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# Chip is an essential cofactor for Apterous in the regulation of axon guidance in *Drosophila*

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## SUMMARY

**LIM-homeodomain transcription factors are expressed in subsets of neurons and are required for correct axon guidance and neurotransmitter identity. The LIM-homeodomain family member Apterous requires the LIM-binding protein Chip to execute patterned outgrowth of the *Drosophila* wing. To determine whether Chip is a general cofactor for diverse LIM-homeodomain functions in vivo, we studied its role in the embryonic nervous system. Loss-of-function *Chip* mutations cause defects in neurotransmitter production that mimic *apterous* and *islet* mutants. Chip is also required cell-autonomously by Apterous-expressing neurons for proper axon guidance, and requires both a homodimerization domain and a LIM interaction domain to function appropriately. Using a Chip/Apterous chimeric molecule lacking domains normally required for their interaction, we reconstituted the complex and rescued the axon guidance defects of *apterous* mutants, of *Chip* mutants and of embryos doubly mutant for both *apterous* and *Chip*. Our results indicate that Chip participates in a range of developmental programs controlled by LIM-homeodomain proteins and that a tetrameric complex comprising two Apterous molecules bridged by a Chip homodimer is the functional unit through which Apterous acts during neuronal differentiation.**

Key words: LIM domain, Homeodomain, *Drosophila*, Axon guidance

## INTRODUCTION

Even simple nervous systems contain remarkably diverse neuronal cell types, each characterized by distinct properties such as axon trajectory and the type of neurotransmitter synthesized. The acquisition of these properties is determined in part by activation of cell-specific repertoires of gene expression. Studies of diverse species have shown that members of the LIM-homeodomain (LIM-HD) family of transcription factors are expressed in subsets of neurons and are required for key aspects of neuronal differentiation (Way and Chalfie, 1988; Lundgren et al., 1995; Pfaff et al., 1996; Hobert et al., 1997; Thor and Thomas, 1997; Benveniste et al., 1998; Sharma et al., 1998; Hobert et al., 1999). In the spinal cord of vertebrates and in the ventral nerve cord (VNC) of *Drosophila*, the expression of specific combinations of LIM-HD family members constitutes a functional "LIM-HD combinatorial code" that dictates axon pathway selection of motor neurons (Sharma et al., 1998; Thor et al., 1999). The *Drosophila* LIM-HD genes *islet* (*isl*), *lim3* and *apterous (ap)* direct axon pathfinding and neurotransmitter identity in subsets of postmitotic neurons of the embryonic VNC. *isl* is required for transmitter production by dopaminergic neurons (Thor and Thomas, 1997), and both *isl* and *lim3* control motor neuron pathway selection in the intersegmental nerve (ISN) (Thor et al., 1999). In addition to its role in wing morphogenesis (Cohen et al., 1992; Diaz-Benjumea and Cohen, 1993; Blair et al., 1994), *ap* is required within the VNC for correct axon guidance of a subset of interneurons and for expression of the neurotransmitter dFMRFamide (dFMRFa) in neuroendocrine cells (Lundgren et al., 1995; Benveniste et al., 1998).

LIM-HD proteins contain a DNA-binding homeodomain plus two cysteine-rich LIM domains (Freyd et al., 1990; Karlsson et al., 1990) that mediate interactions with other proteins (Schmeichel and Beckerle, 1994; Arber and Caroni, 1996). The LIM domains of all LIM-HD proteins, as well as those of nuclear LIM-only (LMO) proteins, are bound by Nuclear LIM Interactor (NLI), first identified in mouse and also known as Ldb1 or CLIM-2 (Agulnick et al., 1996; Jurata et al., 1996; Bach et al., 1997). NLI homodimerizes in vitro and thereby coordinates LIM-HD and LMO transcription factors into homomeric and heteromeric complexes by forming a bridge between two LIM-HD proteins (Jurata et al., 1998). NLI is expressed widely throughout embryonic development of the mouse, with elevated levels in the CNS where broad domains of NLI expression overlap with those of the LIM-HD

proteins Islet-1, Lhx-3 and Lhx-2 among others (Jurata et al., 1996; Bach et al., 1997). Analysis of mice mutant for NLI has revealed that genetic dissection of its function in the CNS will be complicated by an early requirement for NLI at gastrulation (A. Agulnick, personal communication).

Experiments with the *Drosophila* ortholog of NLI, called Chip (dLdb), provided the initial evidence that interactions between these cofactors and LIM domains are required in normal development. Chip interacts with the LIM domains of Apterous (Ap), and genetic experiments indicated that Chip supports Ap activity during wing development (Morcillo et al., 1997; Fernandez-Funez et al., 1998). In vitro biochemical assays and further genetic experiments in vivo suggest that the active form of Chip and Ap in the wing is a tetramer comprising two Ap molecules bridged by a Chip homodimer (Milan and Cohen, 1999; van Meyel et al., 1999). This complex is required for Ap activity in dorsoventral patterning and outgrowth of the wing and is subject to disruption by dLMO, a nuclear LIM-only protein that can compete with LIM-HDs for binding to Chip (Milan et al., 1998; Shoresh et al., 1998; Milan and Cohen, 1999). In the wing, dLMO expression is upregulated by Ap, providing a mechanism for negative feedback upon Chip/Ap tetrameric complexes and modulation of Ap activity (Milan et al., 1998).

Like NLI, Chip is expressed widely during embryogenesis and interacts with all LIM-HD proteins in vitro, suggesting it may be a requisite component of many transcription regulatory complexes involving LIM-HD factors (van Meyel et al., 1999). To determine whether Chip is a general cofactor for diverse LIM-HD functions in vivo, we studied the role of Chip in Apterous and Islet functions in the embryonic nervous system. Here we show that loss-of-function *Chip* mutants have defects similar to *ap* and *isl* mutants including loss of neurotransmitter production in Ap and Isl cells and aberrant axon pathfinding of the Ap neurons. Chip acts cell autonomously during axon guidance of the Ap neurons, and requires intact domains for self-dimerization and for interaction with LIM domains, suggesting that, as in wing development, Chip and Ap are required to interact physically and form a tetrameric complex in Ap neurons. To test this model in the nervous system, we reconstituted the complex using a Chip/Ap chimera lacking domains normally required for their interaction. The Chip/Ap chimera rescues the axon guidance defects of *ap* mutants, of *Chip* mutants and of embryos doubly mutant for both *ap* and *Chip*. Our results provide the first functional evidence in vivo that Chip participates in a range of developmental programs controlled by LIM-HD proteins, and that a Chip/LIM-HD complex is the primary functional unit through which LIM-HD proteins control transcriptional regulation of neuronal differentiation.

## MATERIALS AND METHODS

### Fly strains and genetics

A description of all UAS constructs and the generation of transgenic *Drosophila* lines has been published previously (van Meyel et al., 1999). Multiple lines were generated for each UAS transgene and those that exhibited strongest GAL4-directed c-myc expression were selected for analysis and recombined with a UAS-*tau-lacZ* reporter for axon tracing (Callahan and Thomas, 1994). For mutant analyses, we recombined $Chip^{e5.5}$ onto each of the $ap^{GAL4}$ and $ap^{P44}$ chromosomes, then introduced UAS transgenes into these compound heterozygotes and intercrossed them to achieve the appropriate mutant background. Activation of dFMRFamide expression was assayed using a *Tv-lacZ* reporter on the X chromosome (Benveniste et al., 1998). All fly crosses and embryo collections were carried out at 25°C.

### Immunohistochemistry

Embryo dissection and HRP immunostaining were performed as described previously (Callahan and Thomas, 1994; Thor and Thomas, 1997; O'Keefe et al., 1998). For fluorescence immunostaining to detect Chip and Ap simultaneously, dissected embryos were incubated at 4°C overnight with rat anti-Ap antibody diluted 1:1000 (Lundgren et al., 1995). They were then rinsed, and incubated simultaneously with affinity-purified rabbit anti-Chip antibody (diluted 1:75) and a biotin-conjugated anti-rat secondary antibody (Vector; diluted 1:500) at room temperature for 2 hours. Subsequently, FITC-conjugated streptavidin (Jackson Immunoresearch Labs; diluted 1:400) and Cy-3-conjugated anti-rabbit antibody (Jackson Immunoresearch, diluted 1:500) were incubated for 1 hour at room temperature, rinsed and mounted for microscopy. To simultaneously localize Ap- and dLMO-expressing cells, we double labelled $ap^{GAL4}$/+; UAS-*tau-lacZ*/+ embryos with mouse anti-dLMO sera (diluted 1:100) and rabbit anti-β-galactosidase (diluted 1:1000) as above.

### Quantification of axon pathfinding errors

To compare the occurrence of Ap interneuron pathfinding errors among embryos of different genotypes, one observer (D.D.O'K.) was blinded to the genotype of each embryo and scored abdominal segments A1-A7 for the presence or absence of pathfinding errors. The mean percentage of wild-type segments per embryo was calculated (average $n$=10 embryos per genotype), and for each genotype the mean value was compared with the others in a multiple comparison analysis of variance (ANOVA) using the Student-Neuman-Keuls test ($P$<0.05).

## RESULTS

### Chip expression in the CNS

Chip is expressed in most, if not all, embryonic and larval tissues (Morcillo et al., 1997). In wild-type embryos, we found strong, nuclear Chip expression throughout the developing VNC with no apparent subclasses of neurons excluded (Fig. 1A). A substantial fraction of embryonic Chip is contributed maternally during oogenesis, and this maternally derived expression is required for early embryonic segmentation (Morcillo et al., 1997). To estimate the relative contribution of zygotic and maternally derived Chip to the embryonic VNC, we examined homozygous embryos mutant for $Chip^{e5.5}$, a null allele encoding a truncated protein, and $Chip^{\gamma 230}$, a deficiency that completely removes *Chip*. Derived from an intercross of heterozygous parents, these mutants are expected to retain half of the maternal and no zygotic Chip expression. We observed little reduction of staining in mutant embryos relative to *Chip*/+ heterozygotes (data not shown). Thus it appears a substantial fraction of Chip in the VNC is provided maternally.

Co-labelling embryos with anti-Ap and anti-Chip antibodies revealed that Chip expression overlaps with all the Ap neurons of the developing VNC (Fig. 1A). Based upon previous studies of biochemical interactions and wing development, we propose that, in the nervous system, Ap could function in a tetrameric complex with Chip. However, it is also possible that Ap could

act in a Chip-independent fashion if Chip were sequestered in Ap cells by co-expression of dLMO. Using anti-dLMO antibodies (Milan et al., 1998), we found that dLMO is not expressed in the Ap neurons of the developing VNC (Fig. 1B), suggesting that Chip is free to participate in complex formation and Ap function (Fig. 1C).

## Chip is required for neurotransmitter identities regulated by Ap and Isl

If Chip were required for Ap function, elimination of Chip might be expected to result in an *ap*-like phenotype. The requirement of maternally supplied Chip in segmentation (Morcillo et al., 1997) precluded an examination of the effects of eliminating both maternal and zygotic Chip on neuronal development. Thus, we assayed neurotransmitter expression and axon guidance in $Chip^{e5.5}$ mutants in which half of the maternal and all of the zygotic Chip expression were absent.

In each thoracic hemisegment of the VNC, *ap* is expressed in a lateral cluster of four neurons, one of which is the Tv neuroendocrine cell that expresses the neurotransmitter dFMRFa (Schneider et al., 1993). In wild-type embryos, there are a total of six Tv cells, one in each thoracic hemisegment. In *ap* mutants, the Tv neurons are present, but half of all Tv neurons stochastically fail to express dFMRFa (Benveniste et al., 1998). This regulation of dFMRFa by *ap* is transcriptional, since expression of a fusion transgene comprising a 446 bp Tv neuron-specific enhancer of the *dFMRFa* gene driving β-galactosidase (*Tv-lacZ*, Fig. 2A) is similarly reduced in *ap* mutants. Ap binds in vitro to each of three sequences within the enhancer, and mutagenesis of these sites confirmed that these sequences are important for *Tv-lacZ* expression in vivo (Benveniste et al., 1998).

To determine whether reduction of Chip results in an *ap*-like reduction in transcriptional activation of *dFMRFa*, expression of the *Tv-lacZ* reporter transgene was assayed in wild-type, $ap^{P44}$ and *Chip* mutant embryos (Fig. 2). Both $ap^{P44}$ and $Chip^{e5.5}$ mutant embryos show decreased *Tv-lacZ* activity in Tv neurons relative to wild-type controls (Fig. 2B-D,F), implicating *Chip* in the establishment of this Ap-regulated aspect of neuronal differentiation. The reduction of *Tv-lacZ* activity was less severe in *Chip* null mutants than *ap* null mutants, probably because of the maternally supplied Chip remaining in *Chip* mutants. In embryos homozygous for the antimorphic $Chip^{\gamma 96.1}$ mutation (Torigoi et al., 2000), *Tv-lacZ* expression was reduced further than *Chip* null mutants but not to the level of *ap* mutants (Fig. 2E,F).

Like Ap, the LIM HD protein Isl also regulates neurotransmitter identity of embryonic neurons. There are three dopaminergic cells per segment of the VNC, one unpaired midline cell and a pair of dorsal lateral cells (Fig. 3A,B), all of which express Isl protein and thus represent a subset of the *isl* interneurons. *isl* mutants show loss of expression of tyrosine hydroxylase (TH), a rate-limiting enzyme in the synthesis of dopamine (Thor and Thomas, 1997). Double-labelling immunochemistry showed that dLMO is not expressed in the *isl* neurons of the embryonic VNC (data not shown), suggesting that, in these neurons, Chip is free to associate with Isl. To test the role of Chip in the expression of TH, we stained late-stage wild-type and *Chip* mutant embryos with anti-TH antibodies. Homozygous $Chip^{e5.5}$ mutant embryos retain TH expression in the ventral unpaired midline cells, but few of the dorsal lateral cells express TH, and in those that do, TH levels are significantly reduced relative to wild-type (Fig. 3C,D). In embryos homozygous for the $Chip^{\gamma 96.1}$ antimorph, TH expression was greatly diminished in both the ventral midline and dorsal lateral dopaminergic neurons (Fig. 3E,F). While it is clear that the paired dorsal TH cells are more sensitive to the reduction in Chip dosage than the unpaired ventral cells, the effects of the antimorphic $Chip^{\gamma 96.1}$ allele suggest that TH production in the latter cells is also dependent on Chip. From these results, together with the above results on the expression of FMRFamide, we conclude that Chip is required for both Ap- and Islet-regulated neurotransmitter production in the CNS.

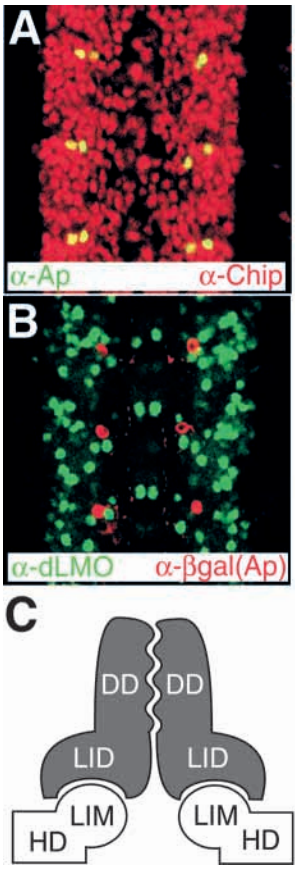


**Fig. 1.** Chip is highly expressed in the CNS. (A) Double immunofluorescence labeling for Apterous (green) and Chip (red) in a dissected embryonic VNC (stage 15-16) of a wild-type embryo. Three adjacent abdominal segments are shown. Ap expression is restricted to the nuclei of three neurons per hemisegment, the two most ventral of which are visible in the focal plane shown. Chip is expressed widely throughout the VNC including the Ap neurons where co-labelling appears yellow. (B) Double-labeling for Ap and dLMO, a LIM-only cofactor known to modulate Chip interaction with Ap in the *Drosophila* wing. The Ap neurons are visualized using $ap^{GAL4}$ to drive expression of the axon targeting UAS-*tau-lacZ* transgene within the Ap neurons. Only the dorsalmost Ap-expressing cell in each hemi-segment is shown. Ap and dLMO are not co-expressed in the VNC at this time. (C) Diagram showing postulated Chip interactions with *Drosophila* LIM-HD proteins to form Chip-bridged tetrameric complexes. The Chip domains responsible for Dimerization (DD) and LIM Interaction (LID) are indicated, as are the LIM- and homeodomains (HD) of LIM-HD transcription factors like Ap.

## Chip is required for correct axon pathway selection of Ap interneurons

For a detailed analysis of Chip's role in neuronal differentiation, we studied axon guidance of the Ap-expressing interneurons. To visualize the axon trajectories of the Ap neurons, we used $ap^{GAL4}$, a P[GAL4] insertion in the *ap* locus, to drive expression of the axon tracing reporter UAS-*tau-lacZ* in the *ap* cells of the CNS (Calleja et al., 1996; O'Keefe et al., 1998). Within each abdominal hemisegment of wild-type embryos, Ap is expressed by three interneurons that extend axons ipsilaterally and anteriorly within each longitudinal connective (Lundgren et al., 1995). Upon reaching the adjacent anterior segment, the Ap neurons tightly fasciculate with their

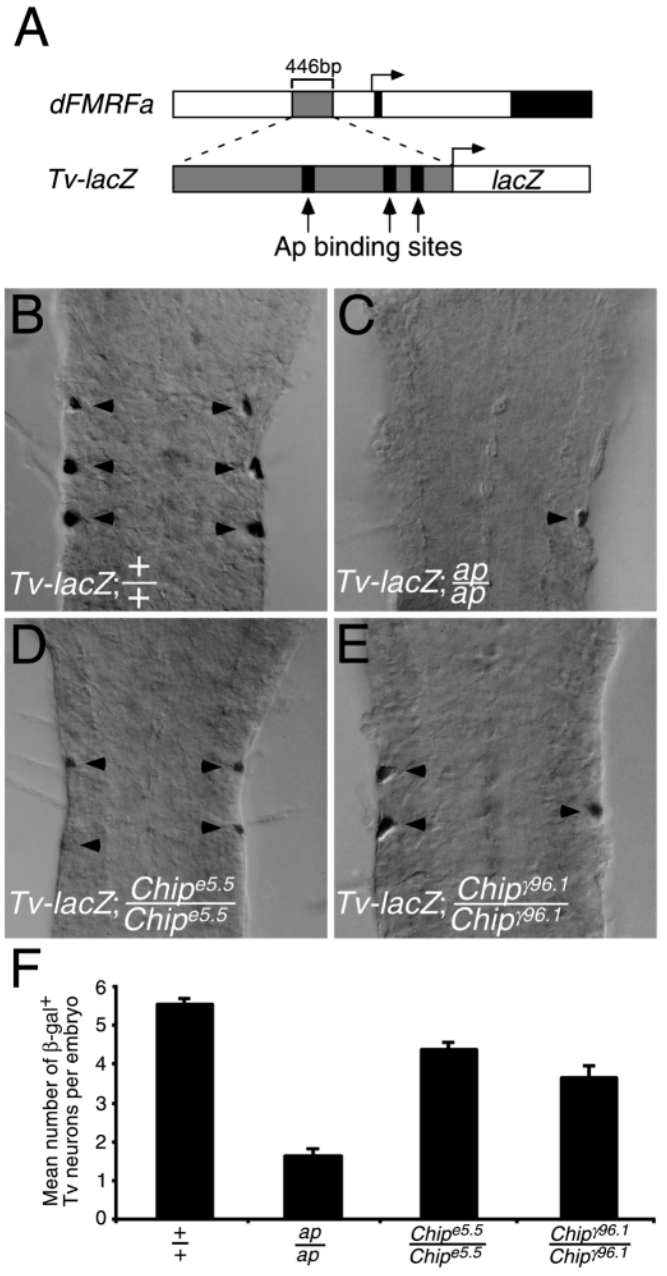


**Fig. 2.** Chip is required for transcriptional activity of an Ap-regulated enhancer of the *dFMRFa* neurotransmitter gene. (A) Schematic representation of the *dFMRFa* neurotransmitter gene and a 446bp Tv neuron specific enhancer fused to the *lacZ* reporter gene (*Tv-lacZ*) (Schneider et al., 1993). The neuronal enhancer contains Ap-binding sites that are required for expression of *Tv-lacZ* in vivo (Benveniste et al., 1998). (B-E) Dissected CNS from late stage 17 embryos using anti-β-galactosidase plus HRP immunochemistry to detect activity of the *Tv-lacZ* transgene. (B) In wild-type (+/+) embryos, there are six β-gal-positive Tv neurons (arrowheads) located laterally within the ventral nerve cord. These cells express Ap, Chip and dFMRFa proteins and project axons to the neurohemal organ, which lies dorsal to the plane of focus along the midline. (C) In $ap^{P44}$ null mutants, Tv neurons stochastically fail to express *Tv-lacZ* as shown in this mutant embryo with only a single β-gal-positive cell (arrowhead). (D) $Chip^{e5.5}$ null mutants also have fewer Tv neurons that express β-gal, as represented here by an individual with only four (arrowheads). (E) *Tv-lacZ* expression is further reduced in embryos homozygous for an antimorphic $Chip^{\gamma 96.1}$allele that is predicted to act in a dominant-negative fashion to disrupt the function of maternally provided Chip (Morcillo et al., 1997; Torigoi et al., 2000). In this individual there are only three β-gal-positive cells (arrowheads). (F) Histogram comparing *Tv-lacZ* activity in wild-type (+/+), $ap^{P44}$ mutant and *Chip* mutant late stage 17 embryos. The mean number of β-gal-positive Tv neurons per embryo was calculated (average $n$=32 embryos). Error bars designate the standard error (s.e.m.). The differences between all four genotypes were statistically significant in a multiple comparison analysis of variance (ANOVA) using the Student-Neuman-Keuls test ($P<0.05$).

homologs, forming a discrete axon bundle running the length of the VNC. $ap^{GAL4}$ is a mutant allele of *ap*, but $ap^{GAL4}$/+ heterozygotes display few pathfinding errors, with 93% of segments showing no defects (Fig. 4B,H). In *ap* mutants ($ap^{GAL4}/ap^{P44}$), all Ap interneurons survive and extend axons, but these axons display pathfinding errors in nearly every segment, including defasciculation of axon bundles, aberrant pathway selection within the longitudinal connectives, occasional misrouting across the midline, and stalling (Fig. 4C,H).

To determine whether reduction of Chip causes *ap*-like defects of axon guidance, we first examined embryos transheterozygous for $ap^{GAL4}$ and $Chip^{e5.5}$. We detected no increase in axon guidance defects over $Chip^{e5.5}$/+ or $ap^{GAL4}$/+ individuals. We next examined homozygous $Chip^{e5.5}$ mutants, in which the levels of Chip should be further reduced, and found they did exhibit the same array of pathfinding errors as *ap* mutants (Fig. 4D). We quantified the incidence of pathfinding errors relative to $ap^{GAL4}$/+ controls and found fewer errors than occur in *ap* mutants (Fig. 4H). Thus, axon guidance defects, like the reduction of *Tv-lacZ* activity, are more moderate in $Chip^{e5.5}$ mutants than in *ap* mutants, likely due to maternally supplied Chip. Pathfinding errors in $Chip^{e5.5}$ mutants occurred in the absence of any discernible disorganization of neurons or neuroblast lineages, as determined by immunochemical staining with antibodies to Engrailed, Even-skipped and Prospero (data not shown). Furthermore, as assayed with an antibody to Fasciclin II (Fas II) (Van Vactor et al., 1993), we detected no defects in the pathfinding of Fas II-expressing cells indicating that errors of axon guidance of the Ap neurons occurred without concomitant errors among large subsets of other neurons.

Introduction of full-length Chip (ChipFL) specifically into Ap interneurons of $Chip^{e5.5}$ mutant embryos using a UAS-*ChipFL* transgene rescued pathfinding errors completely to the level of $ap^{GAL4}$/+ controls (Fig. 4E), showing that Chip activity is required cell-autonomously within Ap neurons for correct axon pathfinding. In contrast, mutant Chip proteins lacking either the LIM Interaction Domain (ChipΔLID, Fig. 4F) or the Dimerization Domain (ChipΔDD, Fig. 4G) failed to rescue the pathfinding errors, despite being expressed at high levels and properly localized to nuclei. This indicates that binding to LIM domains and self-dimerization are both required for Chip function. ChipΔDD, which acts in a dominant negative fashion in wing development (van Meyel et al., 1999), increased the incidence of pathfinding errors (Fig. 4H), presumably because it prevents Ap from associating with maternally supplied endogenous Chip.

To further test the role of Chip in LIM-HD-mediated axon guidance, we examined the Isl-expressing ISN motor neurons

in *Chip*$^{e5.5}$ and *Chip*$^{\gamma 96.1}$ homozygous mutant embryos using the anti-Fas II antibody. In *isl* mutants, these motor neurons fail to innervate their appropriate muscle targets (Thor and Thomas, 1997). In contrast to the Ap neurons, we detected no clear alterations in projections of the Isl motor neurons in *Chip* mutants (data not shown). This suggests that the levels of Chip may not be reduced sufficiently to affect these neurons, similar to the reduced sensitivity of the unpaired ventral TH-expressing Isl cells. Alternatively, Isl might function during pathway selection of these neurons in a Chip-independent fashion, a possibility consistent with our previous finding that Isl, but not Ap, is uniquely able to form heterodimers directly with Lim3 and that these Isl/Lim3 heterodimers may be favored over Isl/Chip complexes (van Meyel et al., 1999).

### Chip and Ap are required to physically interact for function in axon guidance

In *Drosophila* wing development, the active form of Ap appears to be a tetramer, in which the LIM domains of Ap and the LID of Chip permit the homeodomains of two Ap molecules to be spanned by a Chip homodimer (Milan and Cohen, 1999; van Meyel et al., 1999). To determine whether Ap function in the nervous system also requires formation of a complex with Chip, we removed the domains responsible for this interaction and tethered the remainder of each protein together, yielding the chimera ChipΔLID:ApΔLIM. This chimera is capable of rescuing wing defects of *ap* mutants (van Meyel et al., 1999). Using *ap*$^{GAL4}$ to drive expression of a UAS-*ChipΔLID:ApΔLIM* transgene, we tested whether the chimera could also rescue axon guidance defects in *ap* mutants. Axon fasciculation and correct pathway selection were restored in a majority of segments examined from *ap* mutant embryos carrying the chimera transgene, indicating that the axon guidance defects of *ap* mutants were rescued by the chimera (Fig. 5B,F). In contrast, simultaneous introduction of unfused UAS-*ApΔLIM* and UAS-*ChipΔLID* transgenes failed to rescue the defects in *ap* mutants (data not shown).

The observation that reduced Chip expression in the VNC causes defects in axon guidance of the Ap interneurons provided us with an opportunity to assess the ability of the ChipΔLID:ApΔLIM chimera to rescue a *Chip* mutant phenotype. We found the chimera rescued axon defects in homozygous *Chip*$^{e5.5}$ embryos to a level equal to that achieved by full-length Chip and to that of *ap*$^{GAL4}$/+ controls (Fig. 5C,F). This result confirms that the sole function of the 32 amino acid LID domain of Chip is to bind Ap, since the effects of its removal are negligible when the remainder of Chip is tethered directly to Ap.

Although the sole function of the Chip LID appears to be to bind the LIM domains of Ap, the fact that the chimera only partially rescues the axon guidance defects of *ap* mutants suggested a major but not exclusive role for the Ap LIM domains in binding Chip. Therefore we hypothesized that the chimera should also only partially rescue axon pathfinding of embryos doubly mutant for both *ap* and *Chip*. Double mutants exhibit the same types of pathfinding errors as single mutants, and these occur with a frequency equal to that of *ap* mutants (Fig. 5D,F). We indeed observed that the ChipΔLID:ApΔLIM chimera rescued double mutants but, unexpectedly, this rescue was to the level of *ap*$^{GAL4}$/+ controls, significantly higher than

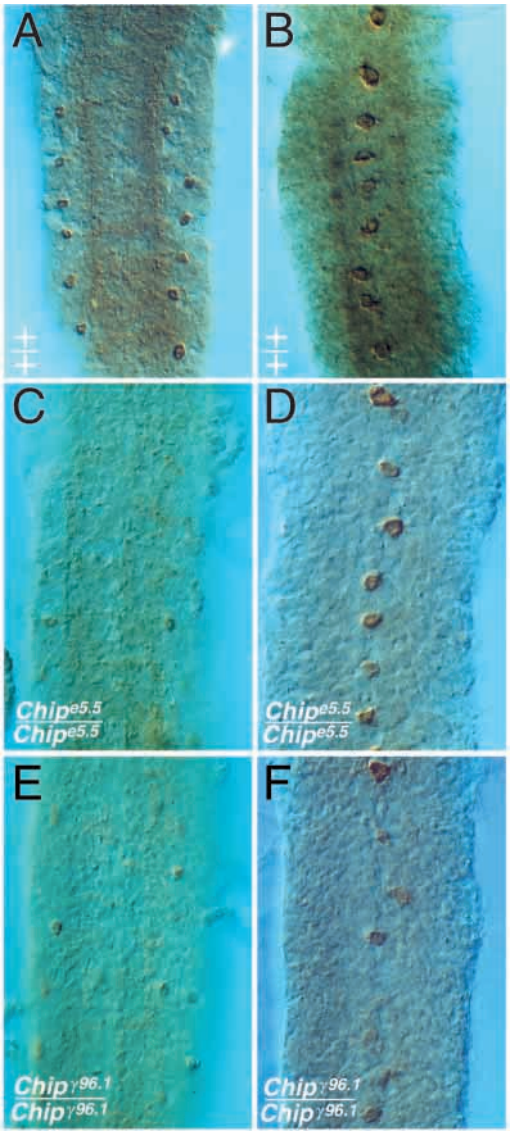


**Fig. 3.** Tyrosine hydroxylase expression is diminished in *Chip* mutants. (A,B) In the VNC of wild-type late stage 17 embryos, tyrosine hydroxylase (TH), the rate-limiting enzyme in the synthesis of dopamine, is expressed in two lateral rows of dorsal cells (A) and in a single row of ventral midline cells (B), as determined using anti-TH antibodies and HRP immunochemistry. TH expression is completely dependent upon the LIM-HD protein Islet (Isl) and misexpression of Islet is sufficient to guide ectopic TH expression in inappropriate cells (Thor and Thomas, 1997). (C,D) In homozygous *Chip*$^{e5.5}$ null mutants, TH expression is dramatically reduced or absent in the dorsal cells (C), but remains unaffected in ventral cells (D). (E,F) TH expression in both the dorsal (E) and ventral (F) cells is reduced or absent in embryos homozygous for an antimorphic *Chip*$^{\gamma 96.1}$ allele.

that achieved in rescued *ap* single mutants (Fig. 5E,F). We suspect that the chimera cannot completely rescue the *ap* phenotype because there remain wild-type levels of endogenous Chip in *ap* mutants and that this pool of Chip is free to dimerize with the chimera and form non-functional complexes with vacant LID sites. Regardless of the mechanism, the complete rescue of double mutants reveals that

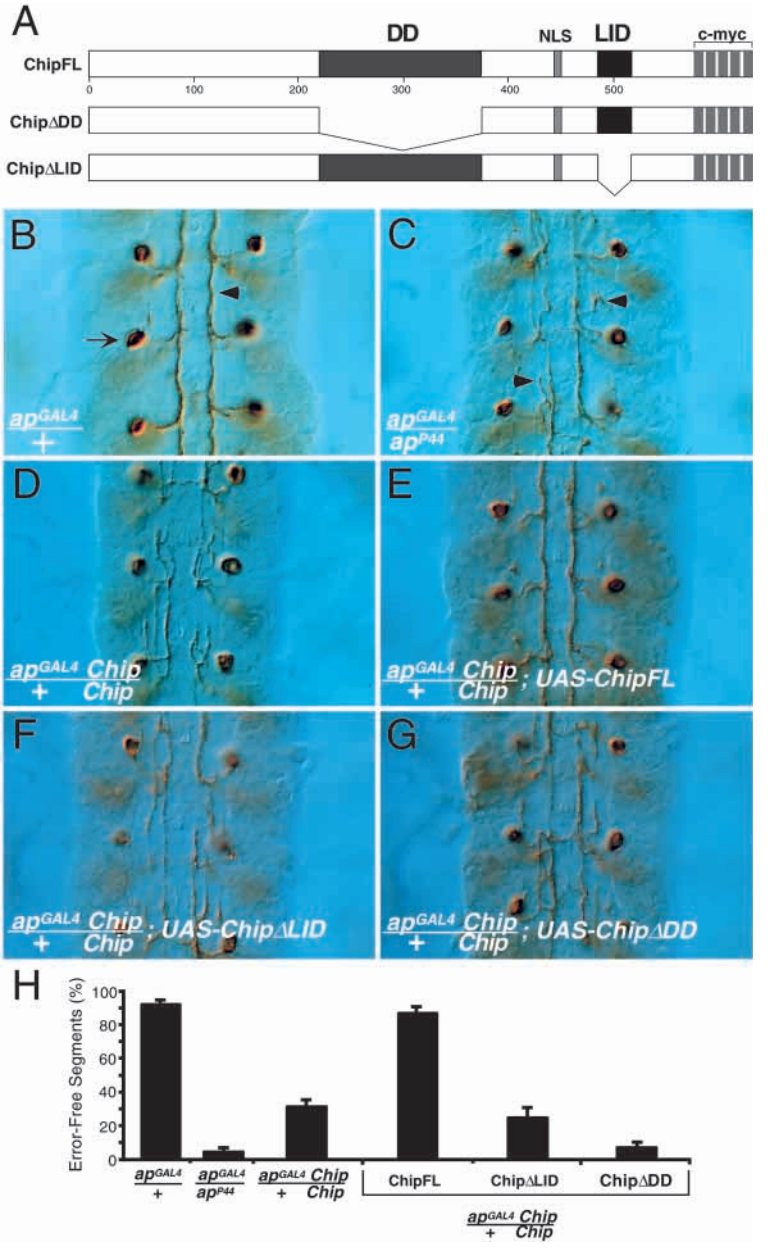


**Fig. 4.** Cell autonomous Chip activity is required for correct axon pathfinding of Ap interneurons, and is dependent upon intact domains for dimerization and LIM-interaction. (A) Schematic diagram of recombinant *c-myc*-tagged Chip derivatives. At top is full-length Chip (ChipFL), showing the Dimerization Domain (DD) and LIM Interaction Domain (LID). The site of a nuclear localization sequence (NLS) is indicated. Below are mutant Chip proteins lacking amino acids 221-376 (ChipΔDD) or 484-515 (ChipΔLID) used in GAL4/UAS studies. (B-G) Dissected VNCs from stage 16-17 embryos carrying both $ap^{GAL4}$ and UAS-*tau-lacZ* to visualize the axon projections of Ap neurons. $ap^{GAL4}$ directs UAS-transgene expression specifically to Ap-expressing cells, and is a mutant allele of *ap*. Embryos were stained for Tau-β-gal using HRP immunohistochemistry. (B) In this $ap^{GAL4}$/+ embryo, the arrow indicates the cell body of a dorsal Ap-expressing interneuron, and the arrowhead shows tightly fasciculated Ap neurons forming a discrete axon bundle running within the connectives along the correct pathway (arrowhead). (C) In $ap^{GAL4}/ap^{P44}$ mutants, the axons of Ap neurons display pathfinding errors, wandering within the connectives and failing to fasciculate (arrowheads). (D) Introduction of a homozygous $Chip^{e5.5}$ null mutation into $ap^{GAL4}$/+ heterozygotes results in pathfinding errors by the Ap neurons similar to $ap^{GAL4}/ap^{P44}$ mutants. The neurons are highly defasciculated, choosing inappropriate pathways within the connectives. (E) Reintroduction of Chip protein specifically into the Ap neurons of $Chip^{e5.5}$ mutants using a UAS-*ChipFL* transgene rescues the pathfinding errors. The Ap neurons re-acquire the ability to fasciculate tightly with their homologs and form a single axon bundle, indicating that Chip is required specifically within the Ap neurons to mediate correct axon guidance choices. (F-G) Pathfinding errors are not rescued by either UAS-*ChipΔLID* (F) or UAS-*ChipΔDD* (G), indicating a requirement for dimerization and LIM-interaction in Chip function. (H) Histogram showing occurrence of pathfinding errors in *ap* and *Chip* mutants, and rescue of pathfinding errors by ChipFL but not ChipΔLID or ChipΔDD. Bars indicate the mean percentage of segments per embryo that exhibited no pathfinding errors, and error bars denote the s.e.m. Exacerbation of pathfinding defects by ChipΔDD indicates dominant-negative activity.

the sole function of the Ap LIM domains in the nervous system is to mediate a complex with Chip.

## DISCUSSION

Control of *Drosophila* wing patterning by Apterous (Ap) is accomplished through complex formation of Ap with dimers of the LIM-binding protein Chip (Milan and Cohen, 1999; van Meyel et al., 1999). Here we provide evidence that formation of this complex is also required for two distinct functions of Ap in the CNS. First, Chip is required to activate transcription from a small Tv neuron-specific enhancer of the *FMRFamide* gene in vivo. Second, physical interaction between Ap and Chip is required for correct axon pathway selection of Ap interneurons. Since we have found that Chip is a necessary cofactor for Ap not only in the wing but also in the establishment of neurotransmitter identity and axon guidance, we hypothesize that Chip is an obligate cofactor for all Ap activities. We also found that Chip is required for at least one function of Islet in the CNS, the activation of tyrosine hydroxylase expression in dopaminergic neurons. Given the widespread expression of Chip in *Drosophila* and of NLI in mouse, and given their high-affinity interactions with all LIM-HD proteins, Chip/NLI family members are likely cofactors for a wide range of LIM-HD activities in the transcriptional control of developmental programs.

*Chip* was first identified in a screen for mutations that reduce the activity of a remote wing margin enhancer in the *cut* locus, and subsequent genetic experiments imply that Chip mediates communication between enhancers and promoters over extremely long distances (Morcillo et al., 1996, 1997). The direct downstream effectors of LIM-HD transcription factors are largely unknown, as are the details of recruitment and

coordination of LIM-HD proteins onto tissue-specific enhancers in vivo. We found that expression of the *Tv-lacZ* reporter transgene was disrupted in *Chip* mutant embryos, similar to *ap* mutants. These results suggest that Chip participates in the elaboration of a specific neuronal phenotype by cooperation with Ap to regulate expression directed by the 446 bp Tv neuron-specific enhancer of the *dFMRFa* gene. That such a small enhancer responds to Chip indicates that Chip can act locally to mediate protein interactions over short distances, presumably by bridging Ap molecules bound to the Ap response elements identified in vitro.

### Additional components of Chip/LIM-HD complexes

It has been proposed that a single LIM domain can serve as a specific protein-binding interface, and that proteins with multiple LIM domains could function as scaffolds for the assembly of protein complexes (Schmeichel and Beckerle, 1994; Arber and Caroni, 1996). Indeed, biochemical assays and yeast interaction experiments have shown that one of the two LIM domains of all LIM-HD and LMO proteins is sufficient to interact with NLI/Ldb1 or the related protein CLIM-1a (Jurata et al., 1996; Bach et al., 1997), but that both LIM domains are required for high-affinity interactions (Jurata and Gill, 1997; Breen et al., 1998). For wing development and axon guidance, we have shown that the sole function of the Ap LIM domains is to bind Chip, and conclude that Ap function in these developmental processes does not entail LIM-mediated interactions with other proteins. However, it is curious to note that the LIM domains of Ap are more closely related to the LIM domains of its vertebrate orthologs Lhx-2 and Lhx-9 than they are to those of other *Drosophila* proteins like Isl or Lim3. This evolutionary conservation is characteristic of LIM-HDs and may reflect specific activities of LIM domains in cells or processes not yet analyzed. In support of this notion, the specificity of the interaction between LIM-HDs and certain transcription factors of other classes resides within the LIM domains. For example, the bHLH transcription factor E47 binds the LIM domains of Lmx-1 but not Islet-1 (Johnson et al., 1997), and Islet-1 binds the LIM domains of Lhx-3 but not other LIM-HDs (Jurata et al., 1998).

We previously demonstrated that, in Ap neurons, the N terminus of Ap, including the LIM domains, is not completely interchangeable with that of another LIM-HD family member (O'Keefe et al., 1998). This stands in contrast with Ap function in the wing disc where the N terminus is interchangeable. Barring technical issues related to protein stability or expression levels in the CNS, this result suggests that, in addition to binding Chip, Ap function in the CNS requires specific interactions with at least one other factor. Since we completely rescued the double mutant pathfinding errors with a chimera lacking Ap LIM domains, we speculate that such specific interactions must involve non-LIM domain sequences in the Ap N terminus (amino acids 1-147). In addition to these Ap-mediated interactions, it is likely that Chip also recruits other proteins of other classes into this multimolecular transcriptional regulatory complex (Bach et al., 1997, 1999).

### The Chip/LIM-HD complex: a source of diversity and a substrate for modulation

Although it is clear that the formation of Chip/Ap complexes is critical in the CNS, as it is in the wing, our results suggest that there are at least two aspects in which control of their formation differs between these two tissues. First, the relative stoichiometry of Ap and Chip is an important determinant of wing patterning and outgrowth, as overexpression of Chip leads to defects that can be suppressed by co-overexpression of Ap (Fernandez-Funez et al., 1998; Milan and Cohen, 1999; van Meyel et al., 1999). In contrast, axon guidance and neurotransmitter expression in *Drosophila* embryos are insensitive to Chip overexpression (data not shown), and we show here that ChipΔLID does not influence axon guidance in a dominant negative fashion, in contrast to ChipΔDD. This suggests that, in the embryonic CNS, where Chip levels are high due to maternal expression, the crucial determinant of

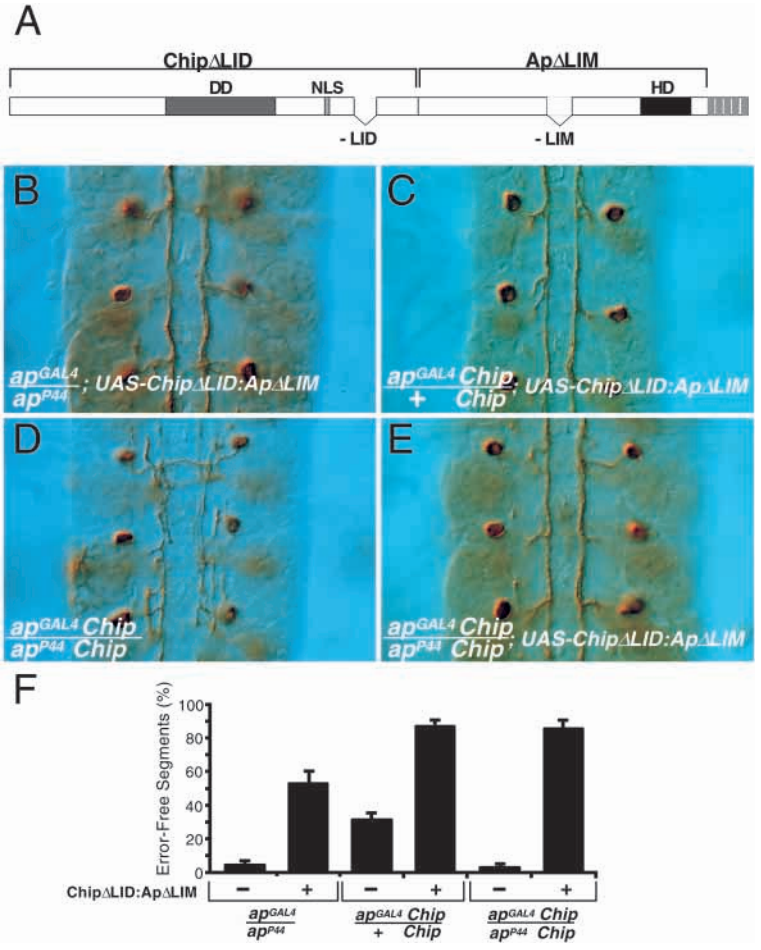


**Fig. 5.** Chip is an essential cofactor for Apterous in neurons. (A) Schematic diagram of the ChipΔLID:ApΔLIM chimera, which retains the Chip self-dimerization domain (DD) and the Ap homeodomain (HD), and is predicted to simulate Chip/Ap complexes. (B,C) ChipΔLID:ApΔLIM restores pathway recognition and axon fasciculation in $ap^{GAL4}/ap^{P44}$ mutants (B), and in $Chip^{e5.5}$ null mutants (C). Compare with Fig. 4C and D, respectively. (D,E) In embryos doubly mutant for *ap* and *Chip*, nearly all segments exhibit severe errors of axon pathfinding (D). ChipΔLID:ApΔLIM rescues all axon pathfinding defects in the double mutants (E). (F) Histogram showing the effect of ChipΔLID:ApΔLIM expression on axon pathfinding errors in *ap* mutants, *Chip* mutants and double mutants for both *Chip* and *ap*.

sufficient Chip/Ap complexes is the absolute levels of Ap rather than the relative stoichiometry of the two proteins.

Second, in the developing wing disc, upregulation of the LIM-only protein dLMO by Ap serves as a negative feedback mechanism by which Chip/Ap complexes are disrupted. However, dLMO is not expressed in the Ap neurons of the VNC, at least during embryonic axon pathfinding. It is possible, however, that another *Drosophila* LMO protein fulfills this task, or perhaps dLMO expression in Ap neurons commences later during larval or pupal development. Alternatively, downregulation of Chip/Ap activity might not be required in the CNS as it is in the wing. This latter alternative appears likely since the Chip/Ap fusion chimera, which is not susceptible to modulation by LMO factors, rescues perfectly the axon guidance errors of double mutants for *Chip* and *ap*. In contrast, in the wing, where dLMO interferes with Chip/Ap complex formation, the dLMO-insensitive Chip/Ap chimera appears to be hyperactive, phenocopying dLMO loss-of-function mutants (Milan and Cohen, 1999; van Meyel et al., 1999).

In Ap cells of the wing disc and CNS, there are as yet no other LIM-HD proteins known to be expressed. This would suggest that the relevant tetramer in these cells consists of two molecules of Ap bridged by two dimerized molecules of Chip. In the dopaminergic cells of the VNC, Isl is the only LIM-HD known to be expressed. It is not yet known whether TH is a direct target for transcriptional regulation by Isl, but the relevant complex for the regulation of TH expression, whether direct or not, may consist of two Chip-bridged molecules of Isl. However, Isl and Lim3 are co-expressed in the motor neurons of the intersegmental nerve branch b (ISNb), analagous to the combinatorial expression of LIM-HDs in vertebrate motor neuron pools (Sharma et al., 1998; Thor et al., 1999). Thus, in ISNb motor neurons, there are several possible combinations, including Chip-bridged complexes of two molecules of Isl, or two molecules of Lim3, or one molecule of each. In addition, *Drosophila* Lim3 and Isl, like their vertebrate counterparts, are uniquely capable of forming heterodimers in the absence of Chip, suggesting they can participate in both Chip-dependent and Chip-independent heterodimeric complexes (Jurata et al., 1998; van Meyel et al., 1999). Thus at least four possible combinations exist in ISNb motor neurons expressing only these two LIM-HD proteins and one cofactor. It is possible that each of these combinations has a unique specificity for target sequence recognition and that, from these four possible complexes, those that are actually formed during development may determine the repertoire of gene expression that gives these motor neurons their particular identities. In fact, biochemical studies in vitro suggest that Isl binds Lim3 with higher affinity than Chip and thus these direct Isl/Lim3 heterodimers may be favored over Isl/Chip complexes (van Meyel et al., 1999). Consistent with this possibility is that, in *Chip* mutant embryos, we detect no clear *isl*-like defects in pathfinding of the Isl motor neurons.

In vertebrates, the complexity of combinatorial expression patterns of LIM-HD proteins and the availability of potential cofactors suggests that the LIM-HD code is a major contributor to neuronal diversity. Recent results, including those presented here, indicate that the complexity of the LIM-HD combinatorial code is determined not only by the availability, concentration and relative affinities of LIM-HDs, but also of Chip/NLI, LMO proteins and possibly other cofactors.

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