The Drosophila LIM-homeodomain protein Islet antagonizes proneural cell specification in the peripheral nervous system

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Abstract

The pattern of the external sensory organs (SO) in Drosophila depends on the activity of the basic helix–loop–helix (bHLH) transcriptional activators Achaete/Scute (Ac/Sc) that are expressed in clusters of cells (proneural clusters) and provide the cells with the potential to develop a neural fate. In the mesothorax, the GATA1 transcription factor Pannier (Pnr), together with its cofactor Chip, activates ac/sc genes directly through binding to the dorsocentral enhancer (DC) of ac/sc. We identify the LIM-homeodomain (LIM-HD) transcription factor Islet (Isl) by genetic screening and investigate its role in the thoracic prepatterning. We show that isl loss-of-function mutations result in expanded Ac expression in DC and scutellar (SC) proneural clusters and formation of ectopic sensory organs. Overexpression of Isl decreases proneural expression and suppresses bristle development. Moreover, Isl is coexpressed with Pnr in the posterior region of the mesothorax. In the DC proneural cluster, Isl antagonizes Pnr activity both by dimerization with the DNA-binding domain of Pnr and via competitive inhibition of the Chip–bHLH interaction. We propose that sensory organ prepatterning relies on the antagonistic activity of individual Chip-binding factors. The differential affinities of these binding-factors and their precise stoichiometry are crucial in specifying prepatterns within the different proneural clusters.

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Introduction

One of the central problems in developmental biology is how patterns of specialized cell types are specified within uniform fields of cells. The patterning of sensory bristles on the thorax of Drosophila constitutes a powerful model to address this question. The position of the thoracic sensory organs reflects the distribution of their sensory organ precursors (SOPs). The SOPs are specified during the third larval instar and early pupal stages from restricted groups of cells, the “proneural clusters”, characterized by the expression of the achaete (ac) and scute (sc) genes. Genes of the ac–sc complex encode proteins bearing a basic helix–loop–helix (bHLH) motif that heterodimerizes with Daughterless (Da) to form a transcriptional complex that activates downstream proneural genes. The spatially restricted expression of ac/sc provides the progenitor cells with the potential to develop a neural fate (Gomez-Skarmeta et al., 2003). A member of the GATA-I family of transcription factors, Pannier (Pnr), appears to be a key morphogenetic gene that specifies dorsal identity in the fly (Calleja et al., 2000) and promotes development of sensory organs in the mesothorax (Ramain et al., 1993; Heitzler et al., 1996a). Studies on GATA factors demonstrate that these proteins carry two zinc fingers: the N-terminal finger interacts preferentially with different protein cofactors whereas the C-terminal finger binds DNA (Liew et al., 2005). Pnr is a direct proneural activator of ac/sc, which binds to the dorsocentral (DC) enhancer (Garcia-Garcia et al., 1999) located 4 and 30 kb upstream to ac and sc, respectively (Gomez-Skarmeta et al., 1995). The proneural transcriptional activity of Pnr is modulated by interactions with specific corepressor or coactivator factors (Cubadda et al., 1997; Haenlin et al., 1997). One of these cofactors, which is also necessary for proper development of the dorsal-most sensory organs, is Chip. Both pnr and Chip loss-of-function alleles remove DC and scutellar (SC) bristles in the median thorax (Ramain et al., 2000). DC proneural activation requires the formation of a Pnr:Chip:Ac/ Da transcriptional complex where Chip acts as a bridge
between Pnr and Ac (Sc)/Da heterodimers to allow enhancer–promoter interactions (Ramain et al., 2000). This bridging activity is negatively regulated by Osa, a component of SWI/SNF chromatin remodeling complexes (Heitzler et al., 2003).

Chip is a ubiquitously expressed nuclear adaptor protein and the only member of the LIM-domain-binding factors (Ldb) in Drosophila (Matthews and Visvader, 2003). Wadman et al. (1997) have described a similar complex involved in vertebrate hematopoiesis, that includes GATA-1, Ldb-1, the bHLH SCL/Tal1 and E2A, and the LIM-only protein LMO2. Sequence comparisons among Ldb factors of distinct species indicate the presence of several well-conserved domains. The most relevant consists in an amino-terminal homodimerization domain (DD) and a carboxyl-terminal LIM interaction domain (LID, Jurata and Gill, 1997). Pnr binds Chip through the DD, preventing Chip from homodimerization (Ramain et al., 2000).

The Ldb factors were first discovered through their physical interactions with LIM-homeodomain (LIM-HD) proteins via the LID (Agullick et al., 1996; Morcillo et al., 1997; Fernandez-Fuzes et al., 1998). In wings, Chip is a necessary cofactor of the LIM-HD factor Apterous (Ap). Ap is a selector gene expressed in dorsal compartment of wing imaginal discs. Ap activity depends strictly on the formation of tetrameric complex containing two molecules of Ap bridged by a homodimer of Chip (Milan and Cohen, 1999; van Meyel et al., 1999).

Chip also plays a pivotal role in organizing other specific transcriptional complexes essential for cell fate decision. Chip-dependent complexes that involve the homeodomain (HD)-containing proteins Bcd and Fz are required for proper segmentation during embryogenesis (Torigoi et al., 2000). Chip-mediated interactions with the HD factors Aristaeless and Bar as well as the LIM-HD Ap and dLim1 regulate proximal–distal leg developmental program (Pueyo and Couso, 2004).

The LIM-HD factors Isl1 and Isl2 are best known for their role in specification of distinct cell subtypes (Thor and Thomas, 1997; Thaler et al., 2002), in endocrine organ differentiation (Hobert and Westphal, 2000) and in odontogenesis (Mitsiadis et al., 2003). Isl interacts with the ubiquitous adaptor protein NLI (Ldb, CLIM, Chip) providing a specific LIM-HD transcriptional code for motor- and interneuron specification (Thor and Thomas, 1997; Thaler et al., 2002). In the present study, we show that Drosophila Isl protein regulates the prepatterning of the sensory organs. We find that Isl is expressed in the posterior region of the thoracic discs within the Pnr expression domain. isl loss-of-function mutants are characterized by increased Ac proneural expression and ectopic sensory organs formation, the opposite phenotype to that seen in Pnr loss-of-function mutants. We show that Isl antagonizes the proneural activity of Pnr through direct heterodimerization with Pnr and by competition with bHLH factor Da for their common cofactor, Chip. We propose that the differential affinities of the LIM-HD, bHLHs and the GATA factor for Chip are critical for specific developmental programs within the different proneural fields and that Isl refines the neuroblast prepattern, which is setting up within the mesothoracic region.

Materials and methods

Origin of isl alleles

We generated new EMS-induced isl alleles. First, we found islE20 and islE41 mutants in two separate screens as dominant enhancers of the Chipþ thoracic cleft and extrascutellar phenotypes. EMS-treated cn bw sp males × b pr cn bw Chipbd females gave islE20 among 106 siblings (Chipþ/+ background). EMS-treated b pr cn bw Chipbd males × w1118,Chipbd females gave islE52 among another 106 siblings (Chipbd/Chipbd background). Second, in order to get more lethal alleles, 2×106 EMS-treated cn bw sp chromosomes were tested in trans with the viable allele islE20 (b islE20 pr cn bw). Thus, islE52, islE53 and islE55 were recovered in F1 as single fly with extrascutellar bristles. islE115, islE57–60 and islE56–63 were obtained from Bloomington and Tübingen stock centers. islE41 and UAS-Isl (Thor and Thomas, 1997) were kindly provided by S. Thor. Isl17–40 and islE56–63 were generated in Luschnig et al. (2004) and Thibault et al. (2004), respectively.

Generation of transformants

P-element transformation was performed as previously described (Rubin and Spradling, 1982). The UAS-IslLIM construct was made by inserting an isl ORF lacking 53–171 aa into vector pUAST (Brand and Perrimon, 1993) carrying the flag-epitope at the carboxyl-terminal end of the protein. The UAS-Ap and UAS-IslHD constructions were kindly provided by Thomas, UAS-Pnr is described by Haenlin (Haenlin et al., 1997). Double UAS-Isl/UAS-Pnr, UAS-Isl/ UAS-Ap strains were obtained by recombination from inserts on chromosome 3. Chipbd/UAS-Isl strain was obtained by recombination of mutation Chipbd and UAS-Isl on chromosome 2.

Clonal analysis

Mutant clones were produced following Heitzler et al. (1996b). The following genotypes were used: (1) y FLP1/y w1118; P[y+,ry+](25F) ckCH52, (2) y FLP1/y w1118; P[y+,ry+](25F) ckCH52, (3) y FLP1/y w1118; P[y+,ry+](25F) ckCH52, (4) y FLP1/y w1118; P[y+,ry+](25F) ckCH52, (5) y FLP1/y w1118; P[y+,ry+](25F) ckCH52, (6) y FLP1/y w1118; P[y+,ry+](25F) ckCH52, (7) y FLP1/y w1118; P[y+,ry+](25F) ckCH52, (8) y FLP1/y w1118; P[y+,ry+](25F) ckCH52, (9) y FLP1/y w1118; P[y+,ry+](25F) ckCH52, (10) y FLP1/y w1118; P[y+,ry+](25F) ckCH52. Isl1 binding sites are underlined. In mutant probe TAAT, nucleotides of TAAAGGCCCCGGGG are critical for specific developmental programs within the different proneural fields and that Isl refines the neuroblast prepattern, which is setting up within the mesothoracic region.

Molecular techniques

Drosophila Isl, Pnr, Chip, Ac, Sc and Da were cloned into pPAC 5C (Krasnow et al., 1989) containing a flag- or myc-epitope tag for transient expression in S2 cells. Ac promoter and DC enhancer were cloned into pGL3 Basic (Promega) for luciferase assay. For in vitro protein expression, Isl and Pnr were cloned in pET-15b (Novagen). For GST pull down assay, LIM domain (53–171 aa), HD (239–300 aa) and the C-terminal (301–534 aa) of Isl containing the ISD domain were cloned into pGEX-2T (Pharmacia). cDNA of the ChipFL, ChipAD, ChipAOID and ChipALID (Torigoi et al., 2000) provided by D. Dorsett were subcloned into pPAC5C containing the myc-epitope. Deletions and point mutations were generated using a PCR-based mutagenesis method (Stratagene).

Electro mobility shift assay

The double-stranded oligonucleotides used as probes were: GCAGGTG-TATAAAAACAAATGCGACTTGCTGCCAA (DC enhancer: 1025–1060) and TAAAGGCCCCGGGGCGATTAGGTATATAGAA (ac promoter: 82–115). Isl1 binding sites are underlined. In mutant probe TAAT, Isl protein was synthesized using the Promega TNT coupled transcription–translation kit. Parallel reactions with S -methylthionine or unlabeled methionine were performed. Labeled protein was analyzed by SDS-polyacrylamide gel electrophoresis (PAGE) to ensure proper synthesis. Binding mixtures for reaction of Isl included 10 mM HEPES (pH 7.6), 75 mM KCL, 2.5 mM MgCl2, 0.1 mM EDTA, 1 mM dithiothreitol, 10% glycerol, 200 ng of poly(dI–dC), 2×106 cpm of each probe and in vitro-translated protein. The reaction mixtures were
incubated at room temperature for 20 min and analyzed by non-denaturing PAGE (5% polyacrylamide) at 4°C.

Cell culture, transient transfection, Western blot analysis

S2 cells were cultured in Schneider cell medium (Gibco BRL) with 10% fetal calf serum under standard condition and transfected using Effectene reagent (Qiagen). For Western blot on S2 cells, the cell pellet was lysed in buffer (50 mM Tris–HCl, 150 mM NaCl, 1 mM EDTA, 1% Triton X100, PIC).

Biochemical interaction assays

For immunoprecipitations, protein extraction from S2 cells, cell lysis, flag fusion protein immunoprecipitation and elution were performed according to the Flag-Tagged Protein Immunoprecipitation Kit protocol (Sigma). GST pull down assays were performed as described in Torigoi et al. (2000). Pnr was expressed using TNT quick coupled transcription/translation kit (Promega).

Immunostaining

Larvae and pupae dissection and immunostaining were carried out according to standard procedures. Primary antibodies (Ab) and dilutions used were: rabbit anti β-galactosidase (1:500) (Sigma, Cappel), mAb Isl 40.3A4 (1:200) (DSHB), mAb atni-Ac (1:200) (DSHB), mAb c-myc (1:200) (DSHB). For fluorescence staining, secondary Ab (Jackson) coupled with Cy3 or Alex (1:200) (DSHB), mAb atni-Ac (1:200) (DSHB), mAb c-myc (1:200) (DSHB).

Microscopy

Images of Drosophila imaginal wing discs were taken on Leica Microscope SP2 at 14–20 focal planes and assembled using an in-house developed software TCSTK.

Results

Genetic screening for modifiers of ChipE identifies isl as an enhancer

The viable mutant ChipE exhibits morphogenetic defects mainly on the adult thorax and is associated with the missense substitution (ChipR504W) in the LIM-interacting domain (LID) known to mediate dimerization with LIM-domain-containing factors (Agulnick et al., 1996; Jurata and Gill, 1997; Bach et al., 1997; Fernandez-Funez et al., 1998; Semina et al., 1998) and bHLH proteins (Ramain et al., 2000). ChipE homozygous flies show loss of the DC sensory organs (Figs. 1A, D) and a mild thoracic cleft. These phenotypes resemble those of Pnr loss-of-function mutants, although physical interactions between Chip and Pnr are not affected (Ramain et al., 2000). In addition to these recessive defects, ChipE exhibits an excess of SC bristles, a dominant phenotype unrelated to loss-of-function Chip or Pnr mutations. These data indicate that DC and SC regions are differentially sensitive to Chip-dependent proneural activity and we expect that additional factors might regulate this activity in a region-specific manner.

In order to identify such factors, we screened for genetic modifiers of ChipE (see Materials and methods). Two new EMS-induced enhancer mutations, E20 and E115, were found to correspond to isl alleles and enhance both the thoracic cleft and excess SC bristle phenotypes of ChipE (Figs. 1D, E). isl encodes an LIM-HD transcription factor, which is a high-affinity binding partner for Chip (Thor et al., 1999; Thaler et al., 2002). The genetic interactions between ChipE and isl alleles suggest that wild-type isl function is required for patterning of the thorax.

Isl mutants affect development of the sensory organ in the mesothorax

In order to investigate the function of the isl gene in vivo, we have characterized an allelic series of isl mutations and their corresponding molecular lesions (Table 1). islIIIB29, islIIIIE16, islE41, islE52, islE53 and islE55 alleles die as embryos with a prominent dorsal hole typical for amorphic alleles (Wright et al., 1976; Nusslein-Volhard et al., 1984; Thor and Thomas, 1997). These isl alleles resemble pnr or ush mutants (Herranz and Morata, 2001). Interestingly, Isl seems to be the only LIM-HD factor required during dorsal closure. islE115 and islE57–40 are strong hypomorphic alleles which die as first-instar larvae, or, occasionally, as embryos with a dorsal hole (not shown). The molecular analysis of lethal isl alleles reveals missense mutations that disrupt the conserved domains. In islE52, a conserved arginin is changed within the homeodomain that is necessary for binding consensus DNA (Gehring et al., 1994). In islIIIB29, islIIIIE53 and islE55 alleles, single conserved cysteine residues required for chelation of zinc ions within the first LIM domain are mutated. islE41, islIIIIE16, islE115 and islE57–40 alleles correspond to splice donor and acceptor mutations (Table 1). These mutations lead to truncation of Isl protein, which lacks most of the functional domains including the HD domain. All these mutations induce homozygous lethal isl loss-of-function alleles and enhance the ChipE phenotype when heterozygous with a wild-type isl allele (Figs. 1B–E).
Table 1
Genetic lesions in isl alleles

<table>
<thead>
<tr>
<th>Allele</th>
<th>Phenotype</th>
<th>Mutation associated with phenotype</th>
<th>Predicted effect</th>
</tr>
</thead>
<tbody>
<tr>
<td>IIIB29</td>
<td>Amorph</td>
<td>Change TGT → TAT</td>
<td>Substitution in LIM domain</td>
</tr>
<tr>
<td>IIIE16</td>
<td>Amorph</td>
<td>Change G → A into the AG acceptor site of splicing of second intron</td>
<td>Severely truncated protein</td>
</tr>
<tr>
<td>E20</td>
<td>Weak hypomorph</td>
<td>Not detected either in the coding sequence nor in 3’ and 5’ UTR</td>
<td>Reduced amount of protein</td>
</tr>
<tr>
<td>E41</td>
<td>Amorph/mull</td>
<td>Change G → A into the AG acceptor site of splicing of first intron</td>
<td>Severely truncated protein</td>
</tr>
<tr>
<td>E52</td>
<td>Amorph</td>
<td>Change CGG → CAG</td>
<td>Substitution in HD domain</td>
</tr>
<tr>
<td>E53</td>
<td>Amorph</td>
<td>Change TGC → TAC</td>
<td>Substitution in LIM domain</td>
</tr>
<tr>
<td>E55</td>
<td>Amorph</td>
<td>Change TGC → TAC</td>
<td>Substitution in LIM domain</td>
</tr>
<tr>
<td>E115</td>
<td>Strong hypomorph</td>
<td>Change T → C into the GT donor site of splicing of third intron</td>
<td>Truncated protein</td>
</tr>
<tr>
<td>57 – 40</td>
<td>Strong hypomorph</td>
<td>Change T → C into the GT donor site of splicing of third intron</td>
<td>Truncated protein</td>
</tr>
<tr>
<td>d03613</td>
<td>Hypomorph</td>
<td>P-insertion in the first exon 343 bp 5’ to ATG</td>
<td>Reduced amount of protein</td>
</tr>
</tbody>
</table>

isld03613 and islE20 alleles are viable hypomorphic mutants. islE20 homozygotes show gain of DC and SC bristles and a slight thoracic cleft (Fig. 2A). We establish, by P-element excision, that this hypomorphic phenotype is associated with an insertion in the 5’UTR of isl. The weakest hypomorphic allele, islE20, also enhances the ChipE phenotype in homozygous and hemizygous conditions (Figs. 1D, E). We were unable to identify any mutation in cDNA from islE20 and suspect that islE20 might affect regulatory sequences of the gene.

To characterize phenotype of null allele, we generated mosaic animals carrying the islE41 allele, which encodes a truncated protein lacking all the functional domains. We observed that islE41-mitotic clones produce extra DC and SC bristles (Figs. 2B, C). Taken as a whole, these results suggest that loss of isl function is correlated with a neoplasia of the sensory organs in the mesothorax.

*Isl is expressed in the dorsal thoracic disc, which includes DC and SC SOPs*

Next we investigated the pattern of Isl expression during the third larval instar when sensory organ precursors are specified. Immunolocalization of Isl protein in wing imaginal disc was performed using a mouse monoclonal anti-Isl antibody raised against the homeodomain of the chicken Isl1 protein (Tsuchida et al., 1994). We found that expression of Isl is restricted to the posterior region of the presumptive notum throughout wing imaginal disc development (Fig. 2D). This result is consistent with the observed phenotypes of isl mutants which are confined to the dorsal region of the thorax.

The proneural activity of bHLH transcription factor Ac promotes the development of the sensory organ precursors during the third larval instar. In order to understand the origin of the observed bristle phenotype, we analyzed the Ac expression in the loss-of-function isl mutants. We found that the gain of sensory organ precursors is associated with an increase of Ac expression in DC and SC clusters (Figs. 2E, F). Neuralized-lacZ is expressed in all imaginal disc sensory organ precursors at the third larval stage. Using this marker, we found that the domain of isl expression includes the DC and SC sensory organ precursors (Fig. 2G) in wild-type wing imaginal discs. In isl mutants, the absence of Isl protein correlates with the formation of ectopic DC and SC precursors (Fig. 2H). The function of Isl is, therefore, to control the specification of region-specific bristle patterns.
The regulation of proneural activity requires the LIM domain of Isl

The coupling of two amino-terminal LIM domains with an homeodomain within the Isl protein suggests that it might regulate the expression of ac/sc by direct binding on specific DNA target sequences and/or via protein–protein interactions within multiprotein transcriptional complexes. Expression of ac/sc genes in medio-dorsal and scutellar regions is regulated by DC and SC enhancers, respectively. The DC enhancer has been extensively characterized (Garcia-Garcia et al., 1999) and contains a cluster of GATA sites, that are critical for activation of ac/sc expression by Pnr. Analysis of genomic sequences showed that both ac promoter and DC enhancer sequences contain one Islet-1 consensus site (CTAATG) (Boam and Docherty, 1989; Karlsson et al., 1990) (Fig. 3A). Cross-genomic comparison of the DC enhancer and ac promoter sequences revealed that the Isl1 consensus site within the DC enhancer is evolutionarily conserved between D. pseudoobscura and D. melanogaster. Interestingly, the Isl1 site within the ac promoter is not conserved in D. pseudoobscura but still present in D. simulans, implying that this site might have no functional significance in vivo. None of the putative Isl-binding sites overlaps known GATA sites, suggesting that no direct competition between Isl and Pnr occurs for binding sites. To determine whether Isl can bind to perfect consensus Isl sites in the DC enhancer and ac promoter, we performed an electromobility shift assay (EMSA) (Fig. 3B). Isl protein binds wild-type probes (lanes 2, 7) and this binding can be competed by excess unlabeled probe (lanes 4, 9), but not by a 500-fold excess of nonspecific competitor (lanes 3, 8). Mutation of TAAT to GCCG in the core sequence of Isl1 consensus sites abolished binding of Isl (lanes 5, 10), suggesting that Isl protein can bind to these sites in a sequence-specific manner in vitro.

To determine whether Isl function in vivo is mediated by DNA binding ability of Isl, we ectopically expressed Isl mutant proteins, in which either the LIM domains (IslΔLIM) or the HD (IslΔHD) was deleted using the pnr-Gal4 driver. The full-length (IslFL) protein was used as a control. Overexpression of IslFL affects development of the DC and SC bristles and also induces a thoracic cleft (Fig. 3C). Overexpression of IslΔHD, which lacks DNA binding domain but still retains LIM domains resulted in similar defects (Fig. 3D). Conversely, overexpressed IslΔLIM has wild-type bristle phenotype and no cleft (Fig. 3E). These data suggest that DNA binding ability of Isl is not crucial for repression of bristles development, and presumably it is mediated via protein–protein interactions.

The ChipE mutation disrupts interaction between Chip and Isl

We identified Isl as specific enhancer of the ChipE mutant. Chip protein functions as a multiple protein adaptor and contains three distinct functional domains. The amino-terminal Dimerization Domain (DD) and the Other Interaction Domain (OID) both mediate homo- and heterodimerization, while the LIM-interacting domain (LID) is required for heterodimerization with different LIM-containing transcription factors Isl, Ap and Lim3 (Jurata and Gill, 1997; van Meyel et al., 1999; Torigoi et al., 2000). In this study, we find that ChipE phenocopies isl loss-of-function in the scutellum and that isl mutants specifically enhance ChipE phenotypes. These genetic data provide support for a functional interaction between the Isl and Chip proteins. Therefore, we expected that the putative protein interactions between Isl and Chip required for normal development might be affected in ChipE flies. To test this hypothesis, a coimmunoprecipitation assay was performed using different deletion and mutant constructs of Chip (Figs. 4A, B), tagged by c-myc epitope at the amino-terminus of each protein for monitoring their expression. We find that Isl is able to bind Chip and that deletion of the LID domain completely abolishes this interaction (Fig. 4B, lanes 2, 5), in agreement with previously published data. In addition, we show that deletion of DD and OID has no effect on the binding of Chip to Isl (lanes 3 and 4, respectively). Interestingly, a substitution RS04W within the LID domain of Chip protein severely
impairs the ability of Chip to interact with Isl (lane 6). The bHLH transcription factors Ac/Sc or Da bind to the LID domain of Chip and the ChipE mutation within the LID domain completely abolishes this interaction (Ramain et al., 2000). Therefore, we propose that Isl and bHLH may recognize the same subdomain of the LID. We hypothesize, that during sensory organs development, the normal function of Isl is to repress the proneural activity of Pnr via competition with the proneural bHLH factors for the LID of Chip. To determine whether Isl is able to antagonize the interaction between bHLH transcription factors and Chip, we performed a competition assay. S2 cells were cotransfected by a constant amount of c-myc-tagged Da plasmid and increasing amounts of Isl plasmid in the presence of flag-tagged Chip. Protein extracts were immunoprecipitated with a mAb-flag recognizing tagged Chip and the relative level of Da protein assayed using myc-epitope tag. Isl protein was detected by mAb-Isl. We found that Isl is able to displace Da efficiently from the Chip-containing complex, while Da was visualized with anti-myc and Isl protein was detected by mAb-Isl. The asterisk indicates an IgG heavy chain loading control. Increasing amounts of Isl decreased level of immunoprecipitated Da protein. (C) Immunoblot of anti-flag immunoprecipitation assay from S2 cell lysates transfected with flag-Da and increasing amounts of Isl as indicated. Anti-flag mAb was used to immunoprecipitate the Chip-containing complex, while Da was visualized with anti-myc and Isl protein was detected by mAb-Isl. The asterisk indicates an IgG heavy chain loading control. Increasing amounts of Isl decreased level of immunoprecipitated Da protein. (D) Immunoblot of anti-flag immunoprecipitation assay from S2 cell lysates transfected with flag-Chip and Isl plasmids. Cells were transfected with a standard amount of flag-Chip (+), Ac and increasing amounts Isl plasmid. Ac protein was visualized with mAb-Ac. Increasing amounts of Isl do not decrease the levels of Ac protein, immunoprecipitated with flag-Chip. The asterisk indicates an IgG light chain loading control. (E–G) Wing phenotype of flies: overexpression of Isl in Ap domain strongly affects wing development (E). Cooverexpression of Ap and Isl (F) rescues the wing phenotype induced by Isl alone. (G) In a ChipE genetic background, the overexpression of Isl does not produce wing defects.
complex (Fig. 4C). Next, we tested whether Isl can compete with Ac or Sc for Chip. Constant amounts Ac or myc-tagged Sc plasmids were cotransfected with varying amounts of Isl plasmid in the presence of flag-tagged Chip. Protein extracts were immunoprecipitated with a mAb-flag recognizing flag-Chip and the relative level of Ac protein was revealed by mAb-Ac. In this assay, Isl was not able to compete with Ac (Fig. 4D) for Chip. Similar results were obtained with Sc (not shown). These data suggest that distinct proneural bHLH factors may have different affinities for the LID of Chip, allowing transcription factor interchange during development.

To confirm the relevance of Isl-Chip interaction in vivo, we ectopically expressed Islet in the wings, using ap-Gal4 driver. In *Drosophila*, the proper wing development requires tetrameric complex comprising a Chip homodimer and two molecules of LIM-HD protein Ap (Milan and Cohen, 1999; van Meyel et al., 1999). Ectopic expression of Isl produces rudimentary wings (Fig. 4E), which mimics ap loss-of-function phenotype. As both LIM-HD proteins, Isl and Ap, can interact with Chip, observed wing phenotype could be explained via quenching of Chip by Isl from the complex with Ap. As expected, simultaneous coexpression of UAS-Ap rescues the phenotype (Fig. 5 C compare with Fig. 3 C), meaning that Isl can interfere with Chip function and to interfere with the Ap function.

Next, we assessed the bristles phenotype in gain of function mutants using the pnr-Gal4 driver. Interestingly, ectopic expression of Isl in ChipE genetic background (Fig. 4I) results in partial suppression of the bristle phenotype (compare with ChipE mutants as a dominant bristles phenotype of *islE41*); or pnr (ChipE/ChipE; +/pnrVE12) background. Since the patterns of Isl and Pnr expression are restricted to the dorsal imaginal wing disc, we examined their colocalization. We found that Isl and Pnr are coexpressed within a restricted subset of cells in the presumptive posterior notum (Figs. 5D–F, D’–F’).

**Isl and Pnr antagonistically regulate the development of sensory organs**

Pnr activity determines the dorsal mesothoracic fate (Calleja et al., 2000) and is required for activation of the proneural *ac/sc* genes in this region (Garcia-Garcia et al., 1999). Since *isl* loss-of-function (*islE41*, *islD10867*) mutants display additional DC and SC bristles whereas loss of Pnr activity induces loss of these bristles, we propose that Isl and Pnr have antagonistic neural activities. We searched for genetic interactions between Pnr and Isl and identified the *islE41* mutant as a dominant enhancer of the ectopic SC bristle phenotypes (5.96 ± 0.12) associated with the *PnrD1* (4.9 ± 0.08) mutant. The *PnrD1* protein acts as a constitutive activator of *ac/sc* (Figs. 5A, B). Furthermore, simultaneous coexpression of Pnr and Isl results in suppression of the loss-of-bristle phenotype produced by UAS-Isl alone (Fig. 5C compare with Fig. 3C), meaning that Pnr is able to overcome effect of Isl-mediated suppression of bristle development. In summary, these genetic data indicate that Isl and Pnr have antagonistic functions consistent with the bristle phenotypes of mutant flies.

Interestingly, the ChipE phenotype is highly sensitive to the dosage of either *pnr* or *isl*. Homozygous ChipE flies are viable in an *islE41* genetic background, but are lethal in a heterozygous *isl (islE41* ChipE/+ * ChipE*), or *pnr* (ChipE/ChipE; +/pnrVE12) background. Since the patterns of Isl and Pnr expression are restricted to the dorsal imaginal wing disc, we examined their colocalization. We found that Isl and Pnr are coexpressed within a restricted subset of cells in the presumptive posterior notum (Figs. 5D–F, D’–F’).

The LIM domain of Isl interacts with Pnr and interferes with the Pnr-driven activation of the DC enhancer in vivo

To test whether Isl-mediated repression results from protein interaction with Pnr, we perform communoprecipitation assays. Pnr protein contains an amino-terminal DNA binding domain (comprising two Zinc fingers, Zf) and a carboxy-terminal heterodimerization domain (encompassing two α-helices). We cotransfected S2 cells with constructs expressing full length Pnr fused to the e-myf epitope and flag-tagged Isl (Fig. 6A). As depicted in Fig. 6B, an anti-flag Ab can communoprecipitate myc-tagged Pnr only in the presence of flag-Isl. To identify the interaction domain of Pnr, we prepared constructs in which either the amino- or carboxy-terminal part of Pnr is expressed. We found that Pnr heterodimerization with Isl is mediated by the amino-terminal part of Pnr protein (lane...
To determine whether the first and second Zinc fingers are required for Isl interaction, we made deletion constructs of Pnr in which either the first, second or both Zinc fingers are deleted. We found that Pnr lacking the first or second Zinc finger is still able to interact with Isl (lanes 5 and 6), whereas Pnr protein that lacks both Zn fingers fails to bind Isl (lane 7).

In order to define the interaction domain of the Isl, GST-tagged LIM, HD and Islet-specific domain (ISD) of Isl protein were expressed in E. coli and their ability to bind in vitro-translated S35-labeled Pnr was tested. We found that Pnr interacts with LIM domain of Isl (Fig. 6C, lanes 3 and 4), but not with HD or ISD (lanes 5 and 6).

Since both Isl and Pnr have antagonistic effect on proneural activity and show overlapping expression patterns, we examined the effects of Isl loss- and gain-of-function on the Pnr-dependent activity in vivo using a lacZ reporter containing a minimal ac promoter fused to the DC enhancer (DC:ac-lacZ; Garcia-Garcia et al., 1999). In isl loss-of-function mutants, exhibiting ectopic DC sensory organs, expression of the DC:ac-lacZ transgene is significantly increased (Fig. 6D) compared to wild-type control (Fig. 6E). In contrast, ectopic expression of Isl under control of pnr-Gal4 driver strongly decreases DC:ac-lacZ expression (Fig. 6F), which is consistent with lack of DC bristles on the notum of flies. These data indicate that Isl can downregulate the Pnr-dependent proneural activity via the DC enhancer of ac/sc in vivo.

**Discussion**

**Islet and the prepatterning of the thorax**

Isl1 was originally identified as a protein that binds to the enhancer elements in the rat insulin I gene (Karlsson et al., 1990) and synergistically activates the insulin I gene expression by interaction with bHLH E47 and E12, forming a functional insulin mini-enhancer complex (German et al., 1992). In vertebrate, Isl1 is expressed in all pancreatic islet cell types and in a variety of other polypeptide hormone-inducing cells of the endocrine system (Thor et al., 1991). In addition, Isl1 is expressed in most of the motor neurons in the spinal cord and the brain stem (Thor et al., 1991). Isl1 contributes to the specification of inter- and motor neuron...
identity through cell type specific protein–protein interactions (for review Bach, 2000; Shirasaki and Pfaff, 2002). In mouse and chicken spinal cord lacking Isl1 function, motor neurons are completely missing (Pfaff et al., 1996). In contrast, the unique Drosophila isl gene is not required for the generation of motor neurons and interneurons. Instead, in isl mutants, these neurons show axon pathfinding defects and fail to exhibit their proper neurotransmitter phenotype (Thor and Thomas, 1997).

Recently, in vertebrate, it has been shown that Isl1 functions also have implications in the specification of nondifferentiated cardiac progenitor cells and left–right asymmetry during heart development (Cai et al., 2003). In addition, Isl1 plays an important role in regulating distal gene expression during jaw and tooth development (Mitsiadis et al., 2003) and during inner ear neurons and sensory epithelia cell differentiation (Li et al., 2004).

During Drosophila development, the expression of transcription factors divides the dorsal thorax into three domains, one median and two lateral domains. The latter domains are specified by the homeobox-containing proteins of the iroquois-complex (iro) (Kehl et al., 1998), whereas the GATA factor Pnr is required to establish the median domain (Calleja et al., 2000). Within the mesothorax, Pnr together with U-shaped (Ush) and Chip plays a key role in dorsal closure (Heitzler et al., 1996a,b; Cubadda et al., 1997; Ramain et al., 2000). In this report, we present evidences that Isl is an essential regulator of the dorso-median patterning of the thorax. isl– clones generated adjacent to the thoracic midline, induce a strong cleft, suggesting that Isl is required for proper dorsal closure during metamorphosis (Zeitlinger and Bohmann, 1999; Usui and Simpson, 2000). Ectopic expression of Pnr leads to wing-to-thorax transformations, consistent with its role as medio-dorsal selector (Calleja et al., 2000). Ectopic Isl expression does not exhibit this phenotype, excluding the LIM-HD factor from a direct function as a prothoracic selector. Pnr is also known to activate wingless (wg) in dorsal thorax (Garcia-Garcia et al., 1999). We found that isl loss-of-function has no significant effect on wg expression. However, overexpressed Isl strongly reduced the size of wg thoracic stripe (not shown). This result is consistent with a repressive activity of Isl on Pnr.

Iro proteins and Pnr are direct activators of the proneural genes (Gomez-Skarmeta et al., 1996; Garcia-Garcia et al., 1999) in their respective domains. Pnr binds directly to the DC enhancer of ac/sc, providing therefore region-specific control of the proneural prepattern. Flies with reduced or lack of Pnr function fail to activate ac/sc and to develop DC and SC sensory organs. The proneural activity of Pnr is antagonized by Ush, the vertebrate homologue of the FOG (friend of GATA) (Cubadda et al., 1997; Haenlin et al., 1997). Ush is expressed only in the dorsal-most cells of the medial region. As a consequence, the segregation of the sensory organ precursors occurs along two stripes at the border of the medial domain of Pnr expression, where Ush is absent or insufficient to repress Pnr (Calleja et al., 1996).

We report here several lines of evidence indicating that Isl interferes with the proneural activity of Pnr as a repressor. (i) isl loss-of-function mutants show an opposite phenotype with regard to Pnr or Chip loss-of-function mutants: an excess of DC and SC sensory organs. (ii) A genetic synergism exists between PnrD and isl– alleles. This genetic interaction is less sensitive than that between PnrD and ush–, implying an alternative route for Isl to modulate the Pnr proneural activity. (iii) Isl is coexpressed with Pnr within the posterior mesothorax. (iv) Isl modulates the activity of a DC:ac-lacZ reporter. Loss-of-function isl mutants expand the DC:ac-lacZ expression like in ush– or PnrD constitutive mutants, whereas overexpressed Isl reduces the DC:ac-lacZ expression.

In the DC region, the regulation of Pnr concentration is critical for the proper position and shape of the DC proneural cluster. Isl expression overlaps with the dorsal-most domain of Pnr and DC proneural activity coincides with the posterior border of Isl expression. Therefore, we propose that both Isl and Ush restrict Pnr activity in the mesothorax. Interestingly, the regulation of the concentration of the mammalian Pnr ortholog, GATA-1, is similarly critical for proper erythroid, megakaryocytic, eosinophilic and mast cell lineages (Migliaccio et al., 2005).

Ush behaves as either an activator, or a repressor, of Pnr, depending on developmental context (Sato and Saigo, 2000). We found no evidence for a direct Isl–Ush interaction by GST pull down assay (not shown), but were able to communoprecipitate Ush, Pnr and Isl from transient transfected S2 cells. Both Ush and Isl may behave as positive cofactors of Pnr for nonneural activities, such as cardiac development (Dodou et al., 2004), embryonic dorsal closure and metamorphosis. Several reports emphasize the role of the Pnr homolog, GATA-1 and Isl1 in human blood disorders (Liew et al., 2005; Shimomura et al., 2000). It seems likely that GATA:Isl interactions represent a conserved mechanism to specify different cell fates in humans and other organisms.

Isl proteins are known as positive regulators of transcription in vertebrates (Wang and Drucker, 1995, 1996). In flies, Isl mediates repression of Pnr-driven proneural activity via binding to the DNA-binding domain of Pnr. Interestingly, these interactions are less specific than for the Pnr–Ush interaction, where the amino-terminal zinc finger of Pnr is specifically involved (Tsang et al., 1997; Lu et al., 1999; Svensson et al., 1999; Tevosian et al., 1999).

Genetic analyses of mutants reveal that the DC and the SC proneural clusters show differential sensitivities during neurogenesis. Ush mutants display ectopic DC bristles and a few additional SC bristles. This phenotype is similar to PnrD constitutive mutants, in which Pnr–Ush interactions are greatly reduced. In contrast, isl mutants show the opposite phenotype, with a large excess of SC bristles and a few additional DC bristles. The ChipE mutant exhibits antagonistic phenotypes: lack of DC bristles, reflecting Pnr loss-of-function (Ramain et al., 2000) and an excess of SC bristles, reflecting Isl loss-of-function. The differential topography of DC and SC enhancer binding sites presumably underlies differential transcription-complex binding affinities.
**LID domain of Chip is a versatile domain of protein interactions that provide different developmental outcomes**

Chip is the ortholog of Ldb factors that are ubiquitous multidaptor proteins in vertebrates (Matthews and Visvader, 2003 for review). Each Ldb-dependent developmental event is specified by modification of the transcriptional complex and is dependent on the stoichiometry of the region-specific Ldb partners (Pueyo and Couso, 2004). During normal development of the thorax, different partners of Chip like Isl, Ap and Pnr are expressed in the same region. We demonstrate that the ChipE mutant is highly sensitive to the dosage of these factors. In ChipE flies, removing one copy of either Pnr or Isl causes pupal lethality associated with extreme morphogenetic phenotypes. Removing one copy of Ap, however, rescues the Pnr-dependent phenotypes of ChipE flies. Taken together, these results indicate selective competition between the different partners of Chip, suggesting that hierarchical protein interactions depending on differential affinities and the strict stoichiometry of Chip and its partners, are critical to establish proper transcriptional codes within different proneural fields.

We isolated isl mutants in genetic screens for dominant enhancers of the ChipE phenotype. We demonstrate that the LIM-HD transcription factor Isl can bind to the LID of Chip (see also Thor et al., 1999). The binding of the LID domain of Chip with LIM domains has been conserved throughout evolution (Jurata and Gill, 1997; Jurata et al., 1998; Thor et al., 1999) as has Chip binding with bHLHs proteins (Ramain et al., 2000). LID contains two subdomains: a small N-terminal hydrophobic β patch (VMVV) followed by a large α helix. ChipE mutation has a single substitution that changes an Arg to Trp (R504W) in the middle of the α helix. This residue is highly conserved among species and mediates high-affinity contact with the LIM domains (Deane et al., 2004). Interestingly, the R504W substitution in Chip abolishes, or strongly reduces, both interactions with the bHLHs (Ramain et al., 2000) and also interactions with Isl. This result implies that bHLHs and Isl recognize the same site within the LID domain of Chip. Our data argue that competition between bHLHs and Isl for the LID domain of Chip may be critical for modulating the activity of transcription complexes during development. In vertebrate, NLI homolog of Chip mediates direct coupling of the proneural transcription complexes during development. In this model, the presence of one of a number of alternative binding factors modifies the specificity of a core transcription complex. This model makes the prediction that, while the core components of the transcription complex will be strongly conserved in evolution, the specificity cassette components will vary significantly between species showing divergent morphogenetic patterns. Comparison of these variable components in related species should provide insights into the fundamental mechanisms of encoding the pattern of differentiated cell types within morphogenetic fields.

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