Drosophila DLMO is a positive regulator of transcription during thoracic bristle development

Shamir Zenvirt, Yael Nevo-Caspi, Sigal Rencus-Lazar and Daniel Segal

Department of Molecular Microbiology and Biotechnology, Tel Aviv University,
Tel Aviv 69978, Israel.
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Corresponding author:

Daniel Segal

Department of Molecular Microbiology and Biotechnology

Tel Aviv University

Tel Aviv 69978

Israel

Phone: 972-3-6409835

Fax: 972-3-6409407

e-mail: dsegal@post.tau.ac.il
ABSTRACT

The Drosophila LIM-Only (LMO) protein DLMO functions as a negative regulator of transcription during development of the fly wing. Here we report a novel role of DLMO as a positive regulator of transcription during the development of thoracic sensory bristles. We isolated new dlmo mutants, which lack some thoracic dorso-central (DC) bristles. This phenotype is typical of malfunction of a thoracic multiprotein transcription complex, composed of CHIP, PANNIER (PNR), ACHAETE (AC) and DAUGHTERLESS (DA). Genetic interactions reveal that dlmo synergizes with pnr and ac to promote the development of thoracic DC bristles. Moreover, loss of function of dlmo reduces the expression of a reporter target gene of this complex in-vivo. Using the GAL4-UAS system we also show that dlmo is spatially expressed where this complex is known to be active. GST-pulldown assays showed that DLMO can physically bind CHIP and PNR through either of the two LIM domains of DLMO, suggesting that DLMO might function as part of this transcription complex in vivo. We propose that DLMO exerts its positive effect on DC bristle development by serving as a bridging molecule between components of the thoracic transcription complex.
INTRODUCTION

The LIM-only (LMO) family of proteins consists of evolutionarily conserved nuclear proteins comprising two tandem LIM domains, which mediate protein-protein interactions. Each LIM domain comprises a cysteine-rich motif akin to double zinc fingers. The LMO proteins contain no other recognizable motifs, whereas various other LIM-containing proteins harbor additional known domains. Most notable among these are the DNA-binding homeodomain (HD) of LIM-HD transcription-activating proteins and protein-binding modules such as SH3, LD and PZD domains (reviewed in Dawid et al. 1998; Bach 2000; Hunter and Rhodes 2005; Zheng and Zhao 2007).

Vertebrates have four LMO proteins (LMO1-4), which have important roles in key developmental processes, including hematopoiesis, neurogenesis and brain development. For example, LMO2 is necessary for normal development of erythroid cells and LMO2-deficient mice lack the yolk sac and die in the embryonic stage (Warren et al. 1994; Yamada et al. 1998; Hansson et al. 2007). Null mutations of LMO4 lead to perinatal lethality due to a severe neural tube defect (Tse et al. 2004; Hahm et al. 2004; Lee et al. 2005). In contrast to LMO2 and LMO4, null mutation of LMO1 or LMO3 alone results in no discernible phenotype (Tse et al. 2004), suggesting the roles of the latter two may be redundant. Indeed, double mutants of both LMO1 and LMO3 die within 24 hours after birth (Tse et al. 2004).

Misexpression of LMO genes contributes to tumorigenesis. For example, misexpression of LMO1 or LMO2, either as a result of chromosomal translocations in humans or by overexpression in transgenic mice, results in T-cell acute lymphoblastic leukemia (Rabbitts 1998; Fisch et al. 1992; McGuire et al. 1992; Neale et al. 1997). Overexpression of LMO3 contributes to the formation of neuroblastoma
(Aoyama et al. 2005), and LMO4 has been implicated in breast cancer (Visvader et al. 2001; Sum et al. 2005).

In both normal cells and cancer cells the vertebrate LMO proteins were found in multi-protein complexes with several transcription regulatory proteins. Thus, LMO2 was found in complexes that include the LIM-domain binding protein Ldb1, as well as transcription-regulating factors of the GATA-type and helix-loop-helix (HLH) DNA-binding proteins (Osada et al. 1995; Wadman et al. 1997; Rabbitts 1998; Grutz et al. 1998; Lécuyer et al. 2002). LMO3 has been demonstrated to interact with the neuronal specific bHLH transcription factor NHLH2 (Aoyama et al. 2005), and LMO4 was shown to participate in a complex comprising BRCA1 and CtIP (Sum et al. 2002). However, the functions of the LMO-comprising complexes, both in normal development and in the formation of cancer, are not fully understood.

The Drosophila LIM-only protein DLMO is the only member of the LMO family of proteins found in Drosophila (Zhu et al. 1995; Shoresh et al. 1998), thus facilitating analysis of the roles of LMO proteins without the confounding effects of redundancy observed in mammals. The association of LMO2 with complexes containing GATA and bHLH proteins in addition to Ldb1 has been suggested to be conserved in Drosophila as well since DLMO was found to interact with the Drosophila GATA factor PANNIER (PNR) in COS cells (Ramain et al. 2000). Yet, the best-characterized function of DLMO is as a negative regulator of a non GATA- non bHLH-containing transcription complex transcription complex that controls the specification of the wing pouch within the wing imaginal disc (Diaz-Benjumea and Cohen 1995; Kim et al. 1995; Shoresh et al. 1998; Milan et al. 1998; Milan and Cohen 2000; Béjarano et al. 2008). Central to this complex is a multimer of the LIM-domain binding protein CHIP, the ortholog of the mammalian Ldb1, with each

The negative regulatory role of DLMO in the developing wing blade is evident in the phenotypes of \textit{dlmo} mutant flies, which fall into two classes: (i) Partial loss of function mutants, termed \textit{heldup} (\textit{dlmo}^{hdp}), which express reduced levels of the DLMO protein. These mutants are associated with recessive erect-wing phenotype and impaired wing morphology, caused by abnormally elevated activity of the CHIP-AP-SSDP transcription complex in the wing pouch (\textit{SHORESH et al.} 1998; \textit{MILAN} and \textit{COHEN} 1999). (ii) Over-expression mutants termed \textit{Beadex} (\textit{dlmo}^{Bx}), which are associated with a dominant phenotype of scalloped wing margins, due to abnormally reduced activity of the CHIP-AP-SSDP complex (\textit{SHORESH et al.} 1998; \textit{MILAN} and \textit{COHEN} 2000). The over-expression of DLMO in \textit{dlmo}^{Bx} mutants was shown to result from over-stabilization of the \textit{dlmo} transcript, caused by disruption of sequences in its 3’UTR, which normally confer mRNA instability (\textit{SHORESH et al.} 1998).

While the best characterized function of DLMO has been as a negative regulator of transcription in the wing, it probably plays additional roles, since expression of \textit{dlmo} is not restricted to the developing wing. It is expressed from early embryogenesis through the adult stage in various tissues including the embryonic posterior midgut,
brain and central nervous system as well as the larval leg, eye and wing imaginal discs (ZHU et al. 1995; SHORESH et al. 1998; ZENG et al. 1998). The present work therefore aimed at identifying new roles of DLMO. Our genetic and molecular analyses suggest that DLMO plays a novel role as a positive transcriptional regulator of development of bristles on the *Drosophila* thorax.
MATERIALS AND METHODS

Fly strains and maintenance:

Flies were grown on a standard medium containing cornmeal, yeast, molasses and propionic acid at 25°C. Flies harboring the mutations \(dlmo^{R590}\) (\(dlmo^{hpR590}\)), \(pnr^{MD237}\) (\(pnr\)-Gal4), \(ac^{I}\) and \(P[lac\ ry^{+}]Dc-ac^{I}\) transgenes were kindly provided by P. Heitzler. For analysis of bristle phenotype, the *forked* marker was crossed off the original \(dlmo^{R590}\) strain. The \(pnr^{VX6}\) mutants and flies harboring the *UAS-pnr* transgene were kindly provided by P. Simpson. Flies harboring the *UAS-Chip* transgene were kindly provided by D. van-Meyel. All other fly stocks were obtained from the *Drosophila* Stock Center in Bloomington. Canton S (C-S) flies were used as wild type.

Mobilization of P elements:

The *EY01065* line contains the P-element \(P\{EPgy2\}\), located 713 bp downstream of *dlmo*. The *MS1096* line contains the P-element \(P\{GawB\}\), located in the 30 kb-long second intron of *dlmo*. Females of the genotypes \(yw P\{w^{+}mC\ y^{+}=EPgy2\}EY01065\) and \(w P\{w^{+}mW.hs=GayB\}MS1096\) were crossed to males harboring the transposase-encoding gene (*P\{Delta2-3\}99B*). Resulting dysgenic males were crossed to \(C(1)DX y f /Dp(1;Y)W39\ y^{+}\) females. Individual male progeny carrying the Y chromosome that harbors the \(Dp(1;Y)W39\ y^{+}\) duplication (which includes a normal copy of *dlmo*) and displaying a white-eyed phenotype (indicating an excision of the P-element), were crossed to \(Df(1)N19 /FM7i\ y B P\{Act-GFP\}\) females. At this stage, *dlmo*\(^{Bx}\)-like scalloped wings could be observed in some of these white-eyed males. We looked for individual crosses in which non-\(Bar\) female offspring had aberrant phenotypes (e.g. deformed wing or thorax, or lethality) and used their *Bar*-displaying female siblings for the generation of balanced lines.
**Phenotypic analysis:**

To identify morphological abnormalities in the mutant flies, 30-100 flies of each genotype were examined under a stereo microscope. To examine genetic interactions between *dlmo* and any other candidate gene, flies carrying a mutation in the candidate gene were crossed to different *dlmo*<sup>hdp</sup> mutants. More than 200 relevant offspring (30-130 flies of each genotype) were examined under a stereo microscope. Interactions between the candidate mutations and *dlmo* gain-of-function (*dlmo*B<sup>x</sup> alleles) were examined in a similar way. Controls consisted of siblings of the relevant progeny, as well as wild-type (C-S) flies.

To examine lethality rates and identify the stage of lethality, eggs from mutant strains balanced over *FM7i y B P{Act-GFP}* were collected. Thirty-six hours later non-GFP-expressing first instar larvae were collected using a fluorescent stereo-microscope and placed in an egg laying plate (3% Bacto-agar, 2% sugar, 1.5 gr/l methylparaben) supplemented with live yeast paste. The plates were examined daily for dead larvae and fresh live yeast paste was added until all animals eclosed or died. Wild type larvae were used as a control.

**Histochemical staining of imaginal discs:**

Wing imaginal discs from late third instar larvae were dissected and fixed in 0.75% glutaraldehyde for 15 min, washed with PBS containing 0.3% Triton X-100, and then stained for 30 min at 37° with 0.2% X-Gal in 10 mM sodium phosphate buffer (pH 7.2); 150 mM NaCl; 1 mM MgCl<sub>2</sub>; 3.1 mM K<sub>d</sub>[Fe<sup>3+</sup>(CM)<sub>6</sub>]; 3.1 mM K<sub>3</sub>[Fe<sup>2+</sup>(CM)<sub>6</sub>]; 0.3% Triton X-100. The reaction was stopped by rinsing with PBS. Discs from all
genotypes were prepared and stained simultaneously in a 96-well plate. The experiment was performed in three biological repeats all leading to similar results.

**DNA cloning and plasmid construction:**

The coding regions of *Chip, pnr* and *dlmo*, as well as fragments of *dlmo*, as described in Table 1, were PCR amplified from cDNA templates and cloned into the pGEM-T Easy vector (Promega). Next they were sub-cloned as *Eco*RI-*Xho*I fragments into pET-17b (Novagen) and into pZEX (a gift from Z. Paroush). The pZEX vector is a derivative of pGEX-2T (Amersham Pharmacia Biotech) whose multi-cloning site was modified to contain an *Eco*RI site in-frame with the Glutathione-S-transferase (GST) coding sequence, followed by a *Xho*I site. All resulting constructs were verified by sequencing.

**Glutathione-S-transferase (GST)-pulldown assays:**

GST-pulldown assays were essentially performed according to TORIGOI *et al.* (2000) with modifications. *Escherichia coli* BL21 strains, each carrying one of the pZEX constructs, as well as the thioredoxin expressing vector pT-Trx (*YASUKAWA et al.* 1995), which increases solubility of foreign proteins, were grown to late log phase. IPTG (1 mM) was then added. After incubation for 2 hr at 30 °C bacteria were spun-down, frozen and thawed and then sonicated in PBS supplemented with 1 mM EDTA, 1 mM EGTA and 1 mM PMSF. The resulting extract was incubated in 1% Triton X-100 for 30 min at 4° and spun-down. The supernatant was incubated with Glutathione-agarose beads (Sigma) for 20 min at room temperature and washed with PBS.
Radioactively labeled $[^{35}\text{S}]$ proteins were generated using the pET-17b constructs in the TnT T7 Quick Coupled Transcription/Translation System (Promega).

Physical interactions between proteins were examined by incubating equal amounts of each GST-fusion protein bound to beads with 1 µl of radio-labeled $[^{35}\text{S}]$ protein for two hours at room temperature in pulldown buffer (PBS supplemented with 5% glycerol, 1 mM EDTA, 0.1% NP40 (Igepal), 0.2 M NaCl, and 1 mM DTT), followed by three repeated washes with pulldown buffer. The bound proteins were then eluted by boiling in SDS-PAGE sample buffer, run on SDS–PAGE gel and quantified using a phosphorimager. As a negative control, physical interactions between the radio-labeled proteins and GST alone were examined.
RESULTS

Generation of new *dlmo* mutants:

In order to identify new roles of DLMO we have generated new *dlmo* mutants by mobilization of either one of the following marked P-elements: *P{EPgy2}EY01065*, which is located 0.7 kb downstream of *dlmo* (Figure 1A) and causes no visible phenotype either in hemizygous males or in homozygous females; and *P{GawB}MS1096*, which is located 0.2 kb downstream to the second exon of *dlmo* (Figure 1D) and causes very subtle alterations in some of the wing veins in hemizygous males and in homozygous females (Milan et al. 1998).

Imprecise excision of *P{EPgy2}EY01065* produced two mutants displaying recessive erect wings (*dlmohdp*-like) and 22 mutants with dominantly scalloped wings (*dlmoBx*-like, Figure 2B). Sequence analysis of the two new *dlmohdp*-like mutants (*dlmohdp48-1* and *dlmohdp185-1*) indicated that both have a deletion that removes exon #5 of *dlmo*, which contains the 3’ UTR region as well as some coding sequences, and extends into the intron upstream to this exon (Figure 1B). These mutants therefore lack the sequences encoding the C’ terminus of DLMO, including the second of the two zinc fingers comprising its second LIM domain. Analysis of five of the new dominant wing scalloping (*dlmoBx*-like) mutants revealed that they have deletions spanning 0.1-0.4 kb of the 3’ UTR of *dlmo* but not extending into its coding region (Figure 1C). This is consistent with previous reports showing that the gain of function of *dlmoBx* mutants is due to disruption of negative regulatory sequences located in the 3’UTR of *dlmo* (Shoresh et al. 1998).

Imprecise excision of *P{GawB}MS1096* yielded 14 mutants displaying recessive erect wing phenotype (*dlmohdp*-like). Sequence analysis revealed that all of them carry deletions that span part of the P element, but retain other parts of the P insert in its
original location. In two of these mutants ($dlmo^{hdp58-1}$ and $dlmo^{hdp67-2}$) the deletion extends into the second exon of $dlmo$, which contains part of the 5’ UTR region of the gene (Figure 1E).

In addition to these new mutants, we examined the $dlmo^{hdpR590}$ mutant which was previously generated by mobilization of the same $P\{GawB\}MS1096$ transposon and was shown to express less DLMO protein than the wild-type (MILAN and COHEN 1999). Our sequence analysis revealed that this mutant has a deletion internal to the P element (Figure 1F). Yet, unlike the original $P\{GawB\}MS1096$ strain, $dlmo^{hdpR590}$ displays an erect wing phenotype (MILAN and COHEN 1999). Our mutagenesis using $P\{GawB\}MS1096$ also yielded several $dlmo^{hdp}$-like mutants with deletions internal to the P element (not shown).

**Phenotype of new $dlmo$ mutants:**

The following novel $dlmo^{hdp}$ mutants were analyzed: $dlmo^{hdp48-1}$ and $dlmo^{hdp185-1}$, which carry deletions spanning parts of the $dlmo$ coding sequences (Figure 1B), as well as $dlmo^{hdp58-1}$ and $dlmo^{hdp67-2}$, which carry deletions spanning part of the 5’ UTR of $dlmo$ (Figure 1E). Flies homozygous or hemizygous for any one of these four mutations display held-up wings with occasional defects in the cross-veins (Figure 2A), resembling the phenotype of the previously described loss-of-function $dlmo^{hdp}$ alleles (SHORESH et al. 1998; MILAN and COHEN 1999). All of the four mutants complement neither each other nor the previously recovered allele $dlmo^{hdpR590}$ (MILAN and COHEN 1999), suggesting that they are also loss of function alleles of $dlmo$. In addition, as expected of loss-of-function alleles of $dlmo$, all four new $dlmo$ alleles partially suppress, in a hetero-allelic state, the dominant wing scalloping of the previously described gain-of-function $dlmo^{Bx2}$ mutant (SHORESH et al. 1998), as well
as of the new \textit{dlmo}^{Bxs-1} mutant (Figure 2D), as does a chromosomal deficiency (\textit{Df(1)N19}) that removes the \textit{dlmo} region (Figure 2E).

While the erect wing phenotype is common to all four new \textit{dlmo}^{hdp} mutants, they differ in wing size. Both mutants carrying a deletion that spans part of the coding region of \textit{dlmo} (\textit{dlmo}^{hdp48-1} and \textit{dlmo}^{hdp185-1}) have normal sized wings, whereas those bearing a deletion that removes part of the 5' UTR of \textit{dlmo} (\textit{dlmo}^{hdp58-1} and \textit{dlmo}^{hdp67-}) have smaller wings than the wild type (Figure 2A). This latter recessive phenotype is also common to the \textit{dlmo}^{hdpR590} mutant (MILAN and COHEN 1999).

In addition to the wing defect, all four new \textit{dlmo}^{hdp} mutants display a novel recessive phenotype: they lack one or two bristles from the anterior pair of the four thoracic dorso-central (DC) bristles (Figure 3B). The penetrance of this bristle defect is 10\%-20\% in mutant \textit{hdp} males (Figure 4B). Similar values were observed for \textit{hdp} homozygous females and higher values (>85\%) were found in hemizygous females carrying any one of these four \textit{dlmo}^{hdp} alleles over a chromosomal deficiency that removes the \textit{dlmo} region (i.e. \textit{dlmo}^{hdp}/\textit{Df(1)N19}; N=117). This indicates that the bristle defect is indeed attributed to the lesions in \textit{dlmo} and suggests that this gene is involved in regulation of the development of the DC bristles. This bristle phenotype is also common to the \textit{dlmo}^{hdpR590} mutant (data not shown).

Two additional abnormalities were observed in all \textit{dlmo}^{hdp} mutants examined: (i) recessive semi-lethality, evident when heterozygous \textit{dlmo}^{hdp}/\textit{FM7} females carrying any of these four alleles were crossed with \textit{FM7}/\textit{Y} males. The proportion of F1 adult hemizygous \textit{dlmo}^{hdp}/\textit{Y} males, expected to be the same as that of the F1 heterozygous \textit{dlmo}^{hdp}/\textit{FM7} female siblings, was in fact less than 25\% of these siblings. The \textit{dlmo}^{hdp}/\textit{Y} hemizygous males died at various larval stages, but displayed no overt
abnormalities. (ii) Defective adult locomotion, evident as difficulties in standing and walking.

In contrast, no apparent abnormalities, other than wing scalloping (Figure 2B), were observed in the new $dlmo^{B_{x}}$ mutants, or in the preexisting mutant $dlmo^{B_{x}2}$.

**Genetic interactions between $dlmo$ and genes encoding components of the CPAD transcription complex:**

As mentioned above, $dlmo^{hdp}$ mutants display DC bristle defects. Like other sensory organs present on the fly's epidermis, each of the four thoracic DC bristles develops from precursor sensory mother cells (SMCs). The SMCs are specified during the third larval instar and early pupal stages from restricted groups of cells, the proneural clusters, which are located at a specific position of the imaginal discs (Modolell 1997). Specification of SMCs that give rise to the DC bristles depends on the expression of the *achaete* (*ac*) gene in the corresponding proneural cluster cells.

Expression of *ac* in the presumptive thorax is regulated by a transcription complex composed of CHIP, the GATA factor PANNIER (PNR), and the bHLH proteins ACHAETE (AC) and DAUGHTERLESS (DA), hereafter termed CPAD complex, which binds GATA sequences in the dorso-central (DC) enhancer of the *ac* gene on one hand, and an E-box motif in its promoter on the other hand (Raman et al. 2000). Indeed, malfunction of the CPAD complex in loss-of-function mutants of its components (e.g. *Chip*, *pnr*, or *ac*) is accompanied by loss of some DC bristles as in the $dlmo^{hdp}$ mutants (Raman et al. 2000). This raises the possibility that DLMO affects the function of the CPAD complex.

If DLMO affects the function of the CPAD transcription complex, $dlmo$ mutants would be expected to genetically interact with mutants in components of this
complex. We therefore looked for genetic interactions between *dlmo* and *pnr, ac* or *Chip*. Two classes of *dlmo* mutants were used for these crosses: The loss-of-function mutants *dlmo*<sup>hdp58-1</sup>, *dlmo*<sup>hdp185-1</sup> and *dlmo*<sup>hdpR590</sup> (Figures 1B, E and F) and the overexpression mutants *dlmo*<sup>Bx2</sup> (a mild hypermorph) and *dlmo*<sup>Bx1002-2</sup> (a severe allele) (Figures 1C and 2B).

**dlmo and pannier (pnr):** The *dlmo* mutants were combined with either the null allele *pnr*<sup>VX6</sup> (RAMAIN et al. 1993) or the partial loss-of-function allele *pnr*<sup>MD237</sup> (HEITZLER et al. 1996). Both *pnr* mutants are homozygous lethal, and less than 1% of the heterozygous flies lack one of the anterior thoracic DC bristles (Figure 4C). Combining any one of the *dlmo* loss-of-function mutants with either *pnr* allele (i.e. *dlmo*<sup>hdp/Y; pnr*/+*) resulted in lack of the anterior pair of the DC bristles (Figure 4D) in >90% of the flies. This high rate of loss of DC bristles, compared to the low rate among either parental mutant (*dlmo*<sup>hdp/Y</sup> or *pnr*/+; 10% and 1%, respectively), indicates synergism between the functions of *dlmo* and *pnr* in the thorax. Over-expression alleles of *dlmo*, i.e. *dlmo*<sup>Bx</sup>, are expected to interact with *pnr* mutant alleles in an opposite manner, namely decrease the frequency of flies lacking DC bristles. Indeed, we found that all males which are hemizygous for either one of the *dlmo*<sup>Bx</sup> over-expression alleles, and are heterozygous for either *pnr* loss of function allele (*dlmo*<sup>Bx/Y; pnr*/+*), have a normal number of thoracic DC bristles (N=399).

These genetic interactions indicate that *dlmo* is required for the development of the thoracic DC bristles, and suggest that DLMO, together with PNR, positively influences the function of the CPAD complex.

**dlmo and achaete (ac):** To examine the genetic interaction between *ac* and *dlmo*, the *ac<sup>c</sup>* allele was used. *ac<sup>c</sup>* is a recessive hypomorph caused by a deletion located upstream to the *ac* gene on the X chromosome (CAMPUZANO et al. 1985). Flies
homozygous or hemizygous for ac\textsuperscript{l} lack all four DC bristles (Figure 3C) while ac\textsuperscript{l}/+ heterozygotes display a wild type thorax with a normal set of the DC bristles (Figure 4F and CULI et al. 2001). Females carrying any one of the loss-of-function \textit{dlmo}\textsuperscript{hdp} alleles as well as ac\textsuperscript{l} (i.e. + \textit{dlmo}\textsuperscript{hdp}/ac\textsuperscript{l} +) lack 1-3 of the DC bristles (Figure 4G), while females heterozygous for any one of the \textit{dlmo}\textsuperscript{hdp} mutations alone (\textit{dlmo}\textsuperscript{hdp}/+; Figure 4E) and females heterozygous for the ac\textsuperscript{l} allele alone (ac\textsuperscript{l}/+; Figure 4F) display a wild type number of DC bristles. Thus, normal \textit{dlmo} activity appears to be required, along with normal ac activity, for the proper function of the CPAD complex.

Given the synergism of loss-of-function mutations in \textit{dlmo} with ac\textsuperscript{l}, one would predict that overexpression of \textit{dlmo}, as in \textit{dlmo}\textsuperscript{Bx} mutants, would act oppositely to suppress the loss of all of the DC bristles which characterizes ac\textsuperscript{l}. Indeed, we found that flies which are homozygous or hemizygous for both ac\textsuperscript{l} and either of the \textit{dlmo}\textsuperscript{Bx} mutations (ac\textsuperscript{l} \textit{dlmo}\textsuperscript{Bx}/ac\textsuperscript{l} \textit{dlmo}\textsuperscript{Bx} and ac\textsuperscript{l} \textit{dlmo}\textsuperscript{Bx} /Y, respectively) have a normal pair of posterior DC bristles and lack only the anterior pair (Figure 4J; compare to the single mutants in Figure 4H, I). Thus, excess DLMO can partially restore the ac\textsuperscript{l}-associated malfunction of the CPAD complex.

Taken together these results support the notion that normal function of \textit{dlmo} is required for proper activity of the CPAD transcription complex.

\textit{dlmo} and \textit{Chip}: Finally, we looked for genetic interaction between \textit{dlmo} and \textit{Chip}.

Two \textit{Chip} mutants were used: 1) The null allele \textit{Chip}\textsuperscript{E5.5} (MORCILLO et al. 1997), and 2) \textit{Chip}\textsuperscript{Enc} (\textit{Chip}\textsuperscript{E}) in which a point mutation causes a single amino acid substitution in the LIM-interacting domain (LID) of CHIP. This mutation decreases the ability of CHIP to bind AC, without affecting its binding to AP and DLMO. The activity of the thoracic CPAD transcription complex in \textit{Chip}\textsuperscript{E} flies is therefore reduced (RAMAIN et al. 2000). The null allele \textit{Chip}\textsuperscript{E5.5} is homozygous lethal, and the heterozygotes have a
wild-type thoracic phenotype. When combined with either one of the $dlmo^{hdp}$ mutants or with the $dlmo^{Bx}$ mutants, the number of thoracic DC bristles remained as in the wild type. Heterozygous $Chip^{E}$/+ flies lack the anterior DC bristles (RAMAIN et al. 2000). This phenotype is not altered by combining $Chip^{E}$ with any one of the $dlmo^{hdp}$ mutants or with the $dlmo^{Bx}$ mutants (data not shown).

While these results reveal no evidence for genetic interaction between $dlmo$ and $Chip$ during the development of thoracic bristles, it is possible that the specific genetic constellations examined here are not sensitive enough to enable phenotypic detection of such interactions. Similarly, no genetic interaction was found in $Chip^{E5.5}$/+ $pnr^{-}$/+ flies (RAMAIN et al. 2000). Nevertheless, taken together, our genetic interaction results indicate that $dlmo$ function is required for normal function of the CPAD transcription complex in regulating the development of the DC thoracic bristles.

**The effect of $dlmo$ on a target gene of the CPAD complex:**

If DLMO is required for normal function of the thoracic CPAD transcription complex, this may also be reflected in the activation of target genes of this complex. For example, expression of the $ac$ gene, mediated by the dorso-central (DC) enhancer and the promoter of the $ac$ gene, is directly regulated by the CPAD complex. Thus, in transgenic larvae carrying the $LacZ$ reporter under the control of the DC enhancer and the promoter of $ac$ ($DC:ac-LacZ$) (GOMEZ-SKARMETA et al. 1995), $LacZ$ expression depends on the thoracic CPAD transcription complex (RAMAIN et al. 2000). We used this reporter transgene to examine the effect of mutations in $dlmo$ on the activity of the CPAD complex in vivo. Histochemical staining of imaginal discs from control larvae carrying this $DC:ac-LacZ$ transgene shows $LacZ$ expression in the wing imaginal disc in the presumptive dorso-central thoracic region (Figure 5A; RAMAIN et
We found that in homozygous DC:ac-LacZ larvae that are also homozygous or hemizygous for either \(dlmo^{hdp590}\) or \(dlmo^{hdp58-1}\) loss-of-function mutations, LacZ is expressed in the wing imaginal disc at lower levels than in larvae homozygous for the DC:ac-LacZ alone (Figure 5B). This result suggests that the activity of the CPAD complex is reduced in the \(dlmo\) loss-of-function mutants. This conclusion is further supported by the observation that larvae that are homozygous for the DC:ac-LacZ reporter and either homozygous or hemizygous for \(ac^{l}\), express less LacZ in their wing imaginal discs compared to larvae homozygous for DC:ac-LacZ alone (Figure 5C). This concurs with the lack of bristles displayed by the \(ac^{l}\) mutants (Figure 4D). However, larvae that are also homozygous or hemizygous for \(dlmo^{Bc2}\) (i.e. \(dlmo^{Bc2}ac^{l}/DC:ac-LacZ\), express LacZ at higher levels than the \(ac^{l}/DC:ac-LacZ\) larvae (Figure 5D). Thus, over-expression of \(dlmo\) results in elevation of the activity of the CPAD complex.

These results further demonstrate a positive effect of DLMO on the function of the thoracic CPAD transcription complex during the development of the thorax, as suggested by our genetic studies.

**Expression of dlmo in the notum:**

To affect the function of the CPAD complex, DLMO has to be present in the same cells where this complex operates. We therefore examined whether \(dlmo\) is expressed in cells that give rise to the thoracic DC bristles. This was accomplished using the \(dlmo\)-GAL4 driver of the \(P\{GawB\}\)MS1096 strain (MILAN and COHEN 2000) to target the expression of either \(UAS-pnr\) or \(UAS-Chip\) in the expression pattern of \(dlmo\). Being components of the CPAD complex, appropriate amounts of both PNR and CHIP are required for proper development of the DC bristles (Romain et al. 1993;
RAMAIN et al. 2000). Thus, overexpression of PNR in the thorax using a pnr-GAL4
driver (in pnr-GAL4/++;UAS-pnr/+ flies) leads to supernumerary DC bristles,
reflecting enhanced activity of the CPAD complex (HAENLIN et al. 1997 and data not
shown). Likewise, we found that driving UAS-pnr under regulation of dlmo-GAL4
leads to excess DC bristles (Figure 3D). Over-expression of CHIP in the thorax in
pnr-GAL4  +/+ UAS-Chip flies causes lack of DC bristles and a thoracic cleft
(RAMAIN et al. 2000 and data not shown). Similarly, targeting UAS-Chip expression
by dlmo-GAL4 results in lack of either one or two bristles from the anterior pair of the
thoracic DC bristles (data not shown). Flies carrying UAS-pnr, UAS-chip or dlmo-
GAL4 alone display a wild-type pattern of thoracic DC bristles, and less than 1% of
the flies carrying pnr-GAL4 alone lack one of the anterior thoracic DC bristles (Figure
4B). Taken together, these results suggest that the spatial pattern of dlmo expression
overlaps the site where activity of the CPAD complex is required for normal
development of the DC bristles. These results concur with published data from in situ
hybridization and immunolocalization experiments (SHORESH et al. 1998; ZENG et al.
1998; ASMAR et al. 2008), indicating expression of dlmo in the dorsal portion of the
wing imaginal disc. Thus, DLMO is likely present where the CPAD complex
functions.

Physical interactions of DLMO with components of the CPAD transcription
complex:

We next considered the possibility that the effect of DLMO on the function of the
CPAD transcription complex is mediated via its direct binding to the complex.
Previous studies have already shown that DLMO binds CHIP during development of
the wing (RINCON-LIMAS et al. 2000; WEIHE et al. 2001). Our in vitro GST-pulldown
assays showed, as expected, that the full length DLMO protein can bind CHIP (Figure 6). Likewise, DLMO bound PNR *in vitro* (Figure 6). This is in agreement with the finding that DLMO co-immunoprecipitates with PNR when both proteins are co-expressed in cultured COS cells (Romain *et al.* 2000). Interestingly, we found that DLMO is capable of binding other DLMO molecules (Figure 6). These observations support the possibility that DLMO physically interacts with the CPAD transcription complex *in vivo* as well.

To study which domains of DLMO can mediate these physical interactions, four different fragments of DLMO were expressed, and their ability to bind the full-length proteins CHIP, PNR and DLMO *in vitro* was assessed. We found that fragments of DLMO that carry either one of its LIM domains, can bind CHIP, PNR and full length DLMO, whereas fragments containing only the N’ terminal or the C’ terminal regions of DLMO are incapable of such binding (Figure 6).

As none of the proteins that were used in this assay binds GST alone, these results indicate that DLMO can physically interact with *bona-fide* components of the thoracic CPAD transcription complex, suggesting that DLMO may be an integral component of this complex.
DISCUSSION

The results presented in this study uncover a novel role of DLMO in regulation of the development of the thoracic dorso-central (DC) bristles. We found that homozygous, or hemizygous, loss-of-function \((dlmo^{hdp})\) mutants lack the anterior pair of the DC bristles. Moreover, these \(dlmo\) mutants displayed genetic interactions with mutants in genes known to regulate DC bristle development, such as \(pnr\) and \(ac\), to reduce the number of DC bristles. Consistently, over-expression alleles of \(dlmo\) \((dlmo^{B5})\) also exhibited genetic interactions with these \(pnr\) and \(ac\) mutants, resulting in an increased number of bristles. In addition, our finding that over-expression of \(pnr\) under the regulation of \(dlmo\)-GAL4 affects DC bristle development suggests that \(dlmo\) is expressed in the region of the wing disc that gives rise to these bristles.

Our results suggest, for the first time, a role of DLMO in positive regulation of transcription. The negative role of DLMO in modulation of transcription during \textit{Drosophila} wing development has been well documented (Milan et al. 1998; Shoresh et al. 1998; Zeng et al. 1998). Our findings indicate that in another context, namely in regulation of DC bristle development by the CHIP-PANNIER-ACEAHTE- and DAUGHTERLESS (CPAD) complex, DLMO has another role, as a positive regulator of transcription. We found that lowering the level of DLMO (in \(dlmo^{hdp}\) mutants) results in a reduction in the expression of a reporter driven by regulatory sequences of a \textit{bona-fide} target gene of the CPAD transcription complex, suggesting that DLMO is a positive regulator of CPAD-dependent transcription. While the mechanism by which DLMO positively regulates transcription in the context of the CPAD complex remains to be elucidated, a first clue to this mechanism may lie in our finding that DLMO can bind constituent proteins of this complex, including PNR and
CHIP, in vitro. Should these interactions also take place in vivo, DLMO may exert its positive role in transcriptional regulation as a component of the CPAD complex.

Insights into the mechanism of positive transcriptional regulation by DLMO can be gleaned from LMO2, one of the mammalian homologs of DLMO. LMO2 was demonstrated to participate in a multiprotein transcription complex that contains Ldb1, a GATA factor (GATA-1 or GATA-2), and the bHLH transcription factors TAL1 and E2A, which are homologous, respectively, to the fly components of the CPAD complex, CHIP, PANNIER, ACEAHTE and DAUGHTERLESS (WADMAN et al. 1997; XU et al. 2003; LAHLIL et al. 2004). Various lines of evidence indicate that in mammals LMO2 serves as a bridge between components of the complex, and silencing of LMO2 causes disruption of the complex and decreases in the activation of transcription of its target genes, just as does silencing of Ldb1 or Tal1 (LAHILL et al. 2004; DELEUZE et al. 2007). Similarly to LMO2, DLMO might serve as a bridge between components of the CPAD complex. LIM domains are protein interaction modules (ZHENG and ZHAO 2007), and could serve DLMO to bind components of the CPAD complex. This suggestion is supported by our finding that each single LIM domain of DLMO is capable of binding components of the CPAD complex in vitro, and it agrees with similar reports on other LIM-containing proteins. Notably, a single LIM domain from LMO2 and LMO4 is sufficient to interact with Ldb1 or the related protein CLIM-1a (JURATA et al. 1996; BACH et al. 1997; DEANE et al. 2003). However, both LIM domains are required for the highest-affinity interactions (JURATA and GILL 1997; BREEN et al. 1998; DEANE et al. 2004; RYAN et al. 2006).

This proposed mode of action of DLMO, as a bridging molecule, which binds a different protein through each one of its LIM domains, predicts that a DLMO molecule with one defective LIM domain and one intact LIM domain would bind
only one protein at a time, and not be able to bridge between molecules. Indeed, in our new dlmo mutants we found that deletions which span the second zinc finger of the second LIM domain of DLMO, namely dlmo<sub>bdp48-1</sub> and dlmo<sub>bdp185-1</sub>, resulted in dlmo loss-of-function mutations. These mutants display partial loss of thoracic DC bristles along with reduced expression of a target gene of the thoracic transcription complex. Interestingly, the wing size of these mutants is normal, unlike the small wings of mutants with lesions in the 5' UTR of DLMO, such as dlmo<sub>bdp58-1</sub>, dlmo<sub>bdp67-2</sub> and dlmo<sub>bdpR590</sub>. This may suggest that the defective DLMO protein, which has only a single intact LIM domain, is sufficient for its function in the context of the wing, where DLMO acts as a negative regulator that binds only one protein (CHIP), but is not sufficient when DLMO acts as a bridging molecule in the thoracic CPAD transcription complex. Finally, our finding that DLMO can bind other DLMO molecules to generate homo-dimers or multimers might provide DLMO with a greater flexibility of bridging between distant components of the complex. This possibility remains to be examined.

In conclusion, DLMO appears to have a dual role in regulation of transcription, depending on the context. Such a phenomenon has been documented for other transcription co-factors, whose dual function in transcription regulation varies according to their binding partners, the specific tissue or the developmental stage (for a review see MA 2005). Likewise, our results indicate DLMO to have such a dual role being a negative regulator with respect to the AP-CHIP complex and a positive regulator in the context of the CPAD complex.

Note added in proof: While this manuscript was in review, a paper reporting essentially similar results was published (ASMAR et al. 2008).
ACKNOWLEDGEMENTS

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Table 1: Proteins and fragments used in GST-pulldown assays

<table>
<thead>
<tr>
<th>Fragment name</th>
<th>Amino acids</th>
<th>Description</th>
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<tr>
<td>CHIP</td>
<td>1-432</td>
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</tr>
<tr>
<td>PNR</td>
<td>1-540</td>
<td>Full length</td>
</tr>
<tr>
<td>DLMO (FL)</td>
<td>1-313</td>
<td>Full length</td>
</tr>
<tr>
<td>DLMO-N’</td>
<td>1-104</td>
<td>No defined domain</td>
</tr>
<tr>
<td>DLMO-L1M1</td>
<td>65-176</td>
<td>First LIM domain (91-145)</td>
</tr>
<tr>
<td>DLMO-L1M2</td>
<td>163-221</td>
<td>Second LIM domain (156-210)</td>
</tr>
<tr>
<td>DLMO-C’</td>
<td>224-313</td>
<td>No defined domain</td>
</tr>
</tbody>
</table>
FIGURE LEGENDS

Figure 1: Schematic maps of the dlmo gene in mutants used. Exons (1a to 5) are outlined as boxes, in which coding regions are black. Sequences encoding the two LIM domains are indicated. Nucleotide numbers start with the first base of exon 1a (corresponding to nucleotide no. 18428698 on the X chromosome according to FlyBase Genome Annotation Release 5.4). P-element insertions are indicated as triangles. Dotted lines represent deleted sequences in the mutants, caused by imprecise excisions of the P-elements. A) The progenitor P-element line EY01065. B) The entire exon 5 is deleted in the loss-of-function mutants dlmo\textsuperscript{hdp48-1} and dlmo\textsuperscript{hdp185-1}. In both mutants the deletion extends into the upstream intron, removing additional 634 bp and 655 bp, respectively. C) In the hypermorphic mutants dlmo\textsuperscript{Bx42-2}, dlmo\textsuperscript{Bx5-1} and dlmo\textsuperscript{Bx1002-2} the deletion removed 114 bp, 273 bp and 375 bp, respectively, from the 3’ UTR of dlmo. D) The progenitor P-element line MS1096. E) The dlmo\textsuperscript{hdp58-1} and dlmo\textsuperscript{hdp67-2} mutants have deletions that extend into exon 2, removing 128 bp and 113 bp from the 5’ UTR, respectively. F) In five other new dlmo\textsuperscript{hdp} mutants, as well as dlmo\textsuperscript{hdpR590} (MILAN et al. 1998), the deletions are confined to the P-element.

Figure 2: dlmo mutations alter wing morphology. All wings are shown at the same magnification. A) Wings from a wild type fly and wings from flies homozygous for dlmo\textsuperscript{hdp} mutations, displaying a short cross-vein. The dlmo\textsuperscript{hdp185-1} mutant represents a class of mutants (including dlmo\textsuperscript{hdp185-1} and dlmo\textsuperscript{hdp48-1}) whose wings have a normal size, while the dlmo\textsuperscript{hdp58-1} mutant represents a class of mutants (including dlmo\textsuperscript{hdp58-1}, dlmo\textsuperscript{hdp67-2} and dlmo\textsuperscript{hdpR590}) whose wings are smaller than the wild type. B) Wings of flies homozygous for new dlmo\textsuperscript{Bx} mutations display different degrees of scalloping. The degree of phenotypic severity correlates with the length of the deleted 3’ UTR.
sequence (See Figure 1C). The severity of wing scalloping of $dlmo^{Bx5-1}$ flies (middle panel) resembles that of the previously described $dlmo^{Bx2}$ mutant (SHORESH et al. 1998). **C** $dlmo^{bdp}$ mutations partially suppress the wing scalloping caused by $dlmo^{Bx}$ mutations. Wings from heteroallelic $dlmo^{Bx5-1}/dlmo^{bdp58-1}$ flies are less scalloped than wings of $dlmo^{Bx5-1}/+$ heterozygotes. A chromosomal deficiency ($Df(1)N19$) that removes the $dlmo$ region similarly suppresses the wing scalloping caused by the $dlmo^{Bx}$ mutation indicating that the $dlmo^{bdp}$ alleles are loss-of-function.

**Figure 3: $dlmo$ is involved in development of the thoracic DC bristles.** **A** Thorax from a wild type fly with two anterior (arrows, top) and two posterior (arrowheads, bottom) DC bristles. **B** Representative thorax from a $dlmo^{bdp}$ homozygous fly (shown here is $dlmo^{bdp185-1}$) lacking the anterior DC bristles, whose presumptive locations are indicated by arrows. **C** Thorax from an $ac^{l}$ homozygous fly lacking the two anterior (arrows) and two posterior (arrowheads) DC bristles (compare to A). **D** Thorax from a $dlmo$-GAL4/++; UAS-pnr/+ fly displaying excess number of DC bristles (compare to A).

**Figure 4: Genetic interactions of $dlmo$ with $pnr$ and $ac$ affect the development of thoracic bristles.** Sketches depicting the thorax of wild type (A) and various $dlmo$-related mutants (B-J). The phenotypic distribution of each genotypic class, according to the phenotypes in Figure 3A-C, is shown below the sketch. Large open circles indicate a thoracic DC bristle that is missing in all flies of the given genotype; small circles indicate a DC bristle that is missing in some flies of the given genotype. **A** Wild type flies have two anterior (a) and two posterior (p) thoracic DC bristles. **B-D** Only 10-20% of the flies that are hemizygous for any $dlmo^{bdp}$ mutation lack both
anterior DC bristles (B), and less than 1% of the \textit{pnr} heterozygous flies (either \textit{pnr}^{VX6/+} or \textit{pnr}^{MD237/+}) lack one anterior thoracic bristle (C). On the other hand, all flies hemizygous for any \textit{dlmohdp} mutation that are also heterozygous for either \textit{pnr} mutation lack the two anterior DC bristles and 10% of them also lack one of the posterior DC bristles (D). \textbf{E-G}) Flies heterozygous for any \textit{dlmohdp} mutation (E), as well as flies heterozygous for \textit{ac} (F), have a normal thoracic phenotype, while all heteroallelic flies lack the anterior DC bristles (G). \textbf{H-J}) Flies hemizygous for \textit{dlmohdp} mutations display a normal notum (H). However, flies homozygous for \textit{ac} (I and \textit{culI} \textit{et al}. 2001), display restoration of the posterior DC bristles when they are also hemizygous for a \textit{dlmohdp} mutation (J).

\textbf{Figure 5: \textit{dlmo} positively regulates the expression of a target gene of the CPAD transcription complex.} Histochemical staining of \textit{LacZ} in wing imaginal discs from late third instar larvae: \textbf{A}) Disc from a larva harboring the \textit{DC:ac-LacZ} transgene. An arrow indicates staining in the presumptive dorso-central thoracic region. \textbf{B}) Partial loss-of-function of \textit{dlmo} (\textit{dlmohdp} mutation) leads to reduced expression of \textit{LacZ}. \textbf{C}) Reduced expression of \textit{LacZ} is also caused by partial loss-of-function of \textit{ac} in \textit{ac} (I) mutants. \textbf{D}) The reduced \textit{LacZ} expression caused by partial loss of function of \textit{ac} is restored by over-expression of \textit{dlmo} (\textit{dlmo}^{Bx}).

\textbf{Figure 6: DLMO physically interacts with components of the CPAD complex \textit{in vitro}.} GST-pulldown assays conducted between bacterially expressed GST-tagged proteins and \textit{in vitro} translated radio-labeled proteins. The input lane contains 1/10 of the amount of the radio-labeled protein used in each assay. The GST lanes contain the control pulldown assays performed with GST alone bound to glutathione beads. The
full-length radio-labeled proteins DLMO, CHIP and PNR bind to the full-length (FL) GST-DLMO fusion protein and to either one of the GST-fused LIM domains of DLMO (LIM1 and LIM2), but not to fragments containing the N' terminal or C' terminal portions of DLMO.
Figure 1
Figure 2
Figure 3
Figure 4
Figure 5
Figure 6