase in *Ubx* expression, even in the wing disc which expresses low levels of *Ubx* endogenously (Figure 4A). Furthermore, *bxd* ncRNA transcribed from the transgene and endogenous *Ubx* RNA are expressed in different cells and in different regions in each of these discs (Figure 4B,C). The lack of overlap between *bxd* ncRNA and *Ubx* expression argues that *bxd* RNA does not activate *Ubx* in imaginal discs.

An approach to sorting nuclei based on *Ubx* expression

The previous experiments suggest that transcriptional elongation of *bxd* ncRNAs has a key role in *Ubx* regulation, but does not suggest how transcription of ncRNAs is regulated molecularly. To address this question, we investigated the role of the TAC1 complex. Embryos contain a mixture of cells expressing and not expressing *Ubx* and *bxd* ncRNAs respectively, so it is not possible to carry out informative biochemistry on whole embryos. As we have shown above, imaginal discs are not suitable for these studies because they do not express *bxd* ncRNAs significantly. Sorting embryonic cells based on expression of *GFP*-expressing transgenes has

not worked for *Drosophila* embryos, mainly because of high levels of fluorescence from yolk proteins in the cytoplasm of embryonic gut cells (Figure 5A). To overcome this problem, we sorted nuclei rather than cells by flow cytometry, based on expression of a *Ubx-GFP* transgene. Our procedure for isolation of highly purified embryonic nuclei in large quantities is simple, reliable, and removes most of the material with non-specific fluorescence (see Experimental Procedures, Supplementary materials and Figure S1). It provides biochemically useful amounts of highly enriched nuclei without significant disruption of chromatin structure, which can be used to detect chromatin-associated proteins, to detect RNA by RT-PCR, and for expression profiling. This technique will be generally useful as nuclei expressing fluorescent proteins controlled by any regulatory region can be sorted from embryos.

The transgene expressing *GFP* under the regulation of 14 kb of DNA from the *bxd* regulatory region illustrated in Figures 2C and S1A was used to sort nuclei into those expressing *GFP* (*Ubx+)* and not expressing *GFP* (*Ubx−*). As shown in Figure 5A, the patterns of *GFP* and *Ubx* expression are very similar, showing that the transgene faithfully reproduces *Ubx* expression. Prior to use in these experiments, all batches of sorted nuclei were tested as shown in Figure S1C to ensure high levels of enrichment.

We first compared expression levels of *GFP*, endogenous *Ubx* and several *bxd* ncRNAs in our *Ubx+* and *Ubx−* nuclei by RT-PCR. One pair of PCR primers was designed for the region that includes the "*bxd*" transcripts, and two other sets were designed for exons 4 and 5 of ncRNAs (Figure 1A; (Lipshitz et al., 1987). Figure 5B shows that in sharp contrast to endogenous *Ubx* and the *GFP* transgene, all three primer sets show the presence of ncRNA transcripts primarily in the *Ubx−* nuclei. Importantly, the results of our RT-PCR analysis of sorted nuclei are consistent with the patterns of expression (Figure 1B,C), and confirm that our sorting procedure allows efficient separation of cells that preferentially express either *Ubx* or *bxd* ncRNA.

TAC1 is involved in transcriptional elongation of *Ubx*

Using sorted nuclei from 7–13 hr embryos in the chromatin immunoprecipitation (ChIP) assay, we asked where TAC1 binds MEs, and at promoters or transcribed regions of either *Ubx* or *bxd* ncRNAs, in *Ubx+* and *Ubx−* nuclei. In *Ubx+* nuclei, the recruitment levels of Trx and Sbf1 are much lower in the *bxd* ME relative to that in *Ubx−* nuclei (Figure 5C,D). The data are simply explained if TAC1 has a role in transcription of both *Ubx* and *bxd* ncRNAs, which as we have shown, occurs in non-overlapping cell populations. As it is often assumed that trxG proteins binds the *bxd* ME only when *Ubx* is transcribed, this result suggests that the *bxd* ME is not the only element that TAC1 binds in the *bxd* region.

Recruitment levels of both Trx and Sbf1 are clearly higher in *Ubx+* nuclei than in *Ubx−* nuclei in the region downstream of the transcription start site, at both endogenous *Ubx* (Figure 5C,D) and the *GFP* transgene (not shown), peaking at about 2 kb from the start site, suggesting that TAC1 binds downstream of actively transcribed promoters. TAC1 components were also detected, albeit at lower levels, in the middle of the *Ubx* gene (24 kb downstream of the start site), but were not significantly enriched at its 3'-end.

This binding pattern is consistent with a specific role for TAC1 in maintaining effective elongation. This idea is supported by RT-PCR analysis of trx^{B11} null mutants (Figure 5E), which shows that synthesis of the 3'-end both of the *Ubx* mRNA and of *bxd* ncRNAs is more strongly reduced than the 5'-end. Such a differential effect implicates *trx* function in the processivity of transcriptional elongation. These results are also consistent with our previous data showing that expression of *Ubx* is not completely abrogated in trx^{B11} mutant embryos (Mazo et al., 1990). Overall, our results show that alternative TAC1 (Trx and Sbf1) binding to

Ubx and to the *bxd* region correlates with a function in transcriptional elongation in these complementary sets of cells.

TAC1 modifies histones in the coding region of *Ubx*

Since the TAC1 complex possesses HMT and HAT activities (Petruk et al., 2001; Smith et al., 2004), we tested whether association of this complex with the transcribed region of *Ubx* in sorted nuclei correlates with increased levels of modified histones. Consistent with the presence of active TAC1, the levels of both acetylated H3 and H3 dimethylated at K4 in the *Ubx* coding region are significantly greater in *Ubx*+ than in *Ubx*− nuclei (Figure 5F). Figure 5G shows that the amounts of methylated H3-K4 and acetylated H3 are significantly reduced in trx^{B11} embryos, demonstrating that modifications of nucleosomes in the coding region of activated *Ubx* are dependent on TAC1. This change in association of TAC1 may be a key determinant of whether *Ubx* expression is maintained in an active or a repressed state.

TAC1 recruitment to the coding regions of *Ubx* and *bxd* ncRNAs depends on elongation factors

If TAC1 is important for transcriptional elongation, then binding of TAC1 within the transcribed regions of *Ubx* and *bxd* ncRNAs and the associated H3-K4 methylation might be affected by mutations in elongation factors, such as Spt16 (a component of the FACT nucleosome assembly complex), Spt4 and Spt6. We examined Trx and Sbf1 binding in these mutants. Homozygous mutant embryos were selected using *GFP*-marked balancers as described previously (Smith et al., 2004). A mutation in *Spt4* did not affect association of TAC1 or H3-meK4 within this region of *Ubx* (Figure 6A). However, binding of TAC1 was strongly decreased in both *Spt6* and *Spt16* mutant embryos (Figure 6A). Methylation of H3-K4 was also decreased in the same mutants. These data suggest that TAC1 may be associated with elongationally engaged Pol II.

TAC1 is essential for recruitment of Spt16

We then asked whether TAC1 is required for the recruitment of Spt16 to *Ubx*. Association of Spt16 with both the promoter and downstream regions of *Ubx* is significantly decreased in homozygous trx^{B11} null mutant embryos (Figure 7A), suggesting that Spt16 recruitment requires the presence of TAC1 during the initial phases of transcriptional elongation.

Components of the FACT elongation complex, Spt16 and SSRP1, were previously shown to be associated with the *bxd* region of *Ubx* (Shimojima et al., 2003). We find that Spt16 association with the *bxd* region is diminished in trx^{B11} embryos (Figure 7A). Therefore, association of FACT with the transcribed regions of both *Ubx* and *bxd* ncRNAs is TAC1-dependent, confirming that TAC1 is involved in transcriptional elongation of both *Ubx* and ncRNAs. Taken together, our results indicate that TAC1 and FACT are coordinately recruited to the elongating Pol II complex downstream of both the *Ubx* and *bxd* ncRNA promoters.

To extend this analysis, we asked whether TAC1 is required for FACT association with target genes on a broader scale by examining binding of Spt16 to salivary gland polytene chromosomes of third instar larvae from wildtype and *trx* RNAi mutant animals. We created a transgenic fly line that carries a Gal4-UAS-driven RNAi construct for the *trx* gene, in which expression of *trx* RNAi can be induced using the *hsp70-Gal4* driver. Induction during the early third larval instar allows these animals to survive long enough to examine polytene chromosomes. In wild type, binding sites of Trx largely overlap with those of Spt16 (Figure 7B). The number of binding sites detected on polytene chromosomes with of anti-Trx antibody depend on the titer of antibody used. In these experiments, we used higher titer of Trx antibody to identify previously undetected minor sites, which are extensive (Figure 7B, compare to Smith et al. 2004). The structure of polytene chromosomes in *trx* RNAi larvae is

indistinguishable from that of the wild type animals. In addition, binding of the control protein ecdysone receptor (EcR) to polytene chromosomes is unaffected (Figure 7B). However, binding of the Trx protein is strongly decreased in these mutants, especially at its characteristic strong binding sites. In the same larvae, binding of Spt16 is also strongly decreased (Figure 7B). Thus, Trx may be required for recruitment of the elongation factor Spt16 to most activated genes, suggesting a global role for Trx in transcriptional elongation.

TAC1 may also have a role in transcriptional initiation of *Ubx* and ncRNAs

Similar amounts of TAC1 are associated with the promoter region of *Ubx* in *Ubx*+ and *Ubx*− nuclei, and the same is true for the region distal to *bxd* ME B, which contains the P1 promoter of *bxd* ncRNAs (Figure 5C,D). We detected a slight but reproducible decrease in the levels of synthesis of the 5'-regions of both *Ubx* and ncRNAs in trx^{B11} mutants (Figure 5E). Interestingly, association of Trx and Sbf1 with the promoter region of *Ubx* is not affected in elongation factor mutants (Figure 6B). All of these results are consistent with the idea that TAC1 recruitment to the promoter is independent of the formation of the elongation complex. These results also show that overall expression of Trx and Sbf1 are not affected in elongation factor mutants. Taken together, our results are consistent with the notion that TAC1 is required primarily for efficient elongation of *Ubx* by Pol II, but that it may well play an additional role in initiation of transcription.

Discussion

An attractive notion has been that transcription of *bxd* ncRNAs, which precedes that of *Ubx* in embryos, facilitates correct spatial expression of *Ubx*. Previous studies showed that transcription through the ME could interfere with silencing (Bender and Fitzgerald, 2002; Hogga and Karch, 2002; Rank et al., 2002); (Schmitt et al., 2005), so it was proposed that *bxd* ncRNA transcription normally prevents recruitment of PcG proteins to the ME. However, our experiments unambiguously demonstrate that *Ubx* and *bxd* ncRNAs are transcribed in different cells in embryos. Our results also suggest that *bxd* ncRNAs do not facilitate *Ubx* expression in larval imaginal discs, as was recently proposed (Sanchez-Elsner et al., 2006). Instead, transcription of ncRNAs correlates with repression of *Ubx*. It is possible that the abnormal transcription induced in previous studies interfered with transcription of ncRNAs in the BX-C, rather than with ME function, a possibility that can be tested experimentally. It will be interesting to use our system of sorting *Ubx*+ and *Ubx*− nuclei to examine binding of PcG proteins in nuclei where *bxd* ncRNAs either are, or are not, transcribed.

Our experiments rule out *trans*-repression by *bxd* ncRNAs, and instead support repression of *Ubx* in *cis* by transcription of these RNAs per se. A likely mechanism of this repression is transcriptional interference, since we show that ncRNA transcription extends into the region just upstream of the *Ubx* initiation site, which may well disrupt protein-DNA interactions required for *Ubx* initiation. However, this does not rule out promoter competition, and both of these mechanisms may contribute to the observed effects. Previous genetic studies (Grimaud et al., 2006a) and the results presented here show that *bxd* ncRNAs do not work by RNAi. An RNAi-based repression mechanism has been described for the miRNA produced by the *iab-4* transcript, which directly interacts with the 3'-untranslated region of *Ubx* and prevents translation (Ronshaugen et al., 2005). These authors show that ectopic expression of *iab-4* leads to homeotic phenotypes in the haltere, but do not show that loss of RNAi prevents this effect, nor has the effect of loss of function mutations of the *iab-4* transcript been tested, so it remains to be seen if the *iab-4* transcript is a bona fide miRNA.

As we did not detect significant levels of *bxd* ncRNAs in imaginal discs, nor do they persist to late embryonic stages, they are unlikely be responsible for repression of *Ubx* throughout development. In fact, Papp and Muller (2006) report that Trx is bound to the *bxd* ME in both

wing and haltere discs, which have low and high levels of *Ubx* expression, respectively. The difference between binding of Trx to the *bxd* ME in embryos and in discs (Papp and Muller, 2006) may be a consequence of the absence of transcription of *bxd* ncRNAs in discs and its presence in embryos, or to other uncharacterized differences between *Ubx* regulation in embryos and discs. Also, as we show that Trx binds constitutively in some areas of the ME, Papp and Muller may have detected such binding in imaginal discs.

Intergenic transcription also cannot explain repression of *Ubx* in the anterior of the embryo, where it is thought that *hunchback* and PcG genes set up and maintain the anterior boundary of *Ubx* expression. However, the pattern of *bxd* ncRNA transcription, which prefigures, in a complementary fashion, the mosaic pattern of *Ubx* expression within the parasegments of the embryonic trunk, appears to be essential for proper *Ubx* initiation. The *Ubx* pattern may then be maintained or modified at later embryonic stages through repression by other Hox proteins (i.e., abdA and AbdB) and by PcG genes. Thus, maintenance of *Ubx* expression likely requires multiple mechanisms that are employed at different developmental stages.

Our data support a role for Trx in transcriptional elongation as a mechanism for maintenance of a developmentally regulated gene. It has been argued that Trx does not have a direct role in activation of homeotic genes in *Drosophila*, but instead prevents repression of transcription by PcG proteins (Klymenko and Muller, 2004). However, our data suggest that *trx* is required for recruitment of elongation factors and for efficient completion of transcripts. Therefore, maintenance of transcriptional activity by Trx may be a consequence of its role in elongation, and a block in elongation might lead to the establishment of PcG-mediated repression. Alternatively, Trx may be required only for normal levels of Hox gene expression, and not for maintenance of low levels of expression, a possibility consistent with at least some aspects of the *trx* mutant phenotype.

This work strongly supports a general role for Trx and TAC1 in transcription, and agrees with our previous findings that TAC1 relocates from other genes to the transcribed region of *hsp70* following induction of the cellular stress response (Smith et al., 2004). The histone methyltransferase activity of Set1, the SET domain protein homologous to Trx, has a role in transcription (Hampsey and Reinberg, 2003), and MLL was suggested to play a similar role in mammals (Guenther et al., 2005; Hughes et al., 2004; Milne et al., 2005; Yokoyama et al., 2004). We suggest that this role is in transcriptional elongation, because Trx and elongation factors are co-ordinately recruited, because Trx binds downstream of the promoter more strongly to the 5’ than the 3’ end, and because transcripts extending to the 3’ end are more strongly affected by *trx* mutations, for both *Ubx* and *bxd* ncRNAs.

TAC1 is also present at the promoter (Figure 5C,D), and this is unaffected by mutations in elongation factors (Figure 6B). Therefore, association of TAC1 with the promoter likely precedes the recruitment of elongation factors. Thus, TAC1 may play several distinct roles, one in initiation, another during the recruitment of the elongation complex and perhaps a third during subsequent elongation, where its ability to modify histones may be required for effective completion of long transcripts.

This work provides the first direct evidence of the involvement of long ncRNAs in regulation of homeotic genes of Drosophila. Repression of *Ubx* is apparently mediated by expression of several intergenic ncRNAs in different germ layers of *Ubx*-expressing parasegments. TAC1 may be required for efficient read-through by Pol II into the region upstream of the *Ubx* initiation site, and as a result, for efficient repression of *Ubx* (see model in Figure 7C). Therefore, we propose a direct link between elongation facilitated by the TAC1 epigenetic complex and repression of *Ubx* by intergenic transcription. A goal for the future will be to

determine if other homeotic genes of Drosophila, and of other organisms, are also regulated by long ncRNAs whose expression is regulated by TAC1 proteins.

Experimental Procedures

Drosophila Genetics

Details on strains used and their construction can be found in the Supplementary material. All strains were maintained on standard medium at 25°C. Homozygous mutant embryos were collected from stocks carrying either the trx^{B11}, *spt4*, *spt6*, or *spt16* mutations over *Kr-GAL4, UAS-GFP*-carrying balancer chromosomes (Smith et al., 2004) based on the absence of *GFP* expression. The wildtype strain Oregon R was used as a control.

Isolation and sorting of embryonic nuclei

Nuclei were prepared using a procedure already described (Petruk et al. 2001), and details are given in Supplementary material. Nuclei were sorted on a Coulter ELITE ESP cell sorter at 4^0C. After sorting, an aliquot of 10,000 nuclei was used to prepare RNA, while the rest of the material was used for ChIP experiments (see Immunoprecipitation).

RNA preparation and RT-PCR

Each batch of sorted nuclei was analysed by RT-PCR for at least a 10-fold enrichment of *Ubx* RNA in GFP-positive nuclei using standard procedures. RNA from 25 wildtype or trx^{B11} mutant embryos or from 15 dissected discs was prepared using the High Pure RNA Isolation Kit (Roche), and RT was performed using random primers. For coordinates of primer sets, see Supplementary material.

Chromatin Immunoprecipitation

ChIP experiments were performed according to the Upstate Biotechnology protocol, using 150,000 to 200,000 sorted nuclei or 50 whole embryos per sample. Details of the procedure, antibodies used, and coordinates of primers are given in Supplementary Experimental Procedures.

In situ hybridization and immunostaining

Embryo and larvae fixation, preparation of labelled RNA probes, and nascent transcript RNA FISH were performed according to (Kosman et al., 2004; Kuzin et al., 1994). cDNA sequences were synthesized by PCR and subcloned into the pGEM-T vector (Promega). For coordinates of probes, see Supplementary material.

RNA probes were labelled with DIG-, and Biotin-conjugated UTP and were detected as follows: DIG: sheep anti-DIG (Roche), Alexa 555 donkey anti-sheep; Biotin: mouse anti-Biotin (Roche), Alexa 488 donkey anti-mouse. Images of embryos were obtained using a confocal microscope in the KCC imaging facility. Preparation and immunostaining of chromosome spreads were performed as described (Tillib at al., 1999).

Injections of dsRNAs

dsRNAs specific to exons 1-3-7 and *bxd* were synthesized from the same constructs that were used for *in situ* hybridization. Sense and antisense RNAs were synthesized using the Riboprobe *in vitro* Transcription System (Promega). Equal amounts of sense and antisense RNAs were annealed by heating at 90^0C for 3 min and cooling down slowly to room temperature. Equal amounts of the exon 1–7 and *bxd* dsRNAs were combined and used for injection at 10ng/ml either in preblastoderm embryos using standard procedures, or in adult females as described (Dzitoyeva et al., 2003). *GFP* (Stratagene) dsRNA was used as a control.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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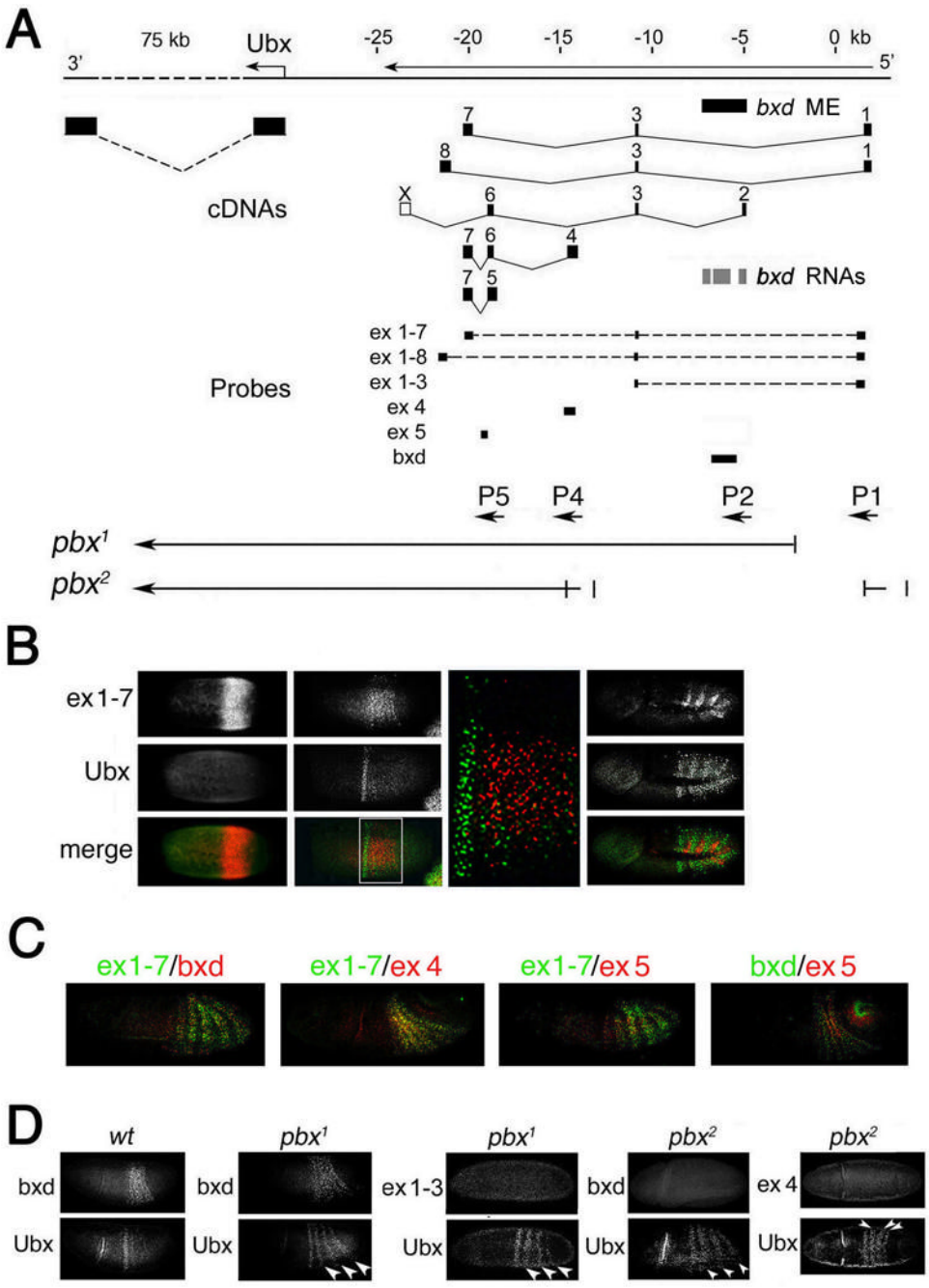

Figure 1. *Ubx* and ncRNAs are expressed in complementary embryonic domains

(A) Map of *Ubx* and intergenic transcripts. The exons of *Ubx* and intergenic transcripts (Lipshitz et al., 1987) are shown in black. *bxd* ME (Tillib et al., 1999) is shown by the black bar below DNA line. Described locations of three *bxd* transcripts (Sanchez-Elsner et al., 2006) are shown by grey bars. Probes used for *in situ* hybridization are diagrammed below the cDNAs as black bars. The bottom map shows the extent of deleted regions in *pbx*^{1} and *pbx*^{2} mutants. Promoters of ncRNAs are indicated by arrows below the probes.

(B) Time-course of nascent *Ubx* and ncRNA transcription. Nascent ncRNA was detected with probes indicated in A. At syncytial blastoderm, RNA 1–7 is expressed earlier than *Ubx* (left panels). The initial expression domain of *Ubx* is anterior to that of RNA 1–7 (middle panels,

including blow-up of merge). During germband elongation, expression domains of RNA 1–7 in each parasegment are anterior to those of *Ubx* (right panels). *Ubx*-expressing cells do not express RNA 1–7, or any ncRNAs detected by probes shown in Figure 1A at any stage (some data not shown).

(C) RNA containing exons 1–7, 4, 5, and *bxd* RNA are expressed within the same intersegmental domains, although their expression differs in different germ layers.

(D) Effects of pbx^{1} and pbx^{2} deletions on expression of the indicated ncRNAs and endogenous *Ubx*.

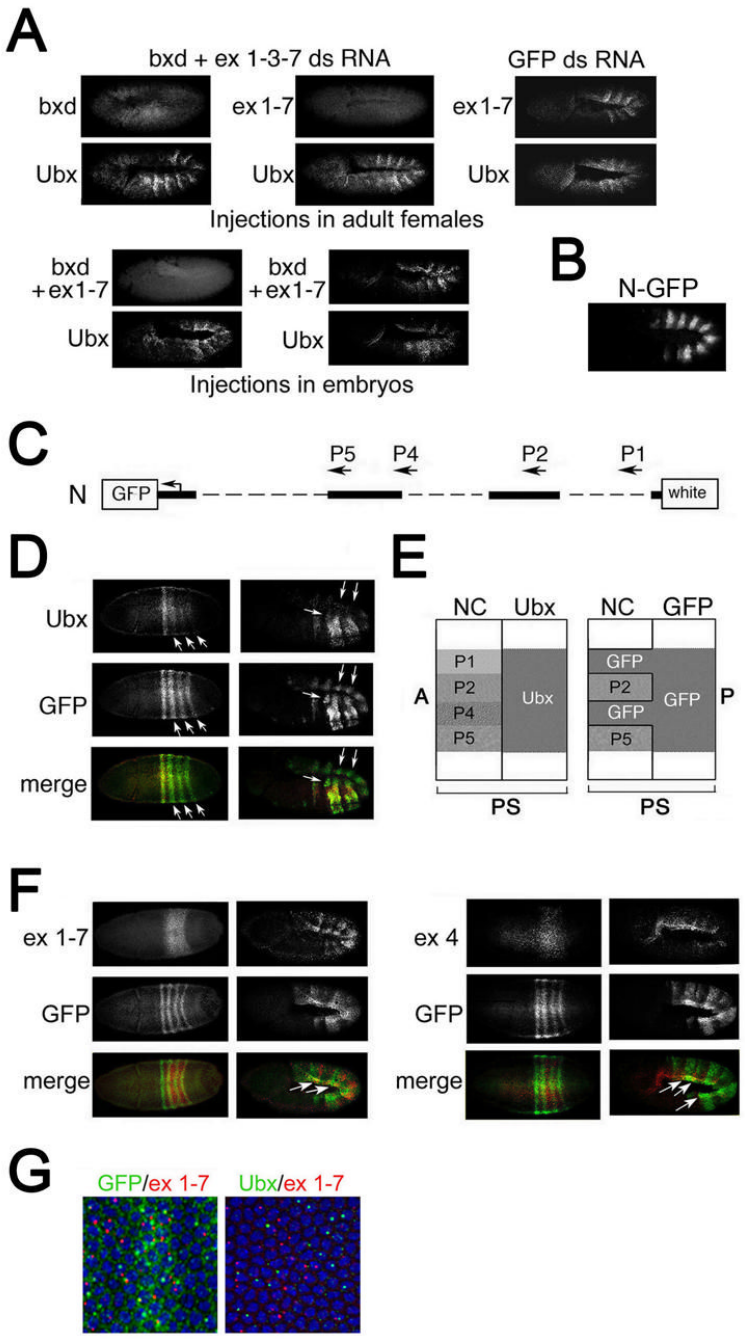

Figure 2. Intergenic transcription represses *Ubx* in *cis*

(A) Products of ncRNAs do not directly repress *Ubx* transcription. A mixture of dsRNAs specific to exons 1, 3, 7, and part of the *bxd* region, and control dsRNA specific to *GFP* were injected either into adult females (top panels) or into preblastoderm embryos. Embryos were analyzed by *in situ* hybridization with probes to *Ubx*, exons 1–7 and the mixture of probes to exons 1–7 and *bxd*, as indicated.

(B) Immunostaining of embryo carrying the *Ubx-GFP* transgenic construct N (see map in C) with GFP antibody.

(C) Map of the *Ubx-GFP* transgenic construct N. The transgene contains ncRNA promoters P2 and P5 but lacks promoters P1 and P4.

(D) The *GFP* reporter gene is ectopically expressed (arrows) at blastoderm and germband extension relative to endogenous *Ubx*.
(E) A diagram of predicted expression patterns of *Ubx* and *GFP*, assuming repression occurs only in *cis*. In the absence of the P1 and P4 promoters, the *GFP* transgene is expected to be expressed in cells where the corresponding endogenous RNAs are expressed. A, P, anterior and posterior of the embryo.
(F) Expression of the *GFP* reporter gene overlaps with expression of ncRNAs that are produced from the P1 (probe ex 1–7 left panels) and P4 (probe ex 4, right panels) promoters.
(G) Comparison of the expression of endogenous *Ubx* and the *GFP* reporter gene with RNA 1–7 at the blastoderm stage. Expression of *GFP* is seen in both cytoplasm and nuclei, and RNA 1–7 is detected in the nuclei of the same cells (left).

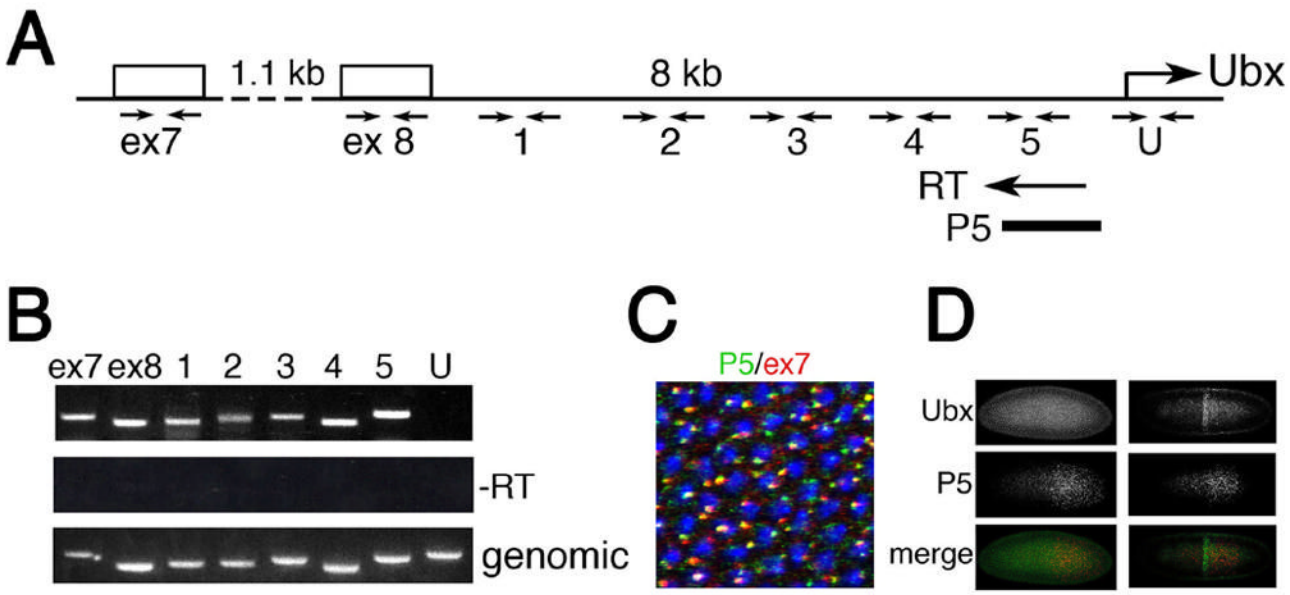

Figure 3. ***Ubx*** **is repressed by read-through transcription from intergenic transcription units**

(A) Map of the intergenic region between the 3'-exons 7 and 8 of ncRNAs and the *Ubx* start site. cDNA was synthesized from the primer close to the *Ubx* start site (RT). Primer sets for PCR amplification are indicated below the map. Probe P5 for *in situ* hybridization is shown at the bottom. For coordinates of primers and *in situ* probes see Supplementary material.

(B) RT-PCR detection of transcripts in the region proximal to the *Ubx* promoter. Primer sets are those shown in (A).

(C) Transcripts from the region proximal to the *Ubx* start site are expressed in the same cells as transcripts of the ncRNAs containing exon 7.

(D) Transcripts from the region proximal to the *Ubx* start site are expressed earlier than *Ubx* in blastoderm embryos (left panels), and they are expressed in cells that do not express *Ubx* (right panels).

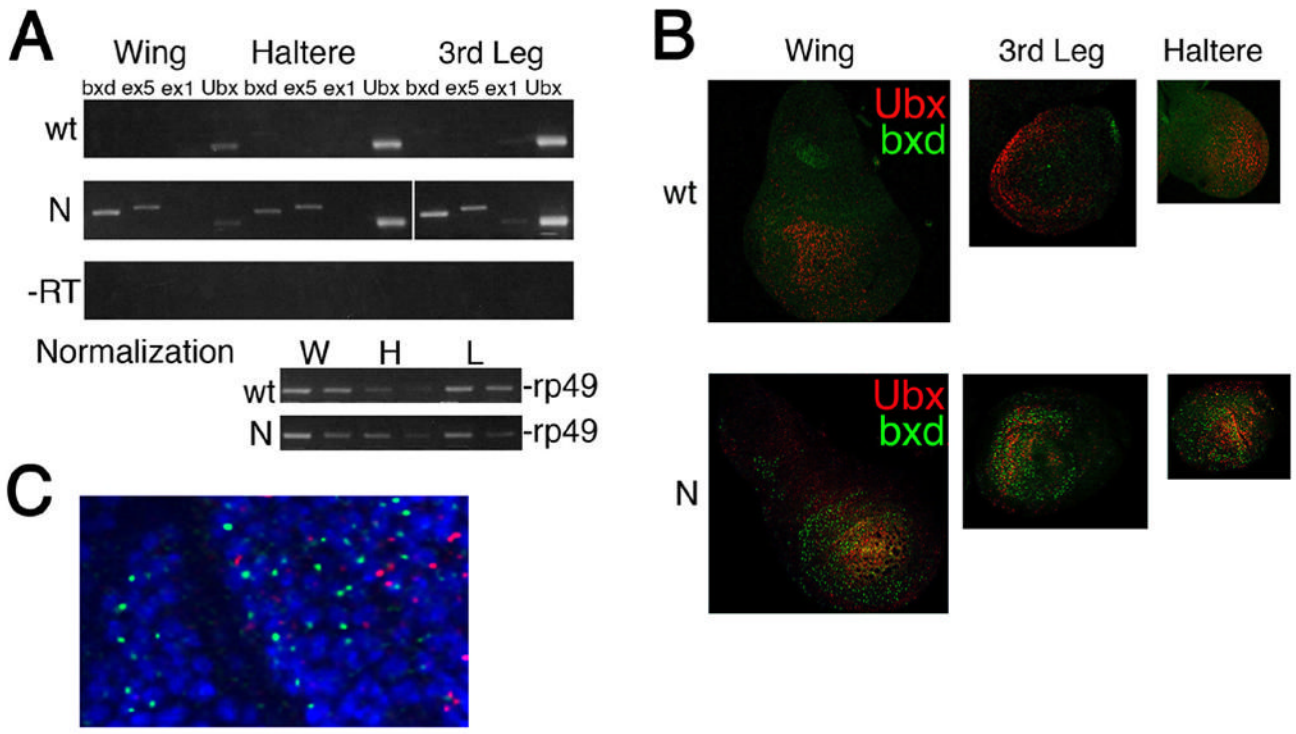

Figure 4. NcRNAs are not required for activation of *Ubx* in larval imaginal discs

(A). RT-PCR analysis of the amounts of ex1, ex5, *bxd*, and *Ubx* RNAs in wing, haltere and 3rd leg imaginal discs in wildtype (wt) and transgenic (N) larvae. Controls without RT are shown in the third row. rp49 was used to normalize the amount of RNA in discs between the two strains (bottom). Primer sets are the same as in Figures 5B,E.

(B) *In situ* hybridization of *Ubx* and *bxd* probes to wing (W), haltere (H) and 3rd leg (L) larval imaginal discs in wildtype (wt) and transgenic (N) larvae. Probes are the same as in Figure 1B,C.

(C) High magnification detection of *Ubx* (red) and *bxd* (green) nascent RNAs by fluorescent *in situ* hybridization in 3rd leg disc of transgenic larvae. Probes are the same as in (B).

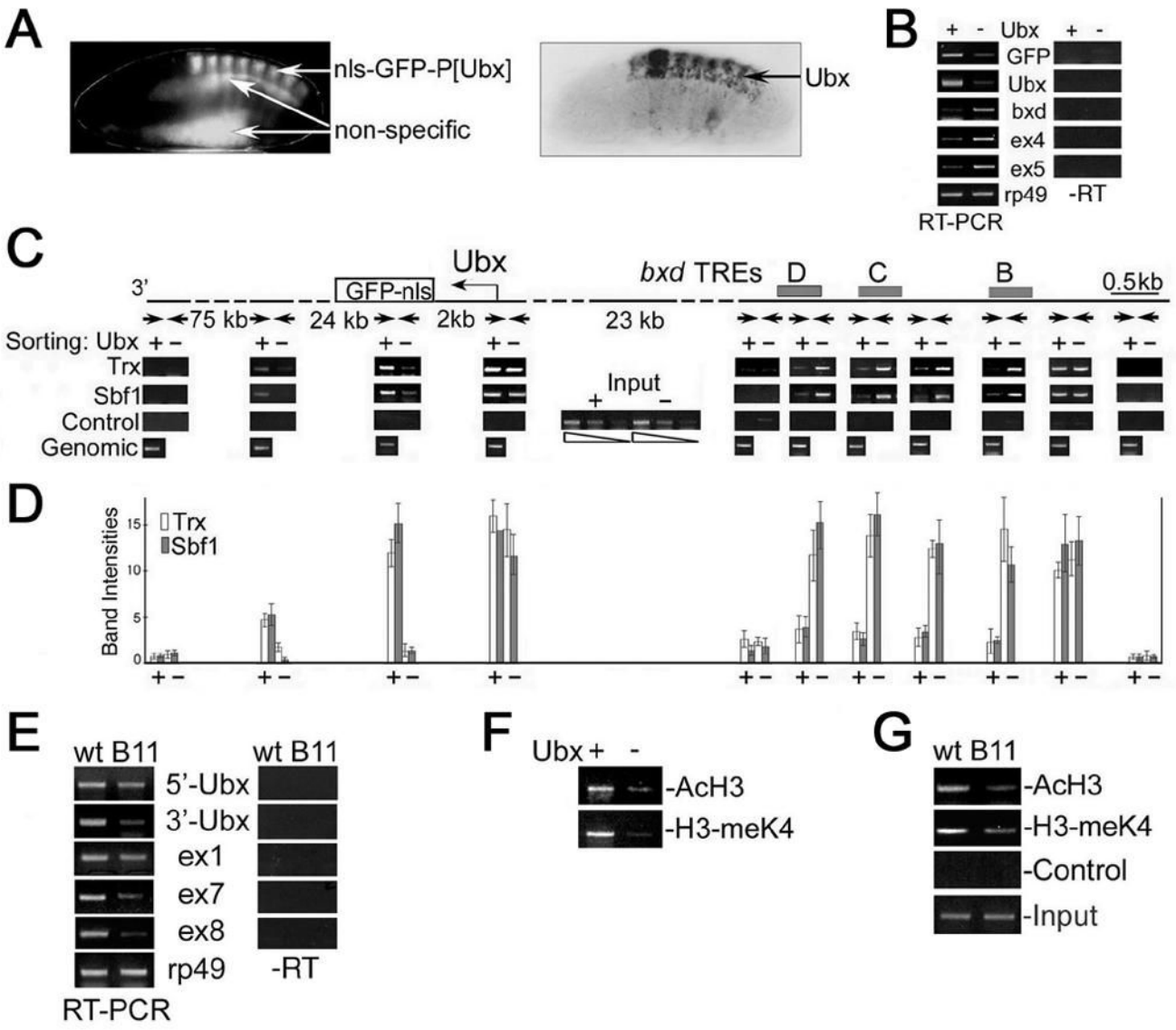

Figure 5. Association of TAC1 complex with active and silenced *Ubx* in embryos

(A) Expression of *GFP* reporter (top) and endogenous *Ubx* (bottom) in mid-stage *Drosophila* embryos. Non-specific fluorescence is indicated.

(B) RT-PCR analysis of the amounts of *GFP*, *Ubx*, intergenic and *rp49* transcripts in RNA from *Ubx/GFP*-positive (+) and *Ubx/GFP*-negative (-) nuclei from 7–13 hr embryos. Primers for exons 4 and 5 and the *bxd* region were designed according to (Lipshitz et al., 1987; Rank et al., 2002). rp49 was used to normalize the amount of RNA. Controls without RT are shown on the right.

(C) Top: map showing the regions of endogenous *Ubx* and the *GFP-Ubx* transgene that were tested for association of TAC1 components. Trx binding sites/response elements (TREs) in the *bxd* region (Tillib et al., 1999) are shown as grey bars, primer sets used for ChIP analysis are indicated by arrows, with distances between them. Bottom: ChIP analyses were performed with antibodies specific to Trx and Sbf1 (as shown on the left) from chromatin isolated from either *Ubx/GFP*-positive (+) or *Ubx/GFP*-negative (–) nuclei. Data shown is for the transcribed region 2 kb downstream of the endogenous *Ubx* start site; indistinguishable results were

obtained with the corresponding primers for the *GFP* transgene. Control, no antibody. Input is shown in the middle.

(D) Graph of the relative levels of Trx and Sbf1 in the *Ubx* transcription unit. Background levels of cross-linking were determined by omitting the precipitating antibody and were then subtracted from the signals.

(E) RT-PCR analysis of the amounts of *Ubx* and ncRNAs in wildtype (wt) and trx^{B11} (B11) mutant embryos. rp49 was used to normalize the amount of RNA. For coordinates of primer sets for *Ubx* and exons 1, 7 and 8 see Supplementary material and Figure 1A. Controls without RT are shown on the right.

(F) ChIP analysis of histone modifications in the coding region of *Ubx*. Immunoprecipitations were performed with antibodies against acetylated H3 (AcH3) and H3 dimethylated at K4 (H3-meK4) from the chromatin isolated from *Ubx/GFP+* and *Ubx/GFP–* nuclei. Immunoprecipitated material was PCR amplified using the primers for the region 2 kb downstream of the *Ubx* start site. Input is the same as in Figure 5C.

(G) Chromatin prepared from wildtype (wt) and trx^{B11} homozygous mutant embryos (B11) was immunoprecipitated with antibodies against AcH3 and H3-meK4. Primers were those for the sequence 2 kb downstream of the *Ubx* promoter. Control, without antibody.

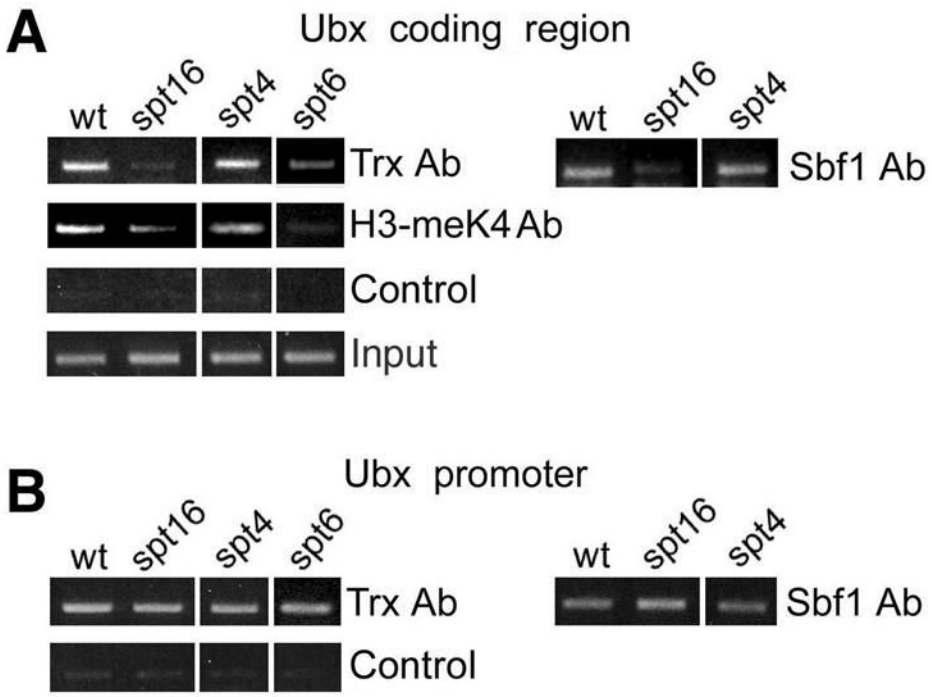

Figure 6. Elongation factors are required for TAC1 recruitment to *Ubx*

Chromatin was prepared from wildtype (wt) and the homozygous mutant embryos indicated above each set, and immunoprecipitated with antibodies against Trx, Sbf1 and histone H3-meK4 as indicated on the right. Immunoprecipitated material was PCR amplified with primers for the region 2 kb downstream of the start site (A) and to the promoter (B). Primer sets are the same as in Figure 5C. Control, no antibody.

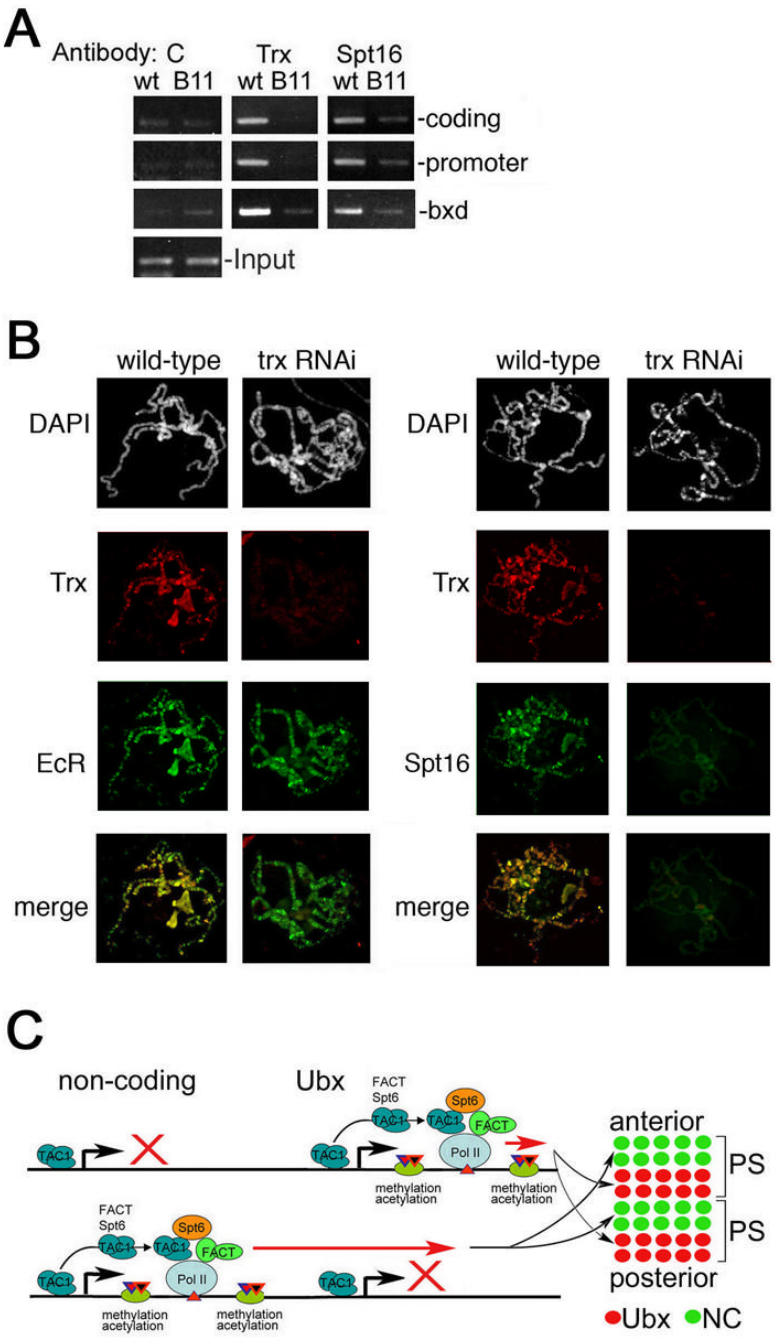

Figure 7. TAC1 is essential for association of FACT

(A) Chromatin prepared from wildtype (wt) and *trx*^{B11} (B11) homozygous mutant embryos was immunoprecipitated with antibodies against Trx or Spt16. Immunoprecipitated material was PCR amplified in with primers for the region 2 kb downstream of the start site (top), for the *Ubx* promoter (middle), and for the central C *bxd* region (bottom) (Figure 5C). C, no antibody control. Input is shown at the bottom.

(B) Salivary gland polytene chromosomes were prepared from wildtype 3rd instar larvae and a line expressing *trx* RNAi. The overall structure of chromosomes is indistinguishable (first row). Binding by Trx (row 2) and Spt16 (right row 3) is almost completely abolished in the

trx RNAi line, while Ecdysone Receptor (EcR) binding is unaffected (row 3 left). Merge is shown at the bottom.

(C) A model for the role of TAC1 in transcription. TAC1 recruitment along with elongation factors and histone modification is necessary for efficient transcriptional elongation of *Ubx* and ncRNAs in complementary cells in the posterior and anterior regions of each parasegment, respectively. Efficient elongation of Pol II from promoters of ncRNAs proceeds through the 5'-regulatory elements of *Ubx*, thus preventing its expression. This generates a mosaic pattern of *Ubx* expression within embryonic parasegments.