The Drosophila melanogaster Mutants ap<sub>blot</sub> and ap<sub>Xasta</sub> Affect an Essential apterous Wing Enhancer

Dimitri Bieli,* Oguz Kanca,* Daryl Gohl,†,1 Alexandru Denes,* Paul Schedl,† Markus Affolter,* and Martin Müller*†2

*Biozentrum, University of Basel, 4056 Basel, Switzerland, and †Department of Molecular Biology, Princeton University, New Jersey 08540

ABSTRACT The selector gene apterous (ap) plays a key role during the development of the Drosophila melanogaster wing because it governs the establishment of the dorsal-ventral (D-V) compartment boundary. The D-V compartment boundary is known to serve as an important signaling center that is essential for the growth of the wing. The role of Ap and its downstream effectors have been studied extensively. However, very little is known about the transcriptional regulation of ap during wing disc development. In this study, we present a first characterization of an essential wing-specific ap enhancer. First, we defined an 874-bp fragment about 10 kb upstream of the ap transcription start that faithfully recapitulates the expression pattern of ap in the wing imaginal disc. Analysis of deletions in the ap locus covering this element demonstrated that it is essential for proper regulation of ap and formation of the wing. Moreover, we showed that the mutations ap<sub>blot</sub> and ap<sub>Xasta</sub> directly affect the integrity of this enhancer, leading to characteristic wing phenotypes. Furthermore, we engineered an in situ rescue system at the endogenous ap gene locus, allowing us to investigate the role of enhancer fragments in their native environment. Using this system, we were able to demonstrate that the essential wing enhancer alone is not sufficient for normal wing development. The in situ rescue system will allow us to characterize the ap regulatory sequences in great detail at the endogenous locus.

The body wall and appendages of the adult fly are generated by specialized clusters of primordial cells in Drosophila larvae called imaginal discs. The patterning of cells in imaginal discs is initiated by establishing cell lineage boundaries, called compartments (Garcia-Bellido et al. 1973; Dahmann and Basler 1999). In the case of the wing imaginal disc, the tissue is subdivided into four different compartments, anterior (A) and posterior (P) as well as dorsal (D) and ventral (V). The A–P compartment is established during the process of segmentation in the embryo. The subdivision into dorsal and ventral compartments takes place later in development during the larval stages when the wing tissue is growing extensively (Wieschaus and Gehring 1976; Lawrence and Morata 1977; Cohen et al. 1992; Williams et al. 1993; Diaz-Benjumea and Cohen 1993). Short-range signaling events between the A–P or D–V compartments specify cells close to the compartment boundaries. These cells, also called organizer, play an important role in patterning the surrounding tissue by secreting long-range signaling molecules, also referred to as morphogens (Struhl and Basler 1993; Diaz-Benjumea and Cohen 1995; Neumann and Cohen 1997; Affolter and Basler 2007).

Compartment specificity is conferred by the cell-autonomous activity of a special class of transcription factors, called selector genes. Selector genes regulate genes important for proper differentiation and genes that control cell—cell interactions at the compartment boundary. apterous (ap), which is expressed in the dorsal compartment of the wing disc, has been shown to act as a selector gene subdividing the wing disc into a D and a V portion (Cohen et al. 1992; Diaz-Benjumea and Cohen 1993; Williams et al. 1994; Blair et al. 1994). Different ap alleles can lead to a wide range of wing phenotypes (Stevens and Bryant 1985). The most striking morphological defect in strong ap alleles is the complete lack of wing and haltere structures (Butterworth and King 1965). Because ap is not essential for the progression through larval and pupal stages, the investigation of adult ap mutant wing phenotypes is possible.

KEYWORDS Drosophila apterous compartment boundary

Copyright © 2015 Bieli et al. 
doi: 10.1534/g3.115.017707
Manuscript received January 27, 2015; accepted for publication March 31, 2015; published Early Online April 2, 2015.
This is an open-access article distributed under the terms of the Creative Commons Attribution Unported License (http://creativecommons.org/licenses/by/3.0/), which permits unrestricted use, distribution, and reproduction in any medium, provided the original work is properly cited.
Supporting information is available online at www.g3journal.org/lookup/suppl/doi:10.1534/g3.115.017707/-/DC1
1Present address: University of Minnesota Genomics Center, Minneapolis, MN.
2Corresponding author: Biozentrum, University of Basel, Klingelbergstrasse 50 / 70, 4056 Basel, Switzerland. E-mail: m.mueller@unibas.ch
The target genes of Ap and their downstream functions in the patterning of the wing disc are relatively well understood. The activity of Ap initiates a bidirectional Notch signaling cascade at the D–V compartment boundary, which subsequently induces the expression of wingless (wg) in a stripe along the compartment boundary (Diaz-Benjumea and Cohen 1993; Williams et al. 1994; Irvine and Wieschaus 1994; Ruhlison and Blair 1995; Kim et al. 1995; Couso et al. 1995). Wg, a ligand of the Wnt family, is responsible for the growth of the wing pouch and patterning along the D–V axis, although its mode of action as a classical morphogen currently is questioned (Neumann and Cohen 1997; Alexandre et al. 2014).

Despite the rather detailed knowledge about the functions of Ap in wing disc development, our knowledge of the mechanisms regulating ap expression is still limited. It has been shown that activation of the epidermal growth factor receptor by its ligand Vein is necessary and sufficient to activate the expression of ap in the dorsal compartment of the wing disc (Zecca and Struhl 2002a,b). Moreover, early ventral wg expression has been shown to restrict the expression of ap to the dorsal portion of the developing wing disc (Williams et al. 1994).

To identify the wing disc-specific cis-regulatory elements of ap, we used several genetic approaches. First, a classical LacZ enhancer reporter study was performed. Second, deletions with defined breakpoints in the ap genomic locus were generated. Third, we have characterized two classical ap alleles, ap1057 and apXasta (apX), at the molecular level and have associated their respective molecular alterations to the minimal cis-regulatory elements in their native environment.

Using these assays, we have defined an essential, but not sufficient, minimal 874-bp ap wing enhancer fragment that drives reporter gene expression in the dorsal compartment of the wing imaginal disc.

**MATERIAL AND METHODS**

**Fly stocks and methods**

Flies were grown on standard cornmeal agar at 25°C, unless otherwise stated. ap1057 (P[Bac(RB)e01573]), ap1057 (P[Bac(WH)f08090]), ap1057 (P[Bac(WH)f00451]), and apXasta (P[Bac(WH)f00878]) were purchased from the Exelixis stock collection at Harvard Medical School. Df(2R)nap1 (BL#1006), apII (BL#1490), w+; T(2;3)apXa, ap1057/CyO, TM3, Sc (BL#2475), P[hsFLP12, y1 w+]/CyO, Df(2R)BSC696 (BL#26548), w+; P[10XUAS-IVS-mCD8:GFP]; P{ actin-Gal4 (BL#32186), Df(3R)EXE1676 (BL#7655), TM3, ryF; St Ser1 P[Δ2-3]99B (BL#1808), Bx-Gal4 (w1118 P[GawB]BAl1096, Blf8866), y+ w+; Mi[y+;+mDint2]=MIC MI00964 (BL#34133), y+ w+; Mi[y+;+mDint2]=MIC MI02330/SM6a (BL#33205), y+ w+; P[EPgy2]EY03046 (BL#15619), ptc-Gal4 (P[GawB]Bptc591), w+; y+ w+; P[GMR ts2CA1]; UAS-GFP/T(2;3)apXasta were all obtained from the Bloomington Stock Center.

fagn-Gal4 (y w+; P[w+;UAS-ns]; scw=Gal4]NP3999 / TM6, P[w=UAS-lacZ; UW23-1]UW23-1, DGCER#104990) was obtained from Kyoto Drosophila Research Center. Dad4-Gal4 was established in our laboratory as described in the sections to follow. actin-Gal4 (y w+; P[actin5c:Gal4, w/+]; CyO) and GMR-Gal4 (w w+; P[GMR:Gal4, w/+]; CyO) were obtained from Steven Henikoff (Ahmad and Henikoff 2001). sal-Gal4 was obtained from the Basler lab via Funun Haramarotoglu (Mosimann et al. 2006). dpp-Gal4 is described in Schaeling-Hampton et al. (1994). UAS-ap was obtained from Marco Milán (Milán and Cohen 1999), y w [vas-int.Dm]zkh-2A, a stock producing ΦC31 inteGRase under the control of the vasa promoter, and insertion platform M[3xP3-RFP, attP2/h-86/Fb] were obtained from Johannes Bischof (Bischof et al. 2007). apII/T(2;3)apXa was obtained from John B. Thomas.

According to our genetic and molecular analysis, apXasta should not be listed as an allele of ap. First, and contrary to a previous report (Bourguinon et al. 1992), hemizygous apXasta flies have normal wings and halteres. Second, although molecular analysis confirmed the presence of a P-element insertion just proximal to usecin on the ap locus chromosome, polymerase chain reaction (PCR), and sequencing failed to provide evidence for a ~200-bp deletion within 1.5 kb of the longest ap cDNA (D. Bieli and M. Müller, unpublished data). The GFP knock-in allele apGFP is described in Caussinus et al. (2011) (BL#38423). apDM has been described in Gohl et al. (2008). It contains an insertion ~400 bp upstream of the longest ap cDNA. Dad4-GFP (P[Dad4:EGFPnuc, w+]) was obtained from Jorgos Pyrowolakis (Vuilleumier et al. 2010). Nuclear enhanced green fluorescence protein (GFP) is expressed under the control of the Dad4 enhancer (Weiss et al. 2010). DadR883832/TM3, Sc was obtained from Tetsuya Tabata. This deletion covers at least 24 kb downstream of the DadR88383 insertion, including the complete Dad open-reading frame (ORF) and three neighboring genes (CG3983, CG5184, and CG5962). T. Tabata, personal communication; Tseunezumi et al. 1997; Henderson et al. 1999). A recombinant between ap1057 and P[y+;7.7]; w;mC1=10XUAS-IVS-mCD8;GFP];P{attP40}, inserted on 2R at 25°C (Pfeiffer et al. 2010), was obtained by meiotic recombination and selection for the dominant Xasto and mini-white markers. The generation of deficiencies apDG1, apDG2, apDG8, and apDG11 is described below in section ΦC31-integrase-mediated transgenesis and generation of deletions.

Adult wings were dissected and mounted in Hoyer’s. Then, wing preparations were baked at 58°C for a few hours. Preparations were allowed to harden at room temperature and flattened by applying a 40-g metal cylinder on the cover slip. Pictures were taken with a Nikon Microphot-FXA microscope with a Sony NEX-5RK digital camera. The notums of adult flies were photographed with a Leica M125 binocular equipped with a Leica DFC420C camera.

**Introduction of ΦC31-integrase targets into the ap locus by gene conversion at the site of apDM**

A method known as direct gene conversion has previously been developed to engineer a desired DNA fragment into the genomic site of a P-element insertion (Gloor et al. 1991; Sipos et al. 2007). Upon exposure of a given P-element insertion to P-element transposase, the transposon is excised and a double strand break is created. It is normally repaired by the cellular machinery using the homologous chromosome as a template. However, the repair process may also use an exogenous plasmid containing the desired DNA fragment flanked by homology arms derived from either side of the P-element insertion site. Such a gene conversion template plasmid containing homology arms flanks the site of apDM insertion, along with hsp70-GFP bracketed by a pair of inverted attB sites was constructed and named pLAPGPIR (see Figure 5A). The construction of this plasmid was a multi-step procedure. Details can be obtained upon request. In brief, left (899 bp long) and right (1981 bp long) ap-homology arms were amplified by PCR. To minimize sequence polymorphism which could decrease the efficiency of gene conversion, apDM genomic DNA (gDNA; isolated as described in Ashburner 1989) was used as the template for PCR. As primers we used apLA-R, apLA-FNotI, apRA-F, and apRA-R (for primer sequences, see Supporting Information, Table S1). Inserts in the proper orientation for subsequent cloning were identified using diagnostic digest and sequencing.

pLAPGPIR was injected (650 ng/mg) along with pTurbo (250 ng/mg) into embryos derived from a cross of y w, ap1057(w+);+/TM3, Sc D2-3/+ males with y w, apDM[w+]; + virgins. Surviving injectees were transferred to fresh vials and carefully tended at 18°C. Among the hatching
adults, males and virgins representing the two desired genotypes (ap^{pG3}[^w]ap^{MM}[^y]) and ap^{pG3}[^w]ap^{MM}[^y]; TM3, Sb Delta-2/3+) were selected for further work. ap^{pG3}ap^{MM} flies have normal wings and halteres. The ap^{pG3} chromosome was included because it lacks the DNA corresponding to the homology arms of pAPGPRα and hence cannot serve as a template for double strand gap repair. A total of 72 fertile crosses involving virgins (in pairs) or single males mated with y w; ap b csp/SfM0u flies could be set up. Originally, it was intended to screen the larval progeny of these crosses for GFP expression. Unfortunately, this elegant approach failed in practice. Therefore, the progeny was screened for y^- w^- males. This phenotype indicates loss of the y marker and therefore most likely also of ap^{MM} and was, in the absence of the positive GFP selection, the only selectable marker to identify putative conversion candidates. A total of 105 y^- w^- males were selected from 32 (out of 72) crosses yielding such males. Balanced lines of potential gene conversion events were established and screened for GFP fluorescence in larval wing discs. Five candidate gene conversion lines with weak GFP expression in wing imaginal discs in an ap-like pattern were obtained from two independent dysgenic crosses (isolation numbers: c1.4a, c1.4b, c1.4d, and c1.4e; c1.13a).

To confirm that the five GFP-positive candidate gene conversion lines had the apt-flanked GFP construct integrated in the ap locus, gDNA was isolated and analyzed by PCR. PCR products were obtained for all five candidates between a primer (SV40out55) within the SV40 trailer sequence (just downstream of GFP) and a primer (apLOF2) in the ap gene outside of the left homology arm. Sequencing of all five lines confirmed the integrity of the ap promoter region and the presence of the ap proximal apt site (data not shown).

On the distal side, PCRs using primers in the hsp70 promoter (just upstream GFP) and several primers outside of the right homology arm initially failed to produce products (data not shown). Later, by use of one of the lines obtained by RMCE (see below), the integrity of the junction between the template plasmid and the right homology arm could be verified by PCR and sequencing using the Mcp-dir-y and ap-dir-3 as primers. Mcp-dir-y primes toward the end of the mini-yellow gene present on our Recombination-Mediated Cassette Exchange (RMCE) insertion cassette. We also tested whether the junction between the right homology arm and the flanking ap sequence is intact by PCR using a primer near the end of the right homology arm (apRAendF), and a primer in the flanking ap sequence (apOR2Z). A product of the expected size was observed, indicating that the junction is intact.

\(ap^{1.4a}, ap^{1.4b}, ap^{1.4d}, ap^{1.4e}, \) and \(ap^{1.13a}\) homoyzgozous all have wild-type wings, indicating that the function of the ap wing enhancer and promoter were not disrupted by the gene conversion event. Gene conversion events \(ap^{1.4a}, ap^{1.4b}, ap^{1.4d}, ap^{1.4e}\) also have a rough eye phenotype when homozygous, but not over \(ap^{pG3}\). The rough eye phenotype can be separated from the ap locus by meiotic recombination. Finally, only \(ap^{1.4b}\) was chosen for further work. One of its applications is the targeted insertion of exogenous DNA into the ap locus by RMCE (Bateman et al. 2006).

**ΦC31-integrase–mediated transgenesis and generation of deletions**

Constructs for ΦC31-integrase–mediated transgenesis were generated based on plasmid pBi-LLFY(BI) [details about the construction of pBi-LLFY(BI) can be acquired upon request]. As required for RMCE, it contains two inverted attB sites. Separating them are the following three genetic components: (1) two LoxP sites in direct orientation with a multiple cloning site in between them; (2) the LoxP cassette is followed by a single FRT site; (3) the mini-yellow transformation marker complements pBi-LLFY(BI). mini-yellow refers to a yellow reporter gene lacking all of its characterized tissue specific enhancers. It consists of the yellow cDNA fused to ~330 bp of 5' genomic DNA, including the yellow promoter and extending up to a KpnI restriction site. The mini-yellow fragment was isolated from plasmid C4yellow (referred to as Dnt in Geyer and Corces 1987; Gohl et al. 2008). In the context of the ap gene and in a y background, mini-yellow activity always manifests itself in phototypically yellow wings. Depending on the transgene and orientation of insert, thoracic bristles may also acquire yellow pigmentation (D. Gohl and M. Müller, unpublished data).

Constrasts were introduced into the ap locus by RMCE into two docking sites, M[y+y+dMi-n][y]=M[C]MICM102330 (Venken et al. 2011) and ap^{1.4b}. DNA was injected at a concentration of 300 ng/μl in 1× phosphate-buffered saline (PBS) into early embryos of the genotype y w M[vas-int.Dm]zh-2A; M102330/CyO or y w M[vas-int.Dm]zh-2A; ap^{1.4b}/CyO. The relevant transgenic lines obtained in this way are ap^{D05.34} and ap^{pF01}, respectively. Their position and the orientation of the FRT are depicted in Figure 1C together with four other FRT containing transposon insertions. Five of the six stocks are homozygous and hemizygous viable. Their wings and halteres are of wild-type appearance. This is not the case for ap^{D08.090}. The lethality of this chromosome cannot be reverted by excision of the PBac[WH], indicating that it is associated with a second site lethal. Rare homozygous revertant escapers as well as frequent hemizygous revertants have normal wings. Therefore, the PBac[WH] insert is responsible for the strong phenotype in hemizygous ap^{D08.090} flies. However, this phenotype is not dependent on the gypsy insulator present in ap^{D08.090} because the wing phenotype is not suppressed in a su(Hw)~ background (M. Müller, unpublished data).

We have noted that in the Drosophila literature, two divergent definitions for FRT orientation are in use! In this study, FRT orientation is indicated according to Thibault et al. (2004).

In Drosophila, the production of deletions by Flpase-catalyzed recombination between two FRT sites either in cis or in trans has enabled the community to obtain a huge collection of tailor-made deficiencies (Golic and Golic 1996; Ryder et al. 2007). We have previously applied this technology to generate a ~27-kb deletion named Df(2R)ap^{DG3} between two FRT sites in ap^{MM} and ap^{pF01} (Gohl et al. 2008). Note that in this study, Df(2R)ap^{DG} is referred to as ap^{DG1}. Applying analogous genetic crossing schemes, we have generated three further deletions:

**Df(2R)ap^{DG1}:** An ~44-kb deletion between two FRT sites located in ap^{D09.080} and ap^{D09.157}. It is referred to as ap^{DG1}. In this deficiency, a large part of the ap transcription unit is lost together with ~27 kb of intergenic DNA separating ap from f(2)99851. Although a considerable part of the ap ORF located proximal to the break in ap^{pG3} remains in place, genetic observations are consistent with it being a true null allele with respect to ap function in wing and haltere tissue. Flanking the new FRT junction are three genetic elements: gypsy insulator and mini-white (of ap^{D09.080}) and a splice acceptor (of ap^{D09.157}). Over several kilobases, the region of the new fusion is identical to PBac(RB)ed10773 and, hence, no adequate ap^{DG1}-specific PCR primers could be designed. Thus, four PCR primer pairs distributed evenly over the ~44-kb interval missing on the ap^{DG1} chromosome were tested on w^- and on ap^{DG1}/Df(2R)nap1 flies (Df(2R)nap1 being a cytologically visible deletion also uncovering ap). The absence of the corresponding DNA in the latter could unambiguously be demonstrated (data not shown).

**Df(2R)ap^{DG2}(w^+):** An ~20-kb deletion between two FRT sites located in ap^{D09.078} and ap^{pF01}. It is referred to as ap^{DG2}. It corresponds to a rather clean deletion of the complete ap transcription unit. Its phenotypes are indistinguishable from those observed for ap^{DG1}. Flanking the new FRT junction are two genetic elements: a UAS-inducible promoter (of ap^{D09.078}) and mini-white (of ap^{pF01}). It was verified by
Df(2R)apDG11, al: An ~11-kb deletion between 2 FRT sites in al ap^P and ap^D33.4. It is referred to as apDG11. Because apDG1 flies have no wings. Both deficiencies share the same proximal break point. Previous transsection studies have suggested that the ap promoter immediately proximal to apDG1 (and hence also of apDG11) remains intact (Gohl et al. 2008). Flanking the new FRT junction are two genetic elements: a LoxP site (of ap^M21) and mini-yellow (of ap^D33.4). The new junction was verified by sequencing. For PCR amplification of the region, the apMM-200fl and yellow5 out primer pair was used. Part of the fragment was sequenced with yellow5 out and Inverseappromfor.

Generation of a \( \Phi C31 \)-integrate based in situ rescue system at ap

Construction of pBSattBattPLoxFRTy: Two complementary oligos (attBPfor and attBPrev) containing attB and attP sites in tandem were purchased from Sigma-Aldrich. These oligos were annealed and cloned between the XhoI and KpnI sites of pBSIIKS. The new plasmid’s name is pBSIIKSattBattP. A XhoI-Gal fragment containing LoxP, FRT, and mini-yellow was isolated from pB-LLFY(BI) and subcloned into pBSIIKSattBattP, thereby generating the pBSattBattPLoxFRTy vector used for \( \Phi C31 \) integrase mediated transgenesis (see Figure 5B). The attB and attP sites on this vector are separated by only 6 bp. It was assumed that therefore the two elements are too close for efficient intramolecular recombination. The fact the two desired insertions (one in each attP site present in ap^1,4b, see Figure 5B) could be isolated seems to support this assumption.

Generation of ap^enh^Deh, a platform for insertion of ap enhancer fragments: pBSattBattPLoxFRTy DNA was injected into \( y \ w M\{vas-int.Dm\}/y \ w M\{vas-int.Dm\}/y \ w \{vas-int.Dm\}/y \ w \{mini-white\} \) embryos. Yellow+ marked flies could be isolated and mated. The desired insert orientation could be identified by PCR using the apdown-forN and aptransch_yw_rev primers. A stock with isolation number 6.1 was selected for correct orientation of insert pBSattBattPLoxFRTy. It is referred to as ap^enh^Deh. We wished to further modify this stock by introducing the same ~27-kb deletion as in apDG1. Therefore, \( y \ w \{ap^enh^Deh\}/y \ w \) males were mated with \( y \ w \hs\Fplp, PBac(RB)e01573\) virgins. Progeny was heat-shocked at three subsequent days during its larval development for 1 hr in a 37°C water bath. Hatchlings were individually crossed to \( y \ w M\{vas-int.Dm\}/y \ w \{mini-white\} \) flies. A total of 7 of 80 single crosses produced phenotypically yellow− and white− flies, indicating the loss of all DNA between the FRT sites of ap^enh^Deh and PBac(RB)e01573, including mini-yellow and mini-white. The newly established deletion was named ap^Deh. The ap^Deh chromosome contains a single functional attP site ready for \( \Phi C31 \)-integrate catalyzed insertion of pEnh-Reentry derived plasmids (see Figure 5D). Insertion events can be further modified by suitably placed LoxP and FRT sites, allowing for the deletion of the yellow marker or the enhancer fragment-yellow+ marker cassette, respectively (see Figure 5E).

PCR and sequencing. Aprec-LA-AscI-F and WARIout#1 primers were used for PCR. For sequencing, we used primers apEnhDelSeq-PBrev and WARIout#2.

Figure 1 LacZ reporter assay and deletion analysis at the apertous locus. (A) Diagrammatic representation of the ap locus. As drawn at the top of the panel, it extends over roughly 50 kb. Its transcribed part is shown in green. ap is flanked by two genes indicated in blue: vulcan on the proximal and I2(0)9851 on its distal side. Arrows above the genomic interval specify the direction of transcription of the three genes. Fragment apC, indicated in orange, has been reported to drive reporter expression in the dorsal compartment of the pouch, the hinge and the notum of the wing imaginal disc, where ap is normally expressed. Below, the relative positions and dimensions of nine fragments tested with our LacZ reporter assay are depicted. Fragments colored in orange (apO, apR, apOR, apOR3, and apRXa) elicit the same expression pattern as apC. Fragments depicted in gray (apP, apC-LacZ, apOR2) do not drive reporter gene expression in the wing disc. (B) X-Gal staining in the wing disc of an apC-LacZ transgenic fly. Scale bar: 100 μm. (C) Deletions generated at the endogenous ap locus with FRT-containing inserts. At the top of the panel, triangles pointing to the de

---

**Note:** The text above contains a mix of natural language and scientific content, indicating it is from a biological research paper. It describes genetic manipulations and their outcomes, highlighting specific deletions and insertions in the apertous gene locus. The text also mentions PCR, sequencing, and the use of specific primers for analysis. The diagrams and figures mentioned (Figure 1) illustrate the reporter assay and deletion analysis. The deletion of ~11 kb between two FRT sites in the al ap^P and ap^D33.4 strains is referred to as apDG11. This deletion results in flies with no wings. Further genetic elements, such as LoxP and FRT sites, are used for targeted insertions into the ap locus, resulting in phenotypic changes. The text also describes the construction of vectors for genetic manipulations using the \( \Phi C31 \)-integrate system and the generation of a platform for insertion of ap enhancer fragments. Specific genotypes and crosses are mentioned to verify the deletion and insertion events. The text concludes with a description of the ap^Deh chromosome, containing a single functional attP site ready for \( \Phi C31 \)-integrate catalyzed insertions.
Generation of pEnh-Reentry constructs: yellow* coding sequence and body cuticle enhancer were subcloned into pBSIKS as a BgIII fragment from C4yellow, thereby generating plasmid pBSIKS-yellow. Please note that the yellow wing enhancer is not part of the BgIII fragment! attB and FRT LoxP fragments were cloned by first annealing and phosphorylating oligos attBiop and attBbottom as well as FRTloxPtop and FRTloxPbottom followed by three fragment ligation with pBSIKS-yellow vector cut with SacI and XhoI. The resulting plasmid was called pEnh-Reentry and served as the backbone for all constructs described below.

The 27-kb full-length enhancer was recombineered in pEnh-Reentry from BACR45O18 (purchased from the Berkeley Drosophila Genome Project). The left homology arm was amplified with PCR with primers containing NotI and XhoI sites (primer pair: aphenrecLA_Not_for and aphenrecLA_XhoI_rev). The right homology arm was amplified with primers containing XhoI and BglII sites (primer pair: aphenrecRA_BglII_rev and aphenrecRA_BglII_rev). Homology arms were cloned in pEnh-Reentry cut with NotI/BglII as 3 fragment ligation. Recombineering was performed according to Thomason et al. 2007. In brief, the pEnh-Reentry-homologyarms vector was linearized with XhoI and transformed into bacterial strain DY380 (purchased from NCI at Frederick) pretransformed with BAC45O18 (purchased from BDGP), and pre-induced at 42°C for 15 min. Recombinants were selected on ampicillin and screened by PCR. The correct recombineering product's name is pEnh-Reentry-Full-length.

Dad enhancer fragments and apRXa were amplified from apRXa gDNA. First, fragments apRXaadDadInt2, DadInt52, and Dad4 were cloned into a pBluescript II KS(+) vector, where the XhoI site was mutated previously into an AvrII site. For apRXaadDadInt2, primers apRX_AvrII_for and dadint52_Xmal_Spel_rev were used. For DadInt52, primers dadint52_Xmal_Spel_rev and XmaXbrkt_Spel_rev were used. To clone Dad4, we used the primer pair dad4_AvrII_for and dad4_Xmal_Spel_rev. These fragments were combined via the respective SpeI or AvrII sites to produce apRXaadDadInt52Dad4 and DadInt52Dad4 fusion fragments. These were subcloned from pBluescript II KS(+) via AvrII and Xmal sites into pEnh-Re-entry cut with AvrII and AgeI. apR, apRXa, apP, and apY were amplified from pEnh-Reentry-Full-length plasmid and cloned into pEnh-Re-entry via NotI, AvrII or AgeI sites. To clone apR, primers apRX_AvrII_for and apRX_Xmal_Spel_rev were used. For apRXa, primer pair apRX_AvrII_for and apRXa_AgeI_rev was used. To amplify apP, primers apP_NotI_for and apF_AvrII_rev were used. apY was amplified using primer pair apY_NotI_for and apY_AgeI_rev.

All pEnh-Reentry derived constructs were brought into the ap locus by F3C1-integrate mediated recombination (see Figure 4, D and E). DNAs were injected at a concentration of 300 ng/µL in 1×PBS into y w M[vas-int.Dm]zh-2A ; apRXa/CyO embryos. Transgenic flies were selected with the help of the yellow* marker and balanced stocks were generated according to standard genetic procedure.

Generation of LacZ-reporter lines

ap regulatory DNA were amplified via PCR from y w67c23 gDNA with primers containing restriction enzyme sites as overhangs, and subsequently cloned into plasmid pAttBlAZ (Weiss et al. 2010) using the respective enzymes. apC was amplified with the primer pair apC_AscI_for and apC_BglII_rev. The apC fragment was defined by Lundgren et al. 1995. The apO fragment was cloned with the primers apC_AscI_for and apO_BglII_rev. For apP, primers apP_AscI_for and apP_AvrII_rev were used. To clone apQR, primer pair apQR_AscI_for and apQR_BglII_rev were used. For apR, the primers apR_AscI_for and apR_BglII_rev were used. apS was cloned with the primers apS_AscI_for and apS_BglII_rev. apOR was amplified with apR_AscI_for and apO_BglII_rev. For apOR2, primers apR_AscI_for and apOR2_BglII_rev were used. apO3 was amplified with apR_AscI_for and apOR3_BglII_rev. apRXa was cloned with apR_AscI_for and apRXa_BglII_rev.

All the reporter transgenes were generated with the F3C1-based integration system using the landing platform M[3xP3-RFP.attP]zh-86Fb (Bischof et al. 2007).

Molecular characterization of apblot

Complementation crosses with apblot over a set of overlapping ap deletions mapped the mutation to am ~11-kb interval upstream of apXa. Therefore, a set of PCR primer pairs was designed to screen for a lesion in that region of apblot gDNA. y w67c23 gDNA served as positive control. With one primer pair, a discontinuity could be identified on the apblot chromosome. It could be best reconciled with the presence of a larger insertion of DNA of unknown origin. Inverse PCR (iPCR) was subsequently used to obtain sequence information about the ends of the putative insertion. Toward that end, apblot gDNA was digested with BsaW1 and ligated with T4 Ligase under conditions as previously described (Ochman et al. 1988). Primer pairs used for iPCR on the proximal side of the insertion were icPcr_for and icPcr_rev. Primer pairs used for iPCR on the distal side of the insertion were K_for and L_rev. Following this strategy, sequence information could be obtained for both ends of the inserted DNA. Sequence comparison identified them as LTRs of the blood retrotransposon (Bingham and Chapman 1986). To verify the insertion, primers out of blood 3’ and 5’ LTR (blood3prime and blood5prime, respectively) were used with primers binding in adjacent ap regions (icPCR_for and L_rev, respectively). Sequencing was performed by Microsynth AG, Switzerland.

Molecular characterization of apXa

The dominant Xasta allele was originally induced by X-ray mutagenesis in a stock already containing two large inversions on 2R and 3R (Serebrovsky and Dubinin 1930; Waddington 1940; Lewis 1951; Hetherington et al. 1968). The new rearrangement was classified as a reciprocal translocation with breakpoints 41P9-41P11;89E8-89F1. Allelism with ap was inferred from noncomplementation with known ap alleles (Butterworth and King 1965; Stevens and Bryant 1985). Complementation crosses with a set of small overlapping ap deletions failed to narrow down the location of apXa. Hence, the whole ap locus was screened by overlapping primer pairs. PCR products obtained from amplification of apXa+/+ and y w67c23 gDNA were compared. The analysis of these reactions identified a difference close to the insertion breakpoint found in apblot. Again, this region was probed by iPCR. apXa+/+ gDNA was cut with NdelI and religated under diluted conditions. For iPCR, the primer pair icPCR_Xa_rev and 19_for was used. Sequencing of the iPCR product revealed that the reciprocal translocation had fused DNA originating from dad locus on 3R to ap-specific sequences. The fusion was confirmed by PCR and sequencing with 19_for and a primer in the dad region (primer dadint52out). The breakpoint associated with Xasta in ap was found to be identical in the two stocks apIT/T(2;3)apXa and w+; T(2;3)apXa, apXa/CyO, TM3, Sb1.

Generation of Dad4-Gal4 fly line

The minimal hsp70 promoter was amplified from the pUAST vector with the primer pair hsp70_Xbal_for and hsp70_BamHI_rev, then cloned into pBluescript II KS(+) via the Xbal and BamHI sites. The Dad4 fragment was amplified from gDNA with the primers dad_NotI_for and dad_NotI_rev, followed by the insertion of the fragment into the NotI and Xbal digested pBS-hsp70 plasmid. Gal4 was amplified from a pCaSpeR4-Gal4 plasmid, obtained from the lab of Konrad Basler, with the primer pair Gal4_BglII_for and Gal4_HindIII_rev. The Gal4
fragment was subsequently cloned into the BamHI and HindIII digested pBS-Dad4-hsp70 plasmid. We amplified the SV40-PA terminator sequence from the pUAST vector, using the primer pair SV40_HindIII_for and SV40_BamHI_ApaI. The SV40_PA was subsequently inserted into the pBS-Dad4-hsp70-Ga4 plasmid using the HindIII and Apal restriction sites. Finally, the Dad4-hsp70-Ga4-SV40_PA sequence was subcloned into the pCaSpeR4 vector, using the Nor1 and BamHI restriction sites. Transgenic flies were generated in a y w^E223 background with the help of the mini-white marker. The Dad4-Ga4 insert used in this study is linked to the X chromosome.

**X-Gal staining of imaginal discs**
Third instar larvae were cut in half, and the anterior part was inverted and subsequently fixed in 1% glutaraldehyde (Fluka) in PBS for 15 min on ice. After fixation, the fixative was removed and the larvae were washed twice with PBST (0.1% Tween 20 in PBS). The tissue was then stained as previously described (Ashburner 1989). Afterward, the imaginal discs were dissected and mounted in 80% glycerol. Discs were analyzed under the Zeiss Axiophot microscope and photographed with a Sony NEX-5RK digital camera.

**In situ hybridization**
A 1.5-kb fragment from the 3’ end of the ap cDNA was amplified from the cDNA clone H1L0212 (purchased from DGRHC) with primers insitu_Scl_for and insitu_Kpn1_rev. The fragment was cloned between Sca1 and Kpn1 sites of pBluescript II KS(+) vector. Then, the resulting plasmid was linearized with Acc65I and digoxigenin(DIG)-labeled RNA was produced from T7 promoter according to the manufacturer’s protocol (Roche, Switzerland). In situ hybridizations were performed as described in Tautz and Pfeifle (1989). Wing imaginal discs were dissected and mounted in 80% glycerol and photographed under a Nikon Microphot-FXA microscope with a Sony NEX-5RK digital camera.

**Immunostaining**
The anterior part of third instar larvae was inverted and fixed with 4% paraformaldehyde in PBS for 25 min at room temperature. Standard protocols were used to perform immunostaining. As primary antibodies, rabbit α-GFP (1:1000; Abcam) and mouse α-Wg (1:120, DSHB, University of Iowa) were used. α-rabbit AlexaFlour488 and α-mouse AlexaFlour568 (Molecular Probes) were used at a 1:750 dilution. Samples were mounted in Vectashield (Vector Laboratories, Inc.). Confocal imaging was performed using a Leica SP5 microscope with a vertical step size of 1 μm. Image processing was done with the ImageJ software.

**RESULTS**
**Defining a short wing-specific enhancer element in apC**
At apterus, four different transcripts starting from three different promoters have been annotated (see www.flybase.org). In this study, the transcription start site for transcripts ap-RA and ap-RC will be referred to as ap TSS.

An ~8-kb DNA fragment named apC located several kilobases upstream of the ap TSS had been shown to drive reporter gene expression in an ap-specific pattern in the wing disc (Lundgren et al. 1995). We used a LacZ reporter assay to analyze the cis-regulatory elements in apC in more detail. apC was first sub-divided into four overlapping fragments, apO, apP, apR and apQ (Figure 1A). Of these, only the two promoter proximal fragments, apO and apR were found to drive reporter gene expression in the wing disc. To further pinpoint the wing disc enhancers, we generated five subfragments that span the DNA sequences covered by apO and apR. As shown in Figure 1A, this analysis defines a minimal 874-bp fragment, apRXa. Because apP and apOR2 together cover the minimal apRXa element, but neither showed any expression in the wing disc, key ap wing enhancer elements are likely to be on both sides of the breakpoint that divides these two fragments.

To determine whether the wing enhancer element identified in the LacZ reporter assay is necessary for the proper regulation of the endogenous ap gene, we generated several deletions with defined breakpoints (Figure 1C; for details see the section Materials and Methods). The largest of these, apDG31, removes almost the entire ap locus, from the 4th intron to a site located about 500 bp upstream of the flanking distal gene, l(2)09851. Previous observations suggested that l(2)09851 activity is not affected by the proximity of apDG31’s distal break (Gohl et al. 2008). As a homoygote or when in trans to a large deficiency, Df(2R)BSC696, that includes the entire ap locus, apDG31 flies displayed a complete loss of all wing and haltere structures (Figure 1D). This result suggests that apDG31 represents an amorphic allele of ap, at least with respect to Ap function during wing development. Furthermore, we generated a deletion, called apDG8, which removes the whole ORF from the end of the 3’UTR to ~400 bp upstream of the ap TSS. Trans-heterozygous apDG8/apDG3 flies again showed a typical ap-null mutant phenotype. Finally, two deficiencies affecting only the 5′ regulatory region were generated, namely apDG2 and apDG11. They share the same proximal breakpoint located ~400 bp upstream of the ap TSS. Previous transvection studies suggested that the activity of the ap promoter is not affected by this breakpoint (Gohl et al. 2008). apDG2 extends ~27 kb distally to the same position as apDG3. apDG11 removes only ~11 kb of upstream DNA, including the whole apC fragment, apDG11/apDG3 as well as apDG11/apDG3 flies lacked all wing and haltere structures (Figure 1D). In these two deletions, the minimal wing enhancer element defined by apRXa is removed, suggesting that elements within apRXa are indeed necessary for the regulation of ap in the endogenous locus (see Figure 1C).

Although ap is expressed in the presumptive notum of the developing wing disc, the phenotypic appearance of the adult dorsal thorax is only mildly affected in flies lacking any ap activity (e.g., apDG3/ apDG3 or homozygous apDG9). Apart from a few missing macro- and microchaetae in the vicinity of the wing appendage, it appears largely normal (Figure 1D and data not shown). The reduced size of the dorsal thorax and the aberrant bristle pattern in apDG11/Df(2R)BSC696 flies can probably be attributed to other genetic loci deleted in Df(2R)BSC696.

Apart from the dominant apDG11 allele, lesions in the ap gene have been reported as recessive in genetic character. Careful inspection of wings obtained from flies heterozygous for any of the 4 deletions presented in Figure 1C corroborated this fact. However, ~2% of them had small margin defects, indicating a mild dominance of strong ap loss-of-function alleles (data not shown).

**Mutations in the apR region result in wing phenotypes**
In the course of investigating the cis-regulatory region of ap, we identified two classical ap alleles, apbrac and apata, that map to the apR region. apbrac was isolated as a spontaneous, hypomorphic mutation that causes notching mostly of the posterior wing margin in homozygous mutant flies, while the anterior wing margin remains largely unaffected (Figure 2A; Butterworth and King 1965; Whittle 1979). To narrow down the genomic site affected by the mutation, intragenic complementation crosses with the aforementioned deletions were analyzed. They showed that apDG11 was the smallest deletion that failed to complement apbrac. This observation suggested that apbrac maps to the ~11-kb interval defined by apDG11. Consequently, this region was screened with a set of overlapping PCR primer pairs. One primer pair did not yield a PCR product and thus identified the site of the putative
lesion on the ap\textsuperscript{blot} chromosome. Using iPCR, we identified the insertion of a retrotransposable element of the blood family in the apRXa sequence (Figure 2B, see the section Materials and Methods for details). This event caused the typical 4-bp duplication at the insertion site characteristic for blood family transposons (Figure 2C; Bingham and Chapman 1986; Wilanowski et al. 1995).

Phenotypes caused by blood insertions at other loci are sometimes temperature-sensitive (Bingham and Chapman 1986). To test this possibility, we raised homozygous ap\textsuperscript{blot} flies at different temperatures and scored their wing phenotypes (Table 1). At 18°C, only 28% of the wings displayed minor defects. In most of these, the posterior cross vein failed to connect with the 4th wing vein (Figure 2A). At greater temperatures, more severe wing phenotypes were detected with a higher penetrance. At 25°C and 29°C, 52% and 70%, respectively, of the wings showed extensive notching within the posterior compartment and reduced wing size (Figure 2A).

The dominant ap\textsuperscript{xa} allele was generated by X-ray mutagenesis and is associated with a reciprocal translocation between chromosome arms 2R and 3R. The breakpoints were mapped to 41F and 89EF, respectively (Serebrovskiy and Dubinin 1930; Waddington 1940; Lewis 1951; Hetherington et al. 1968). When heterozygous, ap\textsuperscript{xa} flies show the characteristic dominant mitten-shaped wing phenotype, in which the distal tip of the wing is missing leading to a deep notching of the wing blade. In hemizygous ap\textsuperscript{xa} flies, only long wing stumps with little or no wing margin and unstructured vein patterns are formed (Figure 2D). The break on 2R has long been known to affect the ap locus (Butterworth and King 1965; Stevens and Bryant 1985). However, our attempt to map ap\textsuperscript{xa} by intragenic complementation was not successful, suggesting that the lesion in ap\textsuperscript{xa} prevents this type of genetic analysis (see also Figure 3D). Thus, we screened the entire ap locus with overlapping PCR primer pairs. We identified a discontinuity in the apR region and determined the molecular nature of the breakpoint (Figures 2, E and F; for details see the section Materials and Methods).

It localized right at the edge of the apRXa fragment, 142 bp distal to the insertion site of the blood transposon in ap\textsuperscript{blot}. Only the proximal 874 bp of apR remain associated with the ap transcription unit (see Figure 2E). The DNA on the other side of the breakpoint is from the daughters against dpp (dad) locus located at 89E on 3R. As predicted from the cytological mapping of the rearranged ap\textsuperscript{xa} chromosomes, the dad locus is inverted compared to its wild-type orientation on 3R (for a comprehensive drawing of the ap\textsuperscript{xa} polytene chromosomes, see Hetherington et al. 1968). We were not able to determine the breakpoint at the reciprocal site of the translocation. Nevertheless, based on its reciprocal nature, it is conceivable that the dad locus is split within its 4th intron and hence destroyed. Because dad is expressed in the imaginal wing disc, it is formally possible that the Xasta phenotype is due to the loss of Dad activity. This possibility was addressed by crossing ap\textsuperscript{xa} with 2 known dad deletions, Df(3R)Exel6176 and dad\textsuperscript{P18841}. The wings of trans-heterozygous animals displayed the characteristic mitten phenotype seen in ap\textsuperscript{xa} heterozygous flies, suggesting that an amorphic dad background does not modify the Xasta phenotype. Hence Dad function is not relevant for the production of the Xasta phenotype (data not shown). This is not unexpected, since dad mutants show no visible phenotype in the adult wing (Ogiso et al. 2011).

The proximity of dad enhancers to the ap transcription unit in the ap\textsuperscript{xa} chromosome suggests a plausible explanation for the Xasta wing phenotype. Two cis-regulatory elements, Dad4 and DadInt52, are located in the dad introns (Figure 2E) and are known to drive reporter gene expression in the wing disc in a stripe along the A–P compartment boundary in response to Dpp signaling (Weiss et al. 2010). Because dad territory encompasses not only the dorsal but also the ventral compartment of the presumptive wing pouch, a likely scenario is that the ap promoter responds to these two dad enhancers, leading to ectopic Ap expression in the ventral compartment of the pouch.

**Ectopic expression of ap in ap\textsuperscript{xa} leads to the ectopic expression of Ap target genes**

To further characterize the effect of the ap\textsuperscript{blot} and ap\textsuperscript{xa} mutations on wing development, we examined ap mRNA and Wingless protein (Wg) expression in 3rd instar larval wing discs (Figure 3, A and B). In wild-type discs, ap mRNA is restricted to the dorsal compartment of the wing pouch, the hinge and the notum (Figure 3A). In the pouch, Ap activity is required to direct the expression of Wg in a stripe along...
the D–V compartment boundary (Figure 3B). This Wg stripe is essential for the proper formation of the wing margin (Couso et al. 1994).

The temperature sensitivity of apblot was faithfully recapitulated by the expression patterns in 3rd instar wing discs. Although ap mRNA levels were reduced at 18°C as well as at 29°C, an obvious deviation of the ap mRNA pattern was only observed at 29°C in the posterior compartment of the pouch. This change correlated with a size reduction of the posterior compartment and the appearance of additional tissue folding in this region (arrow in Figure 3A). Consistent with the sharp boundary of the ap mRNA expression pattern at 18°C, the Wg stripe along the D–V compartment boundary remained unchanged (Figure 3B). In contrast, at 29°C, the fuzzy appearance of the ap mRNA pattern in the posterior compartment correlated with the disruption of the Wg stripe. In summary, these results are consistent with the adult wing phenotypes and provide an explanation for the abnormalities in the posterior wing margin as well as for the reduced size of the posterior compartment in apblot flies raised at elevated temperature.

In apblot heterozygotes, a strong ectopic misexpression of the ap transcript was detected in the ventral compartment of the wing disc, with the highest signal along the medial part of the disc (Figure 3A). As a consequence, the Wg stripe was disrupted in the medial region of the wing pouch (Figure 3B). Remarkably, the disruption of the Wg stripe correlated well with the expression domain of the Dad4-GFP reporter construct (Figure 3C). Wherever GFP was detected, the expression of Wg was either very low or absent. Wing discs of hemizygous ap18/ ap18 flies showed strong ap expression in the entire pouch region. The characteristic Wg stripe in the wing pouch was lost, leaving behind only a small dot of Wg expression in the middle of the pouch. Moreover, the dimension of the wing pouch was reduced to about half the size of a wild-type pouch.

In Drosophila, the somatic pairing of the two homologous chromosomes can lead to a special situation of gene regulation called transvection (Lewis 1954; Sips et al. 1998; Morris et al. 1999; Coulthard et al. 2005). In this case the regulatory elements of a gene can regulate the expression of its homolog in trans. Transvection has been described for many gene loci (for reviews see Wu and Morris 1999; Duncan 2002) including the ap locus (Gohl et al. 2008). Therefore, we decided to test the transvection ability of apXa by crossing it with ap::GFP. In this combination only the gene in trans is labeled with GFP, allowing for the independent detection of the gene product from this chromosome. Trans-heterozygous ap::GFP/apXa flies displayed no ectopic expression of Ap-GFP in the ventral wing pouch (Figure 3D). This result demonstrates that the misexpression of ap is limited to the chromosome affected by the rearrangement.

As a selector gene, ap is known to regulate multiple downstream genes (Bronstein et al. 2010). We wished to know whether the ectopic

| Temperature | Total Wings Scored | Normal Wings | Wings with Phenotypes |
|-------------|--------------------|--------------|-----------------------|
| 18°C        | 294                | 72%          | 28%                   |
| 25°C        | 284                | 48%          | 52%                   |
| 29°C        | 242                | 30%          | 70%                   |

Table 1 Temperature sensitivity of apblot

middle of the pouch is visible. In addition, the size of the pouch is reduced. (C) GFP expression driven by the Dad4 enhancer is detected in the central part of an apXa/+ wing disc. Note that absence of Wg stripe correlates well with higher GFP levels. Therefore, stripe formation is more affected in the anterior than in the posterior compartment. (D) α-GFP and α-Wg antibody staining of an ap::GFP/apXa wing disc. GFP expression is restricted to the dorsal compartment of the wing pouch. In particular, Ap-GFP fusion protein does not spread ventrally where the Wg stripe is interrupted. This indicates that dad enhancers on the apXa chromosome are unable to activate ap::GFP located on the homologous chromosome. (E) Expression of Beadex- and fringe-Gal4 enhancer trap lines in wild type and apXa/+ discs. Note that ectopic expression (white arrows) of these two validated Ap targets in the ventral compartment is only detected where the Wg stripe is interrupted. All scale bars are 100 μm.
Ap expression observed in heterozygous ap\textsuperscript{xa} flies was sufficient to induce its targets also in the ventral compartment of the pouch. Toward that end, we analyzed Gal4-enhancer trap lines of two validated Ap target genes, Beadex (Bx) and fringe (fg) (Irvine and Wieschaus 1994; Mílan et al. 2004). Their activity was monitored with the help of a UAS-CD8-GFP transgene. Under wild-type conditions, Beadex > GFP expression was detected exclusively in the dorsal wing pouch, whereas fringe > GFP was observed predominantly in the whole dorsal compartment with weak ventral GFP outside the wing pouch (Figure 3E). When analyzed in a heterozygous ap\textsuperscript{xa} background, the expression of both reporters extended to the medial-ventral region of the wing discs (Figure 3E). In summary, the data presented in Figure 3 strongly suggest that in ap\textsuperscript{xa}, ap is ectopically expressed due to the juxtaposition of dad wing enhancer elements and the ap promoter. As a consequence, Ap target genes are up-regulated in the ventral compartment of the wing pouch. These molecular events correlate well with the disruption of the Wg stripe in median parts of the wing disc and, finally, the altered adult wing morphology.

Requirements for ectopic wing margin induction

A puzzling observation is that Ap expression does not induce Wg in the ventral part of the pouch, ultimately leading to extra margin formation in adult wings. One explanation for this finding is that compartment and compartment boundaries must be defined by a clear on-off state of selector gene activity. However, the dad gene and its enhancers are regulated by a Dpp concentration gradient (high Dpp in medial parts, low Dpp in lateral parts of the wing disc; for a review see Affolter and Basler 2007). As a consequence, in ap\textsuperscript{xa}, Ap is expressed in the ventral part of the wing pouch in response to the dad enhancers in a gradient-like manner. Thus, no clear selector gene on-off state between neighboring cells would be generated. In this case, the initiation of the signaling cascade that usually induces the Wg gene at the compartment boundary fails to be activated. To explore this possibility in more detail, we used the Gal4/UAS-system (Bártal and Perrimon 1993). Preliminary test crosses indicated that upon Gal4 activation, a UAS-ap transgene leads to lethality or pleiotropic phenotypes with all Gal4 drivers tested except dpp-Gal4. For this reason, we tested an insertion into ap, EY03046, which contains a UAS-driven promoter located several 100 bp upstream of the ap TSS (Figure 4F). In contrast to Gal4 > UAS-ap combinations, Gal4 > EY03046 flies were viable and obvious phenotypes were restricted to the dorsal thoracic appendages. One possible explanation for the difference is that the activation of EY03046 by Gal4 is Schwartz or eliminated by the ap PRE (Schwartz et al. 2006; Tolhuis et al. 2006; Oktaba et al. 2008; D. Biel et al., unpublished data) in most tissues outside of the wing disc. To activate EY03046 expression in the wing pouch, we used the following Gal4 drivers: actin-Gal4, dad\textsuperscript{4}-Gal4, salE-Gal4, dpp-Gal4, and ptc-Gal4. Their expression domains are depicted in Figure 4, A–E. For our purposes, they can be grouped into three classes: (1) actin > GFP is found in all cells of the pouch; (2) Dad\textsuperscript{4} > GFP and salE > GFP expression domains are rather broad with a rather ill-defined edge and centered on the A-P axis; and (3) dpp > GFP and ptc > GFP form a narrow stripe along the A-P axis.

To analyze the effects of ectopic Ap expression, we examined Wg stripe formation along the D-V compartment boundary (see Figure 4, A’–E’) and adult wing morphology (Figure 4, A’–E’). Ubiquitous Ap expression in the pouch using actin:Gal4 prevents Wg activation. As a consequence, margin formation in the tiny adult wings was abolished. As expected from the data in Figure 3, Dad4 and spalt-mediated Ap expressions led to Xasta phenocopies. Wg stripe formation was abolished in the center of the pouch. Occasionally, small ectopic Wg stripes extended into the ventral compartment in Dad4 > EY03046 wing discs. Nevertheless, both Gal4 drivers elicited similar moderate Xasta-like phenotypes in adult wings. Finally, although the expression patterns mediated by the dpp-Gal4 and ptc-Gal4 drivers were remarkably similar, their phenotypic consequences were dramatically different. dpp > EY03046 caused the appearance of a faint ectopic Wg patch on the A-P axis in the ventral compartment. A tuft of ectopic bristles was observed on the ventral side at the intersection of the A-P axis and the wing margin in less than 10% of the adult wings (black arrowhead in Figure 4D’). ptc > EY03046, on the other hand, interrupted the Wg stripe in the center of the pouch and frequently induced a well-defined Wg stripe which traversed the whole ventral compartment. A Wg stripe of variable length also is formed in the anterior compartment. Adult wings of this genotype often formed three-dimensional, balloon-like structures with an oval-shaped posterior margin extending from the proximal edge of the wing appendage to its distal end and back to proximal. In addition, anterior and posterior margins were not continuous at the tip of the wing.

These observations corroborate our expectations. First, the presence of Ap in the ventral compartment at sufficiently high levels impedes the activation of the signaling cascade that induces Wg expression along the D–V compartment boundary. Second, an ectopic compartment boundary can only be formed between cells with sharp on-off levels of Ap. This prerequisite is only satisfied by the ptc-Gal4 driver. In the wing disc, Ptc is expressed in a straight line immediately abutting the posterior compartment where it serves as a receptor for the Hedgehog ligand (Capdevila et al. 1994; Alexandre et al. 1996). Its anterior limit of expression is more graded and less well defined and ectopic anterior margin in the adult wing can only rarely be observed. The question remains why dpp > EY03046 is only marginally active in this experiment. It is possible that ectopic Ap levels remain below a certain threshold because the levels of Gal4 do not suffice. Alternatively, the onset of Gal4 activity in this driver line might be delayed. However, very similar observations were made with a UAS-ap transgene in place of EY03046 and also with a different dpp-Gal4 driver line. In addition, Klein et al. have reported a similar phenotype for ectopic Ser expression by dpp-Gal4 (Klein et al. 1998).

The in situ rescue system

To extend our analysis of the cis-regulatory elements directing ap expression, we decided to characterize and manipulate possible regulatory sequences directly at the endogenous locus. For this purpose, we engineered an in situ rescue system. The establishment of this system was a multistep procedure and is described in detail in the section Materials and Methods. A diagrammatic summary is presented in Figure 5. In brief, we deleted the 27-kb intergenic spacer between the ap and l(2) 09851 loci and replaced it with an attP site located 400 bp upstream of the ap TSS (Figure 5, A–D). This ap allele is referred to as ap\textsuperscript{attP\textsubscript{Enh}\textsuperscript{y}} (Figure 5D). The deleted region is identical to that of ap\textsuperscript{C31}. Therefore, homo- or hemi-hyzygous ap\textsuperscript{attP\textsubscript{Enh}\textsuperscript{y}} flies have no wings (data not shown). The attP site of ap\textsuperscript{attP\textsubscript{Enh}\textsuperscript{y}} serves as docking site for \textPhi\textsubscript{C31}-mediated integration of any desired DNA located on a plasmid containing an attB site and the yellow selection marker (Figure 5, D and E).

As proof of principle, two control plasmids were first introduced into ap\textsuperscript{attP\textsubscript{Enh}\textsuperscript{y}}: (1) the empty pEnh-Reentry vector gave rise to a fly line called ap\textsuperscript{empty}; (2) pEnh-Reentry-full-length contained the complete 27-kb intergenic spacer and the corresponding transgenic line was called ap\textsuperscript{full-length} (Figure 6A). The “wing-forming” activity of these two controls as well as all subsequent transgenic lines analyzed in this study was determined in hemizygous condition. Therefore, balanced ap\textsuperscript{empty} and ap\textsuperscript{full-length} males were crossed with ap\textsuperscript{C31}/SM6 virgins and...
the wings of trans-heterozygous progeny were carefully inspected. As expected, ap<sup>apR</sup>/ap<sup>DG3</sup> flies generated no detectable wing material. In contrast, the reconstituted ap locus produced wild-type wings in ap<sup>apR</sup>/ap<sup>DG3</sup> flies. Taken together, these observations demonstrate the feasibility of our in situ rescue system and suggest that the backbone of the pEnh-Reentry plasmid does not cause any disturbances.

**DISCUSSION**

In the past, cis-regulatory elements were mainly investigated using reporter-based assay systems, in which putative regulatory DNA

**The arPXa enhancer is required but not sufficient for wing formation**

In Figure 1 of this paper, we have presented evidence that the ~8 kb apC fragment harbors an 874-bp wing specific enhancer that is essential for wing formation. However, the experimental approaches we used are not adequate to test whether the enhancer is also sufficient for the formation of a wild-type wing. Therefore, four overlapping fragments covering the whole apC were introduced into the ap locus and the corresponding transgenic lines were obtained: ap<sup>apR</sup>, ap<sup>apY</sup>, ap<sup>apR</sup>, and ap<sup>apRXa</sup>. Their wing enhancer activity was tested in a hemizygous genetic background (Figure 6C). ap<sup>apR</sup>/ap<sup>DG3</sup> flies, which contained the apP fragment that did not yield any LacZ reporter activity (see Figure 1A), also did not develop any wing or haltere tissue and phenotypically resembled ap null alleles. When ap<sup>apR</sup>, a fragment which is shifted by 2 kb toward the ap TSS, was tested in ap<sup>apY</sup>/ap<sup>DG3</sup> flies, wing development was partially restored. However, most of the margin, the alula and the hinge region were poorly formed. Similar phenotypes as for ap<sup>apR</sup> were observed in ap<sup>apY</sup>/ap<sup>DG3</sup> and ap<sup>apRXa</sup>/ap<sup>DG3</sup> flies. Note that these three apC derivatives were sufficient to drive ap-specific LacZ expression in our reporter assay (see Figure 1A). “Homozygotes” obtained by pairwise combinations of ap<sup>apR</sup>, ap<sup>apR</sup> or ap<sup>apRXa</sup> were also studied. Such wings looked improved compared to the phenotypes observed in hemizygous ap<sup>apR</sup> flies (data not shown).

These results demonstrate that the 874 bp arPXa wing enhancer element is required but not sufficient in the endogenous context to correctly regulate ap expression. Our observations imply the existence of further unidentified wing enhancer elements elsewhere in the ap region.
fragments were tested for their ability to drive reporter gene expression when present on a transgene inserted randomly in the genome (Simon et al. 1985; Hiromi and Gehring 1987). Although this method proved to be a highly useful and valuable approach, it has some shortcomings. Enhancer fragments are tested in a genomic environment that may differ considerably from their native position. Additionally, the results of such studies yield little or no information about whether the investigated elements are sufficient, permissive or even dispensable for the regulation of gene expression at their original location. Recently, some improvements were achieved by using bacterial artificial chromosomes to investigate cis-regulatory elements in a broader genomic context (Dunipace et al. 2013).

To circumvent the problem of positional effects, we performed our classical reporter assay at a single \( \Phi C31 \)-system docking site located on 3R. Our laboratory has successfully used this insertion site for the analysis of wing specific enhancer elements (Weiss et al. 2010). Furthermore, we investigated the relevance of the reporter data with two powerful genetic approaches. We used methods from the \( \Phi C31 \)-integrase mediated recombination. By injection of plasmid pBSattBattPLoxFRTy, new attP, LoxP, and FRT sites were introduced into the ap locus. Note that pBSattBattPLoxFRTy can insert in two different attP sites leading to oppositely oriented insertions. \( \text{ap}^{\text{en}1\text{FRT2}} \) is the appropriate one for our purpose. (C–D) Flippase-mediated deletion. Trans-heterozygous \( \text{ap}^{\text{en}1\text{FRT2}/\text{ap}^{01573}} \) animals were repeatedly treated with Flippase during larval stages. Among the progeny of these flies, \( \text{ap}^{\text{en}1\text{FRT2}} \) could be isolated. It lacks the 27kb intergenic spacer but retains a strategically positioned attP site. (D–E) \( \text{ap}^{\text{en}1\text{FRT2}} \) serves as a platform to reinsert enhancer fragments. These are cloned into pEnh-Reentry. This plasmid is injected into young embryos and integrates into the ap locus by \( \Phi C31 \)-integrase mediated recombination.

Transgenics of the type \( \text{ap}^{\text{en}1\text{FRT2}} \) can be isolated thanks to the yellow marker. If desired, yellow can be removed by Cre-treatment. In addition, the complete insert can be excised by Flippase treatment.

Figure 5 Generation of the in situ rescue system at the endogenous ap locus. (A–B) Direct gene conversion at apterous. P-element insertion \( \text{ap}^{\text{MM}} \) located 400 bp upstream of the ap TSS was previously isolated. By mobilization of \( \text{ap}^{\text{MM}} \) and concomitant injection of plasmid pLAPGPRRA, fly line \( \text{ap}^{\text{1.4b}} \) could be isolated. It contains two inverted attP sites flanking a GFP reporter. (B–C) \( \Phi C31 \)-integrase mediated site-specific recombination. By injection of plasmid pBSattBattPLoxFRTy, new attP, LoxP, and FRT sites were introduced into the ap locus. Note that pBSattBattPLoxFRTy can insert in two different attP sites leading to oppositely oriented insertions. \( \text{ap}^{\text{en1FRT2}} \) is the appropriate one for our purpose. (C–D) Flippase-mediated deletion. Trans-heterozygous \( \text{ap}^{\text{en1FRT2}/\text{ap}^{01573}} \) animals were repeatedly treated with Flippase during larval stages. Among the progeny of these flies, \( \text{ap}^{\text{en1FRT2}} \) could be isolated. It lacks the 27kb intergenic spacer but retains a strategically positioned attP site. (D–E) \( \text{ap}^{\text{en1FRT2}} \) serves as a platform to reinsert enhancer fragments. These are cloned into pEnh-Reentry. This plasmid is injected into young embryos and integrates into the ap locus by \( \Phi C31 \)-integrase mediated recombination.

The importance of the apRXa enhancer element is further highlighted through the molecular characterization of 2 classical ap alleles, \( \text{ap}^{\text{blot}} \) and \( \text{ap}^{\text{Xa}} \). \( \text{ap}^{\text{blot}} \) contains an insertion of a retrotransposon from the blood family. This insertion is located within the apRXa enhancer. We have not attempted to prove the presence of the full length 7.4-kb blood element in \( \text{ap}^{\text{blot}} \), but we have completely sequenced both LTRs. So far, all blood elements detected in the Drosophila genome are full-length insertions. None of them was found to be truncated (Kaminker et al. 2002). Hence, it appears likely that \( \text{ap}^{\text{blot}} \) also contains an intact, full length blood element and that it is accountable for the mutagenic effect. For example, it is possible that the insertion destroys an important transcription factor binding site within the apRXa wing enhancer. Alternatively, the inserted DNA might be truncated (Kaminker et al. 2002). If desired, yellow can be removed by Cre-treatment. In addition, the complete insert can be excised by Flippase treatment.

The other ap allele we investigated is \( \text{ap}^{\text{Xa}} \). In this mutant, a reciprocal translocation event between the right arms of the second and third chromosomes caused a breakpoint immediately upstream of the apRXa wing enhancer. This rearrangement juxtaposes the telomeric region of the second chromosome with the apRXa enhancer. The next to \( \text{ap}^{\text{Xa}} \) is the invertible element completes the section of the ventral part of the wing disc, conferring ventral cells with a dorsal cell fate identity.

In this study, the combined application of reporter assay, deletion analysis and in situ rescue system has allowed us to firmly establish the 874 bp apRXa fragment as an essential wing-specific regulatory element for apterous transcription. We show that apRXa is sufficient to drive reporter gene expression within the dorsal compartment of the wing pouch. Flies hemizygous for an 11-kb deletion encompassing the apRXa element develop no wing structures. This observation proves that this larger DNA interval including apRXa is required but not sufficient for ap function. Finally, when tested in the context of the endogenous ap locus, we document that apRXa is required but not sufficient to form wild-type wings.

Volume 5 June 2015 | apterous Wing Enhancer | 1139
This, in turn, likely interferes with signaling at the D-V compartment boundary and causes the disruption of the Wg stripe in the center of the wing pouch. dad-controlled Ap expression also provides an explanation why the anterior compartment is more strongly affected than the posterior one in adult wings. As evidenced by asymmetric Dad4-GFP expression along the A-P compartment boundary, a wider domain with higher levels of GFP is produced in the anterior compartment (see Figure 3C). We propose that a similar asymmetrical distribution of Ap causes differential Wg stripe expression in the two compartments.

Our observations also suggest that dad-mediated transcriptional activation of ap is not the sole cause for the explanation why the anterior compartment is more strongly affected than the posterior one in adult wings. As evidenced by asymmetric Dad4-GFP expression along the A-P compartment boundary, a wider domain with higher levels of GFP is produced in the anterior compartment (see Figure 3C). We propose that a similar asymmetrical distribution of Ap causes differential Wg stripe expression in the two compartments.

Table 2 Penetration of the dominant apXa wing phenotype

| Genotype          | Number of Wings Scored | L2 Junction Present | L2 Junction Absent |
|-------------------|------------------------|---------------------|--------------------|
| apXa/+            | 262                    | 6.5%                | 93.5%              |
| apXaRXodad52.4/+  | 160                    | 98.1%               | 1.9%               |
| apDad52.4/+       | 546                    | 58.6%               | 41.4%              |

* Wings were scored for the presence or absence of the junction between wing vein L2 and the wing margin.
elements (Holdridge and Dorsett 1991; Hagstrom et al. 1996; Scott et al. 1999). Furthermore, the mutagenic effect of an array of Su(Hw) binding sites located on the gypsy mobile genetic element can often be attributed to a similar mechanism (Geyer et al. 1986; Peifer and Bender 1988; Dorsett 1993; Hoggia et al. 2001). Homozygous as well as hemizygous apv0061 flies have been reported to cause a rather strong wing phenotype. Importantly, the phenotype is completely suppressed in a sarHw background (Gohl et al. 2008). This observation suggests the presence of other ap wing enhancer elements distally to P{Bac[WH]}00651. We are currently exploring this possibility with analogous experimental approaches as outlined above, in particular through the use of the in situ rescue system established and described in this study (D. Bieli et al., unpublished results).

ACKNOWLEDGMENTS

We thank Johannes Bischof, John B. Thomas, Jorgos Pyrowolakis, Marco Milán, Steven Henikoff, and Fusun Hamaroglu and the Bloomington and Kyoto stock centers for sending stocks and Jack Bateman for important DNA clones. Stefan Thor is acknowledged for helpful discussions at initial stages of this work. Our direct gene conversion approach benefitted a lot from Laci Sipos’ suggestions. Alex Weiss and Emmanuel Caussinus helped greatly by sharing their injection skills during the screen for gene conversion events at apterous. Thanks are also due to Mario Metzler for verifying the distal foot of P{Bac[WH]}00651. This work would not have been possible without the recent efforts of the following labs: the Basler and Karch labs adopted the F{C31} system for Drosophila and sent us important stocks prior to publication; Hugo Bellen et al. developed the MiMIC tool and shared with us useful insertions in apterous. We would like to thank the Biozentrum Imaging Core Facility for unceasing support. Last but not least, a big thank you to Bernadette Bruno, Gina Evora, and Karin Mauro for constant and reliable supply with world’s best fly food. This study was supported by grants from the Kantons Basel-Stadt and Basel-Land, and the Swiss National Science Foundation.

LITERATURE CITED

Aföltter, M., and K. Basler, 2007 The decapentaplegic morphogen gradient: from pattern formation to growth regulation. Nat. Rev. Genet. 8: 663–674.
Ahmad, K., and S. Henikoff, 2001 Modulation of a transcription factor counteracts heterochromatic gene silencing in Drosophila. Cell 104: 839–847.
Alexandre, C., A. Jacinto, and P. W. Ingham, 1996 Transcriptional activation of hedgehog target genes in Drosophila is mediated directly by the cubitus interruptus protein, a member of the GLI family of zinc finger DNA-binding proteins. Genes Dev. 10: 2003–2013.
Alexandre, C., A. Baena-Lopez, and J.-P. Vincent, 2014 Patterning and growth control by membrane-tethered Wingless. Nature 505: 180–185.
Ashburner, M., 1989 Drosophila: A Laboratory Handbook and Manual (two volumes). Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York.
Bateman, J. R., A. M. Lee, and C. T. Wu, 2006 Site-specific transformation of Drosophila via phiC31 integrase-mediated cassette exchange. Genetics 173: 769–777.
Bellon, H. J., R. W. Levis, G. Liao, Y. He, J. W. Carlson et al., 2004 The BDGP gene disruption project: single transposon insertions associated with 40% of Drosophila genes. Genetics 167: 761–781.
Bingham, P. M., and C. H. Chapman, 1986 Evidence that white-blood is a novel type of temperature-sensitive mutation resulting from temperature-dependent effects of a transposon insertion on formation of white transcripts. EMBO J. 5: 3343–3351.
Bischof, J., R. K. Maeda, M. Hediger, F. Karch, and K. Basler, 2007 An optimized transgenesis system for Drosophila using germ-line-specific phiC31 integrases. Proc. Natl. Acad. Sci. USA 104: 3312–3317.
Blair, S. S., D. L. Brower, J. B. Thomas, and M. Zavortink, 1994 The role of apterous in the control of dorsoventral compartmentalization and PS integrin gene expression in the developing wing of Drosophila. Development 120: 1805–1815.
Bourguin, C., S. E. Lundgren, and J. B. Thomas, 1992 apterous is a drosophila LIM domain gene required for the development of a subset of embryonic muscles. Neuron 9: 549–561.
Bronstein, R., L. Levkovitz, N. Yosef, M. Yanku, E. Ruppin et al., 2010 Transcriptional regulation by CHIP/LDB complexes. PLoS Genet. 6: e1001063.
Butterworth, F. M., and R. C. King, 1965 The developmental genetics of apterous mutants of Drosophila melanogaster. Genetics 52: 1153–1174.
Capdevila, J., M. P. Estrada, E. Sánchez-Herrero, and I. Guerrero, 1994 The Drosophila segment polarity gene patched interacts with decapentaplegic in wing development. EMBO J. 13: 71–82.
Caussinus, E., O. Kanca, and M. Aföltter, 2011 Fluorescent fusion protein knockout mediated by anti-GFP nanobody. Nat. Struct. Mol. Biol. 19: 117–121.
Chan, C. S., L. Rastelli, and V. Pirrotta, 1994 A Polycomb response element in the Ubx gene that determines an epigenetically inherited state of repression. EMBO J. 13: 2553–2564.
Cohen, B., M. E. McGuffin, C. Pfeifle, D. Segal, and S. M. Cohen, 1992 apterous, a gene required for imaginal disc development in Drosophila encodes a member of the LIM family of developmental regulatory proteins. Genes Dev. 6: 715–729.
Coulthard, A. B., N. Nolan, J. B. Bell, and A. J. Hilliker, 2005 Transvection at the vestigial locus of Drosophila melanogaster. Genetics 170: 1711–1721.
Couso, J. P., S. A. Bishop, and A. Martinez Arias, 1994 The wingless signalling pathway and the patterning of the wing margin in Drosophila. Development 120: 621–636.
Couso, J. P., E. Knust, and A. Martinez Arias, 1995 Serrate and wingless cooperate to induce vestigial gene expression and wing formation in Drosophila. Curr. Biol. 5: 1437–1448.
Dahmann, C., and K. Basler, 1999 Compartment boundaries: at the edge of development. Trends Genet. 15: 320–326.
Diaz-Benjumea, F. J., and S. M. Cohen, 1993 Interaction between dorsal and ventral cells in the imaginal disc directs wing development in Drosophila. Cell 75: 741–752.
Diaz-Benjumea, F. J., and S. M. Cohen, 1995 Serrate signals through Notch to establish a Wingless-dependent organizer at the dorsal/ventral compartment boundary of the Drosophila wing. Development 121: 4215–4225.
Dorsett, D., 1993 Distance-independent inactivation of an enhancer by the suppressor of Hairy-wing-DNA-binding protein of Drosophila. Genetics 134: 1135–1144.
Duncan, I. W., 2002 Transvection effects in Drosophila. Annu. Rev. Genet. 36: 521–556.
Dunipace, L., A. Saunders, H. Ashe, and A. Stathopoulos, 2013 Autoregulatory feedback controls sequential action of cis-regulatory modules at the brinker locus. Dev. Cell 26: 536–543.
Fauvarque, M. O., and J. M. Dura, 1993 polyhomeotic regulatory sequences induce developmental regulator-dependent variation and targeted P-element insertions in Drosophila. Genes Dev. 7: 1508–1520.
García-Bellido, A., P. Ripoll, and G. Morata, 1973 Developmental compartmentalisation of the wing disk of Drosophila. Nat. New Biol. 245: 251–253.
Geyer, P. K., and V. G. Corces, 1987 Separate regulatory elements are responsible for the complex pattern of tissue-specific and developmental transcription of the yellow locus in Drosophila melanogaster. Genes Dev. 1: 996–1004.
Geyer, P. K., C. Spana, and V. G. Corces, 1986 On the molecular mechanism of gypsy-induced mutations at the yellow locus of Drosophila melanogaster. EMBO J. 5: 2657–2662.
Gindhart, J. G., and T. C. Kaufman, 1995 Identification of Polyc Comb and trithorax group responsive elements in the regulatory region of the Drosophila homeotic gene Sex combs reduced. Genetics 139: 797–814.
D. Bieli

Gloor, G. B., N. A. Nassif, D. M. Johnson-Schlitz, C. R. Preston, and W. R. Engels, 1991 Targeted gene replacement in Drosophila via P element-induced gap repair. Science 253: 1110–1117.

Goh, D., M. Müller, V. Pirrotta, M. Affolter, and P. Schedl, 2008 Enhancer blocking and transversion at the Drosophila apterous locus. Genetics 178: 127–143.

Golic, K. G., and M. M. Golic, 1996 Engineering the Drosophila genome: chromosome rearrangements by design. Genetics 144: 1693–1711.

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

Henderson, K. D., D. D. Isaac, and D. J. Andrew, 1999 Cell fate specification in the Drosophila salivary gland: the integration of homeotic gene function with the DPP signaling cascade. Dev. Biol. 205: 10–21.

Hetherington, C. M., W. J. Whittington, M. A. Hossain, and W. E. Peat, 1968 The genetics of the Xasta mutant of Drosophila melanogaster. Genet. Res. 12: 285–294.

Hiromi, Y., and W. J. Gehring, 1987 Regulation and function of the Drosophila segmentation gene fushi tarazu. Cell 50: 963–974.

Hogga, I., J. Mihaly, S. Barges, and F. Karch, 2001 Replacement of Fab-7 by the gypsy or scs insulator disrupts long-distance regulatory interactions in the Abd-B gene of the bithorax complex. Mol. Cell 8: 1145–1151.

Holdridge, C., and D. Dorsett, 1991 Repression of hsp70 heat shock gene transcription by the suppressor of hairy-wing protein of Drosophila melanogaster. Mol. Cell. Biol. 11: 1894–1900.

Irvine, K. D., and E. Wieschaus, 1994 fringe, a Boundary-specific signaling molecule, mediates interactions between dorsal and ventral cells during Drosophila wing development. Cell 79: 595–606.

Kaminker, J. S., C. M. Bergman, B. Kronmiller, J. Carlson, R. Svirskas et al., 2002 The transposable elements of the Drosophila melanogaster euchromatin: a genomics perspective. Genome Biol. 3: RESEARCH0084.

Kassis, J. A., and E. P. VanSickle, and S. M. Sensabaugh, 1991 A fragment of engrailed regulatory DNA can mediate transvection of the white gene in Drosophila. Genetics 128: 751–761.

Kim, J., K. D. Irvine, and S. B. Carroll, 1995 Cell recognition, signal induction, and symmetrical gene activation at the dorsal-ventral boundary of the developing Drosophila wing. Cell 82: 795–802.

Klein, T., J. P. Couso, and A. Martinez Arias, 1998 Wing development and specification of dorsal cell fates in the absence of apterous in Drosophila. Curr. Biol. 8: 417–420.

Lawrence, P. A., and G. Morata, 1977 The early development of mesothoracic compartments in Drosophila. An analysis of cell lineage and fate mapping and an assessment of methods. Dev. Biol. 56: 40–51.

Lewis, E. B., 1951 Additions and corrections to the cytolgy of rearrangements. Drosoph. Inf. Serv. 25: 108–109.

Lewis, E. B., 1954 The theory and application of a new method of detecting chromosomal rearrangements in Drosophila melanogaster. Am. Nat. 88: 225–239.

Lundgren, S. E., C. A Callahan, S Thor, and J. B. Thomas, 1995 Control of neuronal pathway selection by the Drosophila LIM homeodomain gene apterous. Development 121: 1769–1773.

Milán, M., and S. M. Cohen, 1999 Regulation of LIM homeodomain activity in vivo: a tetramer of dLDB and apterous confers activity and capacity for regulation by dLMO. Mol. Cell. 4: 267–273.

Milán, M., T. T. Pham, and S. M. Cohen, 2004 Osa modulates the expression of Apterous target genes in the Drosophila wing. Mech. Dev. 121: 491–497.

Morris, J. R., J. Chen, S. T. Filandrinos, R. C. Dunn, R. Fisk et al., 1999 An analysis of transvection at the yellow locus of Drosophila melanogaster. Genetics 151: 633–651.

Mosimann, C., G. Hausmann, and K. Basler, 2006 Parafibromin/Hyrax activates Wnt/Wg target gene transcription by direct association with beta-catenin/Armadillo. Cell 125: 327–341.

Muller, M., K. Hagstrom, H. Gyurkovics, V. Pirrotta, and P. Schedl, 1999 The mcp element from the Drosophila melanogaster bithorax complex mediates long-distance regulatory interactions. Genetics 153: 1333–1356.

Neumann, C. J., and S. M. Cohen, 1997 Long-range action of Wingless organizes the dorsal-ventral axis of the Drosophila wing. Development 124: 871–880.

Ochman, H., A. S. Gerber, and D. L. Hartl, 1988 Genetic applications of an inverse polymerase chain reaction. Genetics 120: 621–623.

Ogiso, Y., K. Tsuneizumi, N. Masuda, M. Sato, and T. Tabata, 2011 Robustness of the Dpp morphogen activity gradient depends on negative feedback regulation by the inhibitory Smad. Dev. Genet. Differ. 53: 668–678.

Oktaba, K., L. Gutiérrez, J. Gagneur, C. Girardot, A. K. Sengupta et al., 2008 Dynamic regulation by polycomb group protein complexes controls pattern formation and the cell cycle in Drosophila. Dev. Cell 15: 877–889.

Peifer, M., and W. Bender, 1988 Sequences of the gypsy transposon of Drosophila necessary for its effects on adjacent genes. Proc. Natl. Acad. Sci. USA 85: 9650–9654.

Pfeiffer, B. D., T.-T. B. Ngo, K. L. Hibbard, C. Murphy, A. Jenett et al., 2010 Refinement of tools for targeted gene expression in Drosophila. Genetics 186: 735–755.

Rulifson, E. J., and S. S. Blair, 1995 Notch regulates wingless expression and is not required for reception of the paracrine wingless signal during wing margin neurogenesis in Drosophila. Development 121: 2813–2824.

Ryder, E., M. Ashburner, R. Bautista-Llacer, J. Drummond, J. Webster et al., 2007 The DrosDel deletion collection: a Drosophila genomewide chromosomal deficiency resource. Genetics 177: 615–629.

Schwartz, Y. B., T. G. Kahn, D. A. Nix, Y. Li, R Bourgon et al., 2006 Genome-wide analysis of Polycumb targets in Drosophila melanogaster. Nat. Genet. 38: 700–705.

Scott, K. C., A. D. Taubman, and P. K. Geyer, 1999 Enhancer blocking by the Drosophila gypsy insulator depends upon insulator anatomy and enhancer strength. Genetics 153: 787–798.

Serebrovsky, A. S., and N. P. Dubinin, 1930 X-ray experiments with Drosophila. Hered. 21: 259–265.

Simon, J. A., C. A. Sutton, R. B. Lobell, R. L. Glaser, and J. T. Lis, 1985 Determinants of heat shock-induced chromosome puffing. Cell 40: 805–817.

Sipos, L., J. Mihály, F. Karch, P. Schedl, J. Gausz et al., 1998 Transvection in the Drosophila Abd-B domain: extensive upstream sequences are involved in anchoring distant cis-regulatory regions to the promoter. Genetics 149: 1031–1050.

Sipos, L., G. Kozma, E. Molnár, and W. Bender, 2007 In situ dissection of a Polycumb response element in Drosophila melanogaster. Proc. Natl. Acad. Sci. USA 104: 12416–12421.

Staeling-Hampton, K., P. D. Jackson, M. J. Clark, A. H. Brand, and F. M. Hoffmann, 1994 Specificity of body morphogenetic protein-related factors: cell fate and gene expression changes in Drosophila embryos induced by decapentaplegic but not 60A. Cell Growth Differ. 5: 585–593.

Stevens, M. E., and J. P. Bryant, 1985 Apparent genetic complexity generated by developmental thresholds: the apterous locus in Drosophila melanogaster. Genetics 110: 281–297.

Struhl, G., and K. Basler, 1993 Organizing activity of wingless protein in Drosophila. Cell 72: 527–540.

Tautz, D., and C. Pfeifle, 1989 A non-radioactive in situ hybridization method for the localization of specific RNAs in Drosophila embryos reveals translational control of the segmentation gene hunchback. Chromosoma 98: 81–85.

Thibault, S. T., M. A. Singer, W. Y. Miyazaki, B. Milash, N. A. Dompe et al., 2004 A complementary transposon tool kit for Drosophila melanogaster using P and piggyBac. Nat. Genet. 36: 283–287.

Thomason, L., D. L. Court, M. Bubunenko, N. Costantino, H. Wilson, S. Datta, and A. Oppenheim, 2007 Recombining: Genetic engineering in bacteria using homologous recombination. Curr. Protoc. Mol. Biol. Chapter 1: Unit 1.16.
Tolbuis, B., E. de Wit, I. Muijres, H. Teunissen, W. Talhout et al., 2006 Genome-wide profiling of PRC1 and PRC2 Polycomb chromatin binding in Drosophila melanogaster. Nat. Genet. 38: 694–699.

Tsuneizumi, K., T. Nakayama, J. L. Christian, and T. Tabata, 1997 Daughters against dpp modulates dpp organizing activity in Drosophila wing development. Nature 389: 627–631.

Venken, K. J. T., K. L. Schulze, N. A. Haeltman, H. Pan, Y. He et al., 2011 MiMIC: a highly versatile transposon insertion resource for engineering Drosophila melanogaster genes. Nat. Methods 8: 737–743.

Vuilleumier, R., A. Springhorn, L. Patterson, S. Koidl, M. Hammerschmidt et al., 2010 Control of Dpp morphogen signalling by a secreted feedback regulator. Nat. Cell Biol. 12: 611–617.

Waddington, C. H., 1940 The genetic control of wing development in Drosophila. J. Genet. 41: 75–139.

Weiss, A., E. Charbonnier, E. Ellertsdóttir, A. Tsirigos, C. Wolf et al., 2010 A conserved activation element in BMP signaling during Drosophila development. Nat. Struct. Mol. Biol. 17: 69–76.

Whittle, J. R., 1979 Replacement of posterior by anterior structures in the Drosophila wing caused by the mutation apterous-blot. J. Embryol. Exp. Morphol. 53: 291–303.

Wieschaus, E., and W. Gehring, 1976 Clonal analysis of primordial disc cells in the early embryo of Drosophila melanogaster. Dev. Biol. 50: 249–263.

Wilanowski, T. M., J. B. Gibson, and J. E. Symonds, 1995 Retrotransposon insertion induces an isoform of sn-glycerol-3-phosphate dehydrogenase in Drosophila melanogaster. Proc. Natl. Acad. Sci. USA 92: 12065–12069.

Williams, J. A., S. W. Paddock, and S. B. Carroll, 1993 Pattern formation in a secondary field: a hierarchy of regulatory genes subdivides the developing Drosophila wing disc into discrete subregions. Development 117: 571–584.

Wu, C. T., and J. R. Morris, 1999 Transvection and other homology effects. Curr. Opin. Genet. Dev. 9: 237–246.

Zecca, M., and G. Struhl, 2002a Subdivision of the Drosophila wing imaginal disc by EGFR-mediated signaling. Development 129: 1357–1368.

Zecca, M., and G. Struhl, 2002b Control of growth and patterning of the Drosophila wing imaginal disc by EGFR-mediated signaling. Development 129: 1369–1376.

Communicating editor: H. D. Lipshitz