Beadex encodes an LMO protein that regulates Apterous LIM–homeodomain activity in *Drosophila* wing development: a model for *LMO* oncogene function

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Formation of the dorsal–ventral axis of the *Drosophila* wing depends on activity of the LIM–homeodomain protein Apterous (Ap). Here we report that Ap activity levels are modulated by dLMO, the protein encoded by the *Beadex* (*Bx*) gene. Overexpression of dLMO in *Bx* mutants interferes with Apterous function. Conversely, *Bx* loss-of-function mutants fail to down-regulate Apterous activity at late stages of wing development. Biochemical analysis shows that dLMO protein competes for binding of Apterous to its cofactor Chip. These data suggest that Apterous activity depends on formation of a functional complex with Chip and that the relative levels of dLMO, Apterous, and Chip determine the level of Apterous activity. The dominant interference mechanism of dLMO action may serve as a model for the mechanism by which *LMO* oncogenes cause cancer when misexpressed in T cells.

[*Key Words*: Beadex; LMO protein; LIM–homeodomain; *Drosophila*; wing development; Apterous]

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Axis formation in *Drosophila* limb development is controlled by localized expression of secreted signaling molecules. Hedgehog (Hh), Decapentaplegic (Dpp), and Wingless (Wg) form activity gradients that define the spatial domains of target gene expression in the developing legs and wings (Diaz-Benjumea and Cohen 1995; Zecca et al. 1995, 1996; Lecuit et al. 1996; Lecuit and Cohen 1997; Nellen et al. 1996; Neumann and Cohen 1996, 1997; Strigini and Cohen 1997). Both spatially restricted expression and appropriate levels of activation of the signaling pathways are critical for normal patterning of the limbs. Misexpression of genes at different levels of these regulatory hierarchies leads to pattern abnormalities. Misexpression of the signaling molecules can lead to axis duplication (Struhl and Basler 1993; Basler and Struhl 1994; Capdevila and Guerrero 1994; Diaz-Benjumea et al. 1994; Diaz-Benjumea and Cohen 1995; Felsenfeld and Kennison 1995; Ingham and Fietz 1995; Zecca et al. 1995). In addition overactivation (or underactivation) of a signaling pathway involved in proper spatial localization of downstream effector genes can also perturb normal limb development (e.g., Johnson et al. 1995; Axelrod et al. 1996; Neumann and Cohen 1996). Finally, misexpression of the effector genes themselves can lead to abnormalities in limb development (e.g., de Celis et al. 1996a; Grimm and Pflugfelder 1996; Gorfinkiel et al. 1997; Sturtevant et al. 1997).

These observations suggest that systematic misexpression of genes in the developing limbs might provide an effective way to screen for genes involved in the cell signaling processes that control limb development. The modular–misexpression system developed by Rørth (1996) was used to carry out a large-scale screen for genes that perturb wing development (Rørth et al. 1998). The system allows conditional misexpression of genes tagged by insertion of a P element that carries a GAL4 regulatable EP (enhancer and a basal promoter) oriented to direct expression of adjacent genomic sequences. When combined with a source of GAL4, the EP element will direct expression of any gene that happens to lie next to its insertion site. The screen identified EP insertions at *hh*, *patched* (*ptc*), and *dpp*, genes with known roles in limb patterning (Rørth et al. 1998) as well as a number of new loci that are implicated in wing patterning by virtue of their overexpression phenotypes.

Here we report the characterization of a gene identified by the EP screen that is involved in dorsal–ventral (DV) patterning of the wing. We present genetic and bio-

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chemical evidence that the product of the *Beadex* (*Bx*) gene regulates Apterous (Ap) activity levels. Gain-of-function mutants reduce Apterous activity. Conversely, loss-of-function mutants of *Bx* appear to increase Ap activity. Ap encodes a LIM–homeodomain protein that specifies dorsal cell fate (Cohen et al. 1992; Diaz-Benjumea and Cohen 1993; Williams et al. 1993; Blair et al. 1994). A LIM-binding protein called Chip has been identified as a possible cofactor for Ap (Morcillo et al. 1997). We show that *Bx* mutants overexpress a LIM-only protein, dLMO, that binds to Chip and thereby interferes with formation of a functional complex between Ap and Chip. We also show that Ap induces expression of its antagonist dLMO, which suggests that Ap and dLMO constitute a feedback mechanism and that the relative levels of dLMO, Chip, and Ap determine Ap activity levels in vivo. These findings suggest a molecular model for the mechanism of action of *LMO* oncogenes in causing leukemia and lymphoma (Fisch et al. 1992; McGuire et al. 1992).

Results

An antagonist of Ap function in dorsal cells

Two independently isolated EP insertions on the X chromosome produced phenotypes suggesting a role in DV axis formation when expressed in the developing wing. Adult wings from flies carrying EP1306 or EP1394 lack most of the normal wing margin and show irregular bits of ectopic margin associated with overgrowths in the dorsal surface of the wing when the EP lines are expressed under control of *optomoter blind–gal4* (*omb–gal4*) (not shown). A milder version of this phenotype is observed when EP1394 is expressed in a narrow stripe of cells in the center of the wing blade under control of *ptc–gal4* (Fig. 1). The dorsal surface of the wing is extensively overgrown and contains an ectopic wing margin along the edge of the overgrowth (Fig. 1B, red arrow). Comparable effects are observed with both EP lines.

The presence of the ectopic wing margin and the overgrowth of the dorsal compartment suggested that EP1394 misexpression might cause ectopic Wg expression. Wg is normally expressed in a stripe along the DV boundary of the wild-type wing imaginal disc (Fig. 1C), where it acts locally to induce nearby cells to adopt wing margin identity (Phillips and Whittle 1993; Couso et al. 1994). Ectopic Wg expression has been shown to induce ectopic wing margin formation and overgrowth of the surrounding tissue (Diaz-Benjumea and Cohen 1995; Kim et al. 1995; de Celis et al. 1996b; Doherty et al. 1996; Neumann and Cohen 1997). In *ptc–gal4*; *EP1306* discs, Wg is ectopically expressed in cells parallel to the *ptc–gal4* stripe in the dorsal compartment (Fig. 1D, red arrow). This ectopic Wg stripe corresponds to the ectopic wing margin shown in Figure 1B.

The observation that EP1306 expression induces ectopic Wg in dorsal cells but not in ventral cells suggested involvement of the *ap* and *fringe* (*fng*) genes. *ap* is expressed in dorsal cells (Fig. 1E), in which it is thought to

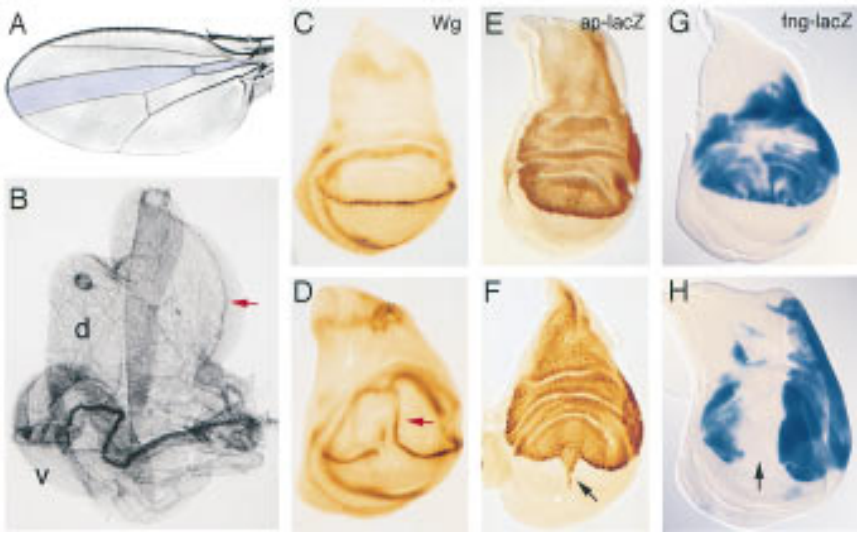

Figure 1. Misexpression of EP1306 interferes with Ap function. (*A*) Cuticle preparation of a wild-type wing colored blue to highlight the region of *ptc–gal4* expression in the region between veins 3 and 4. The wing is viewed from the ventral side, with the anterior margin to the *top*. (*B*) Cuticle preparation of a *ptc–gal4*; *EP1394* wing. The wing is distorted because of the extensive overgrowth of the dorsal compartment. In this example dorsal (d) is to the *top* and ventral (v) to the *bottom*, in effect a 90° rotation perpendicular to the wing in *A*. The anterior margin is viewed edge on. The red arrow indicates an ectopic wing margin running along the edge of the overgrowth in the dorsal compartment. The ectopic margin consists of posterior structures and has both dorsal and ventral elements. Ectopic anterior margins are usu ally incomplete. The topology of these wings is best understood in terms of Wg expression (see *D*). Comparable effects are seen in all cases for *EP1394* and *EP1306*. (*C*,*D*) Wg expression in wild-type (*C*) and *ptc–gal4*; *EP1306* (*D*) wing imaginal discs visualized by antibody staining. Wg expression is interrupted at the DV boundary of the *ptc–gal4*; *EP1306* disc and an ectopic stripe of Wg runs along the edge of the *ptc–gal4* domain (red arrow). Note the relative overgrowth of the dorsal compartment. (*E*,*F*) *ap–lacZ* expression in wild-type (*E*) and *ptc–gal4*; *EP1306* (*F*) wing discs visualized using anti-β-galactosidase. Note that the normally sharp boundary between Ap-expressing dorsal cells and ventral cells is disturbed in the *ptc–gal4* domain (arrow). This suggests a defect in Ap function in cells expressing *EP1306*, because Ap activity is important in generating the DV compartment boundary (Diaz-Benjumea and Cohen 1993; Blair et al. 1994). (*G*,*H*) *fng–lacZ* expression in wild-type (*G*) and *ptc–gal4*; *EP1306* (*H*) wing discs visualized by histochemical activity staining. *fng–lacZ* is not expressed in dorsal cells in which *EP1306* is overexpressed. We verified that Ap regulates *fng–lacZ* by ectopic expression of Ap in the *ptc–gal4* stripe in the ventral compartment in *ptc–gal4*; UAS–Ap flies (data not shown).

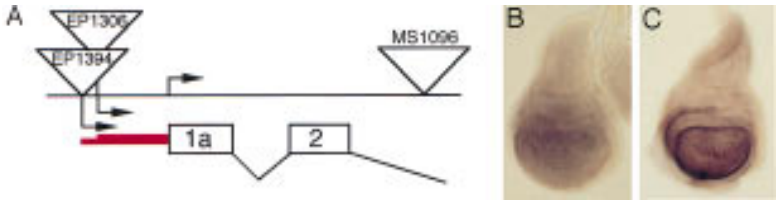

Figure 2. EP1306 directs expression of the *Bx–dLMO* transcript. (*A*) Molecular map of EP1306, EP1394, and MS1096 insertions at the *dLMO* locus. (*B,C*) In situ hybridization to wild-type (*B*) and Bx^1 (*C*) wing discs using an antisense RNA probe prepared from the *dLMO* cDNA. The *dLMO* transcript is overexpressed in *Bx* mutant wing discs. The discs in *B* and *C* were processed in parallel to allow direct comparison of staining intensity. To verify that overexpression of *dLMO* is the cause of the EP1306 and *Bx* mutant phenotypes, a newly isolated *dLMO* cDNA was modified to introduced an epitope tag, cloned into pUAST and expressed under *ptc–gal4* control. The resulting phenotypes were identical to those produced by *ptc–gal4*; *EP1306* (data not shown).

induce *fng* expression (Irvine and Wieschaus 1994). In *ptc–gal4*; *EP1306* discs, an *ap* reporter gene (Fig. 1F) and Ap protein (not shown) continue to be expressed normally in dorsal cells; however, *fng–lacZ* expression is lost in the dorsal compartment where *ptc–gal4* is expressed (arrow Fig. 1H). Loss of *fng* explains the abnormal expression of Wg in EP1306-expressing wing discs. Wg is induced at the interface, where cells that express *fng* meet cells that do not (Kim et al. 1995). Loss of *fng* in cells expressing EP1306 will lead to loss of Wg at the DV boundary and to ectopic Wg along the new boundary of *fng* expression in the dorsal compartment. Removing *fng* activity from a large patch of dorsal cells in somatic mosaic clones produces a comparable effect (Kim et al. 1995). The similarity in these phenotypes suggests that the effects of overexpressing EP1306 can be explained by the observed loss of *fng* expression in dorsal cells. These observations suggest that Ap function is compromised in cells that overexpress EP1306.

EP1306 directs expression of the Bx *gene*

EP1306 and EP1394 were mapped to cytological position 17C1-4 by in situ hybridization to polytene chromosomes. This corresponds to the location of the *Bx* gene. *Bx* alleles are dominant mutations that are thought to increase gene activity (Lifschytz and Green 1979; Mattox and Davidson 1984). The severity of the *Bx* mutant phenotype is enhanced by introducing an extra copy of the wild-type gene and reduced by removing a copy. In addition, increasing the number of copies of the normal *Bx* gene (by tandem duplication) produces the same wing defects as are found in the dominant *Bx* mutant (cited in Lifschytz and Green 1979). This suggests that the *Bx* mutant phenotype is caused by overexpression of the normal gene product. We observe that sequences adjacent to the insertion sites of EP1306 and EP1394 are overexpressed in Bx^1 mutant wing discs (Fig. 2), suggesting that these EP elements direct expression of the *Bx* gene. To confirm that *Bx* mutants produce comparable effects to overexpression of EP1306, we examined Wg, *ap*, and *fng* expression in Bx^1 and Bx^2 mutant wing discs. *ap–lacZ* expression is normal, whereas *fng–lacZ* expression is reduced in dorsal cells of the wing pouch (Fig. 3A,B). Wg expression is reduced and irregular at the margin (Fig. 3C).

Genetic interactions between Bx, ap, *and* chip

Adult wings of homozygous Bx^1 flies show severe scalloping of the wing margin and transformation of dorsal to ventral fate in the alula at the posterior margin of the wing (Fig. 4A,B). The abnormalities in *Bx* wings resemble those produced by reducing *ap* function (Butterworth and King 1965; Wilson 1981). Consistent with the suggestion that *Bx* mutants reduce Ap function, we observed genetic interaction between *Bx* and *ap*, *fng*, and *chip* mutants. Flies heterozygous for Bx^1 and a wild-type copy of the gene show mild notching (Fig. 4C). The severity of this weak Bx^1 phenotype can be enhanced by simultaneously removing one copy of the *ap* gene (Fig. 4D), by removing one copy of *fng* (Fig. 4E), or by removing one copy of *chip* (Fig. 4F), a gene proposed to act as a cofactor for Ap (Morcillo et al. 1997). The strong Bx^1 phenotype can be completely suppressed by increasing the level of *ap* in dorsal cells (in flies of genotype Bx^1/Y; *ap–gal4*; *UAS–ap*; Fig. 4G). Increasing the level of *fng* expression using *ap–gal4*; *UAS–fng* also suppresses the wing margin defects but causes other defects in the internal organization of the wing (Fig. 4H). Taken together, these genetic interactions suggest that the defects caused by overexpressing *Bx* are due to reduced Ap activity.

Bx (dLMO) protein competes for formation of a complex between Ap and Chip

BLAST searches with the DNA sequence flanking the EP1306 insert showed that the EP element is located near the 5′ end of the *dLMO* gene (Fig. 2A). *dLMO* had

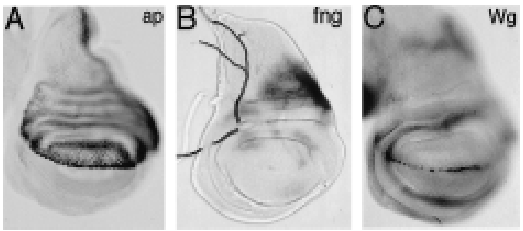

Figure 3. *Bx* interferes with Ap function. (*A*) *ap–lacZ* expression in a Bx^1 mutant wing disc. Ap protein expression is also normal in Bx^1 discs (not shown). (*B*) *fng–lacZ* expression in a Bx^1 mutant wing disc. (*C*) Wg protein expression in a Bx^2 mutant wing disc. Comparable effects on *Wg*, *ap*, and *fng* expression were observed in Bx^1 and Bx^2 mutant discs.

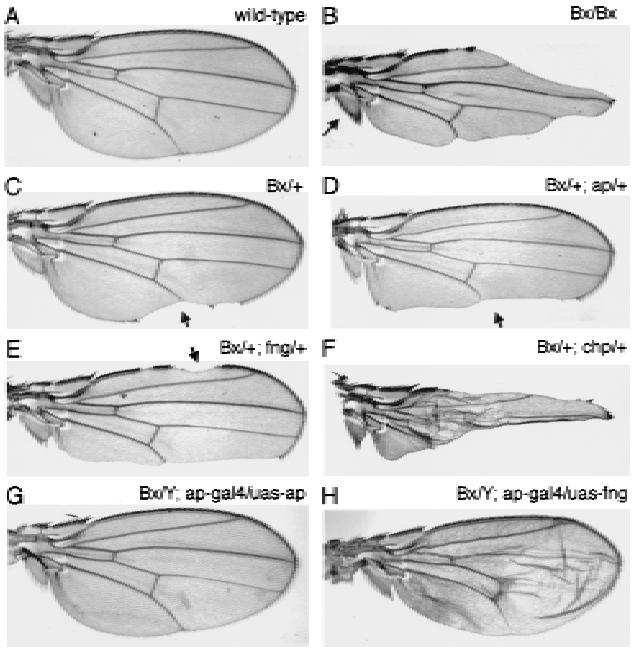

Figure 4. Genetic interactions between *Bx* and *ap*. (*A*) Cuticle preparation of a wild-type wing. (*B*) Bx^1 homozygous mutant wing. Detailed analysis of the Bx^1 phenotype suggests a close functional relationship to *ap*. There is extensive scalloping of the wing margin. In addition, bristles are found on both the dorsal and ventral surfaces of the alula (arrow, the defect in not visible at this magnification). In wild-type wings the alula has bristles only on the ventral surface. The alterations in Bx^1 suggest a partial transformation of dorsal structures toward ventral identity, suggesting that *ap* activity might be reduced. (*C*) Bx^1/+ heterozygous mutant wings typically produce small notches at the posterior margin (arrow). (*D*) Bx^1/+; ap^{UGO35}/+ mutant wing. ap^{UGO35} is a null allele (Cohen et al. 1992). Note that notching is more extensive than in the Bx^1/+ wing. ap^{UGO35} is completely recessive and does not cause a visible phenotype when heterozygous. (*E*) Bx^1/+; fng^{80}/+ mutant wing. Notching of the margin is more extensive than in the Bx^1/+ wing, and also occurs anteriorly. fng^{80} does not produce a phenotype when heterozygous (Irvine and Wieschaus 1994). (*F*) Bx^1/+; $chip^{e55}$/+ mutant wing. Removing one copy of *chip* strongly enhances the loss of wing tissue. $chip^{e55}$ does not produce a phenotype when heterozygous (Morcillo et al. 1997). (*G*) Bx^1/Y; *ap–gal4*; UAS–apterous wing. Both the scalloping and DV fate transformation in the alula are rescued. (*H*) Bx^1/Y; *ap–gal4*; *UAS–fng* wing. The margin scalloping is fully rescued, but DV fate transformation of the alula is not, suggesting that this represents a defect due to underexpression of another Ap target gene.

been cloned previously by homology to the human oncogene encoding the LIM domain protein LMO-2 (Zhu et al. 1995). The EP elements EP1394 and EP1306 are located 280 and 235 residues, respectively, from the 5′ end of exon 1a of *dLMO* and direct expression of *dLMO*. To integrate the genetic and molecular nomenclature we will refer to the protein encoded by the *Bx* locus as the dLMO protein.

LIM domains are thought to mediate protein interactions and are found in a variety of different types of proteins, often in combination with other recognized protein domains, as in the LIM–homeodomain (HD) proteins (for review, see Dawid et al. 1995). dLMO belongs to a class of LIM domain proteins that have two LIM domains and no other recognizable motifs (hence, the designation LMO, for LIM only). In view of the effects of dLMO on Ap function, we asked whether dLMO can interact with Ap and Chip proteins. *ap* encodes a LIM–HD protein (Cohen et al. 1992). *chip* encodes a member of the Ldb (LIM-domain binding) family of proteins (Morcillo et al. 1997). Ldb proteins have been shown to bind to the LIM domains of LIM–HD proteins like Ap (Agulnik et al. 1996; Morcillo et al. 1997). The *Xenopus* Ldb1 protein binds and activates the LIM–HD protein XLim1 in neuraxis induction (Agulnik et al. 1996). Likewise, CLIM1, another Ldb protein, binds and promotes transactivation by LIM3 (Bach et al. 1997). This is thought to occur by alleviating intramolecular repression, perhaps by preventing the endogenous LIM domains of LIM–HD proteins from interfering with homeodomain function (Sanchez-Garcia et al. 1993). LDB proteins also bind to the LIM domains of nuclear LMO proteins of the type encoded by *Bx* (Agulnik et al. 1996).

Genetic interactions between *chip* and *ap* suggest that as for Ldb1 and XLim1, Chip binding might activate Ap function (Morcillo et al. 1997). When overexpressed, *Bx* appears to interfere with Ap function without affecting either Chip or Ap protein expression (not shown). This raises the possibility that dLMO might interfere with binding between Ap and Chip. This was tested using a coimmunoprecipitation assay in which the binding between constant amounts of Chip and Ap proteins was challenged by increasing concentrations of Bx protein (Fig. 5). Chip protein can be immunoprecipitated with T7–epitope-tagged Ap protein and anti-T7 antibody, showing that Ap and Chip proteins bind in vitro (Fig. 5, sample 2, left). Binding between Chip and Ap was challenged by adding increasing amounts of in vitro-translated dLMO protein. The binding reactions (samples 1–6) were split in equal parts and immunoprecipitated with anti-T7 or with antibody to dLMO [Fig. 5, T7 immunoprecipitations (IPs) are on the left; dLMO IPs on the

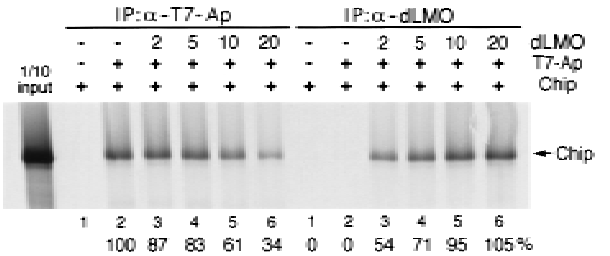

Figure 5. dLMO competes for binding between Ap and Chip. Coimmunoprecipitation of Chip by binding to Ap or to dLMO. Binding reactions (samples *1–6*) contained equal amounts of ^{35}S-labeled Chip protein synthesized in vitro. Samples *2–5* contain 5 µl of Ap lysate; samples *3–6* contain increasing amounts of Bx lysate (µl). After binding the samples were split into equal parts and immunoprecipitated with anti-T7 or anti-Bx. Quantitation of the relative amount of Chip precipitated is indicated below the lanes.

right]. We observed a dose-dependent decrease in the amount of Chip immunoprecipitating with Ap as the amount of dLMO protein was increased (Fig. 5, left, samples 3–6) and a corresponding increase in the amount of Chip immunoprecipitating with dLMO (Fig. 5, right, samples 3–6). These observations indicate that dLMO can bind to Chip in vitro and can compete for binding between Chip and Ap in a concentration-dependent manner. As a further test, the LIM domains of Ap were expressed as a GST fusion protein and tested for binding to full-length dLMO, Chip, and Ap proteins. Ap binds to itself and to Chip but not to dLMO in the GST–pull-down assay (data not shown). This suggests that dLMO interferes with formation of the active Ap–Chip complex by competing with Ap for binding to Chip.

Bx activity in normal wing development

Bx loss-of-function mutants have been described previously under the name *heldup* (*hdp*) because of the abnormal posture of the wings (Lifschytz and Green 1979). Unfortunately, the original *hdp* mutants are no longer available. To study the normal function of dLMO in wing development new mutants were generated by imprecise excision of the MS1096 P element. MS1096 is inserted in the second intron of the *Bx–dLMO* transcription unit (Fig. 2A) and produces a weak phenotype consisting of venation defects (Fig. 6A). New Bx^{hdp} mutants were recovered in P-element excision screens on the basis of their adult wing phenotypes. The wings of the new Bx^{hdp} mutants are reduced in size and show abnormalities in vein pattern (Fig. 6B). In addition the wing posture is abnormal, as described for the original *hdp* mutants (not shown).

hdp mutants behave as dominant suppressors of the *Bx* gain-of-function phenotype (*hdp-a*; Lifschytz and Green 1979). The MS1096 excision mutants completely suppress the *Bx* phenotype in heterozygous females (Fig. 6C), suggesting that they are loss-of-function mutants.

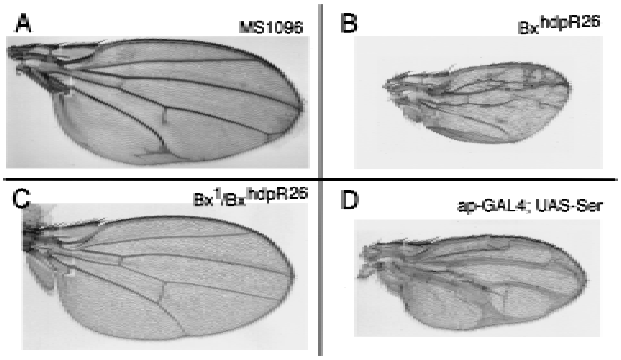

Figure 6. *Bx* loss-of-function mutant phenotypes. (*A*) Cuticle preparation of an MS1096 mutant wing. (*B*) Bx^{hdpR26} wing. *hdpR26* is a lack-of-function *Bx* allele generated by mobilization of the MS1096 P element. Note the small size of the wing and the abnormal thickening of the wing veins. (*C*) Bx^{1}/Bx^{hdpR26} wing. The Bx^{1} phenotype is completely suppressed (cf. Fig. 4C for $Bx^{1}/+$). (*D*) *ap–gal4*; *UAS–Ser* wing.

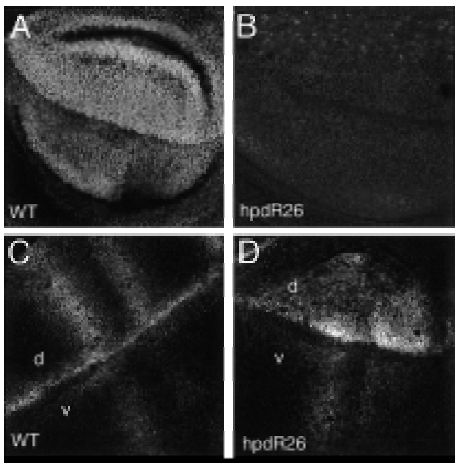

Figure 7. Reduced dLMO activity changes Ser expression. (*A*) dLMO protein expression in the wing pouch of a wild-type third-instar imaginal disc. Expression is higher in the dorsal compartment and near the AP boundary. dLMO protein is nuclear. Previous work using antibody to a vertebrate ortholog suggested that dLMO protein is cytoplasmic in the embryo (Zhu et al. 1995). We have not attempted to resolve this discrepancy. This image is at lower magnification than *B–D*. (*B*) Bx^{hdpR26} wing disc. dLMO protein is expressed at reduced levels compared to wild-type. $Bx^{hdpR590}$ produces a similar wing phenotype but shows less severely reduced dLMO expression. (*C*) Ser protein in a wild-type third-instar wing disc. At this stage Ser expression has resolved to stripes flanking to the DV boundary and along the presumptive wing veins. (d) Dorsal compartment; (v) ventral compartment. (*D*) Ser expression in a Bx^{hdpR26} loss-of-function mutant wing disc. Note the small size of the dorsal compartment and the relatively elevated level of Ser expression dorsally.

This was confirmed by examining dLMO protein, which is expressed at much reduced levels in wing discs of the excision mutant *hdpR26* (Fig. 7A,B). In wild-type discs dLMO protein is nuclear and is expressed at higher levels in the dorsal compartment of the mature third-instar disc than in the ventral compartment. This expression pattern mirrors that of the MS1096 GAL4 enhancer trap line (Capdevila and Guerrero 1994; data not shown). In early- to mid-third-instar discs both MS1096 and dLMO protein are restricted to the dorsal compartment. The observation that dLMO is initially expressed in dorsal cells and maintained at elevated levels in the dorsal compartment suggested that dLMO might be regulated by Ap. To test this possibility we forced ectopic Ap expression using *dpp–gal4* to direct UAS–Ap in a stripe of cells along the anterior–posterior (AP) boundary of the wing disc. dLMO is induced in Ap-expressing ventral cells to the same elevated level typical of cells in the dorsal compartment (Fig. 8). Thus, Ap induces expression of dLMO in dorsal cells. The transition from exclusively dorsal expression to dorsal and ventral expression suggests that dLMO expression is initiated by Ap but comes under an additional control mechanism as the disc matures.

To ask whether the wing abnormalities caused by reducing dLMO levels might be due to an effect on Ap activity, we examined the effects of the Bx^{hdp} excision

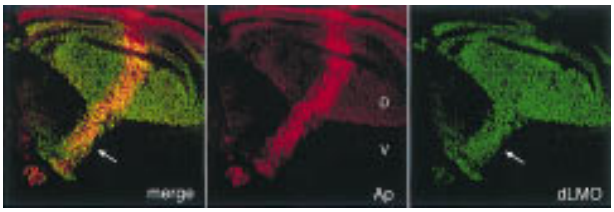

Figure 8. Ap induces dLMO expression. Wing disc expressing Ap in a stripe of cells along the AP boundary (genotype *dpp–gal4 + UAS–ap*). Ap protein is shown in red; dLMO protein in green. Ap directs dLMO expression at dorsal levels when misexpressed in ventral cells (arrow). Note that increased levels of Ap in the dorsal compartment do not obviously increase the level of dLMO protein in dorsal cells.

mutants on Ap target gene expression. In early third-instar *fng–lacZ* and Serrate (Ser) are expressed evenly throughout the dorsal compartment of the wing disc and are thought to be regulated by Ap (Irvine and Wieschaus 1994; Diaz-Benjumea and Cohen 1995). In Bx^{hdp} mutant discs, the size of the dorsal compartment is considerably reduced, consistent with the small wing phenotype (Fig. 7C,D). *fng–lacZ* expression is not affected in Bx^{hdp} discs (not shown). Ser expression is elevated in the dorsal compartment and does not resolve normally into stripes along the DV boundary and wing veins (Fig. 7C,D). Ser expression in the ventral compartment appears normal. The stripes of Ser expression along the DV boundary and wing veins are both dorsal and ventral (Thomas et al. 1991) and are under different regulation than the early dorsal-specific domain (de Celis and Bray 1997; Micchelli et al. 1997). The abnormal pattern of Ser in the dorsal compartment of the Bx^{hdp} may be due to superimposition of the early and late expression patterns. We suggest that this reflects a failure to down-regulate Ap activity as the disc matures.

To ask whether elevated Ser levels might contribute to the defects observed in Bx^{hdp} mutant wings, we overexpressed Ser in the dorsal compartment of an otherwise wild-type disc using *ap–gal4* to direct *UAS–Ser* expression (Fig. 6D). The resulting wings are small and show thickened veins but do not show the abnormalities in vein pattern observed in the Bx^{hdp} mutant wings. Overexpression of *fng* using *ap–gal4* in a wild-type background produces no phenotype (data not shown). These observations suggest that Ser overexpression contributes to the abnormalities observed in Bx^{hdp} mutant wings but that there are likely to be additional factors.

Thus, both gain-of-function and loss-of-function *Bx* mutant phenotypes can be attributed to abnormal regulation of Ap activity. We conclude that Ap induces dLMO expression in the wing disc and that dLMO then functions as part of a feedback system to regulate the level of Ap activity.

Discussion

Ap activity depends on the relative levels of Ap, dLMO, and Chip proteins

Analysis of the LIM–HD proteins has suggested that LIM domains may act as intramolecular negative regulatory domains that block activity of the homeodomain (Sanchez-Garcia et al. 1993; Agulnik et al. 1996). Deleting or mutating the LIM domains activates the homeodomain in LIM–HD proteins. Binding of the LDB protein Ldb1 activates Xlim1, apparently by binding to its LIM domains. This suggests that complex formation between LDB proteins and LIM–HD proteins is necessary to activate LIM–HD proteins (Agulnik et al. 1996). The finding that *chip*, a *Drosophila* relative of Ldb1, shows genetic interaction with *ap* and can bind to Ap protein in yeast (Morcillo et al. 1997) and in vitro (Fig. 5) suggests a similar functional relationship between these proteins in *Drosophila* wing development. Our finding that overexpression of dLMO can functionally inactivate Ap in vivo and that dLMO can interfere with the formation of a complex between Ap and Chip in vitro provides strong support for the proposal that Ap must bind Chip to be activated (represented schematically in Fig. 9).

Biochemical studies have shown that the Chip ortholog NLI (nuclear LIM interactor), binds LIM domains through a short domain in the carboxy-terminal portion of the protein (Jurata and Gill 1997). In addition NLI forms homodimers via an amino-terminal domain. Although dimerization of NLI is not required for LIM-domain binding, higher-order complexes can form, consistent with the possibility that the functional complex might be composed of two NLI/LIM–HD dimers. Whether the active Ap–Chip complex is a dimer or a tetramer, overexpression of dLMO would increase the proportion of Chip bound by dLMO and thereby reduce the pool of Chip protein available for binding to Ap. Conversely, mutants that reduce dLMO levels might allow formation of more than normal levels of functional Ap–Chip complex.

In this context it is striking that the *Bx* mutant phenotype can be completely suppressed in vivo by overex-

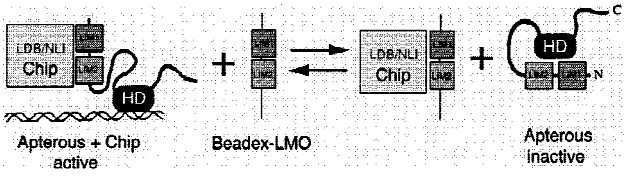

Figure 9. Model for Ap regulation by dLMO. Schematic depiction of complex formation between Chip, Ap, and dLMO proteins. Ap forms an inactive protein when the amino-terminal LIM domains are free. Following the scheme of Dawid et al. (1995), this is depicted as being due to intramolecular binding between LIM and homeodomain regions, but other possibilities are equally plausible. Chip can bind to the LIM domains of Ap or dLMO. Our data suggest that dLMO and Ap compete for binding to Chip and that varying the relative levels of Ap and dLMO will shift the equilibrium between active Ap–Chip complexes and inactive free Ap, by favoring formation of dLMO–Chip complexes. Although Chip is depicted as a monovalent protein here, its ortholog NLI dimerizes and can bind LIM domains as either a monomer or a dimer (Jurata and Gill 1997). This raises the possibility that higher-order complexes involving Ap, Chip, and dLMO might be of functional significance.

pressing Ap. Increasing the concentration of Ap presumably shifts the balance of competition for Chip toward formation of functional Chip–Ap complexes. Likewise, reducing the amount of either Ap or Chip in vivo would shift the balance toward formation of dLMO–Chip complexes and thus enhance the severity of the *Bx* mutant phenotype as shown in Figure 4. We note that the genetic interaction between *chip* and *Bx* is stronger than between *ap* and *Bx*, suggesting that the endogenous level of Chip may be more limiting than that of Ap.

Ap activity is modulated during wing development

Selector genes such as *ap* are often thought of as simple binary switches. Our results suggest that the situation is more complex and that Ap activity levels are modulated during wing development. Ap expression is restricted to dorsal cells. We have identified dLMO as a new target for regulation by Ap and have shown that dLMO functions to modulate Ap activity. These observations suggest that Ap, Chip, and dLMO are components of a regulatory feedback loop that controls Ap activity levels. We have presented evidence that the balance in the levels of these three proteins is important for determining the level of Ap activity in vivo. We note that this regulation occurs at the level of protein activity, not at the level of gene expression, and suggest that this may reflect a requirement to fine-tune activity levels regionally as the wing develops. Genetic analysis suggests that Chip may have additional functions (Morcillo et al. 1997). Given that dLMO is expressed in regions where Ap is not known to function, it is likely that dLMO may have other functions as well. Chip and dLMO may regulate the activity of LIM–HD proteins in other developmental contexts and in postembryonic homeostatic processes.

A possible molecular mechanism for the oncogenic activity of vertebrate LMO *genes*

Gain-of-function mutations that cause misexpression of vertebrate LMO proteins have been implicated in cancers of the lymphoid system. Genes encoding the LMO1 and LMO2 proteins were identified by chromosomal translocations associated with leukemia (McGuire et al. 1989; Boehm et al. 1991). LMO1 and LMO2 have been shown to induce leukemia and lymphoma when misexpressed in T cells in transgenic mice (Fisch et al. 1992; McGuire et al. 1992). We have shown here that overexpression of the *Drosophila* LMO protein causes a dominant interfering activity that reduces activity of the LIM–HD protein Ap. We present evidence that this occurs by competitive inhibition of formation of a functional complex between the LDB protein, Chip, and Ap. We suggest that the molecular mechanism by which LMO proteins cause lymphoid cancers might be similar. It is possible that an as yet unidentified LIM–HD protein is required for proper differentiation or maintenance of the differentiated state in T cells and that loss of its function through overexpression of an LMO protein can lead to a failure in control of cell proliferation.

Materials and methods

Fly strains

The EP collection consists of 2300 independent P-element insertions available through the Berkeley Drosophila Genome Project (Rørth et al. 1998). *ap–lacZ* is described in Cohen et al. (1992); *fng–lacZ* is described in Irvine and Wieschaus (1994).

Molecular analysis of EP1306, EP1394, and MS1096

DNAs flanking EP1306, EP1394, and MS1096 were cloned by plasmid rescue. Sequence analysis showed that EP1306 and EP1394 are located 235 and 280 bp upstream from exon 1 of the *dLMO* gene (Zhu et al. 1995) and that MS1096 is located in intron 2 as indicated in Figure 2. cDNA clones were isolated from an imaginal disc cDNA library using the flanking DNA as probe and used to make antisense RNA probes for in situ hybridization to imaginal discs. Sequence analysis of our *dLMO* cDNA clone and of the genomic flank (accession no.) indicated that the dLMO open reading frame (ORF) differs in the amino-terminal region from that published for *dLMO*, due to a frameshift in the published sequence of the *dLMO* cDNA (x83012; Zhu et al. 1995).

To produce UAS–Bx, three copies of the flu epitope tag were introduced at the amino terminus of the Bx protein by PCR (primers: CATATGTATCCCTATGACGTCCCCGATTATGCCTACCCTTACGATGTACCTGACTACGCGTATCCGTACGACGTTCCGGACTATGCTATGATGACTATGGAC and atggaattcCTCCTCCACCGCCGCCCATTCCTA). The PCR product was cloned into pCRScript (Stratagene) and recloned into pUAST (Brand and Perrimon 1993). *UAS–ap* was prepared by cloning a full-length *ap* cDNA (B8; Cohen et al. 1992) into pUAST.

Bx loss-of-function mutants

MS1096 is inserted in the *dLMO* gene in intron 2. The MS1096 wing venation phenotype can be reverted to wild-type excision of the P element indicating that MS1096 is an insertional mutant (not shown). New *Bx* loss-of-function mutants were generated by imprecise excision of the MS1096 P element. Sixty-two independent excision alleles were recovered. All produce similar phenotypes, though with different severity. Excision mutants derived by mobilization of EP1394 were recovered and produce comparable phenotypes. Complementation mapping of three alleles placed the mutations in the smallest genetic interval known to contain *Bx*—on the basis of failure to complement $Df(1)fu^{E5}$ and on complementation of $Df(1)fu^{B10}$ and $Df(1)os^{UE19}$ (Eberl et al 1992). The excision alleles also fail to complement $maggot^{3E}$, suggesting the *maggot* alleles may be lethal mutations of *Bx*. We refer to these alleles collectively as $Bx^{hdpR\#}$ to indicate that they have the properties previously described for revertants of *Bx*, known as *hdp-a* alleles.

Antibodies

Mouse anti-dLMO: The dLMO ORF was amplified by PCR and cloned into pGEX 2TK (Pharmacia, primers cgcggattcATGATGACTATGGAC and atggaattcCTCCTCCACCGCCGCCCATTCCTA). Fusion protein was purified on glutathione–agarose and used to immunize BALB/C mice. Polyclonal serum was used for histochemistry and IP. Control experiments with pre-

immune serum showed no nuclear staining or IP of dLMO (not shown). Mouse anti-Ser was from Thomas et al. (1991), mouse anti-Wg was from Brook and Cohen (1996), and rabbit anti-β-galactosidase was from Cappell; guinea pig anti-Ap was provided by Juan Botas (Baylor College of Medicine, Houston, TX).

IP

The Ap ORF was cloned into pET23A to introduce a T7 epitope tag at the amino terminus. The Bx ORF was in pSK isolated from a λ–Zap cDNA library. The Chip ORF was excised from a yeast expression plasmid provided by Patrick Morcillo (Morcillo et al. 1997) and recloned into pKS. Proteins were produced using a coupled transcription–translation kit (Promega). For IP Chip was labeled with [^{35}S]methionine, and T7–Ap and dLMO were not labeled. Five microliters of ^{35}S-labeled Chip lysate was incubated with 0 or 5 µl of Ap lysate and with 0–20 µl of dLMO lysate; the final volume of the binding reactions was adjusted to 30 µl with unprogrammed lysate. Binding reactions were assembled on ice and allowed to incubate at room temperature for 30 min. The reactions were diluted to 200 µl with 50 mM HEPES (pH 7.5), 150 mM NaCl, 2 mM $MgCl_2$, 10% glycerol; 1% NP-40 with 1 mM PMSF, and 1 µg/ml each of aprotinin, pepstatin, and leupeptin (IP buffer), centrifuged for 5 min at 4°C to pellet insoluble material. Equal portions were incubated with 2 µg of mouse monoclonal antibody to the T7 epitope tag (Novagen) or with 2 µl of dLMO antiserum for 90 min on ice. Sixty microliters of a 1:1 suspension of protein A–agarose beads in IP buffer was added, and samples were incubated with gentle rocking at 4°C for 30 min, washed three times with IP buffer, and analyzed on a 10% SDS–polyacrylamide gel.

GST pull-downs

The LIM domains of Ap were amplified by PCR and cloned into pGEX2T (primers: atagaattcGACGACTGCTCCGGC; taactcgagACTGGATGAGGCGGTATC, lowercase letters indicate sequences added for cloning using *Eco*RI and *Xho*I). GST and GST–AP–LIM proteins were expressed in bacteria and purified on glutathione–agarose beads. The yield of GST–AP–LIM was very low, compared to GST. Fifty microliters of a 1:1 suspension of GST or GST–AP–LIM beads in IP buffer was mixed with 4 µl of [^{35}S]methionine-labeled in vitro-translated protein in 200 µl of IP buffer, incubated for 1 hr at 4°C, washed three times in IP buffer, and analyzed on a 10% SDS–polyacrylamide gel.

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