An interactive network of zinc-finger proteins contributes to regionalization of the *Drosophila* embryo and establishes the domains of HOM-C protein function

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Summary

During animal development, the HOM-C/HOX proteins direct axial patterning by regulating region-specific expression of downstream target genes. Though much is known about these pathways, significant questions remain regarding the mechanisms of specific target gene recognition and regulation, and the role of co-factors. From our studies of the gnathal and trunk-specification proteins Disconnected (DISCO) and Teashirt (TSH), respectively, we present evidence for a network of zinc-finger transcription factors that regionalize the *Drosophila* embryo. Not only do these proteins establish specific regions within the embryo, but their distribution also establishes where specific HOM-C proteins can function. In this manner, these factors function in parallel to the HOM-C proteins during axial specification. We also show that in *tsh* mutants, *disco* is expressed in the trunk segments, probably explaining the partial trunk to head transformation reported in these mutants, but more importantly demonstrating interactions between members of this regionalization network. We conclude that a combination of regionalizing factors, in concert with the HOM-C proteins, promotes the specification of individual segment identity.

Key words: Hox, Homeotic, Zinc finger, *Drosophila*, Segment identity, Pattern formation

Introduction

Though much is known about HOM-C/HOX control of development (McGinnis and Krumlauf, 1992; Akam, 1995; Biggin and McGinnis, 1997; Mann and Morata, 2000), many unanswered questions remain. Arguably, the most important is how different HOM-C proteins activate or modulate different target genes. In vitro, all HOM-C proteins bind to similar, relatively simple DNA sequences (Hoey and Levine, 1988; Ekker et al., 1994; Walter et al., 1994; Biggin and McGinnis, 1997), and there is evidence that this may be true in vivo as well (Walter et al., 1994; Carr and Biggin, 1999; Li et al., 1999). Surrounding bases can influence binding strength, but there appears to be little specificity or, more appropriately, selectivity, in the DNA-binding properties of different HOM-C proteins. Interactions with co-factors provide the likely resolution of this dilemma, but, currently, few co-factors are known (e.g. Peifer and Wieschaus, 1990; Röder et al., 1992; Chan et al., 1994; Castelli-Gair, 1998; Mann and Morata, 2000; Mahaffey et al., 2001).

Previously, we provided genetic evidence that the C2H2 zinc-finger proteins encoded by *disconnected* (*disco*) and *disco-related* (*disco-r*) are redundant co-factors for the gnathal HOM-C proteins, Deformed (DFD) and Sex Combs Reduced (SCR) (Mahaffey et al., 2001). DFD and SCR are required during development of the *Drosophila* larval gnathal (mandibular, maxillary and labial) segments. Embryos lacking *disco* and *disco-r* develop with a phenotype similar to those lacking these *HOM-C* genes, and this phenotype is due, at least in part, to reduced expression of DFD and SCR target genes. As the gnathal HOM-C proteins are not required for *disco* and *disco-r* activation, and vice versa, we proposed that these redundant proteins were potential co-factors required for DFD and SCR function.

Many questions remain concerning this proposal. For example, are *disco* and *disco-r* required for all DFD functions, and do they have patterning roles independent of the HOM-C proteins? Several studies have shown that ectopic DFD can induce maxillary structures in the trunk segments. Does this indicate that DISCO and DISCO-R are required only for DFD function in the gnathal segments? Interestingly, there are several similarities between *disco* and *disco-r* and the trunk-specification gene *teashirt* (*tsh*). Each encodes a zinc-finger transcription factor and functions as a genetic co-factor during HOM-C specification of segment identity. The DISCO proteins and TSH are required in multiple segments where they interact with different HOM-C proteins. Expression of the *disco* genes and *tsh* abut at the gnathal-trunk boundary, possibly suggesting similar roles, but in different regions of the embryo. Here, we address these issues by examining the role of DISCO, with and without DFD, and the interplay between DISCO and TSH. We conclude the following: (1) alone, DISCO appears to impart a gnathal segment type; (2) cells can respond to the gnathal HOM-C protein DFD only where DISCO is present; and (3) TSH represses *disco* (and *disco-r*) expression in the trunk,

thereby preventing gnathal traits from developing in the trunk segments. These observations lead us to propose a new model for the specification of segment identity within the *Drosophila* embryo.

Materials and methods

Drosophila stocks and culture

Flies were reared on standard cornmeal-agar-molasses medium. The *paired-Gal4* (*prd-Gal4*) stock was obtained from Dr A. Bejsovec (Duke University), the UAS-*Dfd* fly stock from Dr T. Kaufman (Indiana University), the UAS-*tsh-13* and *tsh*^{8} lines from Dr S. Kerridge (CNRS Marseille France), and the *armadillo-Gal4* (*arm-Gal4*) line from Dr W. McGinnis (University of California, San Diego). The *UAS-Scr* fly stock was obtained from the Bloomington, Indiana Drosophila Stock Center.

Induction of UAS-*Dfd* and UAS-*disco*

We induced ectopic expression, at 25°C, using *arm-Gal4* (Sanson et al., 1996) and *prd-Gal4* (Yoffe et al., 1995) drivers with analogous results (referred to as *arm→disco* and *prd→disco* respectively, below).

Cuticle analysis

Embryos were collected and prepared for cuticle examination following procedures described previously (Pederson et al., 1996). Females were allowed to lay eggs for up to 24 hours, and embryos were aged for at least 24 hours before fixing the unhatched terminal larvae.

Expression of *Dfd* in *Df(1)XR14* males

To obtain flies expressing *Dfd* in the trunk segments of embryos lacking *disco* and *disco-r*, we crossed *Df(1)XR14*/*FM7c* females to UAS-*Dfd* (II) homozygous males. The non-*FM7c* female progeny were crossed to homozygous *prd-Gal4* males producing males hemizygous for *Df(1)XR14* and lacking *disco* and *disco-r*. We could recognize those ectopically expressing *Dfd*, as ectopic DFD disrupts anterior head development, thereby further aggravating the phenotype of the *Df(1)XR14* hemizygotes.

In situ localization of mRNA and protein

Localization of mRNA and proteins followed the protocols essentially as described previously (Pederson et al., 1996). Probes for *disco* and *disco-r* mRNAs were from Mahaffey et al. (Mahaffey et al., 2001). For other mRNA localizations, probe templates were obtained from *Drosophila* genomic DNA using PCR. The primers used to generate clones were as follows: *pannier*, ACATTACGGACAGGCGACAC (forward) and TGCAAACAAGGCCGAGTAG (reverse); *salm*, GCATACCAGAGCAAAGCACA (forward) and GATAACCGCGG-CACCCGATCACAGACCA (reverse); *tsh*, GCGTACCTGCACATG-GTGGC (forward) and GATCTCCGCGGCTGACTCTCGGCAGG (reverse).

Results

Both DISCO and DFD are required to specify maxillary identity

In otherwise normal embryos, ectopic DFD induces cirri and, occasionally, sclerotized mouthpart-like material in the trunk segments (Kuziora and McGinnis, 1988; Gonzalez-Reyes et al., 1992). These DFD-induced structures develop at or near regions of *disco* expression, near the Keilin's Organ primordia in the thorax and in analogous positions in the abdominal segments (Fig. 1G, Fig. 3A,D). We suspected this endogenous DISCO was supporting development of the ectopic maxillary structures, and therefore these structures would disappear if embryos lacked *disco* and *disco-r*. To test this, we ectopically expressed *Dfd* in embryos hemizygous for *Df(1)XR14*, and, as expected, found no evidence of ectopic maxillary structures (Fig. 1A,B). We conclude that DFD could not transform the trunk segments toward maxillary identity in the absence of *disco* and *disco-r*.

If, indeed, DFD and DISCO are required for maxillary development, ectopic co-expression should activate maxillary-specific target genes in the trunk segments. We examined the expression of several DFD target genes, *Distal-less*, *Serrate*, *reaper* and *1.28*. Though all appear to be regulated jointly by DFD and DISCO, we present results with *1.28*, as its expression is less complex, and it is a useful marker for maxillary identity (Mahaffey et al., 1993; Mohler et al., 1995; Pederson et al., 1996; Pederson et al., 2000; Mahaffey et al., 2001). We show results using the *prd-Gal4* driver, as this allowed comparison of normal and manipulated segments within the same embryo, but comparable results were obtained with *arm-Gal4*. The pattern of *prd-Gal4* distribution in an early stage 12 embryo is shown in Fig. 1H and diagramed in Fig. 1I.

Alone, *prd→disco* had no effect on *1.28* transcript distribution (Fig. 1D); transcripts accumulated as in wild type embryos (Fig. 1C). *prd→Dfd*, however, caused significant accumulation of *1.28* transcripts in the posterior labial epidermis (Fig. 1E), and we noted slight accumulation in a few cells near the posterior edge of the *prd→Dfd* segments (not visible in the image). That DFD induced *1.28* expression in the labial segment was expected as *disco* is normally expressed in many labial cells (Lee et al., 1991; Mahaffey et al., 2001) and ectopic DFD transforms the labial segment toward a maxillary identity (Kuziora and McGinnis, 1988). The weak expression in the trunk segments was unexpected, but was explained by the fact that ectopic DFD activated *disco* (see below). Co-expression of *disco* and *Dfd* caused significant accumulation of *1.28* transcripts in the posterior epidermis of every other trunk segment (Fig. 1F) overlapping with *prd-Gal4* expression. We conclude from these experiments that the presence of DISCO makes the trunk segments competent to activate DFD target genes, and allows ectopic DFD to function in the presence of the trunk specification system.

The weak expression of *1.28* in *prd→Dfd* embryos did not coincide with *disco* expression in the Keilin's Organ precursors, but was more lateral and posterior. Because all of our other results indicated that DFD and DISCO are required together, we examined *disco* expression in *prd→Dfd* and *arm→Dfd* embryos. In both cases, ectopic DFD activated *disco* (Fig. 3A,C); this induction is likely to be responsible for the low level of *1.28* RNA accumulation in UAS-*Dfd* embryos. Our previous results indicate that DFD is not required for *disco* expression in the gnathal segments (Mahaffey et al., 2001), so we suspect this DFD induction of *disco* reflects that DFD can modulate *disco* expression.

TSH represses DISCO during normal trunk segment development

Because, in an otherwise normal embryo, ectopic DFD causes only a limited trunk to maxillary transformation, and as *disco* is required for this, we suspected that co-expression of *disco* and *Dfd* should yield a more complete transformation. Surprisingly, this was not the case. On average, more cirri

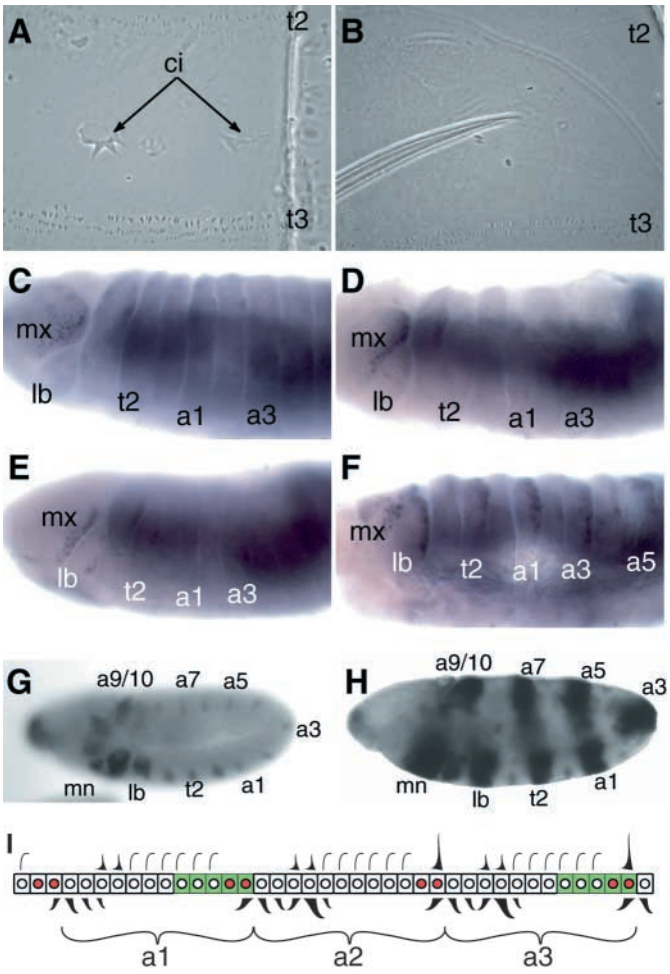

Fig. 1. DISCO and DFD are required for maxillary identity. (A) In otherwise normal embryos, ectopic DFD, which is driven by *prd-Gal4* in this example, induced maxillary structures in the trunk segments. Cirri (ci) and, occasionally, sclerotized rod-like structures (see Fig. 2B) appear in the trunk cuticle. (B) Without *disco* and *disco-r*, no ectopic gnathal structures appeared in *Df(1)XR14/Y* embryos ectopically expressing *Dfd*. (C-F) Together, ectopic DFD and DISCO activate *1.28* transcription. Segment abbreviations in white letters indicate those with ectopic *1.28* RNA. (C) In wild-type embryos *1.28* transcripts accumulate along the posterior edge of the maxillary epidermis, as well as in the gut and anterior spiracles (out of the plane of focus) and in the posterior spiracles (not shown). (D) *1.28* transcript distribution was normal in *prd→disco*. (E) In *prd→Dfd* embryos, in addition to normal transcript accumulation, *1.28* transcripts were detected in the labial segment, which has been transformed toward a maxillary identity, and weakly in the posterior-lateral edge of the expressing segments (not visible in figure). (F) In embryos co-expressing *disco* and *Dfd*, *1.28* transcripts significantly accumulate in the posterior portion of each affected trunk segment, in addition to the maxillary and labial segments. (G) *disco* expression in an early stage 12 wild-type *Drosophila* embryo. The regions of *disco* mRNA accumulation relevant to this study are the gnathal segments (mn, mandibular; mx, maxillary; lb, labial) which make up the visibly segmented region of the head, and the bilaterally symmetric spots along the ventrolateral region of each trunk segment (t2, second thoracic; a, abdominal segments). (H) *disco* mRNA distribution in an early stage 12 *prd→disco* embryo. *prd-Gal4* activates *disco* expression in cells forming the posterior portion of alternating segments, beginning at about stage 10 and continuing through early stage 13. In addition to the regions described in G, *disco* mRNA accumulates in stripes encompassing the posterior half of the mn, lb, t2, a1, a3, a5, a7 and a9/10 segments. (I) *prd→disco* expression in segments a1-a3. Red circles represent nuclei expressing the EN protein. *prd→disco* expression includes the five posterior-most cells (green cells) of every other segment. [Staging is according to Campos-Ortega and Hartenstein (Campos-Ortega and Hartenstein, 1997).]

developed in the trunk segments upon co-activation with *arm-Gal4*, but not with *prd-Gal4* (data not shown). This suggested that DISCO and DFD were not sufficient to induce a stronger transformation; either something else was needed, or the trunk to maxillary transformation was inhibited in a manner that could not be overcome by additional DISCO. Components of the trunk specification program, for example TSH (Fasano et al., 1991; Röder et al., 1992), are likely inhibitors, so we examined the effect of ectopic DFD and DISCO in *tsh* mutant embryos.

Röder et al. (Röder et al., 1992) reported that the trunk segments are partially transformed toward head identity in embryos lacking TSH, as indicated by ectopic sclerotic material in the trunk segments, and changes in the trunk peripheral nervous system. Though we occasionally observed small patches of sclerotic material in the cuticle of homozygous tsh^{8} mutant embryos, we never observed mouth hook-like structures (Fig. 2B). Surprisingly, ectopic DFD caused a stronger transformation when embryos lacked TSH (Fig. 2D) than in otherwise normal embryos (Fig. 2C). Cirri and sclerotized material appeared in most *Dfd*-expressing segments, and the sclerotized material more closely resembled normal maxillary mouth hooks. Co-expression of *disco* and *Dfd* in embryos lacking TSH produced an even more consistent transformation (Fig. 2E,F**)**, where nearly every expressing segment produced cirri and well-formed mouth hooks. We note that no mouthpart structures were produced in embryos lacking *disco* and *disco-r* and *tsh*, regardless of whether or not ectopic DFD was present (data not shown).

These results indicated that TSH hindered the DFD-induced trunk to maxillary transformation, but raised the question why was DFD sufficient to cause a more complete transformation when TSH was absent? As *disco* was still required for the transformation, and because absence of *tsh* causes a partial trunk to head transformation, it seemed likely that TSH may repress head specifying genes, genes such as *disco*. Therefore, we examined *disco* (and *disco-r* with analogous results) mRNA distribution in embryos lacking *tsh* and found it was more widely distributed in these embryos (Fig. 3B). Normally, in the trunk, *disco* mRNA accumulates in the Keilin's Organ primordia and in analogous positions in the abdominal segments (Fig. 3A,D). In *tsh* mutants, the Keilin's Organ primordia were absent, and *disco* mRNA was broadly distributed in the ventral and ventrolateral portion of trunk segments. Ectopic *disco* mRNA did not extend into the dorsal trunk epidermis, where absence of TSH has little or no effect (Röder et al., 1992). Interestingly, *disco* mRNA distribution in *tsh* mutants was very similar to that observed in *arm→Dfd* embryos (see above), with one notable exception. *disco* mRNA was still present in the Keilin's Organ primordia of embryos

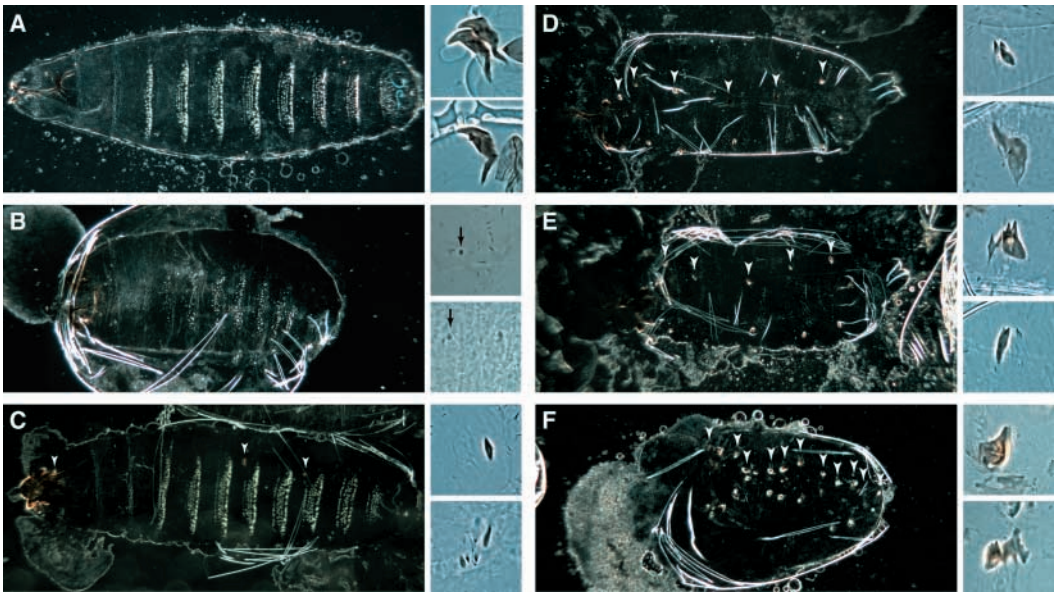

Fig. 2. Trunk to gnathal transformation is more complete in *tsh* mutant embryos. Each panel shows a whole embryo image and higher magnifications from abdominal segments of two independent individuals. Ectopic structures are indicated in the whole embryo images by arrowheads. (A) Wild-type larval cuticle and normal maxillary structures. (B) Ventral cuticle of a terminal homozygous tsh^8 larva. Small spots of sclerotized material (arrows in high-magnification panels) are occasionally present. (C) In the trunk segments of *prd→Dfd* embryos, cirri and, occasionally, rod-shaped sclerotic structures are present. (D) tsh^8, UAS-*Dfd*/tsh^8; *prd-Gal4*/+ embryos demonstrate a more complete transformation. Mouthpart-like material and cirri are present in each affected segment, with well-shaped mouth hooks frequently observed, though the ectopic sclerotized material also can lack a specific shape. (E) tsh^8, *UAS-Dfd, UAS-disco*/tsh^8; *prd-Gal4*/+ embryos exhibit a more consistent transformation. Mouth hooks form in all expressing segments, and we did not observe amorphous sclerotized material as seen in tsh^8, UAS-*Dfd*/tsh^8; *prd-Gal4*/+ embryos shown in D. (F) In tsh^8, *UAS-Dfd, UAS-disco*/tsh^8; *arm-Gal4*/+ embryos, the trunk-to-maxillary transformation is striking, with ectopic maxillary structures appearing in virtually all segments.

ectopically expressing *Dfd*, but not in *tsh* mutants (compare Fig. 3B, *tsh* mutant, with Fig. 3C, ectopic *Dfd*).

To further test the repression of *disco* by TSH, we ubiquitously expressed *tsh* using the *arm-Gal4* driver so that TSH would accumulate in all of the gnathal cells. As shown in Fig. 3E,F, TSH altered normal gnathal expression of *disco*. At the beginning of germband retraction, correlating with the onset of *arm-Gal4* expression, *disco* mRNA levels decreased (Fig. 3E) until, by the end of germband retraction, the normal gnathal distribution was no longer detectable (Fig. 3F). Interestingly, *disco* mRNA was not completely eliminated. In each gnathal lobe, *disco* mRNA accumulated in a small cluster of cells (Fig. 3F) resembling that observed in the thoracic Keilin's Organ precursors (Fig. 3A,D); indeed, ectopic TSH transforms the labial sense organ into one resembling a Keilin's Organ (de Zulueta et al., 1994).

We conclude from the above observations that DISCO and DFD can override the trunk specification system to generate maxillary identity. One manner in which this could occur is for our manipulations to repress expression of the trunk specification system. We noted that ectopic *disco* expression did not repress *tsh* transcription, in contrast to the reverse described above (data not shown). Still, repression could occur through the trunk *HOM-C* genes, and in this regard, it is worth noting that lack of the trunk HOM-C input does give rise to sclerotized material in the trunk segments (Struhl, 1983, Sato et al., 1985). However, our manipulations did not alter the normal distribution of trunk HOM-C proteins (Fig. 4). We examined the distribution of several trunk HOM-C proteins in embryos of all manipulations used in this study, using the *arm*-Gal4 driver to have the broadest possible effect. We found no indication that HOM-C protein accumulation was significantly altered, other than because of the grossly aberrant morphology of later embryos ectopically expressing DISCO. Even then, HOM-C proteins were distributed in the proper register (data not shown). We also examined Labial distribution, as embryos lacking *tsh* were reported to accumulate Labial in small clusters of cells in the trunk. However, we could not detect ectopic *labial* expression in our tsh^8 embryos. We conclude that the combination of DISCO and DFD can override the trunk identity system, redirecting development toward maxillary identity. Clearly, this was more complete when the trunk specification system is compromised, as it is when TSH is absent.

Ectopic DISCO alters trunk development

That *disco* and *disco-r* are ectopically expressed in *tsh* mutants could explain the trunk to head transformation reported in these embryos (Röder et al., 1992). Considering this, we re-examined the effects of ectopic DISCO to see if this would override the trunk specification system, transforming the trunk to a head identity. In embryos ubiquitously expressing *disco*, germband contraction fails and a hole appears in the dorsal epidermis, indicating that DISCO may interfere with dorsal closure (Robertson et al., 2002). We stained *prd→disco* embryos with anti-Engrailed/Invected (EN) antibodies (DiNardo et al., 1985) to monitor the fate of cells in the posterior compartments of the trunk segments, as these cells, and a few cells anterior to these,

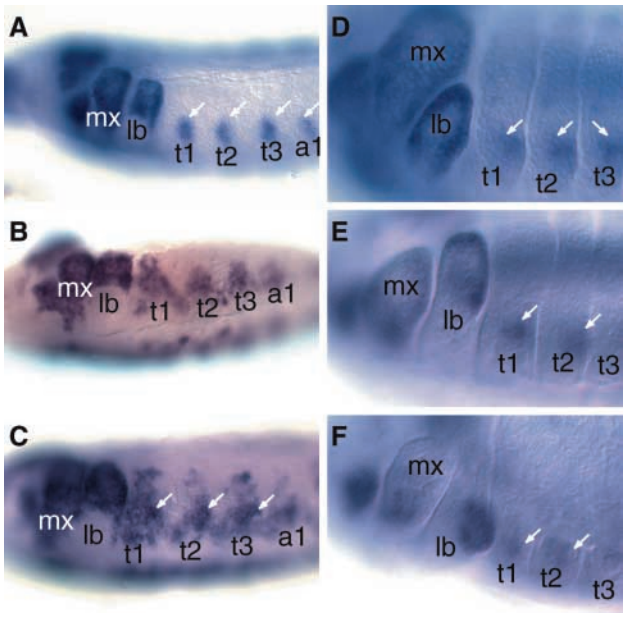

Fig. 3. The role of TSH in *disco* mRNA distribution. (A) *disco* mRNA accumulation in a wild-type embryo as the germband begins to retract. Segment abbreviations are as in Fig. 1. Note the spots of expression in the thorax and abdomen, indicated by white arrows. In the thorax, these demark the Keilin's Organ precursors. (B) In homozygous tsh^{8} embryos, gnathal expression of *disco* is normal, but the trunk distribution is altered. There is a wider distribution in the ventral and ventral-lateral region of trunk segments, particularly notable in the thoracic segments. Note that the spots marking the Keilin's Organs are missing. (C) Ectopic activation of *disco* caused by Gal4-driven DFD. Interestingly, the distribution of *disco* mRNA is quite similar to that in B, above, except that the spots marking the Keilin's Organ precursors are still present in C. (D-F) Ectopic expression of *tsh* represses the normal accumulation of *disco* mRNA in the gnathal segments. (D) Wild-type late stage 12 embryo. *disco* mRNA distribution is fairly uniform in the gnathal segments except where the maxillary and labial sense organs will form and in the salivary primordia (ventral labial). (E) In *arm→tsh* embryos (stage 12), as the germband retracts, *disco* mRNA diminishes in the epidermis of the gnathal lobes. However, staining increases in the central region of the mandibular, maxillary and labial lobes. Later (stage 13) (F), the labial lobe has taken on the appearance of a first thoracic segment, and *disco* is strongly expressed in the sensory precursor, which has been transformed toward a Keilin's Organ. No difference was noted in the trunk *disco* expression.

would be expressing *disco*. In normal stage 13 embryos EN accumulates in dorsoventral stripes about two cells wide marking the posterior compartment of each segment (Fig. 5A). The EN-positive cells can be followed during dorsal closure, when the cells of the trunk segments extend toward the dorsal midline and fuse with cells from the contralateral side. In the affected segments of *prd→disco* embryos, the EN-expressing cells and a few cells anterior to these did not extend toward the dorsal midline (Fig. 5B). Only those cells in the anterior half of the affected segments – those cells not expressing *prd→disco* – completed dorsal closure. We noted that the lack of dorsal closure caused the altered trunk segments to acquire a shape similar to the gnathal lobes.

pannier (*pnr*) encodes a GATA class zinc-finger protein required for dorsal closure (Herranz and Morata, 2001). In

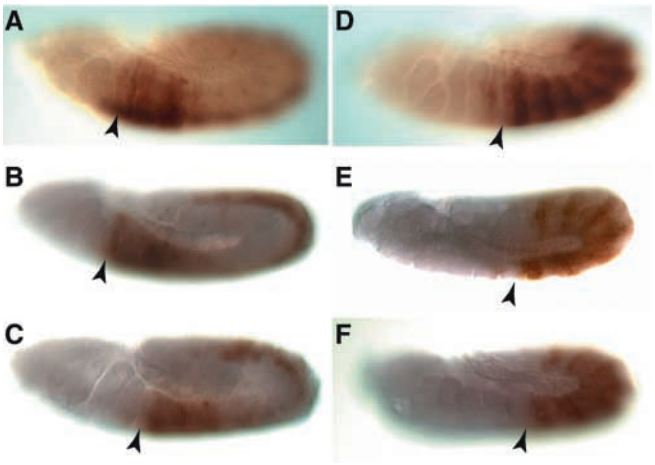

Fig. 4. HOM-C proteins accumulate in the proper register in embryos with ectopic DFD and DISCO but lacking TSH. Stage 12 embryos are shown. In A-C, we show Antennapedia (ANTP) accumulation; the arrowhead marks the beginning of ANTP accumulation in posterior t1. (A) Wild-type embryo. (B) Homozygous tsh^{8} embryo. (C) tsh^{8}, *UAS-disco, UAS-Dfd/* tsh^{8}*; arm-Gal4*/+ embryo. In D-F, Ultrabithorax (UBX) accumulation is shown with the arrowhead marking posterior t3. (D) Wild-type embryo. (E) Homozygous tsh^{8} embryo. (F) tsh^{8}, *UAS-disco, UAS-Dfd/* tsh^{8}*; arm-Gal4*/+ embryo. Note that the register of expression is the same in all cases. The tint to the embryos in B,C,E, F is a consequence of in situ localization of *tsh* mRNA to unequivocally identify *tsh* mutant embryos.

early stage 12 embryos, *pnr* mRNA accumulates along the dorsal edge of the segments, from the posterior maxillary to the eighth abdominal segment (Fig. 5C). In *prd→disco* embryos, this continuous line of *pnr* mRNA accumulation was broken (Fig. 5D). Double-labeling with EN antibodies confirmed that the gaps in *pnr* mRNA accumulation coincided with the cells expressing *prd→disco* (data not shown). Repression of *pnr* was transient. Later in development, as ectopic *disco* mRNA faded (late stage 12 to early 13), *pnr* mRNA was detected in the dorsal limits of the affected segments (data not shown), but apparently, this was too late to rescue dorsal closure. At this time, we do not know whether repression of *pnr* is direct.

Dorsal development is very limited in the gnathal segments, where *disco* is normally expressed. The dorsal ridge is a reduced segment-like structure derived from the gnathal segments (Fig. 5E), and it is the anteriormost structure able to adopt a dorsal fate (Rogers and Kaufman, 1996). Many of the cells that will give rise to the dorsal ridge appear as de novo EN-expressing cells along the dorsal edge of the maxillary and labial lobes (Rogers and Kaufman, 1996). Though *disco* is expressed in many gnathal cells (Lee et al., 1991; Mahaffey et al., 2001), it is not expressed in these dorsal ridge precursors. In fact, the dorsal ridge was quite reduced or eliminated when *disco* was ectopically expressed in these cells (Fig. 5F). This prompted us to ask whether dorsal ridge development was altered in embryos lacking *disco* and *disco-r*, and, indeed, this appeared to be the case. In male embryos carrying *Df(1)XR14*, the dorsal ridge was enlarged and joined with the labial, and sometimes maxillary, lobes (Fig. 5G). We conclude that normal *disco* expression is needed to limit gnathal contribution to the dorsal ridge.

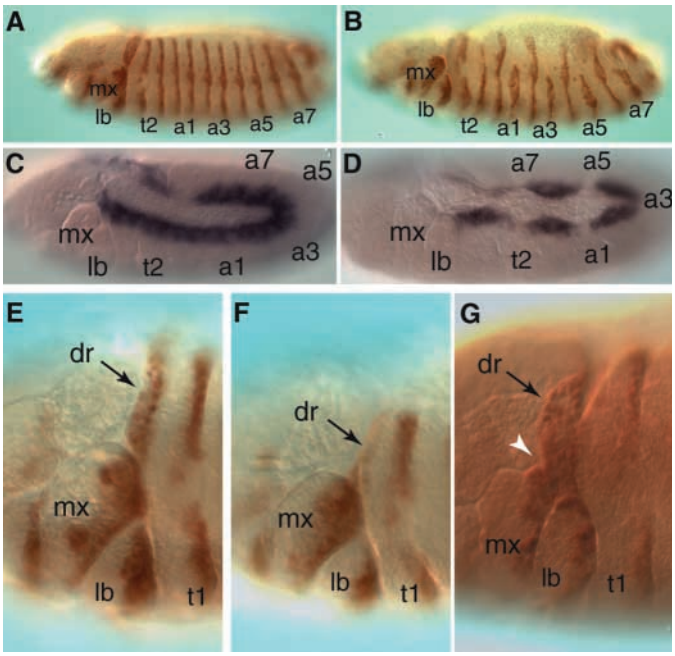

Fig. 5. Dorsal closure is blocked by *prd→disco* expression. (A) In wild-type stage 13 embryos, note the dorsoventral stripes of EN marking the posterior compartment of each segment. (B) In *prd→disco* embryos the segments expressing *disco* do not complete dorsal closure. The EN stripes in these segments extend only about halfway up the embryo. Note that the affected segments are curved and resemble the gnathal lobes. (C-D) *pnr* mRNA distribution in *prd→disco* embryos. (C) In early stage 12 wild-type embryos, *pnr* mRNA accumulates along the dorsal edge of the segments, beginning in the posterior maxillary and extending posteriorly through the eighth abdominal segment. (D) In *prd→disco* embryos, this continuous line of *pnr* mRNA is disrupted. Cells expressing *prd→disco* do not accumulate *pnr*. (E-G) *disco* expression limits the gnathal contribution to the dorsal ridge. (E) Morphology of a normal wild-type stage 13 dorsal ridge. Note the separation of the dorsal ridge from the labial lobe, from which many of the dorsal ridge cells arise. (F) In embryos expressing *prd→disco*, the dorsal ridge is quite reduced. The few EN-positive cells remaining are those that arise from the posterior maxillary/anterior labial where *prd→disco* is not expressed. (G) In *Df(1)XR14/Y* embryos, the dorsal ridge is broadened and contiguous with labial, and sometimes as in this case, maxillary lobes. The embryos were stained to detect EN to facilitate identification of *prd→disco* embryos. dr, dorsal ridge. Anterior is towards the left; dorsal is upwards.

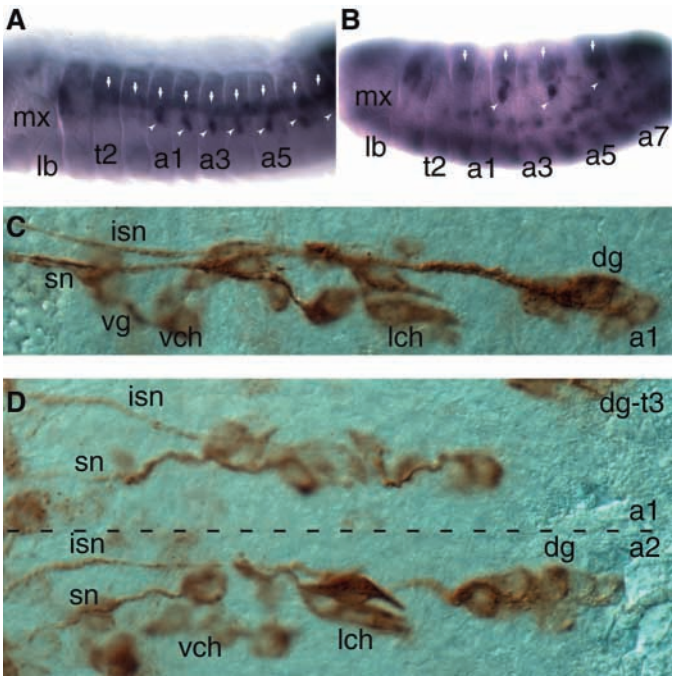

Fig. 6. Ectopic DISCO alters the trunk segments. In an early stage 13 wild-type embryo (A) note the regular appearance of *salm* mRNA in the dorsal tracheal cells (vertical arrows) and the oenocytes (angled arrowheads). (B) In a similarly staged *prd→disco* embryo dorsal tracheal cells and oenocytes are missing in the segments ectopically expressing *disco*. (C,D) The trunk sensory neurons are remodeled by ectopic *disco* expression. Embryo 'fillets' are shown where the gut has been removed and the embryos have been flattened so the neurons are in the same approximate focal plane. Anterior is upwards and dorsal is towards the right in both images. (C) Wild-type sensory neurons of the first abdominal segment. A similar pattern is found in all abdominal segments. Several characteristic neurons and sensory structures are labeled. isn, intersegmental neuron; sn, segmental neuron; vg, ventral sensory organ group; vch, ventral chordotonal organ; lch, lateral chordotonal organ; dg, dorsal sensory group; dg-t3, dorsal group from t3. In *prd→disco* embryos (D), both unaffected (a2, bottom) and affected (a1, top) segments are shown. Note the absence of chordotonal organs in a1, and that the neurons do not extend as far dorsally. The position of the sensory cells in affected segments does not match those in the unaffected or normal trunk segments.

Ectopic *disco* expression disrupted other aspects of trunk development. Previously, we showed that DISCO repressed denticle formation (Robertson et al., 2002), and now we find that other aspects of trunk development are also disrupted. The dorsal trachea and oenocytes were absent (Fig. 6A,B), as indicated by lack of *spalt-major* (*salm*) expression, which is required for formation of these structures (Kühnlein et al., 1994). We note that other regions of *salm* expression were unaffected. The trunk peripheral nervous system was also altered by ectopic *disco* expression. Visualized using anti-22c10/ Futsch antibodies (Hummel et al., 2000), there is a characteristic pattern of sensory neurons produced in each trunk segment (Campos-Ortega and Hartenstein, 1997), and ectopic DISCO altered these in several ways (Fig. 6C,D). The chordotonal organs were absent as were other sensory structures. Ectopic DISCO did not simply eliminate neural structures. Sensory neurons formed, but they did not resemble those normally found in the trunk. We are uncertain of their identity, but suggest that they have a mixed gnathal/trunk identity as both DISCO and TSH are present in these segments. Unknowingly, the role of DISCO in the absence of TSH has been examined previously, while examining *tsh* mutants. As we described above, *disco* and *disco-r* are activated in the trunk of embryos lacking TSH, and Röder et al. (Röder et al., 1992) concluded that the trunk neurons can acquire a gnathal identity in these embryos.

DISCO and SCR can activate SCR target genes

Though our results above deal only with maxillary identity, our prior genetic analysis indicated that *disco* and *disco-r* were

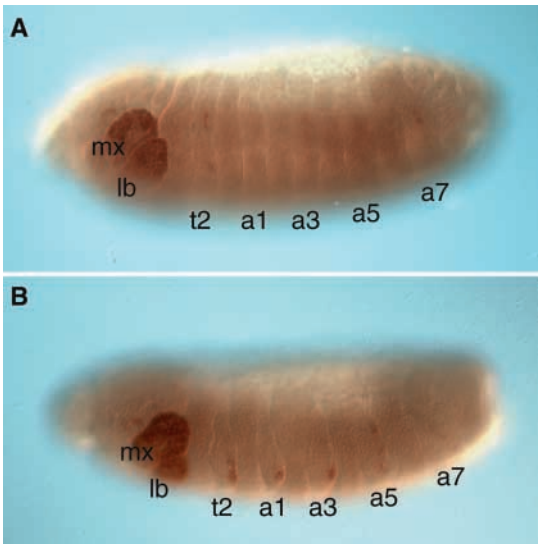

Fig. 7. Activation of the SCR target gene PB by ectopically expressed *disco* and *Scr*. (A) The ectopic expression of *Scr*, using the *prd-Gal4* driver, sometimes results in weak PB activation in some affected segments. The embryo shown was chosen because it was among the most strongly staining for PB accumulation. (B) By contrast, expressing both *disco* and *Scr* in embryos caused significantly increased ectopic PB accumulation, easily visible in each affected segment in all expressing embryos. mx, maxillary; lb, labial; t2, second thoracic; a, abdominal segments.

required for labial development, as well. To determine whether or not this was a general mechanism governing development throughout the gnathal segments, we examined the role of *disco* with *Scr*, using *proboscipedia* (*pb*) as a marker, which has been shown to be a target of SCR in the labial segment (Rusch and Kaufman, 2000). PB ectopically accumulates in the first thoracic segment (T1) of *tsh* mutant embryos (Rusch and Kaufman, 2000), so it seemed likely that this was due to the presence of SCR in T1 and the de-repression of *disco* and *disco-r* in these embryos. To test this, we co-expressed *disco* and *Scr* in the trunk segments using the *prd-Gal4* driver, and indeed, this leads to significant ectopic accumulation of PB, when compared to ectopic *Scr* alone (Fig. 7). This suggests that DISCO has a similar role in maxillary and labial development. We note that expression of *pb* was somewhat spatially limited. This could be due to the use of the *prd-Gal4* driver, the altered morphology of the affected segments, or perhaps other factors limit *pb* expression. A similar enhancement of PB accumulation did not occur with DISCO and DFD.

Discussion

disco was initially identified in a screen for mutations affecting neural development (Steller et al., 1987). It was not until the discovery of *disco-r* that a patterning role was uncovered (Mahaffey et al., 2001). The phenotype of terminal embryos lacking *disco* and *disco-r* is similar to those lacking the gnathal *HOM-C* genes *Dfd* and *Scr*; that is, structures from the gnathal segments (mandibular, maxillary and labial) are missing. This phenotype is due to reduced expression of DFD and SCR target genes. As HOM-C protein distribution is normal in *disco*, *disco-r* null embryos, and vice versa, these factors appear to act in parallel pathways.

We have extended these studies and show that: (1) DFD can only direct maxillary developmental when DISCO and/or DISCO-R are present; (2) TSH represses *disco* (and *disco-r*), helping to distinguish between trunk and gnathal segment types, and thereby establishing domains for appropriate HOM-C protein function; and (3) when ectopically expressed in the trunk, DISCO represses trunk development and may transform these segments towards a gnathal segment type.

Though *HOM-C* genes have a clear role in establishing segment identities, ectopic expression often has only a limited effect. Our data indicate that, for DFD, this restriction arises because of the limited distribution of DISCO in the trunk segments. There are two important conclusions from these observations. First, the spatial distribution of DISCO establishes where cells can respond to DFD, and this is probably true for SCR as well. Cells expressing *disco* develop a maxillary identity when provided with DFD, even though this may not have been their original HOM-C-specified fate. This highlights the second point: the combination of DISCO and DFD overrides normal trunk patterning, without altering expression of *tsh* and trunk *HOM-C* genes. As with the maxillary segment, identity is lost in the mandibular and labial segments when embryos lack *disco* and *disco-r*. This indicates that DISCO and DISCO-R may have similar roles in all gnathal segments. That co-expression of DISCO and SCR in the trunk activates the SCR gnathal target gene *pb* strengthens this conclusion. Therefore, we propose that DISCO defines the gnathal region, and establishes where the gnathal HOM-C proteins DFD and SCR can function.

Alone, ectopic DISCO significantly alters development, indicating that DISCO has a morphogenetic ability, separate from gnathal HOM-C input. As DISCO is required for normal gnathal development, we suspect that *disco* specifies a general gnathal segment type. Definitive identification is difficult because of the lack of morphological or molecular markers that denote a general gnathal segment type. Yet, there is support for the conclusion that *disco* expression establishes a gnathal segment type. Ectopic DISCO can, to some extent, override the trunk specification system and repress trunk development (repressing denticles, oenocytes and trachea). Furthermore, ectopic DISCO blocks dorsal closure, which is similar to the role of endogenous DISCO in the gnathal segments.

Perhaps the most compelling evidence that DISCO specifies a gnathal segment type comes from the observation that *disco* is activated in the trunk segments when embryos lack TSH. The identity of the trunk segments in *tsh* mutant embryos is somewhat uncertain. Fasano et al. (Fasano et al., 1991) and Röder et al. (Röder et al.,1992) suggested that some aspects of the *tsh* phenotype indicate the trunk segments acquire gnathal characteristics; for example, the ventral neural clusters appear to be transformed to a gnathal-like identity (as mentioned above). Röder et al. state that ‘Mutations in the *tsh* gene can therefore be interpreted in two ways; either they partially transform the trunk segments into a gnathal-like identity, and in particular the prothoracic segment into a labial one, or they cause a non-specific change in segmental identity perhaps due to cell death’; however, they also report that the loss of *tsh* and the trunk *HOM-C* genes may transform the trunk cuticle toward anterior head cuticle. Again, the difficulty in assigning an

identity is due to the lack of a readily discernable gnathal morphological or molecular marker. We present evidence that *disco* and *disco-r* are reliable molecular markers for gnathal identity, and we show that *disco* mRNA is present in the ventral and lateral regions of the trunk segments in *tsh* mutant embryos. This expression of *disco* coincides, spatially, with the region of the trunk that is transformed in *tsh* mutant embryos. UAS-driven *disco* does mimic some aspects of *tsh* mutants, denticles are reduced and the ventral chordotonal neurons do not develop, but as TSH is still present, the transformation caused by ectopic *disco* may be incomplete. Finally, DFD cannot induce maxillary structures, even in *tsh* mutants, when *disco* and *disco-r* are absent. This reinforces the role for DISCO in establishing gnathal identity, and indicates that the ectopic DISCO present in embryos lacking TSH is functional. Therefore, considering these arguments, we propose that DISCO and DISCO-R establish the gnathal region of the *Drosophila* embryo, and in this regard, they function similarly to TSH, which specifies the trunk region.

There are other parallels between DISCO/DISCO-R and TSH. They are regionally expressed zinc-finger transcription factors, and they are required in parallel with the HOM-C proteins for proper segment identity. Furthermore, the distribution of these proteins establishes domains in which specific HOM-C proteins can properly direct embryonic development. Our data reveal a regulatory relationship between TSH and *disco* (and *disco-r*), indicating they are part of an interacting network that helps regionalize the *Drosophila* embryo. The HOM-C proteins then establish specific segmental identities in the appropriate region. A schematic of this model is presented in Fig. 8. In the trunk segments, TSH, along with the trunk HOM-C proteins, specifies the trunk segment characteristics, in part by repressing *disco* and, thereby, preventing gnathal characteristics from arising in the trunk segments. Our model requires that *tsh* expression be limited to the trunk segments, and we propose this is accomplished by another C2H2 zinc-finger protein, SALM. Röder et al. (Röder et al., 1992) demonstrated that *tsh* expression expands into the posterior gnathal and posterior abdominal segments in embryos lacking SALM. Therefore, SALM establishes the boundary between the TSH and DISCO domains. We stress that, at this time, we do not know what parts of this regulation are direct. Interestingly, other zinc-finger transcription factors are responsible for positioning *salm* expression (Kühnlein et al., 1997), so that a more extensive hierarchy of zinc-finger transcription factors leads to regionalization, eventually establishing the domains of HOM-C protein function. We also note that TSH has other roles than just repressing *disco*. TSH actively establishes the trunk region, just as DISCO does the gnathal. It is also noteworthy that ectopic TSH activated *disco* in the labial sense organ primordia, leading to a Keilin's Organs fate, as occurs in the thoracic segments. Therefore, for unknown reasons, TSH changes from a repressor of *disco* to an activator in these cells. This observation highlights the complex interplay between factors like TSH and DISCO, and it will be interesting to determine what causes these opposing roles.

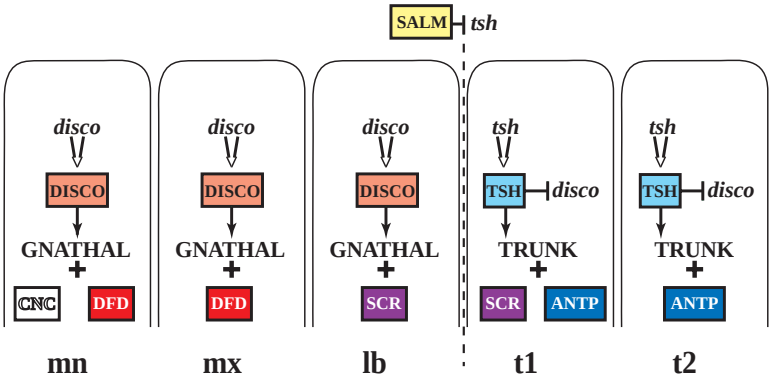

Fig. 8. An interactive hierarchy of zinc-finger transcription factors establishes trunk and gnathal/head segment types. In the trunk segments, TSH represses *disco* expression and directs segments along the trunk developmental pathway. SALM defines the boundary between the head and trunk (broken line) by repressing *tsh* in the gnathal segments. *disco* is expressed in the gnathal segments activating the gnathal development pathway. In this manner, the distribution of TSH and DISCO regionalizes the embryo. When combined with the HOM-C proteins, specific segment identities arise. Note that the HOM-C protein SCR is expressed in both the gnathal and trunk domains and yields a different identity depending upon which co-factor is present. DFD can establish either a maxillary or mandibular identity depending upon the presence of the Cap-n-collar (CNC) protein (Mohler et al., 1995). mn, mandibular; mx, maxillary; lb, labial; t2, second thoracic.

Many other questions remain. For example, how are the expression domains for these factors established? It is clear that SALM could form a boundary separating gnathal from trunk, but in *salm* mutants, *tsh* is only ectopically activated in the posterior labial segment, not in every gnathal segment (Röder et al., 1992). This implies that SALM forms a boundary, not by repressing *tsh* throughout the head, but by, in a sense, drawing a line between the head and trunk regions. What then prevents *tsh* expression from crossing that line and extending further into the gnathal segments in *salm* mutants? Is there an activator of *tsh* that is limiting, another gnathal repressor, or is something else involved? Likewise, what activates *tsh* and *disco*? It is unlikely that lack of TSH is the only requirement for *disco* expression. More likely, this relies on the prior segmentation pathway. With regard to the HOM-C specification of segment identity, questions remain as to how the zinc-finger proteins establish where specific HOM-C proteins can function. Are the zinc-finger proteins co-factors or simply a parallel pathway? Furthermore, if they are co-factors for the HOM-C proteins, how can different HOM-C proteins establish different segment identities with the same co-factor (for example, DFD and SCR with DISCO), or how can different co-factors alter the role of a HOM-C protein (SCR with DISCO or TSH)?

Finally, we are left with the question of whether or not factors such as DISCO and TSH establish head/trunk domains and delimit HOM-C protein function only in the *Drosophila* embryo, in all stages of *Drosophila* or in other animals as well. Though this remains to be tested experimentally, there are indications that this may be a general mechanism. Homologues of these zinc-finger genes are found in vertebrates and in other invertebrates (Caubit et al., 2000; Knight and Shimeld, 2001), and, although only limited data are currently available (Caubit et al., 2000) (M. K. Patel and J.W.M., unpublished), expression data indicate that these genes may have similar roles to their

Drosophila counterparts during embryonic patterning. In an informative experiment by Brown et al. (Brown et al., 1999), they expressed the Tribolium *Dfd* homologue, *Tc-Dfd*, in *Drosophila* embryos lacking the endogenous *Dfd* gene and showed that persistent expression of *Tc-Dfd* could rescue maxillary development. Though, at present, it is not known whether or not a direct interaction is required between DISCO and DFD, this result would indicate that the Tribolium DFD protein can fulfill the same roles as the *Drosophila* protein, and, therefore, it must be able to function with the *Drosophila* regionalization system. In any case, it will be important to investigate and interpret the role of the regionalizing genes as they relate to development and evolution of body pattern in other animals, and to ask whether a similar network is involved in patterning all animals.

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