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Ssdp proteins bind to LIM-interacting co-factors and regulate the activity of LIM-homeodomain protein complexes in vivo

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SUMMARY

LIM-homeodomain transcription factors control a variety of developmental processes, and are assembled into functional complexes with the LIM-binding co-factor Ldb1 (in mouse) or Chip (in *Drosophila*). We describe the identification and characterization of members of the Ssdp family of proteins, which we show to interact with Ldb1 and Chip. The N terminus of Ssdp is highly conserved among species and binds a highly conserved domain within Ldb1/Chip that is distinct from the domains required for LIM binding and self-dimerization. In *Drosophila*, Ssdp is expressed in the developing nervous system and imaginal tissues, and it is capable of modifying the in vivo activity of complexes comprised of Chip and the LIM-homeodomain protein Apterous. Null mutations of the *ssdp* gene are cell-lethal in clones of cells within the developing wing disc. However, clones mutant for a hypomorphic allele give rise to ectopic margins, wing outgrowth and cell identity defects similar to those produced by mutant clones of *Chip* or *apterous*. Ssdp and Ldb/Chip each show structural similarity to two *Arabidopsis* proteins that cooperate with one another to regulate gene expression during flower development, suggesting that the molecular interactions between Ssdp and Ldb/Chip proteins are evolutionarily ancient and supply a fundamental function in the regulated control of transcription.

Key words: LIM domain, Homeodomain, *Drosophila*, Wing development, Apterous, Chip

INTRODUCTION

A diverse set of developmental programs in animals are controlled by members of the LIM-homeodomain (LIM-HD) family of transcription factors. These programs include, among others, early patterning of the embryo, neuronal differentiation, limb and eye formation in vertebrates, and imaginal disc development in *Drosophila* (Curtiss and Heilig, 1998; Hobert and Westphal, 2000). The LIM domains of all LIM-HD proteins, as well as those of nuclear LIM only (LMO) proteins, are bound by a critical co-factor called Ldb1 (also NLI and CLIM-2) in mice or Chip in *Drosophila* (Agulnick et al., 1996; Jurata et al., 1996; Bach et al., 1997; Morcillo et al., 1997). Ldb1/Chip co-factors homodimerize and thereby bridge two LIM-HD proteins to form a tetrameric complex (Jurata et al., 1998). This complex is functional in vivo (Milan and Cohen, 1999; van Meyel et al., 1999; Thaler et al., 2002), but questions remain as to how the complex acts to control transcription of LIM-HD target genes, and whether other proteins also participate in the complex to render it functional.

The developing wing of *Drosophila* has proven a tractable system in which to study the function of complexes formed by LIM-HD proteins and their co-factors (Fernandez-Funez et al., 1998; Milan et al., 1998; Shoresh et al., 1998; Zeng et al., 1998; Milan and Cohen, 1999; Rincon-Limas et al., 2000; Weihe et al., 2001). The wing imaginal disc is divided into distinct lineage-restricted compartments along both the anteroposterior and dorsoventral (DV) axes. In response to signaling via epidermal growth factor receptor (Wang et al., 2000; Zecca and Struhl, 2002a; Zecca and Struhl, 2002b), the LIM-homeodomain protein Apterous (Ap) is expressed in the dorsal compartment of the wing disc where it is required to establish an affinity boundary that partitions the wing along the DV axis (Cohen et al., 1992; Diaz-Benjumea and Cohen, 1993; Blair et al., 1994). The DV boundary of the wing disc differentiates into the wing margin, which lies at the edge of the adult wing blade, and is decorated with mechanosensory and chemosensory bristles distributed in a discrete DV pattern (Palka, 1993). Ap induces Notch activation at the DV boundary through induction in dorsal cells of the Notch ligand Serrate and the glycosyltransferase Fringe (Irvine and Wieschaus, 1994; Kim et al., 1995; Panin et al., 1997; Klein and Arias, 1998; Micchelli and Blair, 1999; Rauskolb et al., 1999; Ju et al., 2000; O'Keefe and Thomas, 2001). This leads to the expression of the secreted morphogen Wingless in a stripe that prefigures the margin, patterns the wing along the DV axis and directs cell proliferation and wing outgrowth (Diaz-Benjumea and Cohen, 1993; Zecca et al., 1996; Neumann and Cohen, 1997). Finally, through activation of the *msh* gene, Ap is required to specify the dorsal identity of cells such as sensory bristles and vein tissue (Milan et al., 2001).

The domains of Ldb1/Chip co-factors that are required for self-dimerization and LIM interaction have been identified (Jurata and Gill, 1997; Breen et al., 1998; van Meyel et al., 1999). In *Drosophila*, it has been shown that Chip and Ap physically interact in vivo to form a tetrameric complex comprised of two molecules of Ap bridged by a Chip homodimer (Milan and Cohen, 1999; van Meyel et al., 1999; van Meyel et al., 2000). This complex is required for Ap activity in DV patterning and outgrowth of the wing and is subject to disruption by *Drosophila* LMO (Bx – FlyBase), a nuclear LIM-only protein that can compete with Ap for binding to Chip and thereby modulate Ap activity (Milan et al., 1998; Shoresh et al., 1998; Zeng et al., 1998). In the wing, LMO expression is upregulated by Ap, thus providing a mechanism for negative feedback upon Chip/Ap tetrameric complexes and modulation of Ap activity (Milan and Cohen, 1999; Milan and Cohen, 2000; Weihe et al., 2001).

Although Chip is required to dimerize and bring two molecules of Ap into a tetrameric complex, we have hypothesized that it may also recruit other proteins or co-factors required for correct transcriptional regulation of target genes (van Meyel et al., 2000). In the present study, we describe the identification and characterization of members of the Ssdp family of proteins in mice and flies, which specifically interact with Chip/Ldb proteins as shown here and by others (Chen et al., 2002). The N terminus of Ssdp contains a recently described LUFS domain, which we find is required for interaction with Chip. Chip binds Ssdp through a highly conserved domain that is distinct from domains for LIM binding and homodimerization, and Chip is required for correct nuclear localization of Ssdp. In vivo, we find that Ssdp is capable of modifying Chip function in wing development. Although null mutations of *ssdp* are cell-lethal in clones of cells in the developing wing disc, clones mutant for a hypomorphic allele of *ssdp* give rise to margin, outgrowth and cell identity defects that are strikingly similar to those produced by mutations of *Chip* and *ap*. Intriguingly, proteins with structural similarity to Ssdp and Chip have recently been shown to cooperate with one another to regulate the expression of a homeotic gene functioning during development of plants (Conner and Liu, 2000; Franks et al., 2002). These results suggest that molecular interactions of the kind between Ssdp and Chip/Ldb proteins are evolutionarily ancient and may supply a fundamental function in the regulated control of transcription.

MATERIALS AND METHODS

Yeast two-hybrid screen

Plasmids, yeast strains and library were from Clontech. The mouse Ldb2-coding region was PCR amplified and cloned into the yeast expression vector pAS2-1. The Ldb2 bait was used to screen approximately 1×10^6 transformants from a mouse embryo E17 cDNA library in the pGAD10 vector, in yeast strain CG1945 according to the Matchmaker system protocol (Clontech). Six His^+ $lacZ^+$ clones were isolated, and the library plasmids were sequenced. Four of these encoded LMO proteins and one clone contained a homolog of the chicken *SSDP* gene, which we have called mouse *Ssdp2*. Mouse cDNAs were identified and sequenced fully for both *Ssdp2* (GenBank Accession Number, AY167988; I.M.A.G.E. CloneID 2088154) and a related gene *Ssdp1* (GenBank Accession Number, AY167987; I.M.A.G.E. CloneID 1193771), which resembles more closely the founding member of the family (chicken *SSDP*).

Yeast qualitative interaction assays

Plasmids were transformed into yeast strain Y187, plated, and colonies were assayed for β-galactosidase activity by the filter lift method as described in the Clontech Matchmaker system protocol. A positive result was scored if blue color developed upon incubation for 3 hours at 30°C. All DBD-Ldb1 constructs and DBD-Ssdp2(1-50) were cloned into pAS2-1, DBD-Ssdp2(1-100) and DBD-Ssdp(1-98) from *Drosophila* were in pGBT9. All AD-Ssdp2 constructs were in pGAD10, and AD-Ldb1 and AD-Chip constructs were in pACT2. The DBD control vector pLAM5′-1 encodes human Lamin C. Negative controls were assayed for each construct to ensure that auto-activation or nonspecific binding did not occur: DBD-Lamin C was tested with most AD constructs, except Chip vectors where empty DBD vector pAS2-1 was used; empty AD vector pACT2 was tested with DBD constructs.

Immunoprecipitation

In vitro transcription and translation were performed according to the manufacturers instructions (TNT Reticulocyte Lysate System, Promega) with or without ^{35}S-methionine (NEN Life Sciences products). Ten µl of each protein was mixed and 20 µl of binding/wash buffer (50 mM HEPES, pH 7.5; 250 mM NaCl; 0.1% NP-40; 200 µM $ZnCl_2$ and $MgCl_2$) was added. After incubation for 2 hours on ice, reactions were cleared with protein A sepharose, and immunoprecipitated with anti-Flag M2 agarose beads (Kodak/IBI). Eluted samples were analyzed on 4-12% acrylamide gradient gels (NuPAGE, Invitrogen), and the results observed using autoradiography.

Fly strains and genetics

The strains EP(3)3004, EP(3)3097 and l(3)neo48 were obtained from the Berkeley *Drosophila* Genome Project collection (Cooley et al., 1988; Rorth et al., 1998). Recently, the strain KG03600 (Roseman et al., 1995) has been identified as an insert in the *ssdp* locus, but was used in only some of the analyses presented here. The $ssdp^{L7}$and $ssdp^{L5}$ alleles were the result of imprecise excisions generated by mobilization of the EP(3)3097 P-element using Δ2,3 as a source of transposase (Tsubota and Schedl, 1986; Robertson et al., 1988). Each of these was fully lethal in complementation tests with $ssdp^{l(3)neo48}$. A number of precise excisions that fully complemented $ssdp^{l(3)neo48}$ were recovered, indicating that lethality in EP(3)3097 was due to insertion of this P-element in the *ssdp* locus. DNA sequencing of the breakpoints of the $ssdp^{L7}$ deficiency revealed that it is a complete null allele of *ssdp* resulting from the deletion of 3363 base pairs (bp) of DNA from the insertion site of the EP(3)3097 P-element through the entire coding region of *ssdp* plus 941 bp of sequence downstream of the 340 bp 3′ untranslated region (UTR). This breakpoint lies 2002 bp away from the nearest predicted gene (CG14313), which is of unknown function. Southern analysis strongly suggests that $ssdp^{L5}$ results from the deletion of ~1750 bp of coding sequence, but the boundaries of this deficiency were not determined by DNA sequencing. In all analyses where it has been examined, $ssdp^{L5}$ has had effects identical to those of $ssdp^{L7}$, suggesting that it too is a null allele. All crosses and embryo/larval collections were performed at 25°C, unless stated otherwise.

Balanced stocks for each of the *ssdp* mutations were maintained over TM3 marked by actin-*lacZ*. This dominant marker was used to score the timing of lethality for various mutant allelic combinations. Homozygous mutants were assessed for viability at the first and third instar larval stages, and upon eclosion of adults.

DNA constructs for transgenic *Drosophila*

We obtained two *Drosophila ssdp* cDNAs (LD23161 and LD37723) and found each to contain the entire open reading frame of the *ssdp* gene (Research Genetics). Using LD37723 as a template for polymerase chain reactions (PCR), SsdpFL and SsdpΔ2-92 constructs were created by a previously described strategy to include five C-terminal Myc epitopes and two stop codons (van Meyel et al., 1999). In a similar fashion, ChipΔ387-426 was created from a Chip cDNA (Morcillo et al., 1997). Each of these was fully sequenced, then cloned into pUASt (Brand and Perrimon, 1993). UAS lines were generated by germline transformation (Rubin and Spradling, 1982) and, for each construct, a minimum of 20 independent lines were created and tested for expression. Those lines that exhibited the strongest, GAL4-directed Myc expression were selected for analysis.

In situ hybridization and immunostaining

In situ hybridization was performed on *Drosophila* embryos in whole mount, and on dissected wandering third instar larvae. A 1.4 kb digoxigenin (DIG)-labeled antisense cRNA probe was synthesized using SP6 RNA polymerase and *Stu*I-cut LD37723 *ssdp* cDNA. For immunofluorescence staining, we drove Myc-tagged UAS transgene expression in muscles 21-24 with ap^{GAL4} (Calleja et al., 1996), and crossed this combination into the $Chip^{e5.5}$ mutant background (Morcillo et al., 1997). We stained dissected embryos with mouse anti-Myc (9E10) at a dilution of 1:50, and secondary antibodies conjugated to Cy3 (Jackson ImmunoResearch) at 1:500. Confocal analysis was performed on a Zeiss confocal station and imaged with the LSM510 software (Zeiss). Images were compiled with Adobe Photoshop 6.0.

Mosaic analysis

Individuals carrying chromosomes recombinant for *ssdp* mutations and FRT inserts at 82B were selected on media containing Geneticin (Invitrogen) and subsequently tested by complementation for viability against mutant alleles of *ssdp*. Timed embryo collections were subjected to heat-shock (1 hour, 38°C) at discrete stages of larval development either 36 hours, 48 hours, 72 hours or 96 hours after egg-laying (AEL). After eclosion, individuals of the genotypes listed below were analyzed for the presence of clones as indicated by the cell-autonomous marker *pwn* (Heitzler et al., 1996), which is seen as pin-shaped hairs (trichomes) with spurs on each mutant cell, and truncated bristles. For microscopic examination, wings were removed, immersed in isopropanol followed by methyl salicylate, then mounted on glass slides in Canada Balsam.

ssdp mutants

hsFLP38*pwn/pwn*;FRT, $ssdp^{L7}$/FRT,Dp pwn^{+}
hsFLP38*pwn/pwn*;FRT, $ssdp^{L5}$/FRT,Dp pwn^{+}
hsFLP38*pwn/pwn*;FRT, $ssdp^{l(3)neo48}$/FRT,Dp pwn^{+}

Control

hsFLP38*pwn/pwn*;FRT, P(w+)90E/ FRT,Dp pwn^{+}

RESULTS

Ssdp proteins interact with Ldb/Chip

From a yeast two-hybrid screen to identify binding partners for mouse Ldb/NLI proteins, we isolated a murine homolog of avian sequence-specific single-stranded DNA-binding protein (SSDP). First identified in an experimental paradigm for induced differentiation of avian chondrocytes in culture, SSDP was shown to selectively bind the promoter of the α2(I) collagen gene (Bayarsaihan et al., 1998). We acquired and sequenced corresponding full-length cDNAs, and identified two mouse genes encoding highly similar proteins, Ssdp1 and Ssdp2, the sequences of which have been deposited in GenBank (Accessions Numbers, AY167987 and AY167988).

To verify the specific interaction between Ldb and Ssdp, we assayed for co-immunoprecipitation of proteins translated in rabbit reticulocyte lysates (Fig. 1). We co-incubated various combinations of Ldb and Ssdp proteins labeled with ^{35}S-methionine and/or tagged with the FLAG epitope. Co-incubation was followed by immunoprecipitation with anti-FLAG antibody-conjugated agarose beads and analysis by SDS-PAGE. We found Ssdp2 protein to be efficiently immunoprecipitated by Ldb1 (lane 2), but not by the LIM-HD protein Lhx3 (lane 4). As expected, Lhx3 was capable of binding Ldb1 (lane 1) and importantly, was able to immunoprecipitate Ssdp2 in the presence of Ldb1 (lane 3), arguing for the formation of a ternary complex in which Lhx3 and Ssdp2 are each bound to Ldb1. Additional control experiments showed that the Ldb1-Ssdp2 complex was not immunoprecipitated in the absence of the FLAG epitope (lane 5), and only a small amount of either Ssdp2 or Ldb1 binds nonspecifically to the beads (lanes 5 and 6).

Domains of interaction between Ldb1 and Ssdp2 were mapped using a qualitative yeast two-hybrid assay. For the first set of assays (Fig. 2A), Ldb1 was fused to the DNA binding domain (DBD) of Gal4, while Ssdp2 was fused to the activation domain (AD). In this configuration, the 375 amino acid full-length Ldb1 protein alone nonspecifically activated the β-gal reporter gene, and therefore could not be tested.

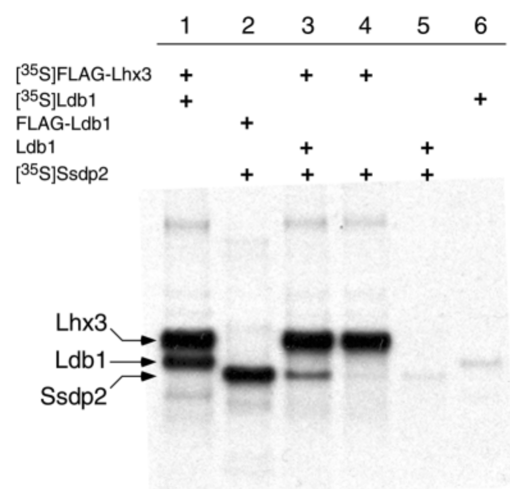


Fig. 1. Ldb1 and Ssdp2 specifically interact in vitro. Ldb1 (from mouse), Ssdp2 (human) and the LIM-HD protein Lhx3 (mouse) were transcribed and translated in rabbit reticulocyte lysates. Proteins labeled with ^{35}S-methionine and/or tagged with the FLAG epitope were mixed in the combinations shown above each lane and then complexes were immunoprecipitated with anti-FLAG antibody-conjugated agarose beads (see Materials and Methods). Ssdp2 protein is efficiently immunoprecipitated by Ldb1 (lane 2), but not by Lhx3 (lane 4). Lhx3 can bind Ldb1 (lane 1) and can immunoprecipitate Ssdp2 in the presence of Ldb1 (lane 3). This indicates the formation of a ternary complex in which Lhx3 and Ssdp2 are each bound to Ldb1, but they do not directly interact with one another. Control experiments show that the Ldb1-Ssdp2 complex is not immunoprecipitated in the absence of the FLAG epitope (lane 5), and only a small amount of either Ssdp2 or Ldb1 binds non-specifically to the beads (lanes 5 and 6).

However, a truncation of Ldb1 that retains amino acids 1-308 does not show this nonspecific interaction, and binds strongly to Ssdp2 as shown in Fig. 2A. This mutant can bind to Ldb1 through the dimerization domain (DD) but cannot bind LIM domains in this assay (not shown) as it removes most of the LIM interaction domain (LID) (Jurata and Gill, 1997; Breen et al., 1998). A truncation leaving only amino acids 1-255 produces a protein that can dimerize with Ldb1 (not shown), removes the entire LID and retains the ability to bind Ssdp2 (Fig. 2A). Further truncation to include only amino acids 1-201 abolishes binding to Ssdp2 (Fig. 2A); however, this mutant can still dimerize with Ldb1 (not shown) (Breen et al., 1998). Therefore, interaction of Ssdp2 and Ldb1 requires Ldb1 residues between 201 and 255, a region distinct from those required for LIM binding and dimerization.

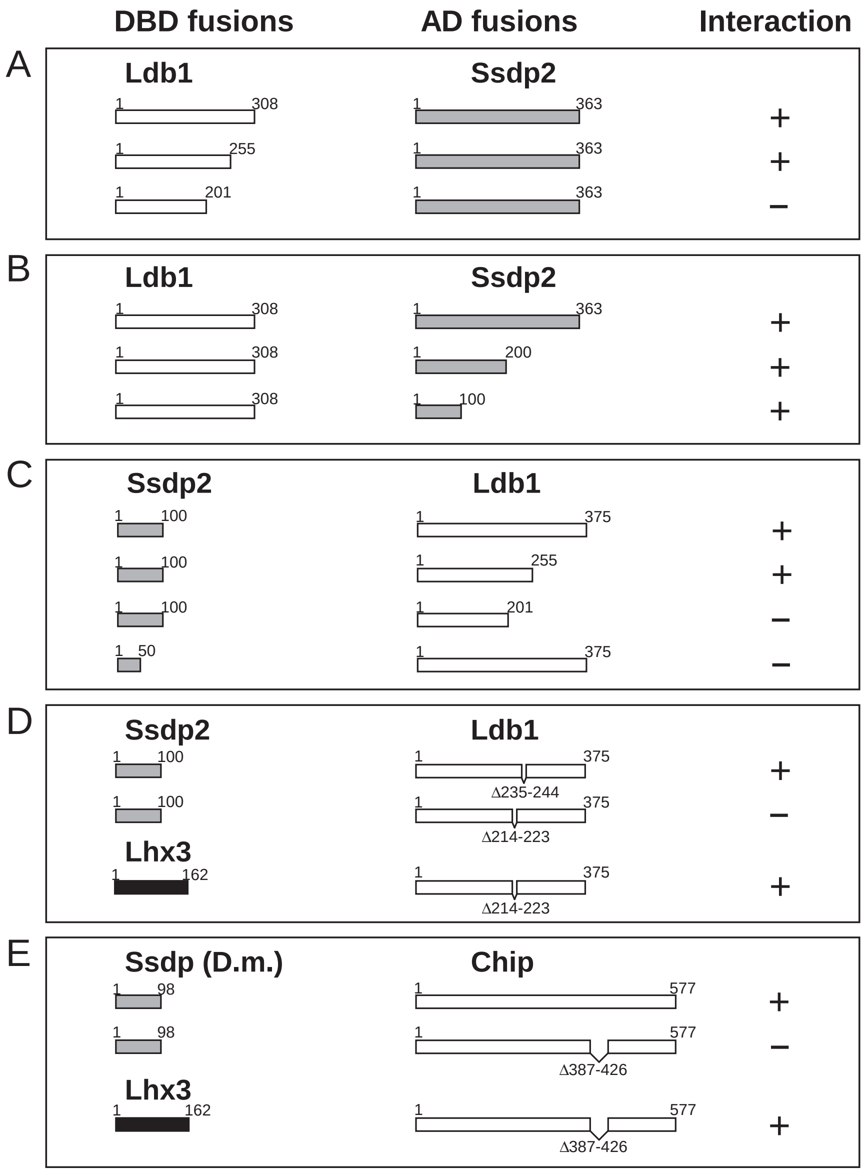


Fig. 2. Qualitative two-hybrid interaction assays in yeast reveal domains required for interactions between Ssdp proteins and Ldb/Chip proteins from mice and flies. Schematic diagram of recombinant proteins fused to the DNA-binding domain (DBD) or activation domain (AD) of GAL4. Mouse Ldb1 and Drosophila Chip are depicted in white, mouse Ssdp2 and Drosophila Ssdp in gray, and mouse Lhx3 as a positive control in black. Interactions between proteins were measured by β-galactosidase activity and were scored as either positive (+) or negative (–). (A) Sequences between amino acids 201 and 255 of Ldb1 are required for interaction with Ssdp2. (B) The N-terminal 100 amino acids of Ssdp2 are sufficient for interaction with Ldb1. (C) Upon switching the configuration of the fusion proteins, the requirements of amino acids 1-100 of Ssdp2 and 201-255 of Ldb1 are reiterated, supporting the specificity of the interaction. (D) Further refinement of Ldb1 sequences required for interaction with Ssdp2 through two deletions of 10 amino acids each. Deletion of amino acids 214-223 disrupts the interaction with Ssdp2, but has no effect on the ability of Ldb1 to bind the LIM domains of Lhx3. (E) The *Drosophila melanogaster* (D.m.) orthologs Ssdp and Chip give similar results to those obtained for the mouse proteins. Ssdp amino acids 1-98 are sufficient for interaction with Chip, and removal of amino acids 387-426 of Chip prevents binding to Ssdp but not to the Lhx3 LIM domains.

Deletion mutants of the Ssdp2 protein indicate that N-terminal amino acids 1-100 are sufficient for strong binding to Ldb1 (Fig. 2B). This fragment was then used as a DBD fusion to verify the Ldb1 residues required for the interaction (Ldb1 as an AD fusion). As shown in Fig. 2C, the same region (201-255) is required for interaction in this configuration. As above, truncation to include only amino acids 1-201 supports dimerization with intact Ldb1 (not shown). Further deletion of Ssdp2 reveals that residues 1-50 are not sufficient for binding to Ldb1.

To map the interaction with Ssdp2 more precisely, two internal deletions of 10 amino acids each were constructed, Ldb1Δ235-244 and Ldb1Δ214-223 (Fig. 2D). Ldb1Δ235-244 binds to Ssdp but Ldb1Δ214-223 does not. As a positive control, the LIM domains of Lhx3 were shown to bind both of these mutants (Ldb1Δ214-223 shown in Fig. 2D).

Both Ssdp2 and Ldb1 have orthologous counterparts in *Drosophila*, called Ssdp and Chip. As shown in Fig. 2E, fly Ssdp residues 1-98 can bind strongly to Chip, and this interaction is dependent upon amino acids 387-426 of Chip. Chip residues 387-435 are 94% identical to Ldb1 amino acids 201-249, and we have named this region the Ldb1/Chip conserved domain (LCCD). Taken together, the results indicate that the N terminus of Ssdp proteins bind Ldb/Chip proteins in a region that is distinct from the two domains needed to form the tetrameric complex, namely the dimerization domain (DD) and the LIM interaction domain (LID).

The interaction domains of Ssdp and of Ldb1/Chip have been highly conserved through evolution

Searches of the NCBI databases indicate that Ssdp proteins comprise a family of highly related proteins in which there are four members in humans (Castro et al., 2002), three in mice and only one in *Drosophila*.

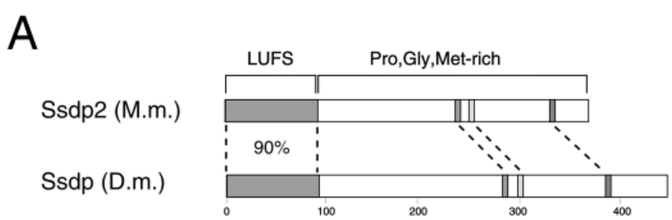
Comparison of primary sequence from Ssdp proteins from these and other species reveals a high degree of amino acid identity, particularly within the first 100 amino acids. A schematic of the overall protein structure comparing mouse Ssdp2 and fly Ssdp is shown in Fig. 3A, and a sequence alignment of the N-terminal sequences for several family members is shown in Fig. 3B. Between flies and mice there is 90% identity over this N-terminal region. As is the case for all family members, the remainders of these proteins are characterized by an unusually high proportion of proline, glycine and methionine residues. For example, of the 352 amino acids of *Drosophila* Ssdp from amino acids 93-445, 21% are proline, 27% are glycine and 9% are methionine, for a total of 57% of all residues. Within this overall architecture, there are three small regions that are highly conserved across species (Fig. 3A).

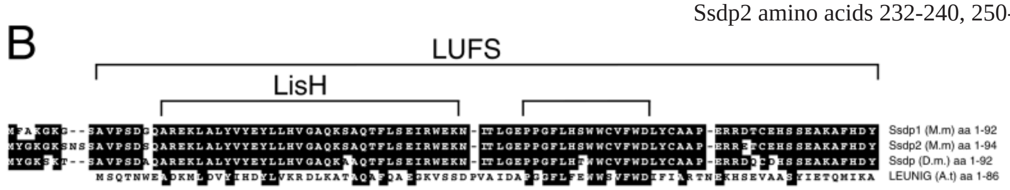
There is also significant similarity in the N terminus of Ssdp family members to LEUNIG, a protein first identified in *Arabidopsis* and for which the N-terminal domain has been termed a LUFS domain, based on its similarity to other proteins in plants, Flo8 in yeast and to Ssdp. Although the LUFS domain remains functionally uncharacterized to date, it contains a Lissencephaly type 1-like homology motif (LisH) with a curious additional motif comprised at its core of the following sequence P-X-GFL-XX-WW-X-VFWD (Fig. 3B).

Like the LUFS domain of Ssdp proteins, the LCCD of Ldb1/Chip has been highly conserved through evolution, with 94% identity between mice and flies over a stretch of 49 amino acids (Fig. 3C).

Nuclear localization of Drosophila Ssdp is dependent upon the LUFS domain and *Chip*

The single *ssdp* gene in flies has been annotated CG7187 by the Berkeley Drosophila Genome Project and a number of corresponding cDNAs have been isolated. The gene consists of two exons, the second of which contains the single open reading frame which encodes a 445 residue polypeptide. We designed epitope-tagged versions of *Drosophila* Ssdp in which five copies of the Myc epitope were fused to the C terminus of full-length Ssdp (SsdpFL) or a mutant lacking amino acids 2-92 (SsdpΔ2-92). These constructs were used to generate transgenic lines in which transgene expression was under the control of UAS sequences (Brand and Perrimon, 1993). We used different GAL4 driver lines to express these recombinant proteins in a variety of cell types, including neurons and muscles. SsdpFL localized to nuclei, with no staining in the cytoplasm (Fig. 3D). By contrast, SsdpΔ2-92 was found throughout the cytoplasm (Fig. 3E). Therefore nuclear localization of Ssdp is dependent upon the Chip-interacting LUFS domain, despite the fact that this region does not appear to encode a nuclear localization sequence (NLS). To address whether Chip, which does have an NLS, is required for translocation of Ssdp into the nucleus, we tested whether SsdpFL is properly localized to the nucleus in *Chip*$^{e5.5}$ null mutants. In contrast to wild-type, SsdpFL was distributed





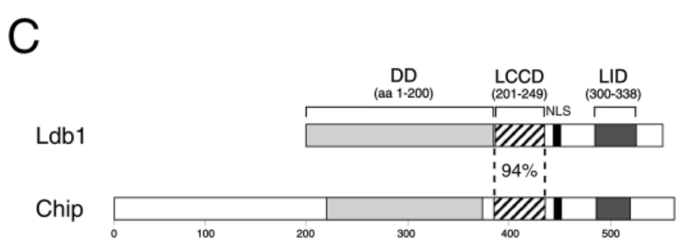


Fig. 3. The LUFS domain of Ssdp proteins mediate interactions with Ldb/Chip that are crucial for nuclear localization. (A) Schematics comparing the amino acid composition and overall structure of mouse (M.m.) Ssdp2 and *Drosophila melanogaster* (D.m.) Ssdp. There is 90% identity of amino acids over the N-terminal region shown to be sufficient for interactions with Ldb1/Chip (gray box), known as the LUFS domain. The remainders of these proteins are rich in proline, glycine and methionine residues, and share three other small domains that are also highly conserved (small gray boxes, corresponding to Ssdp2 amino acids 232-240, 250-262 and 331-337). (B) Alignment of the primary amino acid sequence of LUFS domains of Ssdp proteins from mouse and *Drosophila*, and *Arabidopsis* LEUNIG, showing the LisH domain and the motif P-X-GFL-XX-WW-X-VFWD, which is notable for its conservation in all members and with yeast Flo8. (C) Schematic of Ldb1 and Chip, indicating 94% identity over residues 201-249 of Ldb1 and 387-435 of Chip (hatched boxes). We have called this region the Ldb1/Chip conserved domain (LCCD). Within the LCCD a deletion of 10 residues (Ldb1 amino acids 214-223, corresponding to Chip amino acids 400-409) disrupts the ability of Chip to interact with Ssdp without affecting its ability to homodimerize through the dimerization domain (DD) or bind LIM domains through the LIM interaction domain (LID). The position of the putative nuclear localization sequence (NLS) is indicated.
(D-F) Anti-Myc immunofluorescence staining of ventral muscles of stage 16 embryo. In a wild-type background (D), *ap*^{GAL4}-driven expression of Myc epitope-tagged SsdpFL reveals discrete localization to the multiple nuclei in each of the muscle cells 21-24. By contrast, SsdpΔ2-92 fails to localize to the nucleus and instead is found throughout the cytoplasm (E). SsdpFL fails to localize to the nucleus in a *Chip*$^{e5.5}$ null mutant background (F).

throughout the cytoplasm of cells lacking zygotic Chip (Fig. 3F). Occasionally, we could detect staining in nuclei in addition to cytoplasmic staining. This may reflect residual activity in these embryos of maternally provided Chip. These results argue that nuclear targeting of Ssdp occurs through a Chip-dependent mechanism.

Drosophila ssdp is expressed in neural and imaginal tissues

The pattern of *ssdp* expression was determined using in situ hybridization of digoxigenin-labeled antisense cRNA probes to embryos and third instar larvae. In embryos of syncytial blastoderm stage, *ssdp* transcript was ubiquitous, suggesting that there is maternal contribution. By the time of germband extension, although still widespread, expression appears to be enriched in the developing central nervous system (CNS) (Fig. 4A). During germband retraction this enrichment of transcript in the embryonic CNS is more apparent (Fig. 4A), such that by stage 13-14 *ssdp* expression is largely restricted to the brain and ventral nerve cord (Fig. 4B). Closer examination of the pattern of expression in the ventral nerve cord suggests that expression occurs in all neurons of the CNS, with no major subclasses excluded (Fig. 4C). This pattern of expression is maintained through later stages of embryogenesis (not shown). In third instar larvae, *ssdp* is no longer detected in the ventral nerve cord (Fig. 4D), but moderate *ssdp* expression is observed in the optic lobes of the brain hemispheres (Fig. 4D,E). High levels of *ssdp* expression are observed in imaginal discs, including the anterior region of the antennal-eye disc (Fig. 4E), the wing and haltere discs (Fig. 4F) and all leg discs (not shown), as well as in the salivary gland (not shown). With the exception of the eye-antennal disc, expression in imaginal discs is largely uniform.

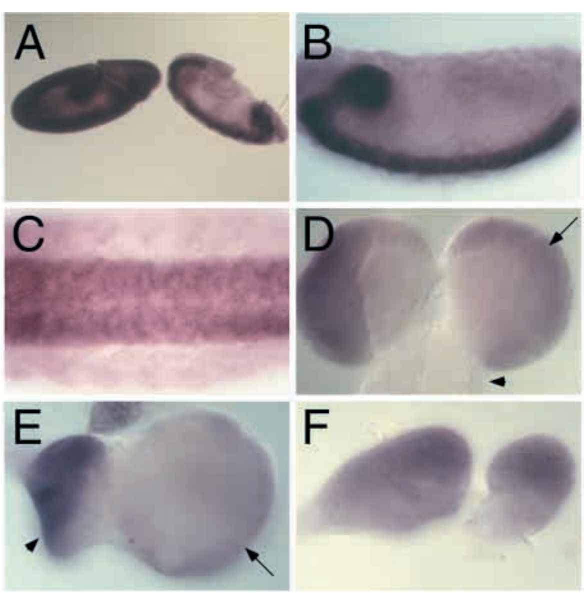


Fig. 4. In situ hybridization of *ssdp* probes to whole-mount embryos and dissected larvae. (A) Embryos at germband extension (stage 11 embryo, left) have widespread expression that increasingly becomes enriched in the developing central nervous system as germband retraction proceeds (stage 12 embryo, right). (B) At stage 13-14 of embryonic development, *ssdp* expression is largely restricted to the brain and ventral nerve cord. (C) Higher power ventral view of the ventral nerve cord showing pan-neuronal expression within the CNS. (D) In third instar larvae, *ssdp* transcripts are not detectable in the ventral nerve cord (arrowhead), but moderate *ssdp* expression was observed in the optic lobes of the brain hemispheres (arrows in D,E). (E,F) High levels of *ssdp* expression are observed in imaginal discs, including the anterior region of the antennal-eye disc (E, arrowhead) and uniform levels in the wing disc (F, left) and haltere disc (F, right).

Generation and analysis of amorphic mutants of *ssdp*

To test the role of Ssdp in vivo, we generated null mutations in the *Drosophila ssdp* gene. P-element transposition was used to imprecisely excise the P-element EP(3)3097 and generate chromosomes carrying deletions that were completely lethal in complementation tests with l(3)neo48. Several deletion lines were thus generated, including *ssdp*^{L7} and *ssdp*^{L5} (Fig. 5A). DNA sequencing of the breakpoints of the *ssdp*^{L7} deletion revealed that it is a complete null allele of *ssdp* (see Materials and Methods) and in all analyses where it has been examined, *ssdp*^{L5} has had effects identical to those of *ssdp*^{L7}, arguing that it too is a null allele.

Each of the P-element and deletion alleles was tested in complementation analyses with the others and viability of the progeny was assessed at first and third instar larval stages using marked balancer chromosomes to distinguish homozygotes. The results are shown in Fig. 5B, and they indicate that the following allelic series exists with respect to increasing severity of the lethal phenotype: *ssdp*$^{l(3)neo48}$<*ssdp*$^{EP(3)3097}$ <*ssdp*$^{EP(3)3004}$<*ssdp*^{L5} and *ssdp*^{L7}. In fact, the combination of EP(3)3097 and l(3)neo48 was not lethal in all cases, with 35% of EP(3)3097/l(3)neo48 individuals surviving through eclosion. Interestingly, most of these viable flies displayed mutant phenotypes, including wing blisters, a mild cleft in the notum along the AP axis, and thin, gnarled macrochaetae on the notum (Fig. 5C,D).

Ssdp can modify Ap/Chip complex activity in the wing

Ap is expressed in the dorsal compartment of the wing disc and is required to establish the DV affinity boundary, the wing margin, wing outgrowth and dorsal-specific wing structures such as sensory bristles (Diaz-Benjumea and Cohen, 1993; Blair et al., 1994). In the absence of Ap, the wing fails to develop (Cohen et al., 1992). We and others have previously shown that Ap functions through a tetrameric complex in which two molecules of Ap are bridged by a homodimer of Chip (Milan and Cohen, 1999; van Meyel et al., 1999). *Chip* mutants interact genetically with *ap* to cause disruptions of the wing margin (Morcillo et al., 1997), and clones of *Chip* mutant cells in the wing disc behave like *ap* mutant clones (Fernandez-Funez et al., 1998; Milan and Cohen, 2000), causing ectopic wing margins and outgrowths.

In contrast to a previous study (Chen et al., 2002), we detected no phenotypes in simple trans-heterozygous combinations of a null allele of *Chip* with any *ssdp* alleles used here, including *ssdp*^{KG03600} and the two null alleles *ssdp*^{L5} and *ssdp*^{L7}. Nor did we detect any phenotypes in transheterozygous combinations of *ap* and *ssdp*. Thus, to address the role of Ssdp in the function of Chip/LIM-HD complexes in vivo, we used the GAL4-UAS system to reduce Ap/Chip complex activity to

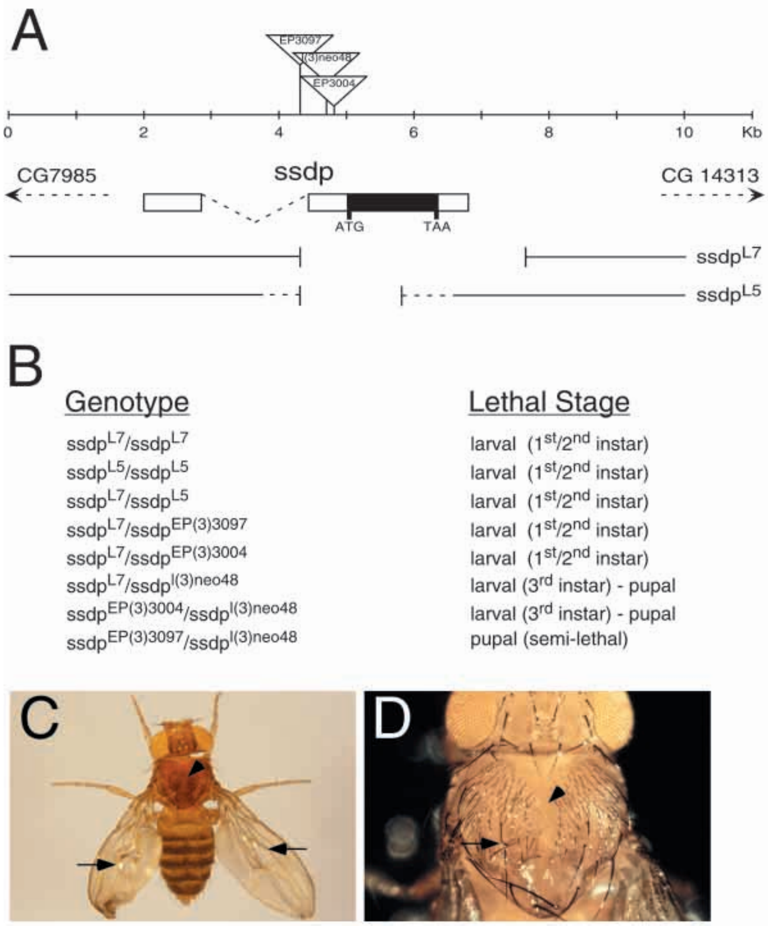


Fig. 5. The *Drosophila ssdp* gene and mutant alleles. (A) Schematic drawing of 11 kb of genomic DNA surrounding the *ssdp* locus. The *ssdp* gene consists of two exons, the second of which contains the entire protein coding sequence. *ssdp* is flanked by two predicted genes of unknown function, CG7985 and CG14313. The insertion sites of three P-elements EP(3)3097, l(3)neo48 and EP(3)3004 are shown, as are the boundaries of two deficiency alleles, $ssdp^{L7}$ and $ssdp^{L5}$, that were generated by imprecise excision of EP(3)3097. (B) Stage of lethality for various allelic combinations of *ssdp*. Viability of individuals of each genotype was assessed at three stages of development: larval first instar, larval third instar and adult eclosion. (C,D) Newly eclosed adults of the genotype $ssdp^{EP(3)3097}/ssdp^{l(3)neo48}$. Note the blistered wings (arrows in C). Other phenotypes for this allelic combination include a cleft along the midline of the notum (arrowhead in C,D), and/or misshapen, mis-oriented, deleted or extra macrochaetae on the notum and scutellum (arrow in D). The phenotype shown in D is frequent and relatively mild, compared with rarer individuals in which the cleft was much more severe (not shown).

levels that would be sensitive to the effects of reducing *ssdp* gene dosage. We used ap^{GAL4}, a GAL4 P-element insertion in the *ap* gene, which faithfully expresses GAL4 in Ap-expressing cells (Calleja et al., 1996), to drive expression of UAS transgenes in the dorsal compartment of the wing disc.

Over-expression of *UAS-Chip* has been shown previously to disrupt wing patterning by titrating endogenous Ap into incomplete complexes in which LID domains of Chip molecules remain vacant (Fernandez-Funez et al., 1998; Milan and Cohen, 1999; van Meyel et al., 1999). Relative to controls (Fig. 6A), such wings are small and lack regular structure, and the wing margin is poorly demarcated (Fig. 6B). These phenotypes resemble hypomorphic *ap* mutants, and can be completely suppressed by simultaneous overexpression of *UAS-ap* (Fernandez-Funez et al., 1998; Milan and Cohen, 1999; van Meyel et al., 1999). This indicates that the stoichiometry between Ap and Chip is an important factor in the formation of functional complexes. We examined the effect of removing one copy of the *ssdp* gene and found that the resulting flies had little or no residual wing tissue, consistent with a further reduction of the activity of the complex (Fig. 6C).

Fusion of Chip and Ap into one chimeric molecule, called ChipΔLID:ApΔLIM, results in a hyperactive complex as it is not susceptible to downregulation of activity imposed by LMO, a LIM-only factor that competes efficiently with Ap for binding with Chip (Milan and Cohen, 1999; van Meyel et al., 1999; Weihe et al., 2001). Flies that overexpress ChipΔLID:ApΔLIM have blistered wings in which the dorsal and ventral surfaces fail to fuse, and which are held upward and away from the thorax in a fashion resembling LMO loss-of-function mutants (Fig. 6D). Removal of one copy of *ssdp* suppresses the blistered wing phenotype and the surfaces fuse properly, although the wings remain held up (Fig. 6E). Thus, Ssdp can modify the activity of Chip/Ap tetrameric complexes of both reduced and hyperactive function.

Finally, we compared the effects of Chip overexpresssion with those produced by expression of a Chip variant lacking the LCCD (ChipΔLCCD). ChipΔLCCD is capable of self-dimerization and binding to Ap, but it cannot bind Ssdp. If Ssdp were required for function of the complex, ChipΔLCCD would be predicted to have a more potent dominant-negative effect on the function of the complex than would Chip itself, as the latter can still recruit Ssdp. Expression of ChipΔLCCD with ap^{GAL4} consistently produced more extreme wing defects than Chip (Fig. 6F). ChipΔLCCD sequesters Ap into nonfunctional complexes, but it cannot bind Ssdp. Therefore, removal of one copy of *ssdp* would not be expected to suppress the phenotype caused by ChipΔLCCD, and indeed it does not (data not shown). Collectively, these results argue that in addition to forming the dimeric bridge for two molecules of Ap, Chip also recruits Ssdp to the complex.

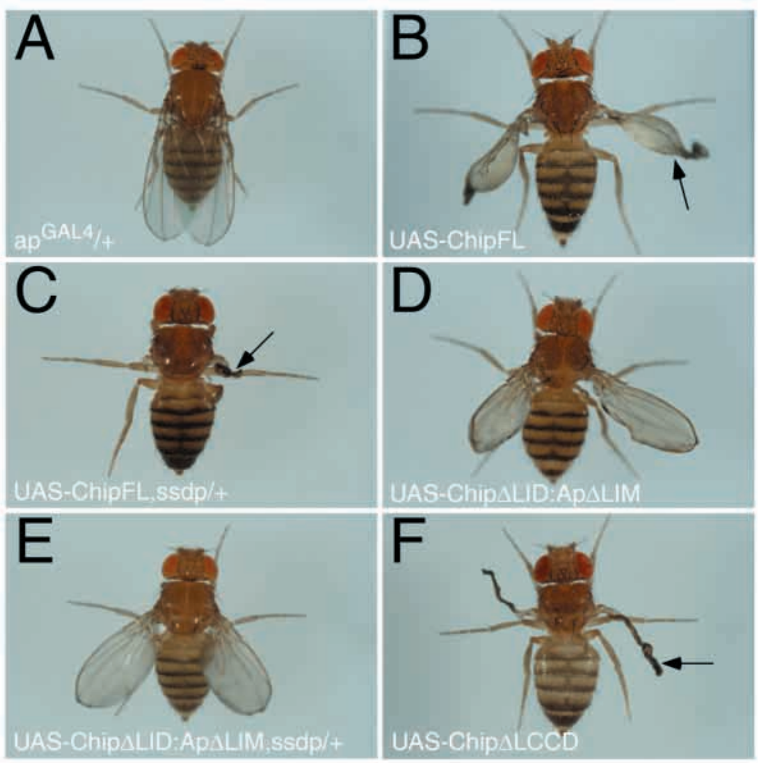


Fig. 6. Ssdp modifies the activity of Chip/Ap tetrameric complexes. Newly eclosed *ap*^{GAL4}/+ flies carrying one copy of each UAS transgene as shown. (A) *ap*^{GAL4}/+ flies exhibit no wing defects. (B) *ap*^{GAL4}/+; UAS-ChipFL/+. Overexpression of full-length Chip (ChipFL) reduces the levels of functional Chip/Ap complexes and results in wings that are small, unfused and with a poorly demarcated margin (arrow). These defects resemble those of hypomorphic *ap* mutants. (C) *ap*^{GAL4}/+;UAS-ChipFL/*ssdp*^{L7}. *ssdp*^{L7} dominantly enhances the wing defects caused by Chip overexpression, leaving little or no organized wing tissue (arrow). (D) *ap*^{GAL4}/+;UAS-ChipΔLID:ApΔLIM/+. The fusion protein ChipΔLID:ApΔLIM results in formation of hyperactive complexes. Flies overexpressing ChipΔLID:ApΔLIM have blistered wings in which the dorsal and ventral surfaces fail to fuse, and which are held upward and away from the thorax in a fashion resembling LMO loss-of-function mutants. (E) *ap*^{GAL4}/+;UAS-ChipΔLID:ApΔLIM/*ssdp*^{L7}. Removal of one copy of *ssdp* from flies expressing ChipΔLID:ApΔLIM suppresses the blistered wing phenotype; the surfaces fuse properly, although the wings remained held up. (F) *ap*^{GAL4}/+;UAS-ChipΔLCCD/+. Expression of ChipΔLCCD, which lacks amino acids 387-426 and thus cannot bind Ssdp but is still capable of homo-dimerization and LIM interaction, results in more severe wing defects than expression of ChipFL, reducing the wing to a ribbon-like process with little discernible structure (arrow).

Generation of *ssdp* mutant clones in the wing disc gives rise to defects that resemble closely those of *ap* and *Chip*

Clones of *ap* mutant cells in the dorsal compartment of the wing disc induce an ectopic wing margin and therefore ectopic wing outgrowth. These *ap* mutant cells differentiate ventral wing margin structures, despite the fact that they remain in the dorsal compartment. *Chip* mutant clones induced in the dorsal compartment give rise to strikingly similar phenotypes (Fernandez-Funez et al., 1998; Milan and Cohen, 2000). The effects of *Chip* clones are influenced both by the timing of their induction as well as their position within the disc (Milan and Cohen, 2000). For example, clones induced later (third instar) resulted in ectopic margin tissue, but did not lead to outgrowth.

If Ssdp were an additional member of the Ap/Chip complex, then mutations of *ssdp* would be predicted to give rise to mutant phenotypes similar to those of *ap* and *Chip*. To test this, we used the FRT/FLP recombinase system to induce clones of cells mutant for *ssdp* in an otherwise heterozygous animal. Clones were generated in larvae at second and third instar by heat-shock induction at 36 hours, 48 hours, 72 hours or 96 hours after egg laying (AEL). The effects of clone induction were observed in newly eclosed adults. Clones of mutant cells were identified by the presence of the cell-autonomous marker *pawn* (*pwn*). Each of the mutant alleles *ssdp*^{L7}, *ssdp*^{L5} and *ssdp*$^{l(3)neo48}$ were tested, as was a control chromosome with no mutation, and the experiment was repeated on four separate occasions, each time observing many individuals of each genotype.

In controls, many clones of various sizes were induced, as evidenced by the presence of *pwn* mutant cells (Fig. 7A). These clones occurred on both the ventral and dorsal surfaces of the wing blade, but no mutant phenotypes were ever observed. By contrast, clones of cells mutant for either *ssdp*^{L7} or *ssdp*^{L5} (as marked by *pwn*) were never observed on either surface of the wing blade, indicating that both alleles have cell-lethal effects in the wing disc. In addition, there were fewer than the expected number of adults eclosing of the appropriate genotype for clone induction, suggesting that the cell-lethal effects, presumably in tissues other than the wing, lead to decreased viability.

In contrast to the cell lethality associated with *ssdp* null alleles, there were striking phenotypes observed in clones of cells mutant for the hypomorphic *ssdp*$^{l(3)neo48}$ allele. We observed many *pwn* mutant clones located both ventrally and dorsally. However, as for *ap* and *Chip* clones, associated phenotypes were found only when the clone arose on the dorsal surface of wing. *ssdp*$^{l(3)neo48}$ clones induced earlier (at 36 hours and 48 hours AEL) gave rise to ectopic margins and occasional wing outgrowth, examples of which are shown in Fig. 7B-F. The outgrowths were associated with *ssdp* mutant cells but were not entirely made up of them, indicating that, as for *ap* and *Chip*, outgrowth resulted from the induction of wild-type tissues in proximity to the clone (Fig. 7D). Clones induced at 72 hours and 96 hours AEL gave rise to margin defects but not outgrowth, indicating that there is a temporal restriction on the extent to which *ssdp* mutation is capable of inducing outgrowth, similar to what has been shown for *Chip*.

Induction of ectopic margin bristles was the most commonly observed effect of dorsal *ssdp* mutant clones. They were primarily observed in proximity to a clone near the native anterior wing margin and comprised at least one row of extra sensory bristles (Fig. 7E,F). Most ectopic bristles were not marked by *pwn*, indicating they were induced by the neighboring mutant (*pwn*) cells. *ssdp*$^{l(3)neo48}$ mutant clones that occurred within the margin, rather than near it, resulted in the

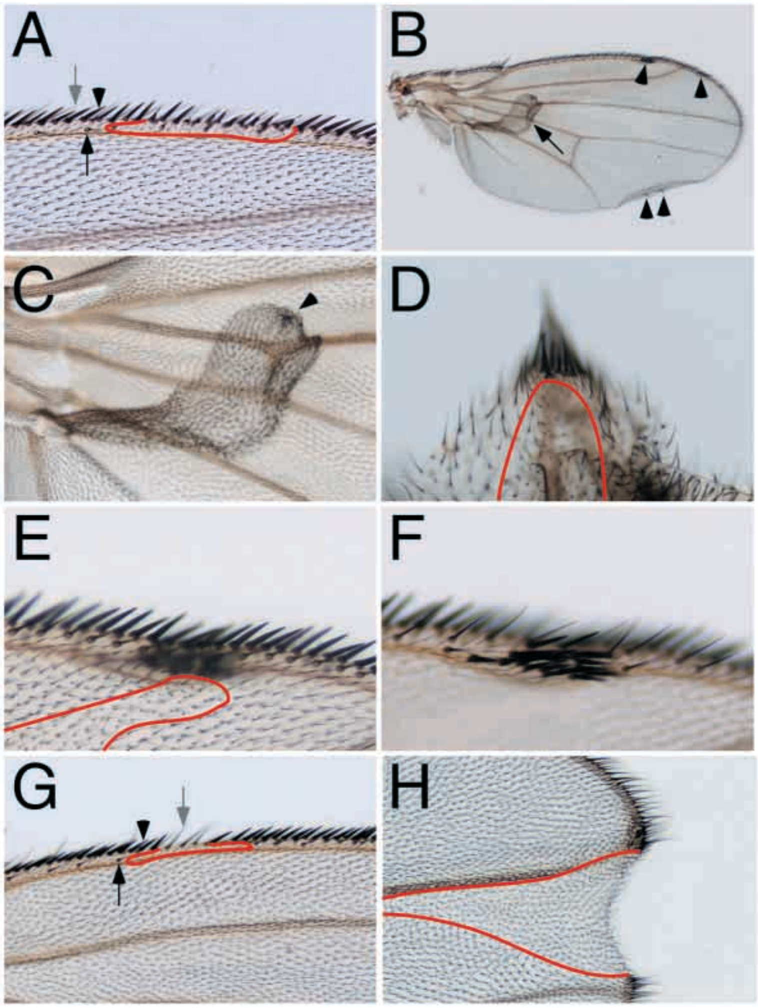


Fig. 7. Mosaic analysis of *ssdp*$^{l(3)neo48}$ mutant clones reveals phenotypes similar to those of *Chip* and *ap*. Mounted wings of newly eclosed adults after heatshock-induced expression of FLP recombinase and subsequent recombination at FRT sites (see Materials and Methods). (A) A control clone along the native wing margin is marked by *pwn*. The border of the clone is outlined in red. Next to the clone the normal arrangement of the triple row of sensory bristles along the margin is shown, including a well-spaced row of dorsal chemosensory bristles (black arrow), a second and more tightly arrayed row of dorsal mechanosensory bristles (arrowhead), and a third row of ventral bristles (out of focal plane, gray arrow). Within the clone, bristles are mutant for *pwn*, but are otherwise normally specified, and there are no associated mutant phenotypes. (B) Low-power image of entire wing showing phenotypes associated with dorsal clones of *ssdp*$^{l(3)neo48}$ mutant cells in an individual in which clones were induced during second larval instar. Phenotypes include ectopic margin formation accompanied by ectopic wing outgrowth (arrow), and ectopic margins in the absence of outgrowth (arrowheads). Phenotypes were never associated with ventral *ssdp*$^{l(3)neo48}$ clones. (C) Higher power view of the outgrowth shown in B. The tip of the outgrowth (arrowhead) has ectopic sensory bristles that resemble the wing margin. (D) Close-up view of outgrowth tip shown in C. The *ssdp*$^{l(3)neo48}$ mutant clone (marked by *pwn* and outlined in red) lies near the tip of the outgrowth and is next to the ectopic margin. (E,F) Two focal planes of the same ectopic margin in the absence of wing outgrowth, the most common phenotype observed for *ssdp*$^{l(3)neo48}$ mutant clones. The extent of the *ssdp*$^{l(3)neo48}$ mutant clone on the dorsal surface of the wing is marked by *pwn* and outlined in red (E). The clone is near but not within the native wing margin, and results in the induction of ectopic bristles shown in the focal plane of F. (G) Loss of dorsal-specific bristles within an *ssdp*$^{l(3)neo48}$ mutant clone (outlined in red) that lies on the native wing margin. Outside the clone both the dorsal-specific chemosensory (black arrow) and mechanosensory (arrowhead) bristles are intact. Within the clone, however, these bristles are lost, despite the fact that the overall structure of the wing is undisturbed. The ventral specific bristles (gray arrow) lie outside the clone and remain intact. (H) A broad *ssdp*$^{l(3)neo48}$ mutant clone (outlined in red) that straddles the margin on both the dorsal and ventral surfaces results in complete loss of wing margin and some wing tissue, resulting in a nicked wing.

loss of dorsal-specific sensory bristles (Fig. 7G). Occasionally a large clone was observed to straddle the dorsoventral boundary, and in these instances, the entire margin, including some nearby non-margin tissue, was lost (Fig. 7H).

In general, there was a striking resemblance between the phenotypes resulting from *ssdp*$^{l(3)neo48}$ mutant clones and those reported for clones of *Chip* or *ap*. This provides strong evidence that Ssdp is an important additional component of Chip/Ap transcriptional complexes in vivo.

DISCUSSION

Ssdp proteins and the function of LIM-HD transcription factors

LIM-HD transcription factors are important regulators of diverse developmental processes in animals. Previously, we and others have shown the importance of the assembly of LIM-HD proteins into tetrameric complexes with the co-factor Ldb1/Chip. We describe the identification and characterization of several members of the Ssdp family of proteins, which specifically interact with Chip/Ldb proteins from both flies and mice. In a recent study, Ssdp1 has been identified as a component of Ldb1-associated nuclear complexes in cultured mammalian cells and has been shown to synergize with Ldb1 and the LIM-HD protein Lim1 to induce secondary axes in *Xenopus* embryos (Chen et al., 2002).

In mice, Ssdp1 and Ssdp2 are expressed broadly (A.D.A, unpublished). Knockout mice for Ssdp2 have been generated and preliminary evidence suggests they die early during embryogenesis (A.D.A., S. Pfaff and S.-K. Lee, unpublished), making it difficult to assess the role of Ssdp2 in LIM-HD functions. Using *Drosophila* as a model, we have shown that mutations in *ssdp* can modify the activities of Chip and the LIM-HD protein Ap in vivo, and that the wing phenotypes caused by *ssdp* mutant clones are strikingly similar to those produced by mutations of *Chip* and *ap*. Our findings provide strong evidence that Ssdp is a functional component of Chip/Ap complexes during development.

The N termini of Ssdp proteins contain a recently described

LUFS domain, which we find is sufficient for interaction with Chip. Within Chip, the highly conserved LCCD is required for Ssdp binding and is a domain that is distinct from those for LIM interactions and homodimerization. It is therefore possible that Chip/Ap tetrameric complexes also include two molecules of Ssdp, each bound specifically to one of the two Chip molecules in the complex.

Ssdp requires the LUFS domain and Chip for correct localization to the nucleus; in the absence of either, Ssdp remains cytoplasmic. Taken together, these results suggest that Ssdp and Chip bind to one another in the cytoplasm, whereupon Ssdp is brought to the nucleus to participate with Chip and Ap in transcriptional regulatory complexes. SSDP was first identified as a DNA binding protein in avian cultured cells, notable because it bound in a sequence-specific manner to a poly-pyrimidine sequence in the promoter region of the α2(I) collagen gene. We do not know whether the ability of Ssdp to bind DNA is required to support the function of the Chip/Ap tetrameric complex in vivo, and as yet the DNA-binding domain of Ssdp is uncharacterized.

Given that Chip/Ldb proteins bind the LIM domains of all LIM-HD proteins, we think it is likely that Ssdp also participates in the function of other LIM-HD proteins in the imaginal tissues and nervous system where it is expressed. However, it is also likely that Ssdp has additional functions outside the context of LIM-HD proteins. The mild cleft observed in the dorsal thorax of adult *ssdp* hypomorphs is similar to that of mutants of the GATA factor *pannier*. Pannier has been shown to complex with Chip and basic helix-loop-helix proteins and promote development of the dorsal thorax (Ramain et al., 2000). It is possible that Ssdp too may play a role in the activity of this complex following recruitment by Chip.

Furthermore, our finding that clones mutant for null alleles of *ssdp* are cell lethal in the wing disc, whereas *Chip* and *ap* clones are not, indicates that Ssdp proteins must have additional functions in wing tissue that are independent of either Chip or Ap.

The LUFS domain is a novel protein interface for transcription regulation in plants and animals

The domains that mediate the interaction between Ssdp proteins and Chip/Ldb are highly conserved, even in plants where the *Arabidopsis* LUFS domain-containing protein LEUNIG cooperates with SEUSS, a protein that shares similarity with Chip/Ldb proteins. Like Ssdp and Chip/Ldb, LEUNIG and SEUSS interact in a yeast two-hybrid assay, although the domains responsible for this interaction have not been mapped (Franks et al., 2002). In addition, genetic analyses has revealed that these proteins cooperate in the transcriptional regulation of AGAMOUS, a homeotic gene functioning in flower development. However, domains within LEUNIG outside of the LUFS domain are different from Ssdp proteins and include glutamate-rich regions and WD40 repeats (Conner and Liu, 2000). LEUNIG is probably a transcriptional co-repressor, based on its regulation of AGAMOUS plus its overall structural similarity to the yeast co-repressor Tup1 (Liu and Meyerowitz, 1995; Conner and Liu, 2000). Given that the effects of *ssdp* mutation in the Drosophila wing phenocopy those of *Chip* and *ap*, we view Ssdp as a likely activator of the complex, not a repressor, and propose that this fundamental difference between LEUNIG and Ssdp proteins lies in the functional domains C-terminal to the LUFS domain where these proteins bear no resemblance to one another.

The intriguing conservation from plants to vertebrates of the interaction between the LUFS domain and sequences within Chip/Ldb and SEUSS proteins suggest a fundamentally important interaction to enable regulated control of transcription. However, unlike Ldb1 and Chip, SEUSS has no LIM interaction domain, nor are there any LIM-HD proteins in plants. It is possible that interactions between LUFS domains and Chip/Ldb/SEUSS proteins exemplify an ancient transcriptional regulatory function that has been recruited by LIM-HD proteins in animals by the addition of a LIM interaction domain to Chip/Ldb.

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