CORRIGENDUM

Development 136, 2309 (2009) doi:10.1242/dev.040022

Drosophila ptip is essential for anterior/posterior patterning in development and interacts with the PcG and trxG pathways

Ming Fang, Hongyan Ren, Jiabin Liu, Ken M. Cadigan, Sanjeevkumar R. Patel and Gregory R. Dressler

There was an error published in *Development* **136**, 1929-1938.

The address for author affiliation 2 is incomplete. The corrected address appears below.

The authors apologise to readers for this mistake.

Department of Genetics and Developmental Biology, Southeast University Medical School, The Key Laboratory of Developmental Genes and Human Disease, Ministry of Education, Nanjing 210009, China.

Development 136, 1929-1938 (2009) doi:10.1242/dev.026559

Drosophila ptip is essential for anterior/posterior patterning in development and interacts with the PcG and trxG pathways

Ming Fang[1,2,*], Hongyan Ren[2], Jiabin Liu[2], Ken M. Cadigan[3], Sanjeevkumar R. Patel[4] and Gregory R. Dressler[1,*]

Development of the fruit fly *Drosophila* depends in part on epigenetic regulation carried out by the concerted actions of the Polycomb and Trithorax group of proteins, many of which are associated with histone methyltransferase activity. Mouse PTIP is part of a histone H3K4 methyltransferase complex and contains six BRCT domains and a glutamine-rich region. In this article, we describe an essential role for the *Drosophila* ortholog of the mammalian *Ptip* (*Paxip1*) gene in early development and imaginal disc patterning. Both maternal and zygotic *ptip* are required for segmentation and axis patterning during larval development. Loss of *ptip* results in a decrease in global levels of H3K4 methylation and an increase in the levels of H3K27 methylation. In cell culture, *Drosophila ptip* is required to activate homeotic gene expression in response to the derepression of Polycomb group genes. Activation of developmental genes is coincident with PTIP protein binding to promoter sequences and increased H3K4 trimethylation. These data suggest a highly conserved function for *ptip* in epigenetic control of development and differentiation.

KEY WORDS: Epigenetics, Polycomb, Trithorax, *ptip*, *Drosophila* development

INTRODUCTION

The establishment and maintenance of gene expression patterns in development is regulated in part at the level of chromatin modification through the concerted actions of the Polycomb and trithorax family of genes (PcG/trxG). In *Drosophila*, Polycomb and Trithorax response elements (PRE/TREs) are cis-acting DNA sequences that bind to Trithorax or Polycomb protein complexes and maintain active or silent states, presumably in a heritable manner (Grimaud et al., 2006; Ringrose and Paro, 2007). In mammalian cells however, such PRE/TREs have not been conclusively identified. Polycomb and Trithorax gene products function by methylating specific histone lysine residues, yet how these complexes recognize individual loci in a temporal and tissue specific manner during development is unclear. Recently, we identified a novel protein, PTIP (also known as PAXIP1), that is part of a histone H3K4 methyltransferase complex and binds to the Pax family of DNA-binding proteins (Patel et al., 2007). PTIP is essential for assembly of the histone methyltransferase (HMT) complex at a Pax DNA-binding site. These data suggest that Pax proteins, and other similar DNA-binding proteins (Shimizu et al., 2001), can provide the locus and tissue specificity for HMT complexes during mammalian development.

In mammals, the PTIP protein is found within an HMT complex that includes the SET domain proteins ALR (GFER – Mouse Genome Informatics) and MLL3, and the accessory proteins WDR5, RBBP5 and ASH2 (Cho et al., 2007; Issaeva et al., 2007; Patel et al., 2007). This PTIP containing complex can methylate lysine 4 (K4) of histone H3, a modification implicated in epigenetic activation and maintenance of gene expression patterns. Furthermore, conventional $Ptip^{-/-}$ mouse embryos and conditionally inactivated $Ptip^{-/-}$ neural stem cell derivatives show a marked decrease in the levels of global H3K4 methylation, suggesting that PTIP is required for some subset of H3K4 methylation events (Patel et al., 2007). The PTIP protein contains six BRCT (BRCA1 carboxy terminal) domains that can bind to phosphorylated serine residues (Manke et al., 2003). This is consistent with the observation that PAX2 is serine-phosphorylated in response to inductive signals (Cai et al., 2003; Cai et al., 2002). In mammals, PAX2 specifies a region of mesoderm fated to become urogenital epithelia at a time when the mesoderm becomes compartmentalized into axial, intermediate and lateral plate (Bouchard et al., 2002; Torres et al., 1995). These data suggest that PTIP provides a link between tissue specific DNA-binding proteins that specify cell lineages and the H3K4 methylation machinery.

To extend our finding to a non-mammalian organism and address the evolutionary conservation of *Ptip*, we asked whether a *Drosophila ptip* homolog could be identified and if so, whether it is also an essential developmental regulator and part of the epigenetic machinery. The mammalian *Ptip* gene encodes a novel nuclear protein with two amino-terminal and four carboxy-terminal BRCT domains, flanking a glutamine-rich sequence (Lechner et al., 2000). Based on the number and position of the BRCT domains and the glutamine-rich domain, the *Drosophila* genome contains a single *ptip* homolog. To understand the function of *Drosophila ptip* in development, we characterized a *ptip* mutant allele that contained a *piggyBac* transposon insertion between BRCT domains three and four. Maternal and zygotic *ptip* mutant embryos exhibited severe patterning defects and developmental arrest, whereas zygotic null mutants developed to the third instar larval stage but also exhibited anterior/posterior (A/P) patterning defects. In cell culture, depletion of Polycomb-mediated repression activates developmental regulatory genes, such as the homeotic gene *Ultrabithorax* (*Ubx*). This derepression is dependent on trxG activity and also requires PTIP. Microarray analyses in cell culture of *Polycomb* and *polyhomeotic* target genes indicate that many, but not all, require

[1]Department of Pathology, University of Michigan, Ann Arbor, MI 48109, USA. [2]Department of Genetics and Developmental Biology, Southeast University Medical School, Nanjing, China 210009. [3]Department of Molecular, Cellular and Developmental Biology, University of Michigan, Ann Arbor, MI 48109, USA. [4]Department of Internal Medicine, University of Michigan, Ann Arbor, MI 48109, USA.

*Authors for correspondence (e-mails: mfang@seu.edu.cn; dressler@umich.edu)

Accepted 20 March 2009

PTIP for activation once repression is removed. The activation of PcG target genes is coincident with PTIP binding to promoter sequences and increased H3K4 trimethylation. These data argue for a conserved role for PTIP in Trithorax-mediated epigenetic imprinting during development.

MATERIALS AND METHODS

Fly genetics

Stocks *Df(3L)fz-GF3b*, *P{wAR}66E* and *Df(3L)ED4543*, *P{3′.RS5+3.3′}ED4543* were obtained from Bloomington stock center. According to Flybase, the two lines have deleted cytological segments 70C2-70D4 and 70C6-70F4, and are referred to as $Df(ptip)^1$ and $Df(ptip)^2$, respectively. PB^{c04574} ($PBac^{ptip}$) was kindly provided by the Exelixis collection at Harvard Medical School (http://drosophila.med.harvard.edu/).

In situ hybridization of the embryos was performed as described (Cadigan et al., 1998), using digoxigenin-labeled antisense RNA probes. For clonal analysis of the $PBac^{ptip}$ allele, it was recombined onto an FRT^{2A} chromosome using standard methods (Xu and Rubin, 1993) and the germline clones were generated using FRT^{2A} P[*ovoD*] as described (Chou and Perrimon, 1996), with heat shocks during larval development. The resulting female virgin flies with the genotype FRT^{2A} $PBac^{ptip}/FRT^{2A}$ P[*ovoD*] were then crossed to $Df(ptip)^1$/*TM6 evelacZ* males. Embryos lacking zygotic *ptip* were identified by the absence of *evelacZ*. Cuticles were prepared and photographed as previously described (Bhanot et al., 1999). Adult flies were frozen overnight and photographed with a Leica M10 microscope.

Zygotic *ptip* mutant wing imaginal discs and salivary glands were prepared from $PBac^{ptip}/Df(ptip)^1$ third instar larvae and immunostained as described (Guan et al., 2007; Parker et al., 2002). Rabbit anti-DLL (1:200) was from J. Kohtz (Northwestern University, Chicago, IL, USA), monoclonal anti-EN (1:20) and anti-WG (1:100) were from the Developmental Studies Hybridoma Bank (Iowa City, IA, USA), rabbit anti-Histone H3 (trimethyl K4, 1:200) and anti-Histone H3 (trimethyl K27, 1:200) were ChIP grade and purchased from Abcam (Cambridge, UK). Cy3- and Alexa Fluor 488-conjugated secondary antibodies were from Jackson Immunochemicals (West Grove, PA, USA) and Molecular Probes (Eugene, OR, USA), respectively. The discs and salivary glands were then digitally photographed with a Nikon Eclipse 800 compound microscope and the images were processed as Abode Photoshop files.

Transgenic rescue experiments

For rescue experiments shown in Fig. 6, P[*UAS*-m*Ptip*] lines were constructed based on the mouse *Ptip* cDNA sequence (m*Ptip*) as described (Lechner et al., 2000) and introduced into w^{1118} hosts by standard P element-mediated transformation. Five independent lines were obtained, and the line mapping to the second chromosome was used for further studies. Flies with a combination of four transgenes were examined. P[*UAS*-m*Ptip*], along with P[*hs-Gal4*], were crossed into a $PBac^{ptip}/Df(ptip)^1$ background, and were subjected to 37°C heat shock for 1 hour/day for 5 days after egg laying.

Cell culture

Kc167 (Kc) cells were routinely cultured at room temperature in Schneider's *Drosophila* media (Invitrogen) containing 5% FBS. RNAi-mediated gene knockdowns were performed essentially as described (Clemens et al., 2000). Briefly, cells were seeded at 10^6 cells/well and a total of 15 μg dsRNA was added. Cells were harvested on the day 7 for total RNA extraction. Templates for dsRNA synthesis were obtained by RT-PCR. The primer sequences are available upon request.

For chromatin immunoprecipitation (ChIP) experiments, Kc cells were seeded at 2×10^6 cells/60 mm dish and control or PcG dsRNAs were added. The next day, cells were transfected with p*UAS*-m*Ptip* and a *Drosophila* β-*actin* promoter VP16-GAL4 activator using FuGENE-mediated lipofection. Chromatin was prepared 72 hours post transfection as described (Patel et al., 2007). For ChIP experiments, 5 μg chromatin was incubated with antibodies against mPTIP, anti-H3K4me3 (Abcam) or rabbit IgG controls overnight at 4°C and processed as described previously. DNA isolated from the ChIP samples was analyzed with primer pairs that recognize the DNA sequences around the transcription start sites of the genes listed. All analysis was done in triplicate using real time quantitative PCR. Primer sequences (shown 5′ to 3′; F, forward; R, reverse) and position relative to the transcription start sites were as follows: *Cad*-F TGAGCCTCTCTGCCTGTTTATCTG (–73) and *Cad*-R TGAAATGTTATTCCGTGCGTGG (+57); *Esp*-F TGAAGAGCCAAGCGACCAGAAC (+25) and *Esp*-R CACTGCCCACAAAACACAAGTTG (+174); *rib*-F TCTCTCGCTCTCACCCTCTGTG (–93) and *rib*-R GTTTTCCGCTCGCCTTGTG (+102); *pnr*-F TCTCTCTTGCTCTTTCGCTCACC (–246) and *pnr*-R CTCACACACACTCACACTCGCTATC (–56); *Ubx*-F TGGTGCGTATCTGTGTTTTGTGC (–1107) and *Ubx*-R TTCTACTTTTCCAACCCCTGCTC (–988); *αTub*-F CAAGCAAAGATTCACGCCCTG (–117) and *αTub*-R CGCCGCATAACCGATAACTG (–2).

Northern blotting

Total RNA was extracted from ~100 μg fly embryos, larvae and adults or 5×10^6 Kc cells using Trizol Reagent (Invitrogen). An aliquot (10 μg) of total RNA was electrophoresed in 1% agarose gel containing formaldehyde, blotted to nylon membrane and probed with ^{32}P-labeled DNA fragments generated by RT-PCR. The primer sequences are available upon request.

Microarrays

Microarray experiments were performed using Affymetrix GeneChip *Drosophila* Genome 2.0 Array in the UMCCC Affymetrix and cDNA Microarray Core Facility at University of Michigan, Ann Arbor. Statistical analyses were done using previous published methods (Irizarry et al., 2006; Smyth, 2004; Yuan et al., 2005). Gene expression patterns were visualized as colorgrams using TreeView (Eisen et al., 1998). The original and normalized data can be accessed at the Gene Expression Omnibus (http://www.ncbi.nlm.nih.gov/geo) accession number GSE15092.

RESULTS

The *Drosophila ptip* gene is ubiquitously expressed in fly

All vertebrates have a single, conserved *Ptip* gene, as based on the position and number of BRCT domains and a glutamine-rich central region located between BRCT domains two and three. A BLAST search of invertebrate genomes also uncovered a *ptip*-related gene in sea urchins, insects and *C. elegans*. An evolutionary distance tree shows the relationship among *ptip* homologs from select invertebrates and vertebrates (Fig. 1A). Note that human *ptip* is more closely related to *Drosophila* (3×10^{-60}) and honey bee (5×10^{-81}) *ptip* than it is to the next most homologous mammalian BRCT domain encoding gene *Mdc1* (2×10^{-16}). The *Drosophila* genome contains a single homologous gene (Flybase ID CG32133), which we believe is the fly *ptip* ortholog based on its markedly similar domain structure to mouse and human *Ptip* (Lechner et al., 2000). The *Drosophila* hypothetical PTIP protein contains two amino-terminal and four carboxy-terminal BRCT domains and an expanded glutamine-rich region (Fig. 1B). Northern analysis reveals that fly *ptip* encodes a single mRNA product of ~7 kb (Fig. 1D), which we have confirmed by RT-PCR and sequencing (data not shown). Fly *ptip* is expressed ubiquitously in the embryo as judged by in situ hybridization (data not shown) and in all developmental stages shown by Northern analysis (Fig. 1D).

Maternally expressed *ptip* is essential for early embryonic development

Three *ptip* alleles are used in this study. Two are deficiencies, referred to as $Df(ptip)^1$ and $Df(ptip)^2$, that have the entire *ptip* locus deleted (see Materials and methods for details). The third allele is a lethal *piggyBac* insertion line, called PB^{c04574} ($PBac^{ptip}$) from the Exelixis collection (Thibault et al., 2004). We used PCR and sequencing to confirm that the $PBac^{ptip}$ insertion mapped to the fifth exon of the *ptip* gene (data not shown) and noted that it is predicted to delete its three carboxy-terminal BRCT domains (Fig. 1C). The

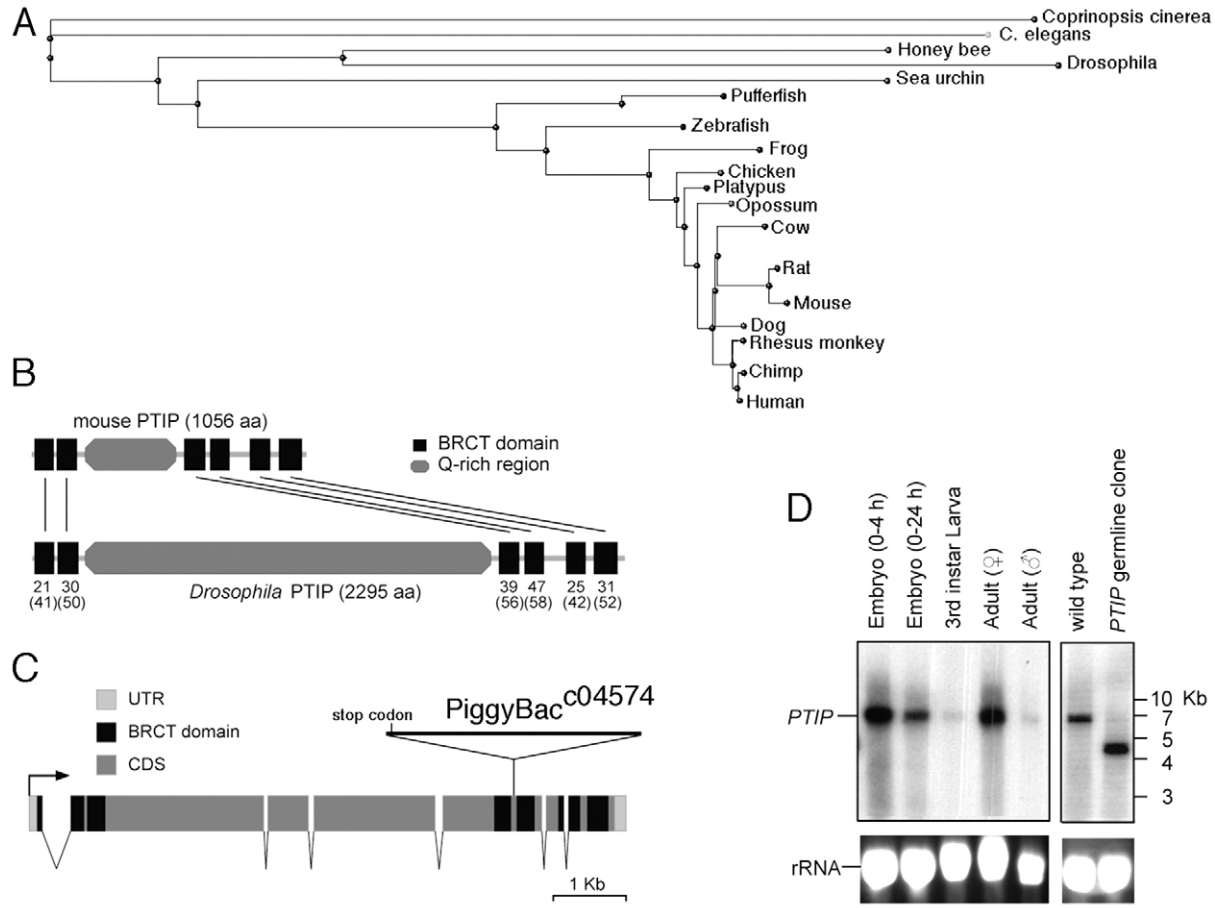


Fig. 1. Conservation of *ptip* gene structure. (**A**) A phylogenetic tree approximating the evolutionary distance of presumptive *ptip* homologs across species. (**B**) Schematic alignment of mouse and *Drosophila* PTIP proteins. The BRCT domains are indicated by black boxes, the percentage amino acid identity and similarity (in parentheses) is shown below. The expanded glutamine-rich region is in gray. (**C**) Genomic structure of the *Drosophila ptip* gene; the position of the *PiggyBac*^{c04574} insertion is indicated. (**D**) Northern blot of total RNA isolated from the stages indicated and hybridized with a *Drosophila ptip* probe. Note the *ptip* germline null embryo clones have lost the full length 7 kb mRNA. Total 18S rRNA is shown by ethidium bromide staining below.

PBac^{ptip} allele is homozygous pre-pupal lethal and is also lethal over *Df(ptip)*^{1} and *Df(ptip)*^{2}, indicating that the disruption of *ptip* is responsible for the lethality.

Since *ptip* mRNA is expressed at particularly high levels in early embryos as well as in female adults, we suspected that it is a maternally expressed gene (Fig. 1D). To remove maternal *ptip* contributions, we recombined *PBac*^{ptip} onto the *FRT*^{2A} chromosome and generated germline mutant clones using *ovoD* for selection (Chou and Perrimon, 1992; Chou and Perrimon, 1996). Indeed, full length *ptip* mRNA was completely removed in early *PBac*^{ptip} germline clone embryos (Fig. 1D), thus confirming a maternal contribution of *ptip*. Instead of the full length *ptip* mRNA, these embryos express a truncated form of *ptip* mRNA, which is consistent with the sequence analysis and indicates a carboxy-terminal disruption of the *ptip* gene (Fig. 1D). We were not able to determine whether a truncated protein product is produced from this transcript but, even if this were the case, the deleted three carboxy-terminal BRCT domains would be likely to abrogate the function of PTIP in flies, since the carboxy-terminal BRCT pairs are crucial for PTIP function (Manke et al., 2003; Patel et al., 2007). Given the embryonic lethality and gene structure, we believe *PBac*^{ptip} is a strong hypomorphic allele, if not a complete null.

As described above, *PBac*^{ptip} homozygotes have a pre-pupal lethal phenotype, as do *PBac*^{ptip}/*Df(ptip)*^{1} and *PBac*^{ptip}/*Df(ptip)*^{2} transheterozygotes. To test the possible roles of maternal and zygotic *ptip* in fly embryo development, we examined the cuticles of embryos derived from *PBac*^{ptip} germline clones. Wild-type embryos have a distinctive patterning of denticles on their ventral cuticle, with each denticle belt arranged in a trapezoidal pattern with intermittent naked cuticle (Fig. 2A). When mothers producing *PBac*^{ptip} mutant eggs are crossed with *Df(ptip)*^{1}/*TM6B* heterozygotes, two classes of mutant phenotype are observed. One mutant displays severely disrupted/complete loss of denticle belts (Fig. 2B), which might indicate early embryonic lethality. In fact, the number of embryos in this class is obviously less than half of the total, but the presence of many empty egg cases indicates that some embryos might die even before they begin to secret cuticle. This phenotypic class was not observed when fathers were wild type, indicating that they are maternal and zygotic *ptip* mutants. By sharp contrast, the other class of embryos exhibit fairly normal cuticle patterns, although frequently with denticle fusions (Fig. 2C). These embryos are maternal *ptip* mutants, as they are similar to what we observed when fathers are wild type (data not shown). Therefore, zygotic *ptip* is sufficient to rescue the cuticle defects caused by the

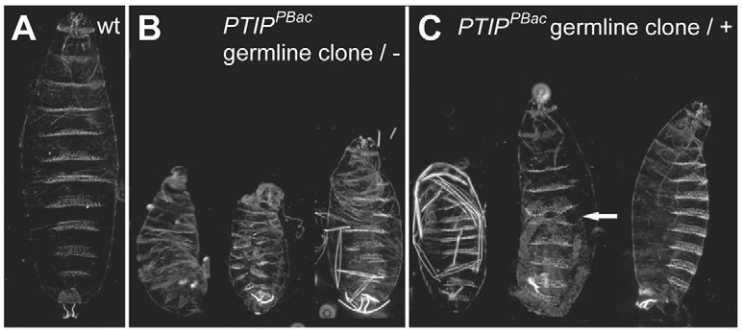


Fig. 2. Analysis of *Drosophila* germline *ptip* mutants in development. Germline null and zygotic rescue embryos were generated by crossing *PBac^{PTIP}/FRT^{2A}* P[*ovoD*] females to *Df(ptip)^{1}/TM6 evelacZ* males. Micrographs of cuticles from wild type (**A**), *ptip* maternal and *ptip* zygotic mutants (**B**) and zygotically rescued *ptip* maternal mutants (**C**) are shown. Note that the loss of maternal and zygotic *ptip* causes severe embryonic segmentation defects that could be partially rescued by providing a paternal *ptip* gene (arrow).

removal of the maternal contribution of *ptip.* We conclude that maternal and the zygotic *ptip* expression are both important for normal fly embryo development.

These cuticle defects in *ptip* mutant flies are consistent with the hypothesis that *ptip* plays a role in early patterning. To characterize the germline null phenotype more precisely, we examined the expression patterns of maternal effect, pair rule and segment polarity genes (Fig. 3). Localization of maternal *bicoid* and *oskar* mRNAs appear unaffected in *ptip* mutant embryos (Fig. 3A,B). However, the expression of the pair rule genes *fushi tarazu* (*ftz*) and *even skipped* (*eve*), which both exhibit a characteristic seven-stripe pattern at the cellular blastoderm stage, was severely affected (Fig. 3C,D). The *ptip* mutants exhibited missing stripes and reduced levels of expression, suggesting failure to activate or maintain transcription from specific enhancer regions of the *eve* and *ftz* genes. Although few apparent *ptip* mutant embryos developed to the extended germband stage, the segment polarity gene *engrailed* (*en*) was not detected in the characteristic 14-stripe pattern in *ptip* mutants (Fig. 3E).

We also examined expression in transheterozygotic *ptip* flies [*PBac^{ptip}/Df(ptip)^{1}*], which lack zygotic *ptip*. These mutants develop further, presumably as a result of maternal rescue. Imaginal discs from late third instar larvae were examined for expression of developmental regulators (Fig. 4). The mutant third instar larvae have no difference in size and locomotion compared with wild type, however, their imaginal discs are always morphologically smaller and are distorted (Fig. 4A,B). *en* is normally only expressed in the posterior compartment of the wing disc and is a widely used molecular marker to examine A/P patterning in the wing disc. However, in the *ptip* mutant discs EN protein is expressed broadly over the entire disc (Fig. 4B), as a result of derepression of *en* in the anterior compartment. This altered EN expression pattern is also observed in third instar leg discs (Fig. 4C,D). The EN protein can repress the Hedgehog effector *cubitus interuptus* (*ci*) through a PcG-mediated mechanism (Dahmann and Basler, 2000; Maschat et al., 1998). Consistent with the ectopic expression of *en*, the expression of *ci* is lost in the anterior half of *ptip* mutants (Fig. 4G,H). By sharp contrast, *wingless* (*wg*), a known signaling determinant of the dorsal/ventral (D/V) axis (Campbell et al., 1993; Galindo et al., 2002), appears to be expressed within normal boundaries (Fig. 4E,F), as is the *wg* target, *Distal-less* (*Dll*). These data suggest that in the absence of *ptip* there is a disruption of A/P patterning in the imaginal disc, although PcG-mediated repression appears intact.

Altered levels of histone methylation

Given the association of PTIP with a histone H3K4 methyltransferase complex, we examined the levels of H3K4 trimethylation in the nuclei of salivary glands isolated from third instar larvae (Fig. 5A-L). Antibodies against trimethyl histone H3K4 (H3K4me3) exhibit strong nuclear staining in wild-type nuclei but show a marked reduction in staining intensity in *ptip* mutants. The reduction in staining intensity observed with H3K4me3 antibodies in *ptip* mutants correlates with an increase in the staining intensity using anti-trimethyl histone H3K27 (H3K27me3) antibodies. To more precisely quantitate these differences, we acid-extracted total histones from wild-type and zygotic *ptip* mutant larvae and probed western blots with methyl-lysine specific antibodies (Fig. 5M).

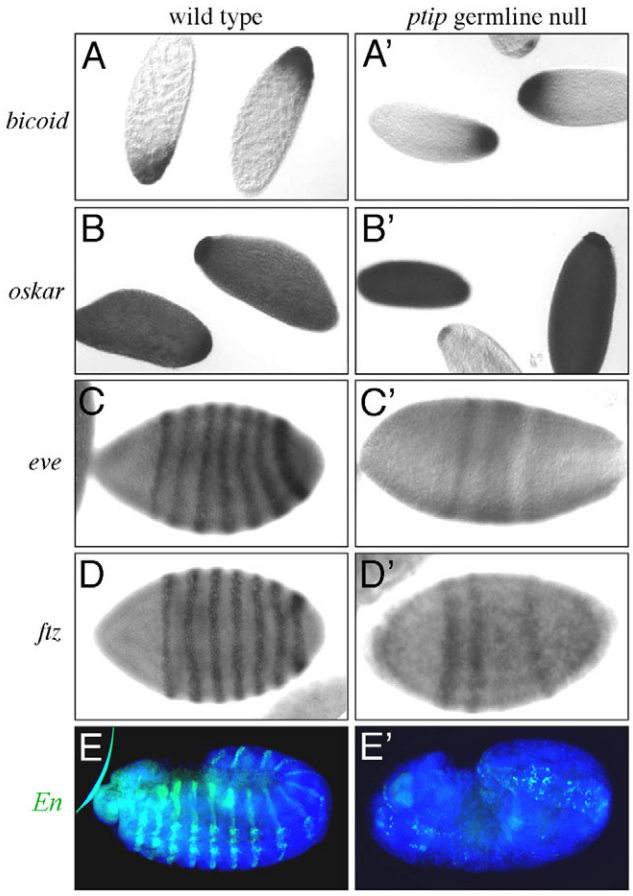


Fig. 3. Patterning defects in *ptip* mutants. (**A-E′**) The expression of maternal effect and segmentation genes is shown in wild type (A-E) and *ptip* germline null mutants (A′-E′). Whole-mount in situ hybridization for *bicoid* (A), *oskar* (B), *eve* (C) and *ftz* (D), and immunostaining for Engrailed (E). Note the normal posterior localization of *bicoid* mRNA in *ptip* mutants (A′) but the loss of some pair rule gene stripes at the cellular blastoderm stage (C′,D′). By the extended germband stage, Engrailed is barely detectable in *ptip* mutants (E′).

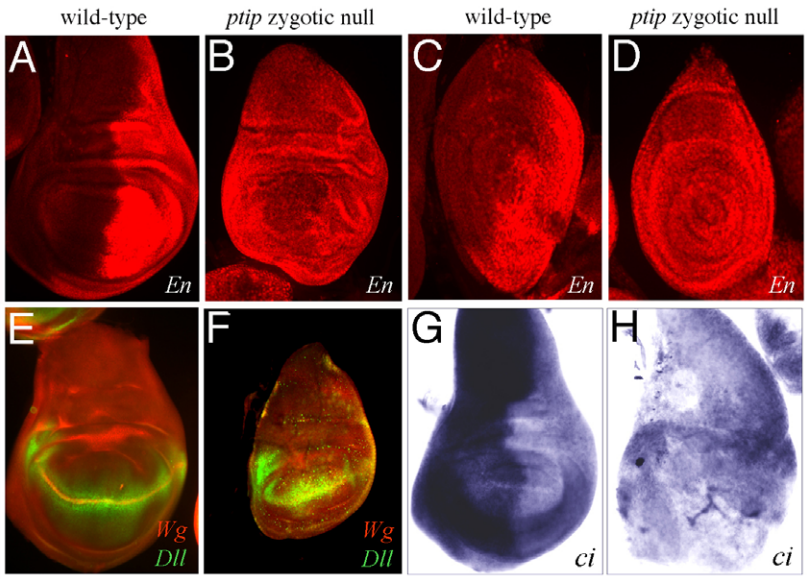


Fig. 4. Patterning defects in zygotic *ptip* null embryos. The imaginal discs from wild type (A,C,E,G) or zygotic *ptip* mutants with genotype $PBac^{ptip}/Df(ptip)^{1}$ (B,D,F,H) were isolated from third instar larvae. (**A**,**B**) Wing imaginal discs stained with anti-Engrailed (red). (**C**,**D**) Leg imaginal discs stained with anti-Engrailed (red). (**E**,**F**) Micrographs of wing imaginal discs immunostained with antibodies against Wingless (red) and Distal-less (green). (**G**,**H**) Whole-mount in situ hybridization for *cubitus interuptus* (*ci*) expression in the wing imaginal discs. Note absence of *ci* expression in *ptip* mutants correlates with ectopic Engrailed protein in the anterior half of wing and leg discs.

Image analysis revealed a ~50% decrease in total levels of trimethyl H3K4 when normalized to total histone H3, whereas levels of trimethyl H3K27 increased more than 80%. These data are consistent with a role for *Drosophila ptip* as a cofactor for trxG-mediated histone methylation.

Mouse *Ptip* rescues the $PBac^{ptip}$ homozygous lethality

Although the structure and number of BRCT domains is similar between the *Drosophila* and mammalian PTIP proteins, a transgenic rescue approach would be a better test of functional equivalence (Fig. 6). Thus, flies were generated that carried the mouse *Ptip* cDNA (m*Ptip*) under the control of the *UAS*-GAL4 promoter, such that mouse PTIP protein could be expressed after heat shock with the hs-Gal4 driver strain (Fig. 6A). The mouse transgene was crossed into the heat shock Gal4 line carrying the $PBac^{ptip}$ allele to generate *w*; P[*UAS*-m*Ptip*]; $PBac^{ptip}/TM6B$ males. These were crossed to females *w*; P[hsGal4]/*CyO*; $Df(ptip)^{1}/TM6B$ to generate P[hsGal4]/P[*UAS*-m*Ptip*]; $Df(ptip)^{1}/PBac^{ptip}$ flies, which express mPTIP, and control *w*; P[*UAS*-m*Ptip*]/*CyO*; $Df(ptip)^{1}/PBac^{ptip}$ flies without the GAL4 activator. Although there were some survivors even in the absence of heat shock, suggesting

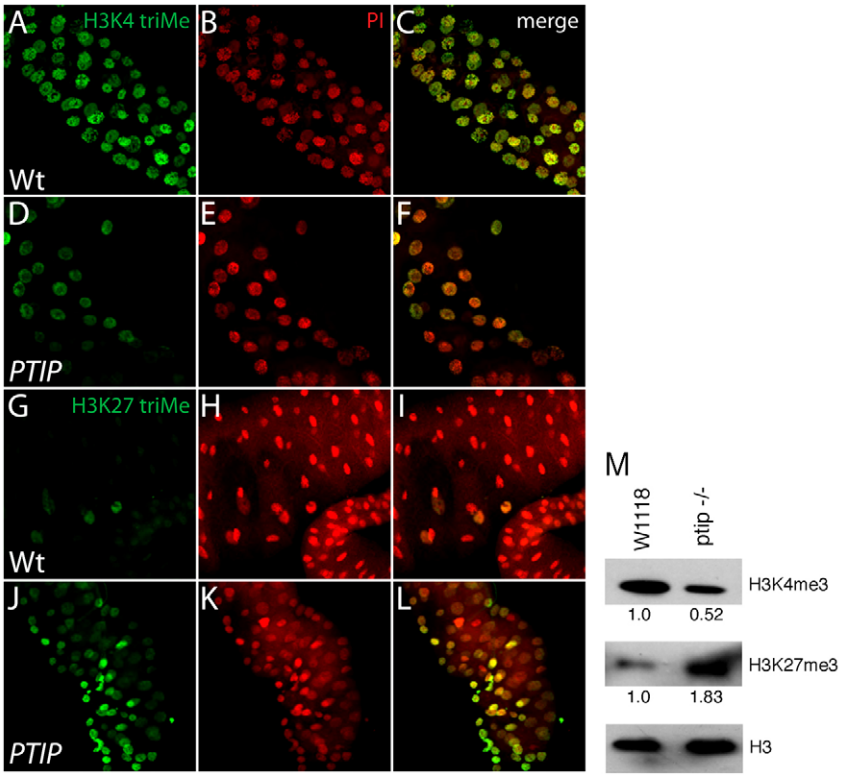


Fig. 5. Immunostaining for histone H3K4 trimethylation. (**A-L**) Salivary glands were dissected free from third instar larvae of wild type (A-C,G-I) or *ptip* zygotic nulls (D-F,J-L) and squashed onto slides for immunostaining with anti-histone H3K4me3 (A,D) or anti-histone H3K27me3 (G,J) antibodies (green). Nuclei were co-stained with propidium iodide (PI, red; B,E,H,K) and merged images are also shown (C,F,I,L). All micrographs were taken at manually set, equal exposure times. (**M**) Western blot of histones extracted from wild-type or zygotic *ptip* mutant larvae probed with the indicated antibodies. Relative protein levels normalized to total histone H3 are indicated; wild-type levels are arbitrarily designated as 1.0.

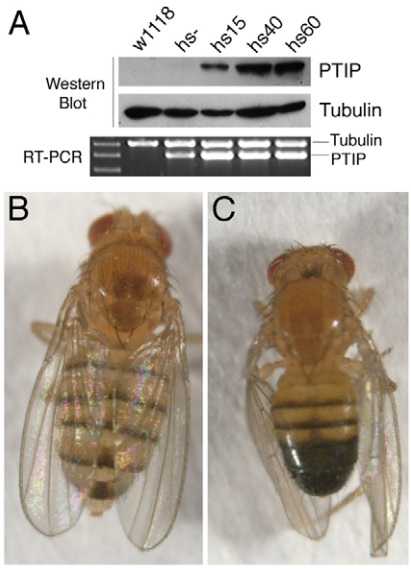


Fig. 6. Mouse *ptip* rescues zygotic *PBac^{ptip}* lethality. (**A**) Western blot using an anti-mousePTIP antibody shows a dose response of mPTIP expression with the increased time of heat shock treatment (above) consistent with the RT-PCR results using a mouse *ptip*-specific primer pair (below). *w^{1118}*, control flies; hs–, *UAS*-m*Ptip* without heat shock; hs15, 15 minute heat shock; hs40, 40 minute heat shock; hs60, 60 minute heat shock. (**B**) Adult female and (**C**) adult male flies with the genotype P[hsGal4]/ P[*UAS*-m*Ptip*]; *Df(ptip)^{1}/PBac^{ptip}* that were derived from heat shocked embryos as described in the Materials and methods section.

leaky expression of mPTIP, activation by heat shock resulted in viable adult flies that carried both *Drosophila* mutant *ptip* alleles (Fig. 6B,C). These rescued flies represented ~4.1% (23/564) of offspring, close to the expected 6.25%. Notably, no adult flies were observed with the genotype P[*UAS*-m*Ptip*]/*CyO*; *Df(ptip)^{1}/PBac^{ptip}* in these crosses, again confirming the lethal nature of the *ptip* homozygotes. Thus, the expression of the mouse *Ptip* cDNA was sufficient to rescue the zygotic null phenotype observed in the *PBac^{ptip}/Df(ptip)^{1}* offspring.

PTIP is required for activation of PcG target genes in Kc cells

Two lines of evidence led us to propose that *ptip* might be involved in the epigenetic control of gene expression by the PcG/trxG pathways. First, mammalian PTIP protein is associated with ALR and MLL3, two SET domain H3K4 methyltransferases similar to *Drosophila* Trithorax (Cho et al., 2007; Issaeva et al., 2007; Patel et al., 2007). Second, analyses of histone methylation in *ptip* mutants revealed global and reciprocal changes in H3K4 and H3K27 methylation levels. To further examine the function of *ptip* in *Drosophila*, we utilized the Kc cell line to assay for gene expression in the presence or absence of Polycomb-mediated repression and *ptip* using double stranded RNA (dsRNA) mediated knockdown. Since *Ultrabithorax* (*Ubx*) is a known PcG target (Castelli-Gair and Garcia-Bellido, 1990; Tillib et al., 1999), we examined whether *ptip* has an impact on the expression of this gene (Fig. 7). The *Ubx* gene is expressed at very low levels in Kc cells, as observed in other *Drosophila* cell lines derived from embryos (Breiling et al., 2001; Schwartz et al., 2006). No significant change in *Ubx* expression is observed when cells are cultured with 3′ or a mixture of 3′ and 5′ *ptip* dsRNAs. However, a slight increase in *Deformed* (*Dfd*) mRNA is observed in *ptip* knockdowns (Fig. 7A). Using microarray analyses to compare untreated Kc cells with *ptip* knockdowns after 7 days of culture revealed 266 genes with significant changes in expression levels (Fig. 8). Of these, 52 were upregulated in the *ptip* knockdowns and 214 were downregulated. The potential *ptip* targets corresponded to genes associated with metabolic processes, transcription, translation and signaling, with no specific pattern observed (see Table S1 in the supplementary material for the normalized dataset). The level of *ptip* knockdown with 3′ and 5′ dsRNAs was consistent at ~70% as measured by mRNA Northern blotting and phosphoimage analysis. Whether this reflects heterogeneity among cells owing to variable uptake of dsRNAs, or the inability of cells to completely eliminate the *ptip* mRNA, could not be determined.

A different pattern emerges when *ptip* was knocked down together with PcG genes. Suppression of the PcG genes *Polycomb* (*Pc*) and *polyhomeotic* (*ph*) by dsRNA in Kc cells resulted in the upregulation of *Ubx* (Fig. 7), indicating that *Ubx* is a target of PcG-mediated repression, as reported previously (Wang et al., 2004). The activation of *Ubx* expression requires the trxG genes, since the activation of *Ubx* is low when *trx* and *ash1* are knocked down along

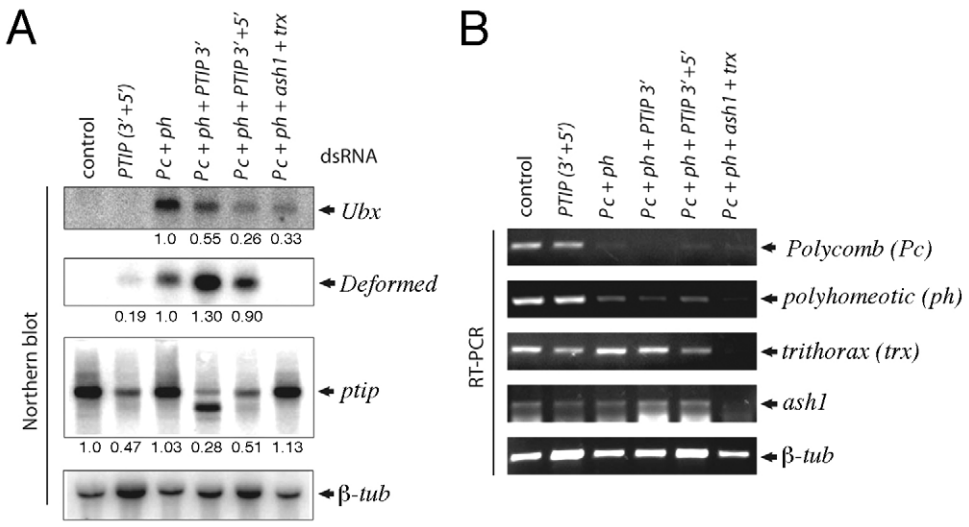
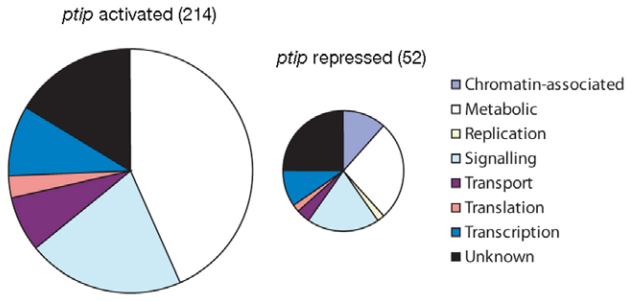


Fig. 7. Derepression of *Ubx* in *Drosophila* Kc cells. (**A**) Kc cells were cultured for 8 days with control dsRNAs or with dsRNA against the indicated genes. Total RNA levels were analyzed by Northern blotting with probes against *Ubx*, *Deformed*, *ptip* and *β-Tubulin*. For *ptip* knockdowns, 3′- and 5′-specific dsRNAs were used. Relative mRNA levels are indicated below each band. Note, *Ubx* activation in response to PcG knockdown requires *ptip*, whereas *Deformed* activation does not. (**B**) Expression levels of the indicated genes were assayed by RT-PCR after the addition of dsRNAs (above) and 8 days in culture.



	up	down	*ptip*-repressed	*ptip*-activated
Chromatin-associated	6	0	12%	0%
Metabolic	14	93	27%	43%
Replication	1	0	2%	0%
Signalling	10	44	19%	21%
Transport	2	16	4%	7%
Translation	1	6	2%	3%
Transcription	5	20	10%	9%
Unknown	13	35	25%	16%
Total	52	214		

Fig. 8. Microarray analysis of *ptip* knockdown in Kc cells. The charts summarize gene expression changes observed in *Drosophila* Kc cells after 7 days of treatment with *ptip* dsRNAs. A total of 52 genes were expressed at levels significantly higher (more than twofold) than controls, whereas 214 genes were expressed at twofold or lower levels. The complete dataset is presented in Table S1 in the supplementary material.

with *Pc* and *ph* (Fig. 7). These data suggest that the derepression of *Ubx* in Kc cells is PcG/trxG pathway-specific. Interestingly, when *ptip* was knocked down together with PcG genes, we found that the activation of *Ubx* was also decreased compared with the PcG knockdown alone. This suggests a role for *ptip* in *trx*-mediated activation of *Ubx* once PcG repression is removed. After relieving PcG repression, the levels of *Ubx* were similarly reduced when cells were cultured either with *ptip* or with *trx* and *ash1* dsRNAs. The homeotic gene *Dfd* was also activated upon PcG repression in a *trx* and *ash1* dependent manner. However *Dfd* expression was not *ptip* dependent, suggesting that *ptip* is required only in a subset of potential PcG/trxG targets. In fact, *Dfd* expression was slightly activated upon *ptip* knockdown and was highest when all three genes, *Pc*, *ph* and *ptip*, were knocked down together (Fig. 7A).

To determine more precisely which genes were *ptip*-dependent and to extend our findings that suggest *ptip* might be a component of the PcG/trxG pathway, we performed Affymetrix microarray analysis to compare gene expression patterns in Kc cells under normal conditions, after *ptip* knockdown, after PcG knockdown and after *ptip* plus PcG knockdown (Fig. 9; see Table S2 in the supplementary material). We obtained a list of PcG targets that are derepressed by PcG knockdown, as judged by statistically significant differences. Many of the genes are known PcG targets and are developmental regulators. However, if both *ptip* and PcG are knocked down, activation of many PcG target genes is significantly reduced when compared with expression levels observed in the PcG gene knockdowns alone (Fig. 9A). This is even more evident when the changes in expression levels are averaged among the top 74 PcG target genes (Fig. 9B). Table 1 outlines a select group of PcG targets and known regulators of development that are derepressed upon treatment with dsRNA against *Pc* and *ph* and require *ptip* for activation upon derepression. These PcG targets are generally unaffected by the *ptip* knockdown alone. In the same experiment, expression levels of the housekeeping genes, such as the tubulins and genes encoding RNA-associated proteins, are minimal (Fig. 9A,B). In addition, the levels of *ptip* are unaffected in the *Pc* knockdowns and the level of *Pc* is unaffected in the *ptip* knockdowns, as judged by Northern analysis (Fig. 9C). These results indicate that the *ptip* mutation might be a suppressor of PcG mutations and might be generally required for the trxG-dependent activation of many genes after removal of PcG repression.

Table 1. Microarray expression levels* of selected PcG targets

Probe ID	Gene	Control	ds*ptip*	dsPcG	ds*ptip*+PcG
1637292	*Esp*	5.98	6.89	11.42	8.41
1635796	*cad*	5.21	5.30	10.00	8.31
1631066	*rib*	5.53	5.86	10.05	7.96
1634370	*pnr*	5.38	5.44	9.38	7.95
1624859	*rpr*	5.08	5.32	8.57	6.70
1639798	*mirr*	5.48	5.77	8.37	7.25
1640296	*Optix*	5.56	5.76	8.28	5.88
1626946	*oc*	5.36	5.27	8.05	6.31
1624744	*nuf*	7.45	6.19	9.97	7.43
1626150	*tsh*	5.45	5.58	7.76	6.58
1631095	*Psc*	8.18	8.00	10.10	8.82
1639500	*nub*	4.43	4.46	6.34	4.59
1639940	*disco*	4.83	4.73	6.69	5.11

*Average of three chips, log scale.

PTIP interacts with PcG target genes after derepression

Given the effects of *ptip* knockdowns on PcG target gene expression in Kc cells, we asked whether PTIP can interact directly with the genes whose expression requires PTIP. At present, we do not have good antibodies against *Drosophila* PTIP nor do we have a full length *Drosophila ptip* cDNA clone. Cloning of the fly cDNA has proved difficult, in part because of the extreme repetitive nature of the glutamine-rich region. Thus, we expressed mPTIP in the fly Kc cells in concert with dsRNAs against *Pc* and *ph* and used these cells for chromatin immunoprecipitation (ChIP; Fig. 10). Cells were transfected with mPTIP and cultured for 3 days with control dsRNAs or dsRNAs against *Pc* and *ph*. The mPTIP protein could be readily detected in chromatin preparations after reverse-crosslinking and western blotting (Fig. 10A). Antibodies against mPTIP and H3K4me3 were used for ChIP experiments to determine if PTIP localized to any of the PcG target genes identified by the microarrays (Table 1). Strikingly, mPTIP could localize near the transcription start sites of all of the PcG genes examined but only when cells were also cultured with PcG dsRNAs (Fig. 10B). Furthermore, trimethylation at H3K4 increased significantly after PcG dsRNA treatment (Fig. 10C). This increase in H3K4me3 was already evident after PcG dsRNA treatment, although the added expression of mPTIP increased H3K4me3 levels even further. Without PcG dsRNAs, mPTIP expression did not increase H3K4me3 levels nor did the mPTIP protein localize to the target genes. As a negative control, a tubulin promoter region did not show changes in H3K4me3 nor did it bind to mPTIP. These data strongly argue for a direct interaction between PTIP and the promoter regions of PcG target genes upon derepression.

DISCUSSION

Embryonic development requires epigenetic imprinting of active and inactive chromatin in a spatially and temporally regulated manner, such that correct gene expression patterns are established and maintained. In this report, we show that *Drosophila ptip* is essential for early embryonic development. We further show that in larval development, *ptip* coordinately regulates the methylation of histone

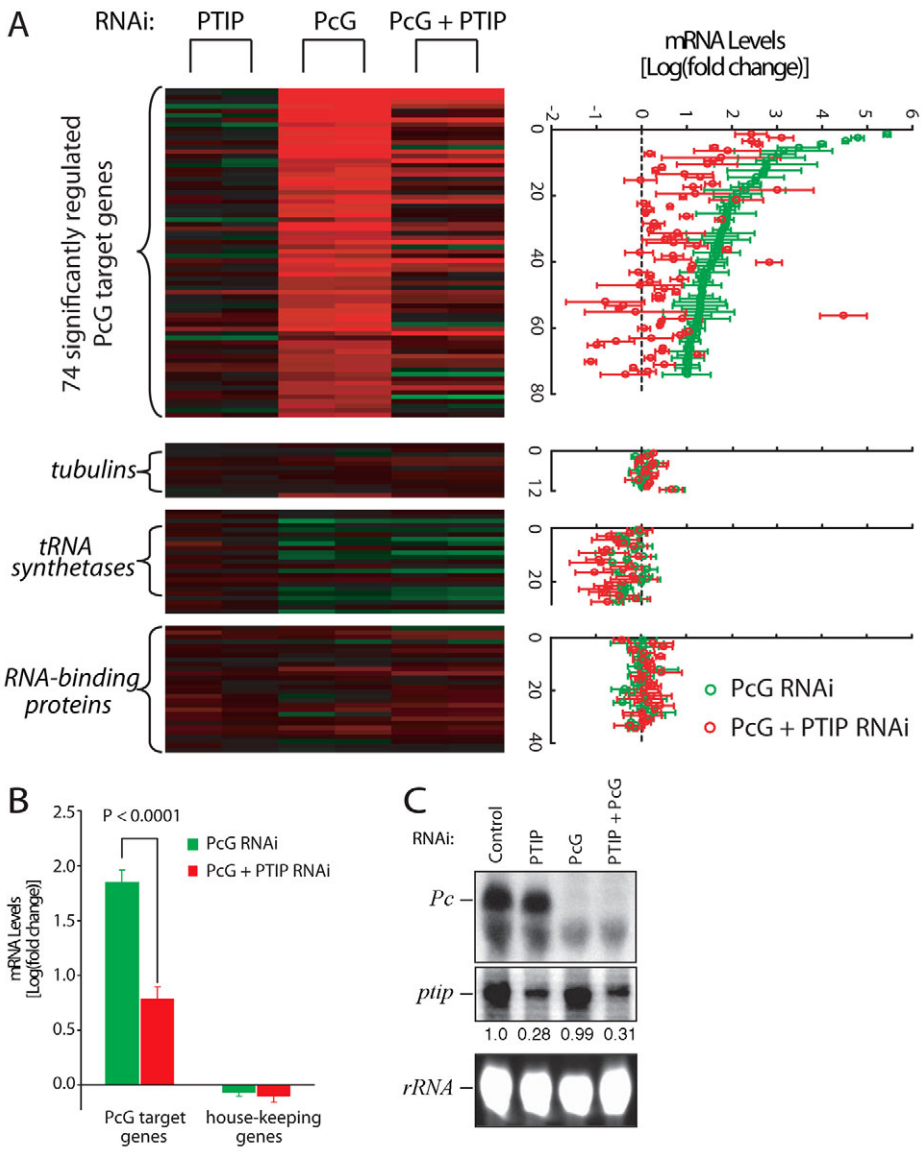


Fig. 9. Microarray analysis of PcG target genes in Kc cells. (**A**) Expression heat diagram for the top 74 mRNA transcripts that demonstrate significant and greater than twofold upregulation of mRNA levels after PcG gene knockdown (red). Each row represents a gene. Each column represents relative expression levels after the combined RNAi treatments as indicated. The level of expression relative to that of control RNAi samples (in triplicates) of each gene is represented using a red-to-green color scale. For reference, expression values for housekeeping genes and RNA-related genes are also shown. Scatter graphs of mRNA expression levels, presented as fold change on a logarithmic scale, are shown to the right. The fold change for PcG knockdowns (green) is compared with the fold change for PcG plus PTIP knockdowns (red). The complete list of Polycomb target genes is shown in Table S2 in the supplementary material. The original array data is available at http://www.ncbi.nlm.nih.gov/geo (accession number GSE15092). (**B**) Averaged expression levels of datasets represented in A. *P* value is calculated by ANOVA analysis followed by Newman-Keul's multiple comparisons. (**C**) Northern analysis of *Polycomb* and *ptip* gene expression levels of samples used in A, rRNA levels are shown for loading controls. Relative mRNA levels of *ptip*, as determined by phosphoimage analysis, are indicated below each band.

H3K4 and demethylation of H3K27, consistent with the reports that mammalian PTIP complexes with HMT proteins ALR and MLL3, and the histone demethylase UTX (Cho et al., 2007; Issaeva et al., 2007). In wing discs, *ptip* is required for appropriate A/P patterning by affecting morphogenesis determinant genes, such as *en* and *ci*. These data demonstrate in vivo that dynamic histone modifications play crucial roles in animal development and PTIP might be necessary for coherent histone coding. In addition, *ptip* is required for the activation of a broad array of PcG target genes in response to derepression in cultured fly cells. These data are consistent with a role for *ptip* in trxG-mediated activation of gene expression patterns.

Early development requires *ptip* for the appropriate expression of the pair rule genes *eve* and *ftz*. The characteristic seven-stripe *eve* expression pattern is regulated by separate enhancer sequences (Jiang et al., 1991; Small et al., 1993), which are not all equally affected by the loss of *ptip*. The complete absence of *en* expression at the extended germband stage also indicates the dramatic effect of *ptip* mutations on transcription. The characteristic 14 stripes of *en* expression depends on the correct expression of pair rule genes (DiNardo and O'Farrell, 1987), which are clearly affected in our *ptip* mutants. However, the maintenance of *en* expression at later stages and in imaginal discs is regulated by PREs and PcG proteins (Breen et al., 1995; Brizuela and Kennison, 1997; DeVido et al., 2008; Maschat et al., 1998; Moazed and O'Farrell, 1992). If *ptip* functions as a trxG cofactor, then expression of *en* along the entire A/P axis in the imaginal discs of *ptip* mutants might be due to the absence of a repressor. This might explain the surprising presence of ectopic *en* in the anterior halves of imaginal discs from zygotic *ptip* mutants. This ectopic *en* expression is likely to result in suppression of *ci* through a PcG-mediated mechanism. Yet, it is not clear how *en* is normally repressed in the anterior half, nor which genes are responsible for derepression of *en* in the *ptip* mutant wing and leg discs.

The direct interaction of PTIP protein with developmental regulatory genes is supported by ChIP studies in cell culture. Given the structural and functional conservation of mouse and fly PTIP, we expressed mPTIP in fly cells and showed that it can localize to the 5′ regulatory regions of many PcG target genes that are activated upon loss of PC and PH activity. Consistent with the interpretation that a PTIP trxG complex is necessary for activation of repressed genes, mPTIP only bound to DNA upon loss of *Pc* and *ph* function.

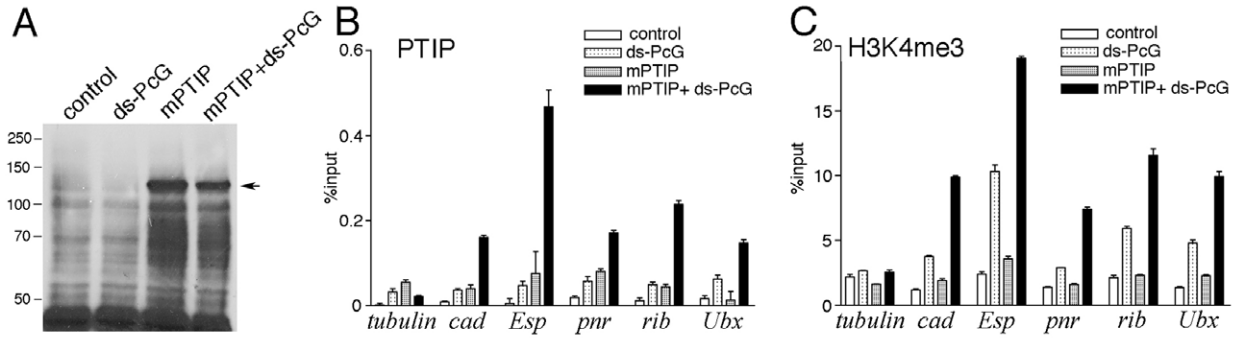


Fig. 10. Chromatin immunoprecipitation of mPTIP in Kc cells. (**A**) Chromatin was prepared from Kc cells transfected with control dsRNAs, dsRNAs against *Pc* and *ph* (ds-PcG), control dsRNAs and m*Ptip*, or ds-PcG and m*Ptip*. Western blot probed with antibodies against mPTIP (arrow) is shown. (**B**) ChIP experiment using antibodies against mPTIP and PCR primer pairs against the indicated genes. The chromatin preparations are as described in A. Data are normalized to control rabbit IgG and presented as the percentage input as calculated from standard curves. Note that mPTIP localizes to PcG target genes only when ds-PcG is present. Experiments were done in triplicate; error bars are one standard deviation from the mean. (**C**) ChIP experiment using antibodies against H3K4me3 and the chromatin preparations described in A. Note the increase in H3K4me3 levels when ds-PcG is present. This increase is enhanced with exogenous mPTIP. Experiments were done in triplicate; error bars are one standard deviation from the mean.

In the Kc cells, suppression of both *Pc* and *ph* results in the activation of many important developmental regulators, including homeotic genes. A recent report details the genome-wide binding of PcG complexes at different developmental stages in Drosophila and reveals hundreds of PREs located near transcription start sites (Oktaba et al., 2008). Strikingly, most of the genes we found activated in the Kc cells after PcG knockdown also contain PRE elements near the transcription start site.

In vertebrates, PTIP interacts with the Trithorax homologs ALR/MLL3 to promote assembly of an H3K4 methyltransferase complex. The tissue and locus specificity for assembly may be mediated by DNA-binding proteins such as PAX2 (Patel et al., 2007) or SMAD2 (Shimizu et al., 2001), which regulate cell fate and cell lineages in response to positional information in the embryo. In flies, recruitment of PcG or trxG complexes to specific sites also can require DNA-binding proteins such as Zeste (Dejardin and Cavalli, 2004), DSP1 (Dejardin et al., 2005), Pleiohomeotic (Brown et al., 2003; Wang et al., 2004) and Pipsqueak (Huang et al., 2002). Whereas PcG complexes have been purified and described in detail (Ringrose and Paro, 2007; Schuettengruber et al., 2007; Shao et al., 1999), much less is known about the *Drosophila* trxG complexes. Purification of a trxG complex capable of histone acetylation (TAC1) revealed the proteins CBP and SBF1 in addition to TRX (Petruk et al., 2001; Petruk et al., 2004). By contrast, the mammalian MLL/ALL proteins are components of large multi-protein complexes capable of histone H3K4 methylation (Cho et al., 2007; Hughes et al., 2004; Nakamura et al., 2002). Although the mutant analysis, the reduction of H3K4 methylation and the dsRNA knockdowns in Kc cells all suggest that *Drosophila ptip* has trxG-like activity and hence might be a suppressor of PcG proteins, a more definitve biochemical analysis awaits the generation of antibodies and the delineation of in vivo DNA-binding sites for PTIP and its associated proteins at specific target genes.

Mammalian PTIP is also thought to play a role in the DNA damage response based on its ability to bind to phosphorylated p53BP1 (Munoz et al., 2007). PTIP also binds preferentially to the P-SQ motif, which is a good substrate for the ATR/ATM cell cycle checkpoint regulating kinases (Manke et al., 2003). Several reports demonstrate that PTIP is part of a RAD50/p53BP1 DNA damage response complex, which can be separated from the MLL2 histone H3K4 methyltransferase complex (Cho et al., 2007; Patel et al., 2007). Both budding and fission yeast contain multiple BRCT domain proteins that are involved in the DNA damage response, including Esc4 (Rouse, 2004), Crb2 (Saka et al., 1997), Rad9 (Hammet et al., 2007) and Cut5 (Saka and Yanagida, 1993). All of these yeast proteins have mammalian counterparts. However, neither the fission nor budding yeast genomes encodes a protein with six BRCT domains and a glutamine-rich region between domains two and three, whereas such characteristic PTIP proteins are found in *Drosophila*, the honey bee, *C. elegans* and all vertebrate genomes. These comparative genome analyses suggest that *ptip* evolved in metazoans, consistent with an important role in development and differentiation.

In summary, *Drosophila ptip* is an essential gene for early embryonic development and pattern formation. Maternal *ptip* null embryos show early patterning defects including altered and reduced levels of pair rule gene expression prior to gastrulation. In cultured cells PTIP activity is required for the activation of Polycomb target genes upon derepression, suggesting an important role for the PTIP protein in trxG-mediated activation of developmental regulatory genes. The conservation of gene structure and function, from flies to mammals, suggests an essential epigenetic role for *ptip* in metazoans that has remained unchanged.

This work was supported in part by grants from the Chinese 973 Project (2007CB512304) and the Natural Science Foundation of China (30771068 and 90608019) to M.F. and from the National Institutes of Health (DK073722) to G.R.D. Deposited in PMC for release after 12 months.

Supplementary material

Supplementary material available online at
http://dev.biologists.org/cgi/content/full/136/11/1929/DC1

References

Bhanot, P., Fish, M., Jemison, J. A., Nusse, R., Nathans, J. and Cadigan, K. M. (1999). Frizzled and Dfrizzled-2 function as redundant receptors for Wingless during Drosophila embryonic development. *Development* **126**, 4175-4186.

Bouchard, M., Souabni, A., Mandler, M., Neubuser, A. and Busslinger, M. (2002). Nephric lineage specification by Pax2 and Pax8. *Genes Dev.* **16**, 2958-2970.

Breen, T. R., Chinwalla, V. and Harte, P. J. (1995). Trithorax is required to maintain engrailed expression in a subset of engrailed-expressing cells. *Mech. Dev.* **52**, 89-98.

Breiling, A., Turner, B. M., Bianchi, M. E. and Orlando, V. (2001). General transcription factors bind promoters repressed by Polycomb group proteins. *Nature* **412**, 651-655.

Brizuela, B. J. and Kennison, J. A. (1997). The Drosophila homeotic gene moira regulates expression of engrailed and HOM genes in imaginal tissues. *Mech. Dev.* **65**, 209-220.

Brown, J. L., Fritsch, C., Mueller, J. and Kassis, J. A. (2003). The Drosophila pho-like gene encodes a YY1-related DNA binding protein that is redundant with pleiohomeotic in homeotic gene silencing. *Development* **130**, 285-294.
Cadigan, K. M., Fish, M. P., Rulifson, E. J. and Nusse, R. (1998). Wingless repression of Drosophila frizzled 2 expression shapes the Wingless morphogen gradient in the wing. *Cell* **93**, 767-777.
Cai, Y., Lechner, M. S., Nihalani, D., Prindle, M. J., Holzman, L. B. and Dressler, G. R. (2002). Phosphorylation of Pax2 by the c-Jun N-terminal kinase and enhanced Pax2-dependent transcription activation. *J. Biol. Chem.* **277**, 1217-1222.
Cai, Y., Brophy, P. D., Levitan, I., Stifani, S. and Dressler, G. R. (2003). Groucho suppresses Pax2 transactivation by inhibition of JNK-mediated phosphorylation. *EMBO J.* **22**, 5522-5529.
Campbell, G., Weaver, T. and Tomlinson, A. (1993). Axis specification in the developing Drosophila appendage: the role of wingless, decapentaplegic, and the homeobox gene aristaless. *Cell* **74**, 1113-1123.
Castelli-Gair, J. E. and Garcia-Bellido, A. (1990). Interactions of Polycomb and trithorax with cis regulatory regions of Ultrabithorax during the development of Drosophila melanogaster. *EMBO J.* **9**, 4267-4275.
Cho, Y. W., Hong, T., Hong, S., Guo, H., Yu, H., Kim, D., Guszczynski, T., Dressler, G. R., Copeland, T. D., Kalkum, M. et al. (2007). PTIP associates with MLL3- and MLL4-containing histone H3 lysine 4 methyltransferase complex. *J. Biol. Chem.* **282**, 20395-20406.
Chou, T. B. and Perrimon, N. (1992). Use of a yeast site-specific recombinase to produce female germline chimeras in Drosophila. *Genetics* **131**, 643-653.
Chou, T. B. and Perrimon, N. (1996). The autosomal FLP-DFS technique for generating germline mosaics in Drosophila melanogaster. *Genetics* **144**, 1673-1679.
Clemens, J. C., Worby, C. A., Simonson-Leff, N., Muda, M., Maehama, T., Hemmings, B. A. and Dixon, J. E. (2000). Use of double-stranded RNA interference in Drosophila cell lines to dissect signal transduction pathways. *Proc. Natl. Acad. Sci. USA* **97**, 6499-6503.
Dahmann, C. and Basler, K. (2000). Opposing transcriptional outputs of Hedgehog signaling and engrailed control compartmental cell sorting at the Drosophila A/P boundary. *Cell* **100**, 411-422.
Dejardin, J. and Cavalli, G. (2004). Chromatin inheritance upon Zeste-mediated Brahma recruitment at a minimal cellular memory module. *EMBO J.* **23**, 857-868.
Dejardin, J., Rappailles, A., Cuvier, O., Grimaud, C., Decoville, M., Locker, D. and Cavalli, G. (2005). Recruitment of Drosophila Polycomb group proteins to chromatin by DSP1. *Nature* **434**, 533-538.
DeVido, S. K., Kwon, D., Brown, J. L. and Kassis, J. A. (2008). The role of Polycomb-group response elements in regulation of engrailed transcription in Drosophila. *Development* **135**, 669-676.
DiNardo, S. and O'Farrell, P. H. (1987). Establishment and refinement of segmental pattern in the Drosophila embryo: spatial control of engrailed expression by pair-rule genes. *Genes Dev.* **1**, 1212-1225.
Eisen, M. B., Spellman, P. T., Brown, P. O. and Botstein, D. (1998). Cluster analysis and display of genome-wide expression patterns. *Proc. Natl. Acad. Sci. USA* **95**, 14863-14868.
Galindo, M. I., Bishop, S. A., Greig, S. and Couso, J. P. (2002). Leg patterning driven by proximal-distal interactions and EGFR signaling. *Science* **297**, 256-259.
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.
Guan, J., Li, H., Rogulja, A., Axelrod, J. D. and Cadigan, K. M. (2007). The Drosophila casein kinase Iepsilon/delta Discs overgrown promotes cell survival via activation of DIAP1 expression. *Dev. Biol.* **303**, 16-28.
Hammet, A., Magill, C., Heierhorst, J. and Jackson, S. P. (2007). Rad9 BRCT domain interaction with phosphorylated H2AX regulates the G1 checkpoint in budding yeast. *EMBO Rep.* **8**, 851-857.
Huang, D. H., Chang, Y. L., Yang, C. C., Pan, I. C. and King, B. (2002). pipsqueak encodes a factor essential for sequence-specific targeting of a polycomb group protein complex. *Mol. Cell. Biol.* **22**, 6261-6271.
Hughes, C. M., Rozenblatt-Rosen, O., Milne, T. A., Copeland, T. D., Levine, S. S., Lee, J. C., Hayes, D. N., Shanmugam, K. S., Bhattacharjee, A., Biondi, C. A. et al. (2004). Menin associates with a trithorax family histone methyltransferase complex and with the hoxc8 locus. *Mol. Cell* **13**, 587-597.
Irizarry, R. A., Wu, Z. and Jaffee, H. A. (2006). Comparison of Affymetrix GeneChip expression measures. *Bioinformatics* **22**, 789-794.
Issaeva, I., Zonis, Y., Rozovskaia, T., Orlovsky, K., Croce, C. M., Nakamura, T., Mazo, A., Eisenbach, L. and Canaani, E. (2007). Knockdown of ALR (MLL2) reveals ALR target genes and leads to alterations in cell adhesion and growth. *Mol. Cell. Biol.* **27**, 1889-1903.
Jiang, J., Hoey, T. and Levine, M. (1991). Autoregulation of a segmentation gene in Drosophila: combinatorial interaction of the even-skipped homeo box protein with a distal enhancer element. *Genes Dev.* **5**, 265-277.
Lechner, M. S., Levitan, I. and Dressler, G. R. (2000). PTIP, a novel BRCT domain-containing protein interacts with Pax2 and is associated with active chromatin. *Nucleic Acids Res.* **28**, 2741-2751.
Manke, I. A., Lowery, D. M., Nguyen, A. and Yaffe, M. B. (2003). BRCT repeats as phosphopeptide-binding modules involved in protein targeting. *Science* **302**, 636-639.
Maschat, F., Serrano, N., Randsholt, N. B. and Geraud, G. (1998). engrailed and polyhomeotic interactions are required to maintain the A/P boundary of the Drosophila developing wing. *Development* **125**, 2771-2780.
Moazed, D. and O'Farrell, P. H. (1992). Maintenance of the engrailed expression pattern by Polycomb group genes in Drosophila. *Development* **116**, 805-810.
Munoz, I. M., Jowsey, P. A., Toth, R. and Rouse, J. (2007). Phospho-epitope binding by the BRCT domains of hPTIP controls multiple aspects of the cellular response to DNA damage. *Nucleic Acids Res.* **35**, 5312-5322.
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.
Oktaba, K., Gutierrez, L., Gagneur, J., Girardot, C., Sengupta, A. K., Furlong, E. E. and Muller, J. (2008). Dynamic regulation by polycomb group protein complexes controls pattern formation and the cell cycle in Drosophila. *Dev. Cell* **15**, 877-889.
Parker, D. S., Jemison, J. and Cadigan, K. M. (2002). Pygopus, a nuclear PHD-finger protein required for Wingless signaling in Drosophila. *Development* **129**, 2565-2576.
Patel, S. R., Kim, D., Levitan, I. and Dressler, G. R. (2007). The BRCT-domain containing protein PTIP links PAX2 to a histone H3, lysine 4 methyltransferase complex. *Dev. Cell* **13**, 580-592.
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., Smith, S. T., Krajewski, W., Nakamura, T., Canaani, E., Croce, C. M. and Mazo, A. (2004). Purification and biochemical properties of the Drosophila TAC1 complex. *Methods Enzymol.* **377**, 255-266.
Ringrose, L. and Paro, R. (2007). Polycomb/Trithorax response elements and epigenetic memory of cell identity. *Development* **134**, 223-232.
Rouse, J. (2004). Esc4p, a new target of Mec1p (ATR), promotes resumption of DNA synthesis after DNA damage. *EMBO J.* **23**, 1188-1197.
Saka, Y. and Yanagida, M. (1993). Fission yeast *cut*5+, required for S phase onset and M phase restraint, is identical to the radiation damage repair gene *rad*4+. *Cell* **74**, 383-393.
Saka, Y., Esashi, F., Matsusaka, T., Mochida, S. and Yanagida, M. (1997). Damage and replication checkpoint control in fission yeast is ensured by interactions of Crb2, a protein with BRCT motif, with Cut5 and Chk1. *Genes Dev.* **11**, 3387-3400.
Schuettengruber, B., Chourrout, D., Vervoort, M., Leblanc, B. and Cavalli, G. (2007). Genome regulation by polycomb and trithorax proteins. *Cell* **128**, 735-745.
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.
Shao, Z., Raible, F., Mollaaghababa, R., Guyon, J. R., Wu, C. T., Bender, W. and Kingston, R. E. (1999). Stabilization of chromatin structure by PRC1, a Polycomb complex. *Cell* **98**, 37-46.
Shimizu, K., Bourillot, P. Y., Nielsen, S. J., Zorn, A. M. and Gurdon, J. B. (2001). Swift is a novel BRCT domain coactivator of Smad2 in transforming growth factor beta signaling. *Mol. Cell. Biol.* **21**, 3901-3912.
Small, S., Arnosti, D. N. and Levine, M. (1993). Spacing ensures autonomous expression of different stripe enhancers in the even-skipped promoter. *Development* **119**, 762-772.
Smyth, G. K. (2004). Linear models and empirical bayes methods for assessing differential expression in microarray experiments. *Stat. Appl. Genet. Mol. Biol.* **3**, Article3.
Thibault, S. T., Singer, M. A., Miyazaki, W. Y., Milash, B., Dompe, N. A., Singh, C. M., Buchholz, R., Demsky, M., Fawcett, R., Francis-Lang, H. L. et al. (2004). A complementary transposon tool kit for Drosophila melanogaster using P and piggyBac. *Nat. Genet.* **36**, 283-287.
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.
Torres, M., Gomez-Pardo, E., Dressler, G. R. and Gruss, P. (1995). Pax-2 controls multiple steps of urogenital development *Development* **121**, 4057-4065.
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.
Xu, T. and Rubin, G. M. (1993). Analysis of genetic mosaics in developing and adult Drosophila tissues. *Development* **117**, 1223-1237.
Yuan, D. S., Pan, X., Ooi, S. L., Peyser, B. D., Spencer, F. A., Irizarry, R. A. and Boeke, J. D. (2005). Improved microarray methods for profiling the Yeast Knockout strain collection. *Nucleic Acids Res.* **33**, e103.