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The appendage role of insect *disco* genes and possible implications on the evolution of the maggot larval form

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Abstract

Though initially identified as necessary for neural migration, Disconnected and its partially redundant paralog, Disco-related, are required for proper head segment identity during *Drosophila* embryogenesis. Here, we present evidence that these genes are also required for proper ventral appendage development during development of the adult fly, where they specify medial to distal appendage development. Cells lacking the *disco* genes cannot contribute to the medial and distal portions of ventral appendages. Further, ectopic *disco* transforms dorsal appendages toward ventral fates; in wing discs, the medial and distal leg development pathways are activated. Interestingly, this appendage role is conserved in the red flour beetle, *Tribolium* (where legs develop during embryogenesis), yet in the beetle we found no evidence for a head segmentation role. The lack of an embryonic head specification role in *Tribolium* could be interpreted as a loss of the head segmentation function in *Tribolium* or gain of this function during evolution of flies. However, we suggest an alternative explanation. We propose that the *disco* genes always function as appendage factors, but their appendage nature is masked during *Drosophila* embryogenesis due to the reduction of limb fields in the maggot style *Drosophila* larva.

Keywords: *Drosophila*; Segment identity; Zinc finger; Appendage; Limb; Pattern formation

Introduction

The arthropod body is composed of a series of repeated segments along the anterior/posterior body axis, and though sometimes similar in appearance, different segments often have differing morphological characteristics. The differences in segment morphology are often evident as variations in the form of appendages extending from each segment. The wormlike *Drosophila* larva lacks appendages except for small sensory organs that are thought to be the remnants of larval appendages. Adult appendages arise from the imaginal discs, which are blocks of cells set aside during embryogenesis. The genetic hierarchy governing appendage development has been extensively studied in *Drosophila* (see reviews by Kojima, 2004; Morata, 2001; Panganiban, 2000). Many factors have been identified that have proximal to distal domains of expression and control of appendage development. Some factors have roles in all appendages, while others are specific for a particular class of appendage. Comparative studies in other insects indicate that much of this process has been conserved (for examples, see Abzhanov and Kaufman, 2000; Angelini and Kaufman, 2005a, 2005b; Beermann et al., 2004; Jockusch et al., 2004; Prpic et al., 2001; Williams and Nagy, 2001).

Previously, we presented evidence that the redundant genes *disconnected* (*disco*) and *disco-related* (*disco-r*) (together referred to as the *disco* genes below) function in parallel with the head Hox genes to specify identity in the larval head segments during *Drosophila* embryogenesis (Mahaffey, 2005; Mahaffey et al., 2001; Robertson et al., 2004). In this manuscript, we describe our investigation into the role of the *disco* genes during development of the adult fly. Earlier work (Lee et al., 1991) demonstrated that *disco* is expressed in some

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of the imaginal discs. Furthermore, *lacZ* from the enhancer trap line C50.1S1 (reported to reside near *disco*) has been used as a marker for studies of leg joint formation (Bishop et al., 1999; Mirth and Akam, 2002). Below we demonstrate that the *disco* genes play a prominent role in appendage specification where they are necessary in order for cells to contribute to the medial and distal portions of the ventral appendages. Highlighting the substantial role these genes play during appendage development, ectopic expression transforms dorsal appendages to ventral fate; for example, ectopic activation of *disco* transforms the wings towards legs. We also demonstrate that the appendage specification role is conserved in the beetle, *Tribolium castaneum*. However, we found no evidence for an embryonic head specification role in *Tribolium*. We discuss this apparent discrepancy between appendage and segmentation roles for *disco* genes and propose that, in actuality, there may only be an appendage role. We offer that the reduction of appendage fields during evolution of the derived, limbless maggot larval form may be the reason these genes to appear to be regional head specification factors in the embryo.

Materials and methods

Drosophila and Tribolium stocks and culture

Flies were reared on standard cornmeal-agar-molasses medium. Deficiency *Df(1)ED7355* (14A8–14B7) (FBst0008899) was generated by the DrosDel Project (Ryder et al., 2004) and was obtained from the Bloomington Stock center. *Tribolium* (GA1 wild-type) were reared at 30 °C on whole-wheat flour supplemented with 5% yeast powder (Berghammer et al., 1999).

Induction of UAS-disco

We induced ectopic expression of *disco* with the *UAS-disco* lines described by Robertson et al. (2002) at 17 °C, 25 °C and 29 °C. We tried several imaginal disc drivers, *P{GawB}E132*, (Halder et al., 1995), *P{GAL4}klu*^{G410} (Klein and Campos-Ortega, 1997), *P{GAL4-dpp.blk1}* (Staehling-Hampton et al., 1994) and *P{GawB-ΔKE}Bx*$^{MS1096\text{-}KE}$ (Capdevila and Guerrero, 1994). Only *P{GawB-ΔKE}Bx*$^{MS1096\text{-}KE}$ and *P{GAL4-dpp.blk1}* supported development to produce some pharate adults that could be dissected for examination. Even with these drivers, most died before pupation.

Cloning of Tc-disco

The *Drosophila disco* sequence was compared to *Tribolium* genome trace files available at NCBI (http://www.ncbi.nlm.nih.gov/) using BLAST (Altschul et al., 1997). Overlapping regions were assembled to obtain the contiguous genomic region using Biolign 2.0.9 and the CAP contig assembly program (Huang, 1992). ORF Finder (http://www.ncbi.nlm.nih.gov/gorf/gorf.html) was used to identify the predicted open reading frame. For in situ probes and RNAi studies, primers were designed to amplify the ORF as a DNA template for RNA transcription. Genomic DNA was isolated from *Tribolium* pupae with the Qiagen DNeasy Tissue Kit. Two sets of primers were necessary to amplify overlapping regions of the *Tc-disco* ORF. The first pair amplified a 2220-bp region: forward—5′ ATGTCACCTAACCATCGCC 3′; reverse—5′ GTAATGCGTTTTCACGCCGA 3′. The second pair amplified a 2177-bp region: forward—5′ AGACGTTTTGCGACAAAGG 3′; reverse—5′ TCACGAACTCTCCGAACTCTT 3′. The resulting PCR products were cloned into Promega's pGEM-T Easy vector. Both clones were digested with *Xba*I and *Sac*II, and the appropriate adjoining fragments were isolated and ligated together yielding a 2931-bp *Tc-disco* ORF. Sense and antisense transcripts were generated using T7 or SP6 polymerase (Promega).

RNAi injections

Injections were performed as described for parental RNAi (Bucher et al., 2002). Approximately 0.2 μl of dsRNA (0.5 μg/μl to 3 μg/μl) was injected into each pupa. Injected females were allowed to complete pupation and were mated to wild-type males. Larval offspring and unhatched eggs were collected. To control for the mechanical effects of injection or effects coming from the injection buffer, control injections were performed with buffer only. To control for effects that may arise from activation of the RNAi pathway, but not specific to *Tc-disco*, we also performed control injections using dsRNA prepared from a portion of the *Drosophila giant*coding region. Unhatched or newly hatched *Tribolium* larvae were prepared for cuticle examination essentially as described for *Drosophila* (Pederson et al., 1996). Efficacy of RNAi was assayed by in situ hybridization with the *Tc-disco*probe to embryos collected from injected females.

In situ localizations of mRNA and protein

Drosophila imaginal tissues were dissected from larvae and pupae in phosphate-buffered saline and placed into the standard in situ fixative. Digoxigenin-labeled antisense RNAs were prepared and in situ hybridizations were done essentially as in Tautz and Pfeifle (1989). For *Tribolium*, the partial *Tc-disco* ORF comprising the first PCR generated clone described above was used as a template. Overnight hybridization was done at 55 °C. Probes for *disco*, *disco-r* and *Dll* mRNAs were described in Mahaffey et al. (2001). The *wingless*clone was kindly provided by Dr. Amy Bejsovec (Duke University). Other probes were obtained from *Drosophila*genomic DNA using PCR. The primers used to generate clones were as follows: *bifid*, AGATACGACGTCCAGGAGCTG (forward) and TGCCGCTCTTGGTGATGA (reverse); *apterous*, TTGGTACTCGCCGATGCT (forward) and CAAGTTAAGTGGCGGTGTGC (reverse); and *scalloped*, CTATGTGTTTGAGGTGGCGG (forward) and GCTGAACTAAAGTCGGTT (reverse). Immunological detection of proteins followed the protocol described in Pederson et al. (1996).

Imaginal discs for fluorescent in situ hybridization were dissected and fixed as mentioned above. Probes for fluorescent in situ hybridization were made essentially as described with different haptens. The hapten used for *disco* probes was digoxigenin and for *teashirt* probes was biotin. The protocol used for hybridization is essentially described in Kosman et al. (2004); however, there was no treatment with xylenes or proteinase K for imaginal discs. For detection of *disco*, a sheep anti-DIG HRP (Roche) antibody (1:400) was used, and for *teashirt* a mouse anti-Biotin (1:400) was used as a primary then a goat anti-mouse HRP (1:250) as a secondary. All antibodies were preabsorbed against fixed *Drosophila* embryos. In both cases, the Cy3/Flourescein tyramide signal amplification kit from Perkin Elmer was used. For *disco* the tyramide reaction was for 25 min, and for *teashirt* was 5 min in imaginal discs. For embryos both tyramide reactions were 15 min. Detection of bound Dachshund antibodies was with goat anti-mouse labeled with fluorescein. Images were obtained using the Zeiss Pascal confocal microscope and then subsequently processed using the 3D imaging software Volocity LE (Improvision) and Adobe Photoshop.

Mosaic analysis of Drosophila

We used the FLP/FRT recombination system (Xu and Rubin, 1993). *Df(1) ED7355* (FBab0030966) females were crossed with *w*^{1118}*,sn3,P{neoFRT}19A* (FBst0001740) males to generate recombinants between the deficiency and the *19A FRT* site. We generated both *sn* marked and non-marked *Df(1) ED7355*, *FRT* bearing chromosomes, permitting us to mark in separate experiments either the homozygous deficient cells or the twinned wild-type cells with the *sn* bristle marker. The recombinant females were crossed with *P{neoFRT}19A, P{tubP-GAL80}LL1,P{hsFLP}1,w** (FBst0005132) males. To increase the rate of mosaicism, *w*^{1118}*,sn3,P{neoFRT}19A* and *P{neoFRT}19A,P{tubP-GAL80}LL1, P{hsFLP}1,w** females were crossed with *w*^{1118}*;P{70FLP}10* (FBst0006938) males that carry *FLPase* on second chromosome. Male progeny were mated with the deficiency-FRT recombinant females. Females were allowed to lay eggs for either 4 or 24 h followed by heat shock for 4 h by submerging vials in a 37 °C water bath. Resulting female progeny of appropriate genotype were analyzed.

To mark the clones with green fluorescent protein (GFP), we generated a second chromosome that carried *UAS-GFP*, *arm-Gal4* and *hsFLP* by following the standard recombination methods. This chromosome was homozygosed in *sn-, Df(1)ED7355, FRT* background as well as in the *P{neoFRT}19A,P{tubP-GAL80} LL1,P{hsFLP}1,w** flies. Females of the first genotype were mated with males of the latter genotype to create mosaic flies. Heat shock treatment was the same as described before.

Results

The disco genes are widely expressed in ventral imaginal discs of Drosophila

Prior work mentioned that *disco* is expressed in many of the imaginal discs (Lee et al., 1991). Below, we extend the description of *disco* and include *disco-r* expression in the imaginal discs. In early third instar larvae, the *disco* genes are expressed in the ventral imaginal discs (Fig. 1): the antennal and maxillary palp regions of the eye-antennal discs, the labial discs (data not shown) and in the leg discs. In addition, they are expressed in a small patch of cells in the future scutellum of the wing discs, in a similar position in the haltere discs and weakly in an anterior region of the eye disc. We did not detect any difference in the spatial expression of the two genes, though *disco-r* appeared to be more abundant in the discs which is opposite to what we previously observed in embryos (Mahaffey et al., 2001).

To help define the spatial and temporal aspects of expression, we compared *disco* with two other known appendage factors, *teashirt* (*tsh*) and *dachshund* (*dac*). *tsh* is required for proper development of the trunk segments during embryogenesis (de Zulueta et al., 1994; Fasano et al., 1991; Roder et al., 1992) and of the body wall and proximal regions of ventral discs during development of the adult (Erkner et al., 1999; Wu and Cohen, 2000). In accord with this, *tsh* is expressed in the outer ring of the leg discs (Figs. 2A and E′). *disco* mRNAs were co-expressed in many of the *tsh* expressing cells of the leg discs, except in the dorsal (stalk) region (Figs. 2E, E′ and E″). In the medial and distal portions of the discs (where *tsh* is not expressed) there was uniform expression of the *disco* genes.

dac is required for medial appendage development (Mardon et al., 1994). We used an antibody to detect Dac while detecting *disco* mRNA. Since Dac is a nuclear protein and the nuclei of the columnar disc cells are basally located, Dac staining appeared below the cytoplasmic staining of the *disco* mRNA. However, *z*-section reconstructions (Figs. 2F, G) permitted unequivocal demonstration that Dac positive cells also expressed *disco*. As above, we present data from the leg discs as an example. The distribution of Dac protein was entirely contained within the *disco* domain. In the dorsal region, the expression boundaries are nearly congruent. However, ventrally the *disco* mRNA was more proximally expressed, in the region where *disco* and *tsh* overlap (Fig. 2F). Furthermore, while Dac accumulated only in the medial portion of the discs, *disco* expression extended through the distal region (Figs. 2F and G), thus overlapping the *Distal-less* (*Dll*) domain (data not shown). This distal expression was more pronounced during eversion of the leg (Fig. 2G). We also note that both *disco* genes are weakly

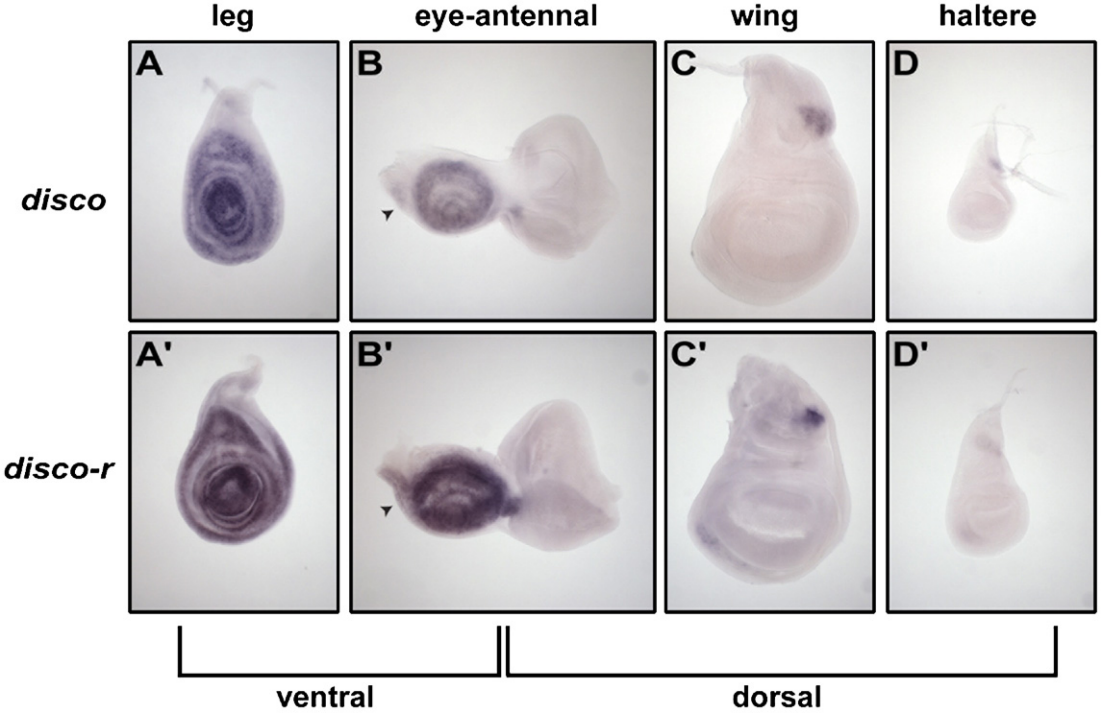


Fig. 1. Expression of *disco*and *disco-r* in third instar imaginal discs. The top panels show expression of *disco* and the lower panels *disco-r* (denoted by ′). The *disco* genes were expressed throughout most of the leg discs (A and A′), and the antennal and maxillary palp (arrowhead) portion of the eye-antennal discs (B and B′), and there was weak expression in the anterior-ventral fold of the eye discs. In the wing (C and C′), the *disco* genes were expressed in a small region of the scutellum. Expression was observed in an equivalent region of the haltere disc (D and D′). The legs and antennal and maxillary portion of the eye-antennal disc are ventral appendages while the wing and haltere are dorsal appendage discs.

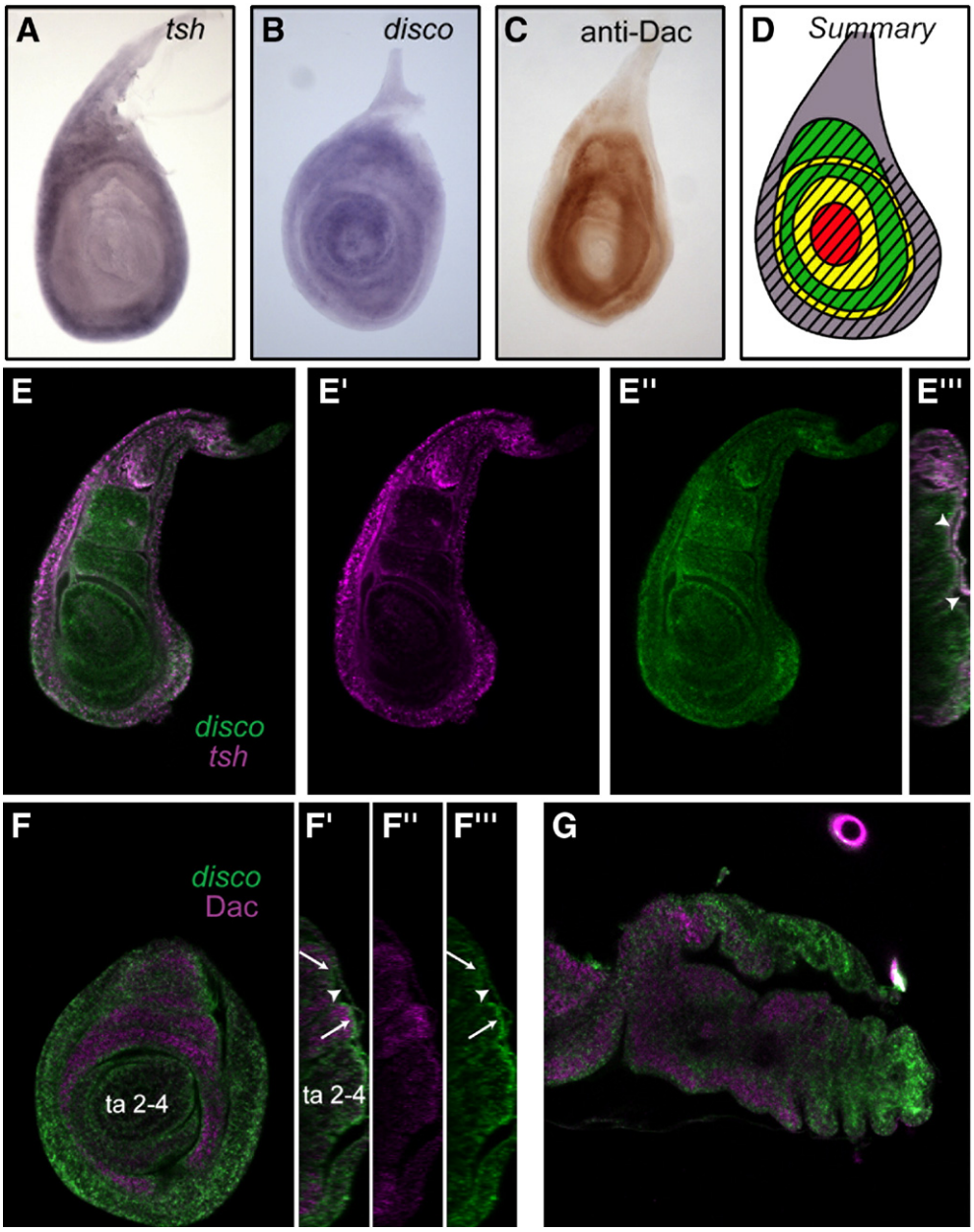


Fig. 2. Relationship between *disco* and other appendage factors in the *Drosophila* wandering third instar discs. (A) *tsh*, (B) *disco* and (C) Dac. (D) A summary of our interpretation of gene expression in the leg discs; *tsh* (violet), Dac (green), *Dll* (red), Dac+*Dll* (yellow) and *disco* (striped region). (E–E‴) Fluorescent in situ detection of *disco* (green) and *tsh* (magenta) mRNAs. E, composite of both; E′, *tsh*; E″, *disco*; and E‴, a *z*-section of the stacked images. White arrowheads point to the peripodial membrane. F–F‴, Dac protein (magenta) and *disco* mRNA (green). F, composite XY section; F′ composite YZ section. Arrows point to the apical location of the *disco* mRNA, while the nuclear Dac protein is more basally located. F″, Dac; F‴ *disco* mRNA. (G) Extending leg labeled as in panel F.

expressed near the joints of completely extended legs, while the C50.1S1 enhancer trap is strongly expressed making it a good marker for studies of joint formation (see Supplementary Fig. 1). In sum, the *disco* genes are widely expressed throughout the majority of the leg disc cells, indicating these genes may have a prominent role during development of these tissues.

Cells lacking the disco genes do not contribute to the ventral appendages

Embryos homozygous for loss of both *disco* genes die during late embryogenesis, so mosaic analysis, generating small clones of homozygous mutant cells in a heterozygous background, is needed to assess the role of these genes during later stages. However, redundancy requires that we eliminate both genes, and our attempts to generate double mutants have been unsuccessful so far. Therefore, we took advantage of a small deficiency generated by the DrosDel project (Ryder et al., 2004), *Df(1)ED7355*, which removes both *disco* and *disco-r* and only a few neighboring genes (see Supplementary Fig. 2). We used this deficiency with the FLP/FRT system (Xu and Rubin, 1993).

We generated an FRT-bearing chromosome containing *Df(1)ED7355* and with the *sn* bristle marker to mark homozygous

Table 1

Fly no.	Eye	Dorsal head	Ventral head	Ant	Max	Prob	Femur	Coxa/ Troch	Thorax	Abd
(A) sn,Df(1)ED7355, FRT/FM7h × FLP, FRT/y;+/FLP										
208	188	39	16	0	0	0	0	3	83	134
%	90	18	8	0	0	0	0	1	40	64
(B) Df(1)ED7355, FRT/FM7h × sn, FRT/y;+/FLP										
91	88	66	55	31	25	20	38	36	75	80
%	96	72	60	34	27	21	41	39	82	88

In the upper table (A), homozygous *Df(1)ED7355* clones were marked with *sn* bristles. In the lower table, *sn* bristles mark the homozygous non-*Df(1)ED7355* clones. In both, eye clones are marked by combinations of mini-white. The three clones listed as coxa/trochanter in (A) were all in the coxa. The reduced number of clones in the dorsal and ventral head capsule (reduced when compared to the reciprocal cross, B) could indicate that the *disco* genes play some role in specification of this region. In both tables, the top row shows absolute numbers from the experiment, and the bottom row shows the percent of total flies scored. We did note that even where homozygous *Df(1)ED7355*cells survived, non-deficiency *sn* clones were often larger, possibly indicating there was somewhat of a growth disadvantage to the homozygous deficiency cells. This may have accounted for the lower numbers of total clones identified when marking the deficiency cells. Abbreviations: Ant=antenna; Max=maxillary palps; Prob=-proboscis; Troch=trochanter; Abd=abdomen.

deficiency clones in adults (see Materials and methods for more details). Furthermore, *Df(1)ED7355* contained the mini-white marker, so dark red sectors would mark homozygous deficiency clones in the eyes, while their "twinned" homozygous normal cells would be white. Using this technique, we were able to generate homozygous *Df(1)ED7355* clones in many tissues (Table 1A). *sn* bristles appeared in the dorsal and ventral body wall of the thorax, in the abdomen and in the dorsal and ventral head capsule (see thoracic example in Figs. 3A and B). Dark red sectors accompanied by white "twinned" regions were observed in the eyes (Fig. 3C). In contrast, we never observed *sn*-marked bristles in the antennae, maxillary palps or proboscises (Table 1A). In the legs, we did find three flies each with one or two *sn*bristles in the coxa (i.e. the proximal-most portion of the legs), but we never observed *sn* bristles in the trochanter or femur (Table 1A). The occasional clone in the coxa could reflect that the *disco* genes are expressed only in part of the proximal regions of the legs, or that these were late-occurring recombinants arising after *disco* function. As a control, we used *sn* to mark bristles in cells homozygous for the non-deficiency chromo-

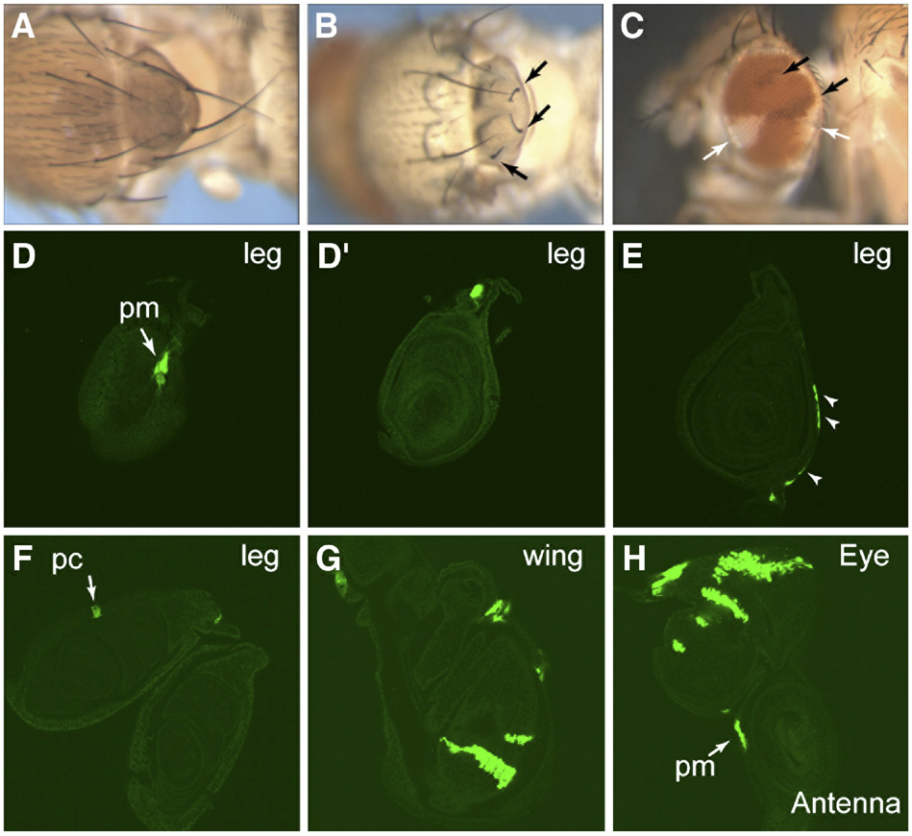


Fig. 3. Clonal analysis of *disco* genes. (A) Scutellar bristles on the thorax of a wild-type *Drosophila* adult. (B) *sn* marked bristles developing in a clone of cells that are homozygous for *Df(1)ED7355*. (C) Clones of cells in the eye. Homozygous wild-type cells are white, while those homozygous for *Df(1)ED7355* are dark red. (D–H) GFP marked clones in imaginal discs. (D and D′) Two different *z*-sections from the same leg disc. (D) A clone in the peripodial membrane (pm) while panel D′ shows there are no clones in the columnar cells of the disc proper. (E) Another leg disc with clones in cells along the outer edge of the disc (arrowheads). These appear to be hematocytes and are not disc-specific cells. (F) Paired first thoracic leg discs. Note the clone (pc) in the columnar cells within the proximal region of the disc. In contrast to the leg discs, marked cells appear in many regions of the dorsal imaginal discs. (G) A wing disc, (H) An eye-antennal disc with several clones in the eye, a dorsal structure, but only in the peripodial membrane (pm) of the antennae, a ventral disc.

some (see Materials and methods), resulting in *sn* bristles in all regions of the fly (Table 1B).

Since *sn* only marked bristle cells, we established a variation of the MARCM system (Lee and Luo, 2001) so that we could mark homozygous *Df(1)ED7355* clonal cells with green fluorescent protein (GFP) (see Materials and methods for details). In agreement with the *sn* marker, we were able to generate numerous GFP-positive clones in the eyes, abdomen, thoracic and head capsule of adult flies (data not shown). The only GFP-positive clones in the legs, antenna, maxillary palps or proboscis were in neurons that ran down the center of the appendages (data not shown). We also examined clone positions in third instar larval imaginal discs. Overall, clones were prevalent in the dorsal imaginal discs (wing and eye, Figs. 3G and H, respectively). GFP-positive cells were present in all parts of these discs. By contrast, GFP-positive cells were rare in ventral discs, and they were not observed in the medial and distal portions of the ventral discs (Figs. 3D′, E and F). Marked clones were observed in the peripodial membranes (Figs. 3D and H) and occasionally in the proximal portions of the disc proper (Fig. 3F), but never in the medial or distal regions. We also detected what appeared to be GFP-labeled hemocytes/macrophage in and around the leg discs (Fig. 3E). The GFP-tagged data agrees with our *sn* marked clonal results, and we conclude that homozygous *Df(1)ED7355* clones can survive in many regions, but not where the *disco* genes are expressed. We suspect that clonal cells lacking the *disco* genes were generated, but that they cannot survive or are removed from the medial and distal portions of the ventral appendages.

Ectopic Disco transforms dorsal appendages to ventral identity

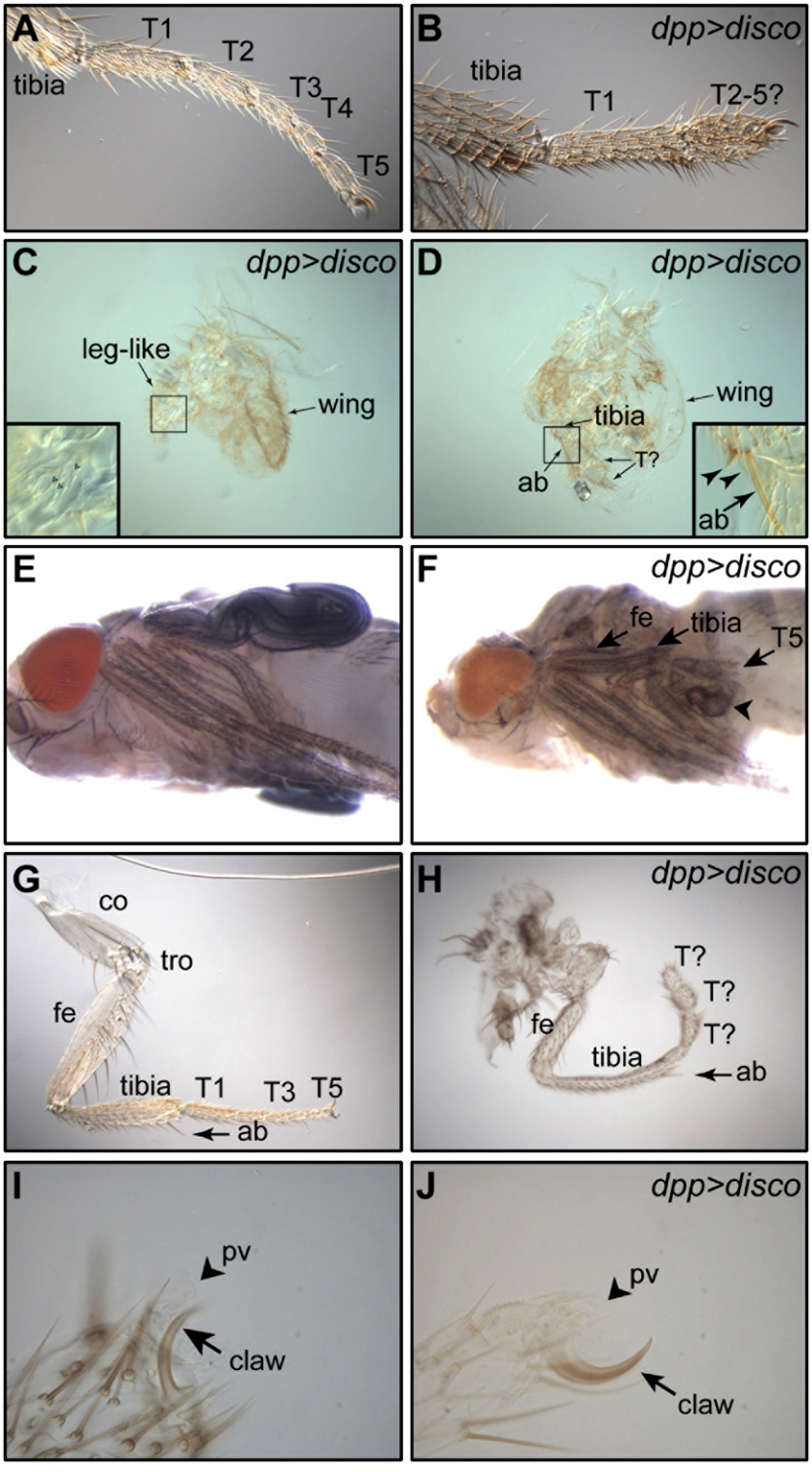
That cells lacking the *disco* genes were not found in the ventral appendages could imply that either these genes are required to establish this region of the appendage, or that they are required for cells to remain viable in this region. To distinguish between these possibilities, we ectopically expressed *disco* (Robertson et al., 2002) using the UAS/Gal4 system (Brand and Perrimon, 1993). All flies ectopically expressing *disco* died prior to eclosing as adults and most died during larval stages. However, when they did survive to form pupae, we dissected them from the pupal case to examine the consequences of ectopic *disco* expression. Using the *P{GAL4-dpp.blk1}* driver, we noted that most ventral appendages (antenna, mouthparts and legs) were nearly normal. The only defect noted was that the number of tarsal segments in the legs was sometimes reduced (Figs. 4A and B). It was not clear if this was caused by fusion or deletion of the tarsal subsegments.

In contrast to the limited effect on ventral appendages, ectopic *disco* had an impressive effect on dorsal appendages; for example, wings and halteres were transformed into leg-like appendages (Figs. 4C–H) and antenna-like structures occasionally developed in the eyes. The transformation was sensitive to growth temperature. No larvae survived to form pupae at 29°, and most did not form pupae even at 25 °C. For those that did form pupae at 25 °C, the wings were in various states of transformation toward legs. About equal numbers were of the weak and moderate phenotype (Figs. 4C and D, respectively). Though the extent of transformation did vary, in all cases, what should have been wing contained easily recognized leg tissues. Bracted bristles, a characteristic of legs, were found on all of the transformed wings. In many cases tarsal segments were present, and often we could identify apical bristles and spurs, specialized bristles found on second thoracic legs, indicating that the leg tissue had appropriate second thoracic segment identity. About 20% of the transformed wings developed as well-formed leg-like structures (Figs. 4E–J). In these cases, the identity of tissues in the proximal wing was difficult to determine, though it was possibly a combination of wing and leg tissues. We could not rule out the presence of some coxa- and/or trochanter-like material. Clear femur, tibia, tarsal and pretarsal segments were formed, and the pretarsal segment contained at least one claw-like bristle and a pulvillus (Fig. 4J). Well-formed joints separated the femur and tibia and the tibia from the terminal region. The *P{GawB-ΔKE}Bx*$^{MS1096\text{-}KE}$ driver also generated leg-like tissues in the wing (data not shown). Regions of bracted bristles were always observed and occasionally a bristle resembling the apical/preapical bristle was observed. In some cases, regions of bracted bristles were separated by what might have been vestiges of leg segmentation.

Ectopic expression has been used to study other limb patterning genes (Chen et al., 1997; Duncan et al., 1998; Estella et al., 2003; Gorfinkiel et al., 1997) and several have been proposed to transform the wings into leg tissues. We examined *spineless*, *homothorax* (*hth*), *dac* and *tsh* using the *dpp*-Gal4 driver (data not shown). All of those we tested, and those described in the literature, had rather modest transformations compared with what we observed resulting from ectopic *disco*. From these observations, we conclude that *disco* is a potent member of the ventral appendage network, capable of transforming dorsal appendages toward ventral fate.

That such complete legs formed from the wing imaginal disc was surprising considering *P{GAL4-dpp.blk1}* activates expression only in a narrow line of cells along the anterior/posterior compartment boundary of the wing discs (Staehling-Hampton et al., 1994). To investigate how limited *disco* expression could cause such a complete wing-to-leg transformation, we examined expression of several leg and wing factors following ectopic activation of *disco* in the wing discs. As expected with the *dpp* driver (Staehling-Hampton et al., 1994), *dpp*-driven *disco* mRNA accumulated to high levels along the A/P border (Figs. 5A and A′). However, we noted that ectopic *disco* (Fig. 5A′) and *disco-r* (Figs. 5B′, F and G) mRNAs accumulated anterior to this stripe, in the anterior wing blade region. Prior cell lineage tracing studies (Weigmann and Cohen, 1999) indicated that these anterior wing blade cells arise as daughters of cells initially in the *dpp* stripe. These cells cease expressing *dpp* as they progress anteriorly. Lee et al. (1999) demonstrated that *disco* can autoactivate, so perhaps the continued ectopic *disco* and *disco-r* expression may be due to autoactivation of the endogenous genes. This region continues to grow producing a large number of cells with high levels of ectopic expression of the endogenous *disco* genes

(Figs. 5F and G). Dac (Figs. 5C and C′), required for medial appendage development (Beermann et al., 2004; Mardon et al., 1994), and *Distal-less* (*Dll*) (Figs. 5D and D′), required for distal appendage (Cohen et al., 1989; Cohen and Jurgens, 1989), were also activated in these cells. As the transformed region continued to expand, a region composed of cells with high levels of *disco* (data not shown), *disco-r* (Fig. 5F), Dac (Fig. 5G) and *Dll* expression arose (data not shown). We also examined expression of *hth* and *tsh*, both encoding proximal appendage factors, and neither was induced by ectopic *disco* (data not shown). This indicates that activation of *disco* does not just induce leg, but it induces the portions of the leg normally controlled by *disco*, the medial and distal regions.



Since dramatic changes were observed in the expression of the *disco* genes and Dac, we followed the expression of these genes in more detail, using *disco-r* as a probe so that the *dpp*-driven expression of *disco* would not complicate the analysis (Figs. 5E–H). It appeared that the Dac-positive cells completely overlapped the *disco-r*expressing cells, though many cells expressed *disco-r* but not Dac. And though there was no way to determine whether or not the discs we examined could have formed pharate adults, in all cases the expression patterns were similar.

We also examined expression of several wing-determining genes, *bifid* (*bi*), *apterous* (*ap*), *scalloped* (*sd*) and *wingless* (*wg*) (see Supplementary Fig. 3). Though *bi* and *ap* appeared unchanged, *sd* was reduced, though the distribution appeared normal, and *wg* was at normal levels but the normally closed line of expression along the dorsal wing blade boundary was broken. It was likely that this break was caused by the ectopic growth in the wing blade induced by *disco* expression. Overall, at this stage of development, wing-specific gene expression was not altered as much as we might have expected.

The appendage role of disco is conserved in the beetle, Tribolium

To investigate the role of *disco* homolog in another insect, we turned to the red flour beetle, *T. castaneum*. Unlike the fly maggot larva, which lacks visible appendages, appendages are present on the head and thoracic segments of *Tribolium* larvae. These appendages develop during *Tribolium* embryogenesis. Furthermore, unlike the maggot fly larvae with internalized head segments, the beetle head segments with appendages remain external. These differences make comparisons between the fly and beetle informative.

Our analyses of the *T. castaneum* genome identified only a single *disco*-like gene (referred to as *Tc-disco* below). The structure of the encoded Tc-Disco protein was more similar to the vertebrate homolog Basonuclin (Tseng and Green, 1992), containing three paired zinc finger domains, in contrast to the one and two pairs of Disco and Disco-r, respectively. Still, the N-terminal pair of zinc fingers, nearly identical in all animals examined, permitted unequivocal identification. We used a fragment from the largest *Tc-disco* exon as a probe to visualize mRNA distribution during *Tribolium* development (Fig. 6). *Tc-disco* transcripts were detected in the medial portions of all appendages, at all stages of embryogenesis. Other than the transient expression in the abdominal segments, *Tc-disco* mRNA was not detected in other regions of the body or head of the beetle. There were obvious similarities between *disco* expression in *Tribolium* and *Drosophila*. Transient expression in the abdomen was observed during embryogenesis in both organisms. As appendages everted, concentric rings of stronger expression arose both in beetles and flies (for fly images see Supplementary Fig. 1). Yet, there were also differences. Both the *Tribolium* and *Drosophila disco* genes were expressed in the medial portions of the legs, but only in the fly did expression extend to the distal tip of the leg (for fly images see Supplementary Fig. 1). However, the most significant difference was that, in the beetle, only an appendage role was evident.

To investigate the effect of reducing *Tc-disco* function during *Tribolium* development, we used parental RNA interference (RNAi) (Bucher et al., 2002). Efficacy of RNAi was determined by in situ hybridization with the *Tc-disco* probe to fixed embryos collected from injected females. Eggs laid by injected females hatched, but the larvae were nearly immobile and unable to feed due to severe truncations of all appendages (Fig. 7). Similar medial deletions were observed in all appendages. We highlight the alterations in the legs and antennae (Figs. 7C–F). In a typical collection of 115 larvae from injected females, 82 (71%) had the strong phenotype. The legs were markedly shorter due to loss of the medial regions (Fig. 7D) as were the antenna (Fig. 7F). The distal-most portion of the leg, the pretarsal claw, was present as were the sensory structures characteristic of the distal tibiotarsus, immediately above the claw. The proximal-most portion of the leg was more difficult to characterize, but remaining sensory organs and bristles indicated that the coxa and perhaps a small portion of the trochanter were present. Twenty-five larvae (22%) had a less severe phenotype with three clear leg segments, but lacking the trochanter. The remaining 8 larvae appeared to have normal larval legs. All RNAi defects were restricted to the regions of *Tc-disco* expression. Careful comparison between RNAi larvae and wild-type larvae revealed no other differences, such as a

Fig. 4. Effects of ectopic, *dpp*-driven *disco* expression. Except for panel C, all panels on the left are of wild-type flies and those on the right (along with panel C are of *dpp*-driven *disco*. Normal *Drosophila* legs (for example, see panel G) are composed of six segments—coxa, trochanter, femur, tibia and tarsi (divided into five subsegments) and the claws and pulvilli are found on the distal pretarsus. In *dpp>disco* pharate adults, the tarsal subsegments of the normal legs are altered (compare panels A and B). It is not clear whether this represents a fusion or loss of subsegments, occasionally only two subsegments were present. (C) An example of a weaker wing-to-leg transformation. Bracted bristles (see insert, a few are marked by arrowheads) indicate leg identity. Often distinctions between leg segments are visible, though individual segment identities are unclear. (D) An example of a moderately transformed wing. In addition to bracted bristles, specific leg segments are visible. Often, as is the case here, an apical bristle (ab) and spurs (arrowheads) are present (see insert) permitting identification of distinct leg segments (for example, the apical bristle and spurs are on the tibia). T?=tarsal segments. (E) A wild-type fly just before eclosing that was dissected from the pupal case. Note the wing is not inflated, which occurs after eclosion. Also note the three folded legs. (F) A *dpp>disco* pharate adult that was dissected from the pupal case. Note the absence of the wing and the fourth leg above the three normal legs. The femur (fe), tibia and tarsal segments of the ectopic leg are labeled. The arrowhead points to the haltere, which is also transformed toward a leg-like identity. (G) Higher magnification of a second thoracic wild-type leg, which consists of the coxa (Co), trochanter (tro), femur (fe), tibia and the five tarsal segments (T1–T5). The apical bristle (ab) is also marked. (H) Higher magnification of the transformed wing from a *dpp>disco* fly. The femur and tibia are clearly identified based on the presence of specific bristles, and several tarsal segments are present. Normal femurs have both bracted and non-bracted bristles, which was also the case for the femur-like segment of the transformed wings. Additionally, there are distinguishing bristles at the distal end of the normal second thoracic tibia-stout bristles referred to as spurs and two longer bristles, the apical (closest to the spurs) and preapical bristle. The presence of these bristles on the transformed wings indicated second thoracic identity, as would be expected since wings are second thoracic structures. (I) In a wild-type fly, the pretarsal segment contains the terminal claws (arrow) and the pulvilli (arrowhead, and out of the focal plane). (J) In the *dpp>disco* transformed wing, one of the terminal claws is clearly visible (arrow) as is a pulvillus (arrowhead).

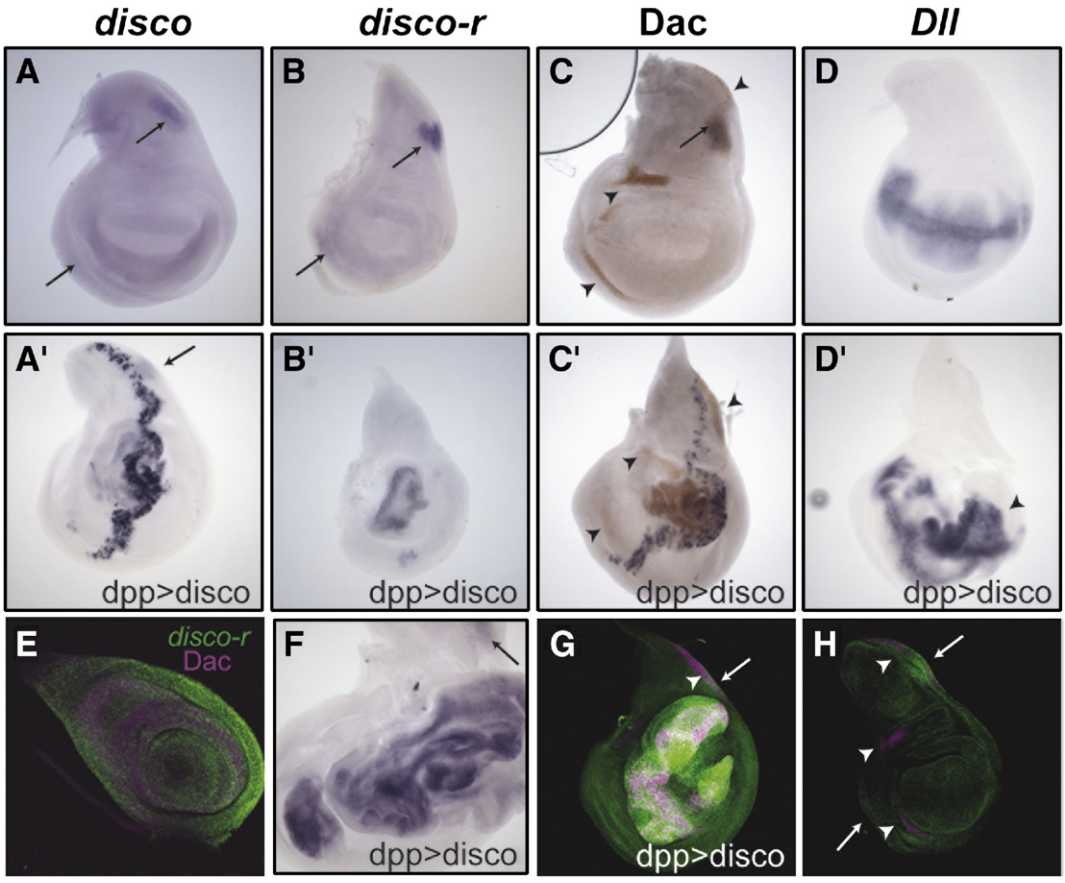


Fig. 5. Expression of leg determining genes in *dpp>disco* transformed wing. (Note, except for *Dll*, expression in normal leg discs are shown in Figs. 1 and 2.) Top row, normal wings; second row, *dpp*-driven *disco* wing discs (Denoted by ′), third row examining *disco-r* and Dac in more detail. (A, A′) *disco*. In normal wings, *disco* is expressed in a small region of the wing disc that will give rise to a portion of the scutellum. In the transformed wings, *disco* mRNA accumulates in the *dpp* stripe along the anterior/posterior border, as would be expected for this driver. However, note the weaker staining in what should be the wing blade region, anterior to the *dpp* stripe. These cells are the daughters of cells initiating in the *dpp* stripe (Weigmann and Cohen, 1999). As they divide, they move away from the anterior/posterior border and cease expressing *dpp*. However, it appears that *disco* expression continues. (B, B′) *disco-r* expression mirrors that of *disco* except that the gene is not activated by *dpp*-Gal4; hence, no stripe of staining along the anterior/posterior border. However, *disco-r* mRNA accumulated in the anterior portion of the wing blade region in daughter cells that have moved from the *dpp*-driven stripe. (C, C′) Staining to detect *disco* mRNA and Dac protein demonstrated that Dac was ectopically activated in the region of the wing blade where there was ectopic expression of the endogenous *disco* and *disco-r* genes (also see panel G below). (D, D′) *Dll* mRNA accumulation was enhanced in the region of *dpp>disco* expression. The region of ectopic *Dll* activation is marked with an arrowhead. (E) *disco-r* (green) and Dac (magenta) in a wild-type leg disc. Dac and *disco-r* appear distinct only because Dac is localized to the nucleus (see text). (F) *disco-r* mRNA localization in a *dpp*-driven *disco* transformed wing from a late third instar larva. Note the extensive proliferation that has occurred in the *disco-r*-expressing wing blade region. Additional growth is also observed in the region expressing *disco-r* below the wing blade. This disc was photographed at the same magnification as those above. (G) Dac and *disco-r* in a transformed wing disc from a somewhat younger third instar larva. Note that ectopic Dac protein was completely within the *disco-r* region. (H) Dac and *disco-r* in a wild-type wing disc. In all panels, arrows point to the regions of *disco* or *disco-r* mRNA accumulation; arrowheads point to normal Dac accumulation.

more general disruption of head development as might have been expected from the role attributed to *disco* during *Drosophila* embryogenesis.

We also examined the *Tc-disco* RNAi phenotype on a molecular level using the *Tribolium dachshund* (*Tc-dac*) homolog as a marker for medial leg (Figs. 7G and H). At the fully contracted germ band stage, normal *Tribolium* legs have three regions of *Tc-dac* expression (Prpic et al., 2001), an intense ring around the middle of the leg, a weaker stained proximal band and a spot at the junction of the leg and the body wall. In the strongest class of *Tc-disco* RNAi embryos, little or no *Tc-dac* mRNA was detected. It was not clear whether the slight staining that did remain was from the weaker proximal band or a small portion of the normally larger distal band. However, we can conclude that the leg deletion caused by reducing *Tc-disco* eliminates most, if not all, of the *Tc-dac* and medial regions of the *Tribolium* appendages.

The discogenes operate as appendage factors during Drosophila embryogenesis

Though wormlike in appearance, *Drosophila* larvae are thought to have remnants of appendages in the thorax, the paired ventral Keilin's organs (Angelini and Kaufman, 2005b; Bolinger and Boekhoff-Falk, 2005; Cohen et al., 1991). In addition, the cells that will give rise to the thoracic imaginal discs also arise from this region of the thorax (Bolinger and Boekhoff-Falk, 2005; Cohen et al., 1991, 1993). Development of the Keilin's organs requires many of the appendage network genes. In the embryo, both *disco* genes are expressed in the

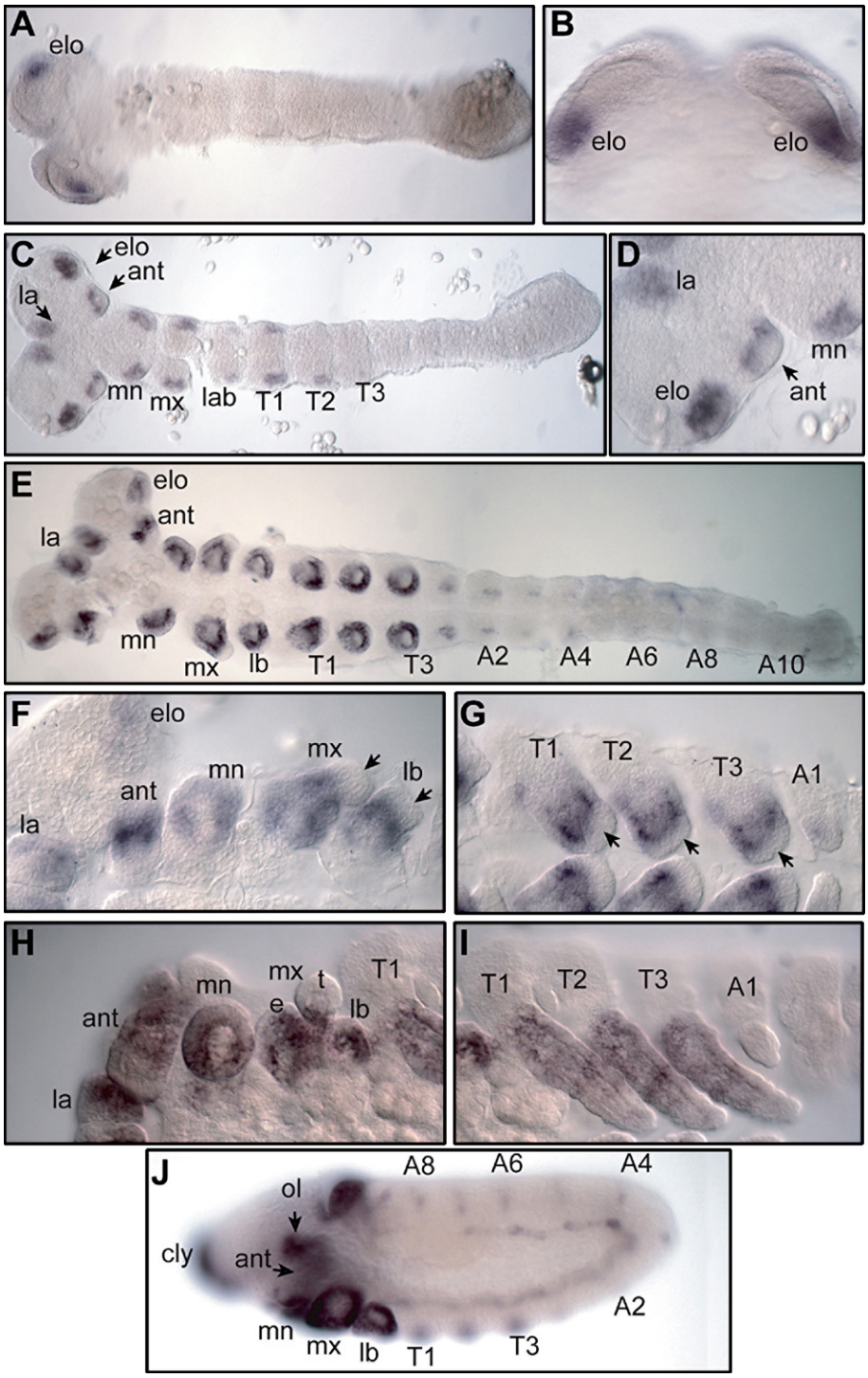


Fig. 6. Expression of *Tribolium disco* genes. *Tc-disco* mRNA distribution during *Tribolium* development. Transcripts were first detected during early germ band elongation in the eye lobes (A, B), but as germ band elongation continued, transcripts accumulated in an anterior to posterior manner in the primordia of each head and trunk appendage—beginning first with the labral segment, then appearing in the antennal, mandibular, maxillary, labial, first thoracic (T1), second thoracic (T2) and the third thoracic (T3) segments (C–E). Expression was also detected in the region that will give rise to the pleuropodia. Weak, transient accumulation was also detected in the second through tenth abdominal segments (E). As the appendages began to evert, *Tc-disco* mRNA became limited to the medial portion of each appendage (F–I), with stripes of higher accumulation apparent in the antenna and legs (H, I). Transcripts were not detected in the body portion of the head or trunk segments or in the distal-most portions of the appendages. (J) *disco* expression in a germ band extended *Drosophila* embryo for comparison. Abbreviations: elo, eye lobe; la, labrum; ant, antenna; mn, mandible; mx, maxillary; lb, labial; T1–3, thoracic; A1–10 abdominal; cly, clypeolabrum; ol, optic lobe.

region of the thorax where the Keilin's organs appear (Figs. 8A–D), so we suspected these genes might also be required for their development. Indeed, it appeared that Keilin's organ development was incomplete in *Df(1)ED7355* hemizygous embryos (Figs. 8E and F). An opening in the cuticle is present; however, the remainder of the organ is absent. Therefore, as in

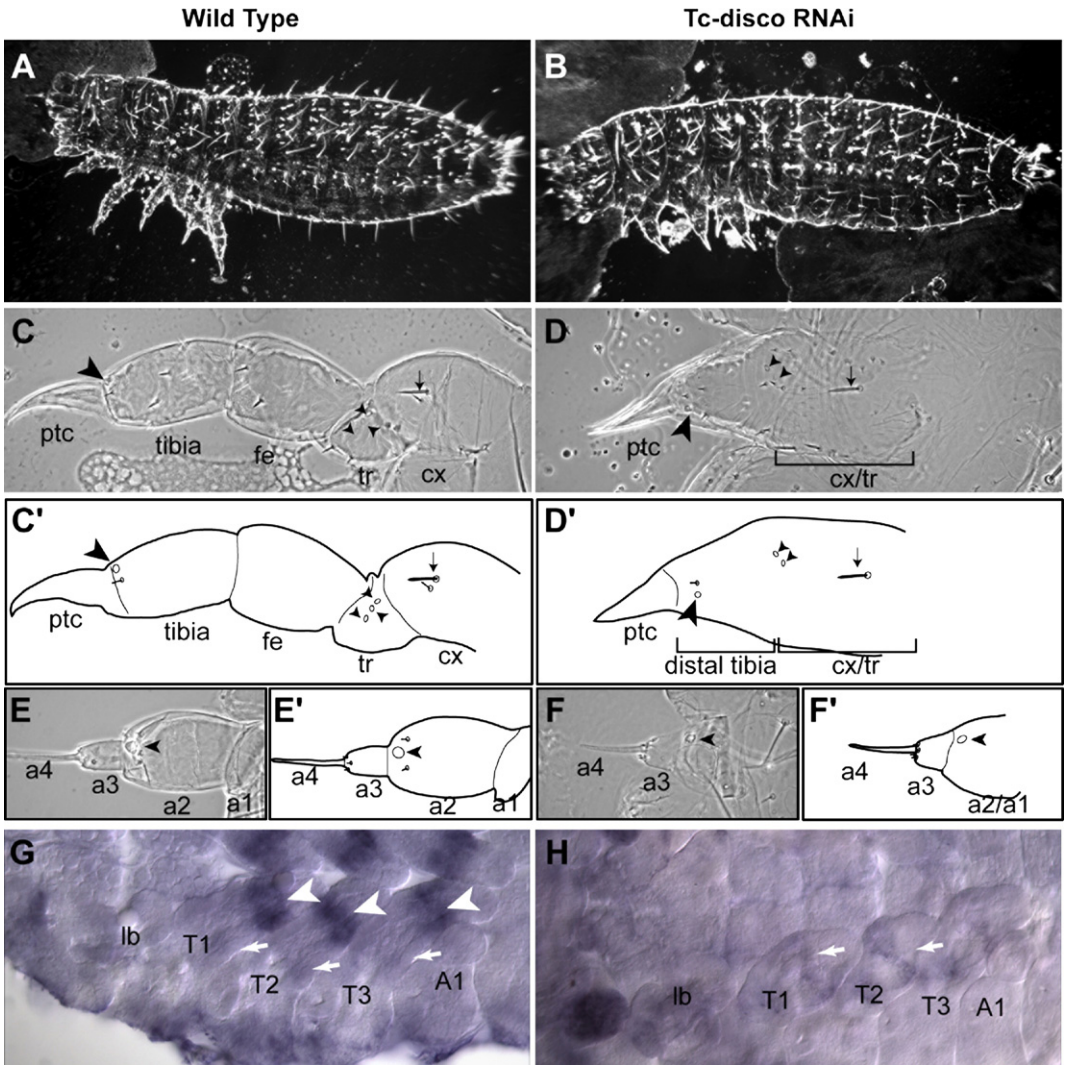


Fig. 7. *Tribolium* RNAi analysis. (A) A dark field image of a wild-type *Tribolium* larval cuticle. (B) Similar image of a larva progeny of a female that was injected as a pupa with *Tc-disco* double-stranded RNA. The normal *Tribolium* larval leg has, from proximal to distal, the coxa, trochanter, femur, tibiotarsus and pretarsal claw. *Tc-disco* RNAi legs (B, D, D′) were markedly shorter than wild-type (A, C, C′) due to loss of the medial region. (C) A higher magnification of a wild-type larval leg and (C′) a drawing that highlights specific leg segment markers. (D) High magnification view of an RNAi leg and (D′) a drawing to highlight the changes. Specific sensory organs (arrow heads) and bristles (arrows) were used to determine the extent of the deletion. The pretarsal claw was present and well formed in the RNAi leg indicating that the distal-most portion of the leg was present. The large bristles of the coxa were also present. (Note the smaller bristle is out of the focal plane.) (E and E′) Wild-type antenna. (F and F′) antenna from RNAi embryos. Note the shorter structure of the antenna with reduced proximal to mid domain. (G) and (H) *Tc-dac* mRNA in wild-type and *Tc-disco* RNAi developing legs, respectively. In panel G, large arrowheads point to the major band of *Tc-dac* mRNA accumulation; smaller arrows denote lower intensity bands. In panel H, the arrows point to the remaining reduced expression of *Tc-dac* after reducing *Tc-disco* via RNAi. Abbreviations: ptc, pretarsal claw; fe, femur; tr, trochanter; cx, coxa; a1–a4, antennal segments a1–a4; lb, labial segment; T1–T3, thoracic legs; A1, first abdominal segment with pleuropodia.

development of the adult appendages, the *disco* genes are necessary for development of the remnant of larval legs, the Keilin's organs, indicating that, as in development of the adult, these genes have an appendage role.

Discussion

disco as an appendage factor

The *Drosophila disco* gene was initially identified as required for proper neural migration (Steller et al., 1987), and our later work demonstrated that *disco* along with the paralog *disco-r* were required for embryonic pattern formation (Mahaffey et al., 2001; Robertson et al., 2004). Yet because mutations in *disco* affected migration of neurons during development of the adult and work by Lee et al. (1991) indicated that *disco* was expressed in many of the imaginal discs, we suspected that the *disco* genes would have a role after embryogenesis. Furthermore, *disco-lacZ* from the enhancer trap line C50.1S1 has been used as a marker for leg joint formation (Bishop et al., 1999; Mirth and Akam, 2002). Here, we present evidence that *disco* genes are conserved members of the insect proximal/distal appendage specification network. In both *Drosophila* and *Tribolium*, these genes are expressed in the

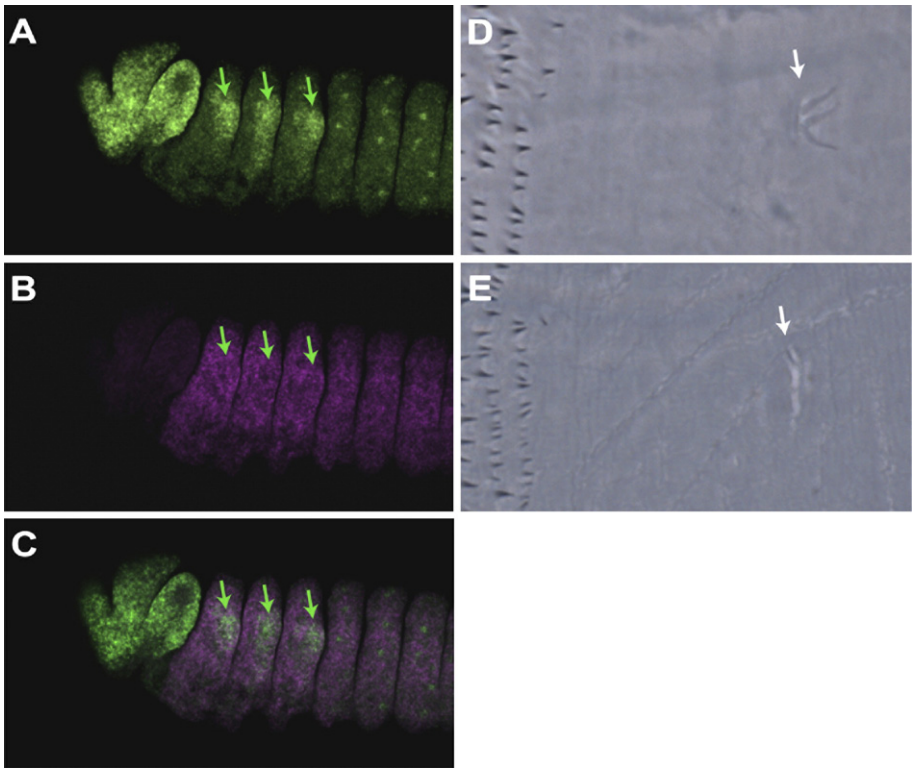


Fig. 8. Role of the *disco*genes during appendage development in embryos. (A–C) Comparison of *disco* (green) and *tsh* (magenta) mRNA distribution in stage 12 *Drosophila* embryos. Note the spots of *disco* accumulation in the thoracic segments (arrows). This expression marks the position where the Keilin's organs and leg disc primordia are located. The *disco* genes are expressed in cells of both (Mahaffey et al., 2001). Note, further, that many of the central *disco*-positive cells are not expressing *tsh*. This arrangement of *disco* and *tsh* is similar to that in the third instar imaginal discs. (D, E) Keilin's organ phenotype in embryos lacking both *disco* genes. (D) Wild-type third thoracic segment Keilin's organ. Note the three bristles extending from the opening in the cuticle (arrows). (E) In embryos hemizygous for *Df(1)ED7355*, the only visible sign of the Keilin's organs are openings in the cuticle. There are no sockets or bristles. This may indicate that the outer regions of the organ develop normally, but that the inner components, those that would be more distal in an appendage scenario, are absent.

ventral appendages. Loss-of-function evidence from *Drosophila* and *Tribolium* indicates that the *disco* genes are required for ventral appendage development. Clonal analyses indicated that cells homozygous for *Df(1)ED7355* were lost from adult tissues that developed from *disco*-expressing regions of the ventral imaginal discs, though such cells were viable elsewhere. There is no evidence that any genes in *Df(1)ED7355* other than *disco* and *disco-r* affect viability or pattern formation. The proteins encoded by these other genes are unlikely to have such effects, and none are known to be expressed in a pattern similar to *disco*and *disco-r*. Therefore, it would be quite unlikely that one of these other genes would have an effect only in *disco* expressing regions while being viable elsewhere. Furthermore, our clonal analysis in *Drosophila* and RNAi experiments in *Tribolium* yielded complementary results. It is unlikely that two non-specific processes could yield such similar results. Gain-of-function studies in *Drosophila* demonstrate that ectopic *disco* transforms dorsal appendages to ventral fates. Altogether, we feel this is strong evidence that the *disco* genes are ventral appendage factors. Though the C50.1S1 enhancer trap has been used by others to mark the region of leg joint formation, we could not test for a direct role for the *disco* genes during leg joint formation, since the clones of *Df(1)ED7355* cells did not survive in the leg discs. However, we did not observe any indication of additional leg joints forming due to ectopic expression. The high levels of β-gal from the *disco-lacZ* enhancer trap C50.1S1 present in the leg joint regions may reflect the perdurance of β-gal.

That cells lacking *disco* function either fail to proliferate or die when they are in the medial to distal portions of the appendage primordia implies that they are recognized as aberrant or inappropriately determined cells which are removed through autonomous actions or by their normal counterparts. Cell communication is important in establishing appendage regions (for example, Goto and Hayashi, 1999), and our observations demonstrate that there must be some form of communication between *disco*-expressing and non-expressing cells in the developing medial appendage region. Identifying the mechanism responsible is an important quest for the future.

Though we have not attempted to extensively address the regulatory interactions between the Disco proteins and other appendage factors, some insights are apparent. Tsh represses *disco* and *disco-r* during *Drosophila* embryogenesis (Robertson et al., 2004). That *disco* transcripts were not expressed throughout all of the proximal leg discs may indicate that Tsh represses the *disco* genes during appendage morphogenesis as well. In addition, that ectopic *disco* induced *dac* expression in the wing discs might indicate that *dac* is a target of Disco,

though other explanations are certainly possible. At this time it is not known whether these regulatory events are direct.

The Drosophila embryo and appendage factors

During *Drosophila* embryogenesis, the *disco* genes act in parallel with the *hox* genes to establish proper segment identity in the head (Mahaffey, 2005; Mahaffey et al., 2001; Robertson et al., 2004). In this role, the *disco* genes are similar to the *teashirt* gene (tsh), which during embryogenesis encodes a trunk segment specification factor (de Zulueta et al., 1994; Fasano et al., 1991; Roder et al., 1992). Both the *disco* genes and *tsh* encode regionally expressed zinc finger transcription factors and, strictly from studies of *Drosophila* embryogenesis, they appear to establish zones along the anterior posterior axis of the embryo (Disco, head; Tsh, trunk) (Mahaffey, 2005; Robertson et al., 2004). Interaction between these two systems is evident in that *tsh* expression represses *disco* and *disco-r*, limiting their expression in the trunk segments (Robertson et al., 2004).

The newly discovered appendage role for the *disco* genes is intriguing in light of the segment specification function during *Drosophila* embryogenesis. As mentioned above, *tsh* also is required for proper proximal development of adult appendages. As we found with *disco*, the appendage role of *tsh* appears to be conserved, while the trunk segment specification role is found only during fly embryogenesis (Herke et al., 2005; Peterson et al., 1999). It is possible that the embryonic and appendage functions are distinct. If so, this would indicate that, for *disco*, either the head specification role was newly acquired in the fly lineage, or that it was lost in the beetle. Yet even during embryogenesis, we do find that the *disco* genes are required for the appendage primordium and for the Keilin's organs, which are proposed to be remnants of larval appendages (Angelini and Kaufman, 2005b; Bolinger and Boekhoff-Falk, 2005; Cohen et al., 1991). Therefore, even during *Drosophila* embryogenesis, *disco* is functioning as an appendage factor. But what about the expression in the head segments? To address this, we consider the differences between *Drosophila* and other insects as well as between the larval and adult forms of the fly. We suggest that, perhaps, *disco* is always an appendage factor, including during specification of the *Drosophila* larval head segments.

Most insects have well-formed appendages when they hatch as larva, but this is not the case for the worm-like larva of higher dipterans. In these insects visible appendages do not arise until the pupal stage when the adult body develops. Appendages arise from the imaginal discs, which are blocks of cells set aside during embryogenesis. Certainly, reduction of the distal and medial appendage domains could account for the Keilin's organs being derived from larval legs. Less obvious, but perhaps more significant in terms of novelty, are the changes that occurred to generate the internalized larval feeding apparatus of fly larvae. In *Drosophila* larvae, the embryonic head segments are highly reduced and internalized, unlike most other insects (Diederich et al., 1991; Jurgens et al., 1986; Rogers and Kaufman, 1996). Perhaps, to form an internalized, multi-segmental feeding apparatus, the mouthpart appendages (mandibles, maxilla and labial palps) have been reduced, as occurred with the legs. However, instead of reducing the medial and distal portions of the appendage, perhaps in the head the proximal tissues were reduced so that the medial appendage domain, governed by *disco*, remains and is prominent in these head segments. In this regard, it is interesting to note that in less highly derived insects with external larval head appendages, homologs of *tsh* are expressed in the ventral portion of the head segments (Herke et al., 2005; Peterson et al., 1999). If this model of the evolution of the *Drosophila* larval head is correct, then the *disco* genes would have analogous roles as appendage factors in the head and trunk segments, also uniting their roles in establishing appendages in the larva and adult.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.ydbio.2007.06.017.

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