Patterning of the adult mandibulate mouthparts in the red flour beetle, *Tribolium castaneum*

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Abstract

Specialized insect mouthparts, such as those of *Drosophila*, are derived from an ancestral mandibulate state, but little is known about the developmental genetics of mandibulate mouthparts. Here, we study the metamorphic patterning of mandibulate mouthparts of the beetle *Tribolium castaneum*, using RNA interference to deplete the expression of 13 genes involved in mouthpart patterning. These data were used to test three hypotheses related to mouthpart development and evolution. First, we tested the prediction that maxillary and labial palps are patterned using conserved components of the leg-patterning network. This hypothesis was strongly supported: depletion of *Distal-less* and *dachshund* led to distal and intermediate deletions of these structures while depletion of *homothorax* led to homeotic transformation of the proximal maxilla and labium; joint formation required the action of Notch signaling components and *odd-skipped* paralogs; and distal growth and patterning required EGF signaling. Additionally, depletion of *abrupt* and *pdm/nubbin* caused fusions of palp segments. Second, we tested hypotheses for how adult endites, the inner branches of the maxillary and labial appendages, are formed at metamorphosis. Our data reveal that *Distal-less*, Notch signaling components and *odd-skipped* paralogs, but not *dachshund*, are required for metamorphosis of the maxillary endites. Endite development thus requires components of the limb proximal-distal axis patterning and joint segmentation networks. Finally, adult mandible development is considered in light of the gnathobasic hypothesis. Interestingly, while EGF activity is required for distal, but not proximal, patterning of other appendages, it is required for normal metamorphic growth of the mandibles.
Article summary

Specialized insect mouthparts are derived from an ancestral mandibulate state, with chewing mandibles and maxillary and labial palps. Little is known about the patterning of mandibulate mouthparts. Through functional study of 13 genes in the beetle Tribolium castaneum, we examine patterning in mandibulate mouthparts and test several hypotheses about the evolution of insect mouthparts. The maxillae and labium in this beetle share many patterning features with legs. Endites, the inner mouthpart branches, require the same genes involved in axial patterning and joint formation. Intriguingly, the mandibles of T. castaneum, which evolved through loss of distal identity, nonetheless require EGF signaling, which is required for development of distal regions of other appendages.
Introduction

Arthropods share a body plan of serially homologous body segments (Snodgrass 1928; Boxshall 2004; Brusca et al. 2002). Within this body plan a great diversity of forms has been generated in several ways, including changes in the number of body segments, patterns of regionalization (tagmosis), and appendage morphology. While insects are the most diverse arthropod group in terms of species numbers, they have a fixed pattern of tagmosis and little variation in the number of body segments (Brusca et al. 2002). Diversification of appendage morphology, however, has played a key role in the diversification of insects; mouthpart morphology is especially variable across lineages, which has enabled exploitation of diverse dietary niches.

Early insects evolved a generalized set of mouthparts useful in biting, chewing, and manipulation of food. Over the last 350 million years, insect mouthparts diversified along with new food sources, and many specialized mouthparts have been derived from the ancestral mandibulate form (Grimaldi and Engel 2005). Some examples of mouthpart specializations include the piercing stylets and beak of bugs (Hemiptera), the elongated galea tubes used by Lepidoptera in nectar feeding, and the sponging proboscis of brachyceran flies. The developmental genetics of several of these specialized mouthparts has been studied (Abzhanov et al. 2001; Angelini and Kaufman 2004; Joulia et al. 2005), and it is assumed that these specialized morphologies evolved by modification of an ancestral leg-like appendage-patterning network (see Boyden 1947; Roth 1984; Angelini and Kaufman, 2005b). However, much less attention has been directed toward the development of mouthparts that retain the ancestral mandibulate morphology. Thus, the hypothesis that ancestral insect mouthparts developed using a general arthropod appendage-patterning network remains to be fully tested.

Mandibulate mouthpart morphology is characterized by a robust pair of unsegmented mandibles followed by two pairs of multi-branched appendages, the maxillae and labium (Fig 1; Grimaldi and Engel 2005; Snodgrass 1928). The short, unsegmented mandibles are believed to have been derived from segmented appendages by loss of distal regions, rendering them serially homologous to only the proximal regions of other appendages (Panganiban et al. 1994; Snodgrass 1935). The maxillary and labial appendages have an outer palp and inner branches called endites. Endites are an ancestral feature of arthropod appendages, but the maxillary and labial endites are the only endites unambiguously present in living insects (Boxshall 2004). It has
been suggested that maxillary and labial palps were modified from distal regions of ancestral appendages that were more leg-like in morphology and function (Snodgrass 1928). This notion is based in part on the presence of leg-like palps in the Archaeognatha, the sister group to all other insects (Regier et al. 2010).

The mandibulate mouthpart morphology is retained with only minor modifications in many living groups, including beetles. Here, we use developmental genetic data from the red flour beetle, Tribolium castaneum, to address three questions about the metamorphic patterning of mandibulate mouthparts. First, we test the hypothesis that the main axis of the adult maxillary and labial appendages is patterned using genes also required in the leg development network. Key aspects of this network are conserved in the legs of numerous arthropods, suggesting that they were likely components of the leg patterning network of ancestral arthropods (e.g. Abzhanov and Kaufman 2000; Angelini et al. in revision; Angelini and Kaufman 2005b; Beermann et al. 2001; Inoue et al. 2002; Jockusch et al. 2000; Palopoli and Patel 1998; Prpic and Damen 2009; Rogers et al. 2002; Schoppmeier and Damen 2001). Second, we test several hypotheses about the mechanism that governs branching of the PD axis to give rise to endites. The development of endites has been attributed variously to reiteration of the main PD patterning network (Giorgianni and Patel 2004; Williams 1998), to action of the joint formation network (Olesen et al. 2001), or to initiation of a dachshund-dependent mechanism (Sewell et al. 2008). Third, we test the extent to which mandible metamorphosis depends only on proximal components of the limb-patterning network, as predicted by the gnathobasic mandible hypothesis (Snodgrass 1928).

To test these hypotheses, we have investigated the function of 13 genes during mouthpart metamorphosis in T. castaneum using RNA interference (RNAi). This set of genes includes the canonical “leg gap genes” Distal-less (Dll), dachshund (dac), and homothorax (hth); the epidermal growth factor (EGF) signaling pathway, another important regulator of distal development; Notch and its ligands and the odd-skipped (odd) family of transcription factors, which regulate growth and joint formation of large regions of the limbs. Our results show that, as predicted, palp development parallels leg development. Leg gap genes and genes used in joint formation are functionally conserved. The data are partially consistent with two of the three hypotheses for endite development, but do not suggest a role for dac in endite metamorphosis. Mandible development was affected in surprising ways. The EGF ligand encoded by Keren
(Krn) regulates distal development of most appendages, but is required to promote axis elongation in the mandibles at metamorphosis. These data suggest that patterning of mandibulate mouthparts retains greater similarity with patterning in legs than does patterning of mouthparts in species with specialized mouthpart structures.

Materials and Methods

Beetle culture

Wildtype cultures of Tribolium castaneum were obtained from Carolina Biological Supply Company and maintained at 32°C under conditions recommended by the supplier.

Preparation of double-stranded RNA and RNA interference

Candidate genes were identified for study in T. castaneum (Table 1) based on data from other arthropods, especially D. melanogaster. Candidates were amplified from mixed late larval/pupal cDNA with custom primers, cloned, and sequenced to confirm identity. All primer sequences are available upon request. Knockdown phenotypes were generated in adult beetles using RNA interference (Tomoyasu and Denell 2004), and knockdown of gene activity was verified using realtime RT-PCR.

To synthesize double-stranded RNA (dsRNA), a template DNA was amplified from a cloned gene fragment, using specific primers with the T7 promoter sequence added at the 5’ end. Table 2 lists dsRNA sizes for each target gene. Double-stranded RNA was transcribed using the Megascript Transcription Kit (Applied Biosystems) with T7 RNA polymerase, then treated with DNase I to remove plasmid DNA. The product was annealed by cooling and purified by precipitation in ammonium acetate and ethanol. After resuspension in pure water, dsRNA concentrations were determined through triplicate measurements on a nanoscale spectrophotometer (Thermo Scientific NanoDrop).

Prepupal larvae were selected for injection in the last instar just as they cease movement at the onset of apolysis. This is the earliest stage at which individuals can reliably be identified as committed to metamorphosis, and untreated individuals typically molt within 1-2 days. Approximately 0.167 µl of 2 µg/µl dsRNA was injected into the dorsal thorax using a pulled-glass capillary needle (Tomoyasu and Denell 2004). In treatments with multiple dsRNAs, the
total mass of injected dsRNA was held constant (333 ng). For odd family genes, dsRNA constructs were designed to minimize sequence similarity between paralogs.

**RNAi validation**

The extent of gene knockdown was determined by comparing realtime (rt)-PCR amplification of target gene sequences from treatments with gene-specific and nonspecific control (green fluorescent protein, GFP) dsRNAs. For each dsRNA treatment, total RNA was isolated from pools of 8-10 randomly selected pupae, three days after molting to the pupal stage, using the PureLink RNA Mini Kit (Life Technologies). Total RNA concentration was determined by triplicate measures on a nanoscale spectrophotometer (GE Life Sciences NanoVue) and the RNA was diluted to 100 ng/µl immediately prior to assays. This RNA was used as template in reverse transcription SYBR Green rt-PCR reactions (Quanta BioSciences). Primers were designed using the Primer3 algorithm (Rozen and Skaletsky 2000), avoiding conserved functional domains. Several reference genes (Lord et al. 2010) were tested for stable expression across multiple developmental stages, and rps18 (NC_007420) was identified as the most suitable reference. Dissociation curves for each reaction were used to verify that a single product was produced. To produce single-stranded quantitative template standards, clones were linearized and transcribed in vitro from T7 promoters. Regions used as quantitative standards had no overlap with regions used in dsRNA synthesis. Standard RNA was treated with DNase I to remove template DNA and purified by precipitation in ammonium acetate and ethanol. Immediately before rt-PCR assays, the RNA concentration was determined in triplicate and the molar quantity was calculated. Dilution series containing 10^3, 10^5 and 10^7 RNA molecules were used to produce a standard curve (Pfaffl 2004). In cases where quantitative standards could not be used (because the injected dsRNA fragment encompassed the entire cloned fragment), primer pairs that amplified other regions of the gene were used in relative rt-PCR, and expression levels were normalized as described by Pfaffl (2001). In relative rt-PCR, ratio distribution standard errors were calculated from the C_t values of triplicate reactions in R (R Development Core Team 2010). This calculation was made using a re-sampling method (source code available on request).

**Characterization of RNAi effects**
Morphology was examined after clearing in a solution of 20% glycerol in glacial acetic acid (Van der Meer 1977). Eclosed adults and pharate adults dying prior to eclosure were scored for anatomical characters related to the mouthparts, including the presence, absence, or fusion of segments and changes in size, shape and bristle patterns (Supplemental File 1). A random subset of mandibles were also assessed quantitatively. Three measurements were taken using an ocular micrometer (Fig. 3A): the distance from the distal tip to the base of the molar surface (medial length), the distance from the distal tip to the lateral condyle (lateral length), and the distance from the base of the molar surface to the lateral condyle (proximal width). The distance across the head between the outermost edges of the eyes (ocular distance; Fig. 3D, inset) was used to normalize for head size. Treatment effects were tested for using one-way ANOVA, and post-hoc Tukey’s HSD tests were used to identify treatments that differed significantly from nonspecific GFP dsRNA controls. No significant differences were found between left and right mandibles; therefore, all subsequent tests were restricted to the left mandible to avoid pseudoreplication. All statistical tests were conducted in R (R Development Core Team 2010).

Phenotypic severity for dsRNA specimens (Table 2) was calculated based on 81 characters related to the anatomy of antennae, mouthparts, and legs. Severe, moderate and mild categories were defined as at least two-thirds, one-third, or less (respectively) of the maximum percentage of defects observed for that dsRNA treatment.

Photomicrographs of dissected mouthparts were obtained with an Olympus digital camera on a Zeiss Axioskop compound microscope. Specimens were prepared for electron microscopy by overnight dehydration in ethanol, followed by immersion in hexamethyldisilazane for 15 min. A Zeiss DSM982 Gemini field emission scanning electron microscope was then used to image specimens coated in gold palladium.

**Results**

Knockdown of candidate genes resulted in informative phenotypes in the mouthparts (Figs. 2-4). The average degree of knockdown in RNAi treatments ranged from 24% to 75% reduction of control transcript levels (Table 2). Table 2 also lists the proportion of phenotypes falling into mild, moderate and severe categories for each gene. An advantage of RNAi is that the range of severity in phenotypes is akin to a hypomorphic series of mutant alleles, which allows
assessment of gene function from a spectrum of reduced activity. Phenotypic penetrance varied across appendage types; Table 3 details the penetrance of dsRNA treatments in each mouthpart. The effects of these genes on metamorphic development of antennae, genitalia and legs in *T. castaneum* appear elsewhere (Angelini *et al.* 2009; Aspiras *et al.* 2011; Angelini *et al.* in revision). Other genes were also targeted for RNAi in those studies, and while they produced defects in other appendages, informative phenotypes were not produced in the mouthparts. These genes included *apterous, apterous-related, aristaless, bric-à-brac, clawless, decapentaplegic, Lim1, rotund*, and *spineless*.

**Anatomy and metamorphosis of the mouthparts**

Detailed descriptions of *Tribolium* mouthparts are available in El-Kifl (1953) and Sokoloff (1972). Here we briefly describe their morphology and metamorphosis to aid in the interpretation of RNAi results. Mouthparts and other appendages are present in *T. castaneum* larvae. Experiments using both amputation (Huet and Lenoir-Rousseaux 1976) and cautery (Daly and Sokoloff 1965) in the tenebrionid beetle *Tenebrio molitor* confirm that the entire larval appendage gives rise to the adult appendage and that specific adult structures within each appendage generally arise by transformation of their corresponding larval structure. Thus, based on its relatively close relationship with *Tenebrio*, the adult mouthparts of *T. castaneum* are assumed to be formed by transformation of their larval precursors. This contrasts with *Drosophila*, where larval appendages exist as small sensory organs and adult appendages develop from imaginal discs that are set aside during embryonic development.

The adult mandible is a short, highly sclerotized appendage that articulates with the head capsule through two condyles (Fig. 1G). No evidence of segmentation is present either internally or externally. There are two enlarged lobes, a distal incisor and a more proximal molar. These regions are all identifiable in the larval mandible (Fig. 1A-B), which grows substantially at metamorphosis, but undergoes a less extensive change in shape than the other mouthparts.

The base of the maxilla (Fig. 1H) consists of two segments, the cardo and stipes. The cardo contains a single sclerite, which is roughly semicircular in shape in the adult; the main sclerite of the stipes is triangular and articulates with the cardo along its diameter. Two medial endites, the lacinia and galea, and the maxillary palp articulate with the stipes. The galea is broadened and rounded distally with a brush of long setae extending across the distal edge. The
lacinia is curved, with a sharply pointed distal tip bearing sclerotized teeth. A fringe of long setae extends along the medial edge of the lacinia. The maxillary palp consists of four segments. The distal palp segment is elongated and terminates in a field of sensory receptors. The larval maxilla differs from the adult maxilla in having only three palp segments. Two medial maxillary lobes are present transiently during embryogenesis, but these fuse before hatching to form the single larval endite (Jockusch et al. 2004). This larval maxillary endite (Fig. 1A,C) is then presumed to give rise to both the adult lacinia and galea.

The adult labium (Fig. 1F,I) is a mid-ventral structure formed by the fusion of paired embryonic labial appendages (Snodgrass 1935). Its base consists of the mentum, which contains a single sclerite with relatively straight proximal and distal edges that are approximately equal in length and joined by slightly convex lateral edges. Distal to the mentum is the prementum, to which the lateral labial palps are attached. Between the labial palps is the ligula, a structure fringed with long distal setae and formed from the fused labial endites. The adult labial palps consist of three segments and, like the maxillary palps, terminate in a field of sensillae. In the larval labium (Fig. 1A,D), two-segmented palps are separated by a very small ligula bearing long setae.

**Mandible RNAi phenotypes**
The average lengths and width of adult mandibles in dsRNA treatments are given in Figure 2. ANOVA confirmed a significant effect of dsRNA treatments on mandibles, for both absolute measures of size and after normalization by ocular distance. The only gene whose depletion produced a significant reduction of mandible length was *Krn* (Fig. 3C). Tukey’s HSD supported a significant difference in the medial and lateral mandible lengths of *Krn* RNAi specimens relative to those of *GFP* RNAi controls (Fig. 2A-C). Ocular distance was also significantly smaller in *Krn* RNAi specimens (Tukey’s HSD test of *GFP* vs. *Krn*, *p* < 10⁻⁷), although this was largely due to reduction of the eyes (Fig. 3D-E). Nevertheless, lateral mandible length in *Krn* RNAi specimens was significantly reduced even after normalization based on ocular distance (Fig. 2B, black bars). The molar and incisor regions of *Krn*-depleted mandibles were both reduced, with the distal incisor area more severely affected. This distal bias is consistent with the *Krn* RNAi phenotypes of other appendages, including the legs (Angelini et al. in revision).
Maxillae and labium

*homothorax*

*homothorax* is a homeodomain transcription factor required for normal development of proximal leg segments in *T. castaneum* (Angelini *et al.*, in revision), *D. melanogaster* (Abu-Shaar and Mann 1998; Wu and Cohen 1999; Yao *et al.* 1999) and other insects (Angelini and Kaufman 2004; Ronco *et al.* 2008). It is one of the “leg gap genes” (Rauskolb 2001). *hth* was required for development of the most proximal regions of the maxilla and labium in *T. castaneum*. Depletion of *hth* caused dramatic shape changes in the proximal palps, stipes, cardo, prementum and mentum suggestive of partial homeotic transformations (Fig. 4B-C). Proximal-to-distal homeotic transformations were observed in the legs of *T. castaneum* (Angelini *et al.*, in revision).

In mildly affected *hth*-depleted specimens, the proximal segment of the maxillary and labial palps was enlarged (Fig. 4B). In unmanipulated beetles the proximal palp segment is distinctly smaller than more distal palp segments (Fig. 4A); thus, this phenotype may represent a homeotic transformation of the proximal palp towards a more distal identity. In more severely affected *hth* RNAi specimens (Fig. 4C), the maxillary and labial palps were each missing one segment (38 of 62 scored). The remaining segments had morphology consistent with that of the distal three maxillary palp segments or distal two labial palp segments. In the maxilla, the region immediately proximal to the palp also was abnormal. Based on the presence of stout bristles and fusion with the galea, we interpret this region as the palpifer (a component of the stipes). Its shape indicates that it has been transformed toward palp segment identity. The galea and lacinia were slightly reduced in size but otherwise normal in appearance. Both the cardo and main sclerite of the stipes were misshapen, and the suture between these elements appeared incomplete.

Patterning of the proximal portion of the labium was also greatly altered in response to *hth* depletion (38 of 50 scored). The mentum was shorter than in the wildtype, with straighter lateral edges and thinner mid-line cuticle (Fig. 4B, C). The proximal portion of the prementum was elongated, while the distal portion was much narrower than in wild type. The palps were inserted into rounded sockets on this transformed prementum. The ligula was also slightly reduced.
**dachshund**

*dachshund* is homologous to the human Ski/Sno transcription factor (Caubit *et al.* 1999), and its expression intercalates between the *hth* and *Dll* domains where it is required for development of an intermediate leg region in *T. castaneum* (Angelini *et al.* in revision; Suzuki *et al.* 2008) and *D. melanogaster* (Abu-Shaar and Mann 1998; Lecuit and Cohen 1997). Although not expressed in the maxillary or labial primordia of *D. melanogaster* (Abzhanov *et al.* 2001; Joulia *et al.* 2006), *dac* is expressed in all three mouthparts in *T. castaneum* embryos (Prpic *et al.* 2001). We found that *dac* was required for palp metamorphosis in *T. castaneum*. Both the proximal- and distalmost regions of the palps (including the sensory fields) appeared normal in *dac* RNAi individuals. However, depletion of *dac* reduced the length of palps and caused the fusion of palp segments (Fig. 4D), suggesting that *dac* is normally required for the development of an intermediate region of the palps. In the maxillary palps, the first segment always retained its characteristic short, narrow shape and both adjacent joints. This palp segment articulated to a single distal segment that was somewhat more elongated than the wildtype distal segment, suggesting that it was formed by the fusion of two or more of the remaining segments. In the labial palp, all joints were lost, although the proximal end retained the approximate shape of the first palp segment and a normal connection to the prementum. The endites and proximal portions of the maxilla and labium appeared normal in *dac* RNAi specimens.

**Distal-less**

*Distal-less* is the third classical leg gap gene, required for development of distal regions of the leg in *T. castaneum* (Beermann *et al.* 2001; Suzuki *et al.* 2009; Angelini *et al.* in revision) and *D. melanogaster* (Cohen and Jürgens 1989), as well as distal appendage regions of other species (reviewed by Angelini and Kaufman 2005b). In *T. castaneum*, depletion of *Dll* led to distal truncations of the palps, with only a small proximal nub remaining in the most severely affected labial palps, and a single, unjointed segment in the maxillary palps (Fig. 4F). In milder *Dll* depletion phenotypes, palp segments were partially fused to each other and the distal sensory field remained (Fig. 4E).

*Dll* was also required for normal development of endites. The maxilla of severe *Dll*-depleted individuals retained a single medial endite in the adult (Fig. 4F). It was squared off distally and shorter than wildtype endites; however, it retained a fringe of long distal setae,
suggesting that endite growth was reduced, but that distal tip identity remained. Two interpretations of this phenotype are possible. The single larval maxillary endite is normally transformed into the two endites of the adults, and the reduced maxillary endite in Dll RNAi individuals may result from retention of the larval structure without distinct identity of either galea or lacinia. Alternatively the shape and pattern of terminal bristles suggest that the lobe is the galea and that no lacinia is present. In the most severely affected Dll RNAi specimens, the ligula was also reduced and did not extend beyond the base of the truncated labial palps. As in the maxillary endite, a fringe of distal setae remained (Fig. 4F). Proximal regions of the maxilla and labium appeared unaffected in Dll RNAi specimens.

The EGF ligand encoded by Keren

The T. castaneum genome has only one activating EGF ligand, with greatest sequence similarity to Krn (Tribolium Genome Sequencing Consortium 2008). EGF signaling is required for development of the distal leg in T. castaneum (Angelini et al. in revision) and D. melanogaster (Campbell 2002; Galindo et al. 2002, 2005). Depletion of Krn in T. castaneum had pronounced effects on the mouthpart metamorphosis. The distal two segments of the maxillary and labial palps were reduced and fused together (Fig. 4G). The distal sensory fields at the tips of the palps were present, but reduced and disordered, suggesting a role for Krn in patterning the distal tips of the palps. More proximal appendage regions appeared wild type.

Genes of the odd-skipped family

Both T. castaneum and D. melanogaster possess four closely related zinc-finger transcription factors in the odd-skipped (odd) family: odd, brother of odd with entrails limited (bowl), sister of odd and bowl (sob), and drumstick (drm). These genes are required for proper leg growth and joint formation in T. castaneum (Angelini et al. in revision) and D. melanogaster (de Celis Ibeas and Bray 2003; Greenberg and Hatini 2009; Hao et al. 2003).

Because of the substantial sequence similarity between odd, bowl and sob (up to 37% in T. castaneum), it is possible that a dsRNA construct matching one gene could lead to the simultaneous down-regulation of multiple family members. For example, while bowl expression was not significantly changed in response to RNAi targeting sob (Welch’s t-test, p = 0.083), it was reduced by 41% relative to controls after RNAi targeting odd. Our experiments do not
distinguish between off-target effects and regulatory interactions as causes of this reduction. Because of the possibility of off-target effects, our experiments are able to assess the overall role of *odd* family members in mouthpart patterning but not to identify roles of particular paralogs. In *D. melanogaster*, *odd*, *sob* and *drm* are identically expressed and act redundantly during leg development, while *bowl* has a distinct expression pattern and role (Hao *et al.* 2003).

Knockdown phenotypes were similar in all *odd*-related dsRNA treatments (Fig. 4H-L). Defects included reduced length and impaired joint formation in the maxillary and labial palps, as well as defects in the endites and some proximal portions of the maxillae and labium. In severely affected *odd*-related RNAi specimens, all palp segments were fused and the length of the palp was severely reduced (Fig. 4H,K,L). The palp was also fused proximally (to the stipes or prementum) and medially (to the endites). Although these fusions made the limits of the palp difficult to determine, the proximal-most portion appeared broader than in wildtype specimens, suggesting that the narrow first palp segment was deleted. However, the distal tip of the palps including the sensory field appeared relatively unaffected. In milder *odd*-related RNAi phenotypes, adjacent palp segments were fused. There was no obvious bias for the fusion of segments at any particular PD level of the palps.

Additional fusions occurred in the proximal portions of the maxilla and labium of *odd*-related RNAi specimens. The mentum and prementum of moderately and severely affected specimens were reduced and fused (Fig. 4I,L). Thus, *odd*-related transcription factors are required for the formation of all joints in the adult labium. The ligula was reduced in some individuals, but a distal fringe of setae occurred between the labial palps, indicating that some ligula identity remained. One interpretation of this phenotype is that an intermediate region, including the prementum and proximal portions of the ligula and palps, was reduced and lost all joints. Interestingly the stipes and cardo were never as affected as their labial homologs, and their intervening suture was always retained. However, reductions in the maxillary endites and palps, and loss of the joints separating these structures from the stipes, are consistent with a deletion of a smaller intermediate region than was deleted in the labium. Additionally a single fused maxillary endite, with both distal and medial setae and sclerotized teeth, developed in response to depletion of *odd*-paralogs. The morphology of this endite indicates that it has both lacinia and galea identity, and therefore likely originated by failure of the two endites to separate
at metamorphosis. In the most severe phenotypes, this single endite was fused to the stipes (e.g. Fig. 4H,K,L).

**Notch signaling**

We assayed the effects of depletion of the receptor encoded by Notch and its ligands, encoded by Serrate (Ser) and Delta (Dl). Notch signaling is required for both growth and joint formation in the legs of *T. castaneum* (Angelini *et al.* in revision) and *D. melanogaster* (Bishop *et al.* 1999; de Celis *et al.* 1998; Rauskolb and Irving 1999). In *T. castaneum*, all mouthpart joints were lost in the most severely affected Notch RNAi specimens, and the ligula did not flare out laterally as it does in wildtype specimens (Fig. 4M). Compared to Ser RNAi, Notch RNAi caused less reduction in palp length and the retained of lacinia and galea with more wildtype appearance. Ser depletion phenotypes closely resembled odd-paralog depletion phenotypes (Fig. 4N-O), with reduction of the palps to short, unsubdivided single segments in the most severely affected individuals, fusion of the maxillary endites, proximal reductions in the maxilla, and extensive reduction of the prementum and ligula (Fig. 4O). Delta RNAi produced extensive eye defects and fusions of antennal (not shown) and tarsal segments (Angelini *et al.* in revision); however, the maxillae and labium appeared normal (Table 3). RNAi simultaneously targeting Ser and Delta (Fig. 4P) produced mouthpart phenotypes indistinguishable from those resulting from Ser depletion alone.

**Additional genes affecting palp development**

We identified two other genes whose depletion led to alterations in mouthpart morphology: abrupt (*ab*) and pdm. abrupt is required for the normal development of the legs and antennae in *T. castaneum* (Angelini *et al.* in revision; Angelini *et al.* 2009) and *D. melanogaster* (Diaz-Benjumea and Garcia-Bellido 1990; Grieder *et al.*, 2007; Hu *et al.* 1995). The homeobox transcription factor encoded by *pdm* is known for a role in patterning the wing of *D. melanogaster* (Ng *et al.* 1995) and is also required for leg patterning of diverse insects, including *D. melanogaster* (Cifuentes and Garcia-Bellido 1997; Hrycaj *et al.* 2008; Turchyn *et al.* 2011). Knockdown of *ab* or *pdm* affected palp segmentation (Fig. 4Q-S). *ab* RNAi had low penetrance in the mouthparts (Table 3) and resulted in fusions of the distal maxillary (Fig. 4Q) or labial palp segments (Fig. 4R). In contrast, *pdm* depletion produced a more penetrant and specific mouthpart
phenotype: a 3-segmented maxillary palp (Fig. 4S). The size and shape of the palp segments and the presence of the distal sensillae suggest that the third maxillary palp segment was deleted. We did not observe any defects in the labial palps of pdm RNAi specimens (n=62), suggesting that pdm is deployed during the development of maxillary, but not labial, palps.

**Discussion**

Arthropod appendages are serial homologs and are assumed to share many underlying aspects of their development. The mouthparts represent important appendage types, thought to be derived from ancestrally leg-like structures (Snodgrass 1928; Kukalová-Peck 1998). Furthermore, it has been argued that mandibles evolved by loss of distal appendage regions (Snodgrass 1928; Boxshall 2004). Here we explore the implications of the adult mouthpart phenotypes generated by RNA interference in *T. castaneum* (summarized in Fig. 5A-C) for hypotheses of mouthpart development and evolution.

**Conservation and divergence of maxillary, labial and leg patterning**

Figure 5 compares functional domains of genes in the adult maxilla, labium and legs of *T. castaneum*, as well as homologous structures of *D. melanogaster*. The similarity between knockdown phenotypes across appendage types and species suggests conserved roles for the three leg gap genes (*Dll, dac* and *hth*) in establishing distinct PD axis domains and for *Notch* and *odd*-family pathways in the growth and segmentation of appendages (Fig. 4; Bishop *et al.* 1999; de Celis *et al.* 1998; de Celis Ibeas and Bray 2003; Greenberg and Hatini 2009; Hao *et al.* 2003). An association between *odd, Notch* and boundary formation is also observed during generation of body segments in the three major segmented phyla, arthropods, annelids and chordates (Rivera and Weisblat 2009), suggesting that this association is evolutionarily ancient.

In *D. melanogaster*, *Dll* mutants lack maxillary structures and portions of the proboscis (i.e., labium), although *Dll* expression in the maxillary anlagen is weaker than in the leg or antennal discs (Abzhanov *et al.* 2001). Paralleling our results for *T. castaneum*, in the horned beetle *Onthophagus taurus* distal regions of the adult mouthparts were deleted with larval *Dll* RNAi (Simonnet and Moczek 2011). The embryonic and metamorphic functions of *Dll* in *T. castaneum* are also similar: the gene is required for the development of distal structures at both stages, and during embryogenesis *Dll* is expressed throughout the developing palps (Beermann *et
Interestingly, removal of *T. castaneum Dll* expression earlier during larval life led to delayed metamorphosis, as well as changes in appendage morphology (Suzuki et al. 2009). Many insects delay molting after appendage loss to allow time for regeneration, and this dual role of *Dll* suggests a mechanism linking these processes.

The data from *T. castaneum* provide evidence for a conserved gap gene role of *dac* during patterning of mouthparts and legs of this species. *Dachshund* is not expressed in or required for development of the labial and maxillary anlagen of *D. melanogaster* (Abzhanov et al. 2001). In *T. castaneum* embryos *dac* is expressed strongly in the proximal maxilla and part of the developing endite. Embryonic *dac* expression is weaker in the distal maxillary palp and the labium (Prpic et al. 2001). Our data show a clear metamorphic requirement for *dac* in the intermediate regions of the maxillary and labial palps (Fig. 4D), as does a recent study of *O. taurus* (Simonnet and Moczek 2011). A function for *dac* in the development of an intermediate portion of the maxillary and labial appendages has so far only been observed in these two beetles, while data from two species with specialized mouthparts (the milkweed bug *O. fasciatus* and *D. melanogaster*) found that *dac* is not required for PD patterning of the mouthparts (Abzhanov et al. 2001; Angelini and Kaufman 2004). Thus, comparative data from other species do not support the hypothesis that this mouthpart patterning role is ancestral. However, if mandibulate mouthparts evolved from leg-like structures, as proposed (Kukalová-Peck 1998; Manton 1964; Snodgrass 1928), similarities in the expression and function of genes patterning both legs and mouthparts are expected to be plesiomorphic. This hypothesis can be further tested by examining the role of *dac* in mouthpart development in additional insect orders, particularly those that retain mandibulate mouthparts, and in other arthropods.

The effects of *hth* depletion are distinct in different species, but typically involve some degree of homeotic transformation. In *D. melanogaster*, *hth* is expressed in the labial discs, but without nuclear expression of its cofactor extradenticle (Abzhanov et al. 2001). Maxillary palps are retained in *hth* loss-of-function flies, but they may possess bristles typical of legs, indicating a partial proboscis-to-leg transformation (Inbal et al. 2001). In the cricket *Gryllus bimaculatus*, which has mandibulate mouthparts, *hth* depletion causes transformation of proximal mouthpart structures towards antennal identity, with a loss of endites, while distal structures are transformed towards leg identity (Ronco et al. 2008). *hth* RNAi in *T. castaneum* transformed intermediate regions of the maxilla and labium towards distal mouthpart identity (Fig. 4B-C).
Proximal regions also appeared transformed, but their identity could not be established, while distal regions appeared wild type. In the beetle *O. taurus*, proximal regions of the labium are transformed towards maxillary endite identity, but distal regions of the labium and the entire maxilla remain relatively unaffected (Simonnet and Moczek 2011).

Our results highlight the similarity between patterning of the maxilla, labium and legs in *T. castaneum* (see also Angelini *et al.* in revision). Functional data from two species with highly derived mouthpart morphologies, *D. melanogaster* (Abzhanov *et al.* 2001; Joulia *et al.* 2006) and the milkweed bug *Oncopeltus fasciatus* (Angelini and Kaufman 2004; Angelini and Kaufman 2005a; Hrycaj *et al.* 2008), suggest only limited similarity between mouthpart and leg patterning. One explanation for this low degree of conservation is that evolution of the ancestral patterning mechanism has occurred in concert with the functional and morphological diversification of these mouthparts. A correlation between generative mechanisms and structural morphology has been used as a common null hypothesis (Raff 1996), although exceptions in which similar morphologies result from different developmental pathways are documented (e.g., Abouheif and Wray 2002; True and Haag 2001). Nevertheless, this hypothesis predicts that developmental patterning should be more highly conserved across appendage types in species that retain the ancestral mandibulate mouthpart morphology.

Serial homology of the maxillae and labium

The maxillary and labial palps are an interesting case of serial homology. Despite a difference in overall size, their shape and arrangement of sensillae are similar. The intermediate segments of each palp type are also similar, but differ in number, which suggests that segment number is regulated independently from other morphological traits. The RNAi depletion of *pdm* in *T. castaneum* caused the reduction and deletion of the third maxillary palp segment, producing a phenotype closely resembling the wildtype morphology of the labial palps. While a role for *pdm* in the labium cannot be excluded, the absence of observed labial phenotypes was significant compared to maxillary results (McNemar’s $\chi^2$ test, $p = 1.519 \times 10^{-8}$). Therefore, we hypothesize that the difference in the number of palp segments results from specific activation of *pdm* in the maxillary palp. Loss of function in the Hox gene *Deformed* during *T. castaneum* embryogenesis causes a transformation of the larval maxillae towards labial identity (Brown *et al.* 1999; Brown *et al.* 2000). Since Hox genes are the primary determinants of body segment identity, we propose
that \textit{pdm} is activated by \textit{Deformed}, and repressed by the labial \textit{Hox} gene \textit{Sex combs reduced}. RNAi targeting \textit{pdm} in another mandibulate insect, the cricket \textit{Acheta domesticus}, generated defects in the antenna and legs, but no defects in the mouthparts, despite similar \textit{pdm} expression in these appendages (Turchyn \textit{et al.} 2011).

**Metamorphic development of endites**

Endites are a primitive component of arthropod appendages, and they are retained in insect mouthparts, as well as in the mouthparts and thoracic appendages of many crustaceans (Boxshall 2004). At least three hypotheses have been put forward for how endites are patterned, and these hypotheses are not mutually exclusive. The first hypothesis states that multiple PD axes result from redeployment of a PD axis patterning mechanism shared by palps and endites. A second hypothesis posits that endites and appendage segments form by the same mechanism (Olesen \textit{et al.} 2001), \textit{Notch}-mediated in-folding of the cuticle. A third hypothesis states that \textit{dac} expression initiates endite branching from the main appendage axis (Sewell \textit{et al.} 2008).

The axis redeployment hypothesis predicts that depletion of genes involved in PD axis patterning will have similar effects on the development of palps and endites. Some support for this hypothesis comes from studies of endite morphogenesis and the expression and function of leg gap genes in the embryos of \textit{T. castaneum} and the orthopteran \textit{Schistocerca americana} (Beermann \textit{et al.} 2001; Prpic \textit{et al.} 2001), but not all data are consistent with it (Jockusch \textit{et al.} 2004). The segmentation hypothesis predicts that endites will fail to differentiate if genes required for joints are depleted. This hypothesis was posed based on a comparative developmental study of segmented and phyllopodous crustacean limbs (Olesen \textit{et al.} 2001). Finally, the \textit{dac}-mediated hypothesis predicts that depletion of \textit{dac} will lead to reduced endites. This hypothesis emerged from the observation that \textit{dac} expression is reiterated along the medial edges of larval endites in the crustacean \textit{Triops longicaudatus} (Sewell \textit{et al.} 2008). Comparative expression data from the isopod \textit{Porcellio scaber} (Abzhanov and Kaufman 2000) are also consistent with the \textit{dac}-mediated hypothesis.

Our data are consistent with predictions of the axis redeployment and segmentation hypotheses but do not support a role for \textit{dac} in endite metamorphosis. We found that adult endites were disrupted by depletion of \textit{Dll}, \textit{Krn}, the \textit{odd}-related genes, and Notch signaling, and to a lesser degree \textit{hth} (Fig. 4). In the maxilla depletion of most of these genes led to the failure of
the single larval endite to divide into two distinct branches, while in the labium, their depletion caused reduction of the ligula. Their requirement in the endites is consistent with the hypothesis that these structures are generated by redeploying appendage PD axis determinants. Depletion of Notch signaling components and the odd paralogs produced reductions and fusions between palp segments, between the palps and endites, and between the lacinia and galea (Fig. 4H-P). Thus, these data are compatible with both the hypothesis that a reiterated PD axis is used to pattern the endites and the hypothesis that endite formation is linked to joint formation. Normal endite development in dac-depleted specimens is inconsistent with the dac-mediated hypothesis.

It is noteworthy that endite specification and the division of the single larval endite into the adult galea and lacinia appear to be separable functions. For example, Ser RNAi resulted in a single endite lobe with lacinia identity medially and galea identity laterally (Fig. 4N). In contrast, severe Dll RNAi individuals had a single endite that lacked also obvious lacinia identity.

**Patterning of the mandibles**

The mandibulate structure of Tribolium mouthparts is the pleisomorphic state for insects and is shared by a majority of insect orders. These mouthparts are characterized by robust mandibles, lacking segmentation (Brusca et al. 2002; Daly et al. 1998). A classic debate in arthropod morphology concerns whether the mandibles of insects and myriapods are derived from a whole appendage (Manton 1964) or only from proximal appendage regions; the latter are called gnathobasic mandibles (Boxshall 2004; Snodgrass 1935). Palps are retained on the mandibles of many crustaceans, making it clear that the biting regions of their mandibles are gnathobasic (Kukalová-Peck 1998). Phylogenetic support for the gnathobasic hypothesis comes from phylogenetic studies that place insects nested within crustaceans (Regier et al. 2010). The first developmental genetic support for the gnathobasic hypothesis came from the discovery that insect mandibles lack Dll expression (Panganiban et al. 1994; Popadić et al. 1998; Scholtz et al. 1998). Furthermore, neither mutations in Dll nor its depletion through RNAi have been observed to alter mandible development in insects (Angelini and Kaufman 2004; Moczek and Rose 2009; Niimi et al. 2005), including T. castaneum (Fig. 3B; Beermann et al. 2001). This evidence has led to widespread acceptance of the gnathobasic hypothesis (vis-à-vis Manton 1964). Of the 13 genes depleted in this study, two (Krn and hth) produced results that would not be predicted by the most straightforward form of the gnathobasic hypothesis for mandible origins.
Loss of EGF function in insects leads to distal appendage defects, including pretarsal or tarsal deletions (Campbell 2002; Galindo et al. 2002). The role of EGF signaling in distal appendage regions is conserved in *T. castaneum* metamorphosis, since depletion of the EGF ligand *Krn* leads to reduction of the antennal flagellum (Angelini et al. 2009), and maxillary and labial palps (Fig. 4G), as well as to deletion of the pretarsus and malformation of the tarsus (Angelini et al. in revision). In light of the restriction of *Krn*’s role to distal appendage regions and regulation of distal EGF ligand expression by *Dll* in *D. melanogaster* (Galindo et al. 2005), the gnathobasic hypothesis predicts that *Krn* should not be required for normal development of the mandible in *T. castaneum*. In contrast to this prediction, depletion of *Krn* produced a significant reduction in mandible length (Figs. 2A-B; 3C).

The hypothesis of a gnathobasic mandible also predicts that *hth* depletion should produce effects in the mandible similar to those in the proximal regions of other appendage types. In *T. castaneum*, *hth* RNAi during metamorphosis caused homeotic transformation of proximal regions of the maxilla, labium (Fig. 4B-C) and legs (Angelini et al. in revision). However, the mandibles were not affected by *hth* depletion. In the beetle *O. taurus*, *hth* depletion slightly altered mandible shape, but also without apparent homeosis (Simonnet and Moczek 2011). In contrast, *hth* RNAi in embryos of the cricket *G. bimaculatus* transformed the mandible towards a leg-like structure distally and an antenna-like structure proximally, paralleling the transformation observed in other appendages (Ronco et al. 2008). Because these results come from only two lineages and from different life stages, additional data are needed to determine whether a homeotic role for *hth* was present ancestrally in insect mandibles.

We caution that these data must be weighed alongside other evidence bearing on the gnathobasic hypothesis. In *T. castaneum*, the lack of phenotypic effects on mandible metamorphosis of other genes studied here is consistent with the gnathobasic hypothesis. In particular, we observed that mandible metamorphosis was normal following depletion of genes involved in distal growth and patterning or joint formation. Moreover, homology at one biological level, such as anatomy, does not preclude divergence at other levels, such as development (Sommer 2008; True and Haag 2001; Wagner 2007). Nevertheless, since developmental genetic studies of *Dll* and other appendage-patterning genes have been used as strong support for the gnathobasic homology of the insect mandible, our findings of *Krn* function highlight the difficulties in establishing serial homology based solely on developmental data.
Conclusions

This study provides a genetic model of adult mouthpart development in *Tribolium castaneum* based on 13 genes. While previous studies have examined patterning in species with derived mouthpart morphologies, *T. castaneum* retains the pleiomorphic, mandibulate state of insect mouthparts. Our results demonstrate the conservation of many gene functions in the maxilla and labium, relative to the legs, thus supporting the interpretation of novel gene functions in groups with derived mouthpart morphology as indicative of their specialized morphogenetic roles in those species. Mandibulate mouthparts such as those of *T. castaneum* include medial maxillary and labial endites, and our data are consistent with hypotheses of reiteration in the PD axis and specification by Notch signaling, but rule out a direct role for *dac* in branch generation or patterning at metamorphosis. Additionally our results demonstrate that a regulator of distal leg development, *Krn*, which encodes an EGF ligand, is required for normal mandible elongation. This finding underscores the complex relationship between homology at the levels of anatomy and developmental patterning.

Acknowledgments

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### Tables

**Table 1.** Candidate genes. The chromosomal linkage group (LG) is listed, as well as the GenBank accession number of the known or predicted transcript. Abbreviations: BTB, Bric-a-brac/Tramtrak/Broad complex domain; EGF, epidermal growth factor; TXF, transcription factor.

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<tr>
<th>gene name</th>
<th>symbol</th>
<th>protein class</th>
<th>LG</th>
<th>GenBank</th>
<th>clone source</th>
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<td>homothorax</td>
<td>hth</td>
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<td>7</td>
<td>NM_001039400</td>
<td>ANGELINI and KAUFMAN, 2004</td>
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<td>dac</td>
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<td>XM_964678</td>
<td>PRPIC et al., 2001</td>
</tr>
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<td>NM_001039439</td>
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</tr>
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<td>7</td>
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<td>This study</td>
</tr>
<tr>
<td>Delta</td>
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<td>X</td>
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</tr>
<tr>
<td>odd-skipped</td>
<td>odd</td>
<td>Zn-finger TXF</td>
<td>8</td>
<td>XM_966993</td>
<td>ANGELINI et al., 2009</td>
</tr>
<tr>
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<td>Zn-finger TXF</td>
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Table 2. Summary of overall effects of RNA interference. The level of target gene knockdown was determined by realtime PCR comparisons of pooled pupae to nonspecific GFP dsRNA controls.

<table>
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<tr>
<th>dsRNA</th>
<th>size (bp)</th>
<th>target gene knockdown</th>
<th>number scored</th>
<th>phenotypic severity</th>
<th>maximum defects</th>
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<td></td>
<td></td>
<td></td>
<td></td>
<td>unaffected</td>
<td>mild</td>
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<tr>
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<td>83</td>
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<td>4%</td>
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<tr>
<td>lth</td>
<td>335</td>
<td>44% ±8%*</td>
<td>204</td>
<td>18%</td>
<td>56%</td>
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<tr>
<td>dac</td>
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<td>41%</td>
</tr>
<tr>
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<td>75% ±5%*</td>
<td>39</td>
<td>9%</td>
<td>9%</td>
</tr>
<tr>
<td>Krm</td>
<td>188</td>
<td>67% ±9%*</td>
<td>14</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
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<td>24% ±9%*</td>
<td>31</td>
<td>10%</td>
<td>26%</td>
</tr>
<tr>
<td>Ser</td>
<td>329</td>
<td>n/a b</td>
<td>151</td>
<td>3%</td>
<td>13%</td>
</tr>
<tr>
<td>Dl</td>
<td>180</td>
<td>71% ±14%*</td>
<td>70</td>
<td>63%</td>
<td>32%</td>
</tr>
<tr>
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<tr>
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<td>65% ±7%*</td>
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<td>56</td>
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<tr>
<td>sob</td>
<td>321</td>
<td>61% ±8%*</td>
<td>15</td>
<td>0</td>
<td>33%</td>
</tr>
<tr>
<td>drm</td>
<td>225</td>
<td>n/a b</td>
<td>30</td>
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</tr>
<tr>
<td>odd, bowl, sob</td>
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</tr>
<tr>
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<tr>
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<td>26</td>
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<td>pdm</td>
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<td>45</td>
<td>7%</td>
<td>89%</td>
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* Significant difference from gene expression in GFP control specimens (Welch’s t-test, p < 0.05).

a Note that 0% represents no reduction in activity, while 100% is complete suppression.

b Suitable primers for rt-PCR targeting Ser and drm were unavailable.

c Mild, moderate and severe phenotypic categories were defined as specimens having defects of any kind in the lower, middle and upper third of scored characters, relative to the specimens with the maximum number of defects scored for that dsRNA. Notch and its ligands shared the same scale of severity, as did dsRNAs targeting the odd-related genes.

d Some control specimens had a fusion between antennal segments, or amputation of a leg. Similar defects may be seen in unmanipulated beetles.
Table 3. The effects of dsRNA treatments on the maxilla and labium during metamorphosis. Percent phenotypic penetrance in each appendage type is listed as the number of abnormal specimens (given in parentheses) relative to the number scored for that structure.

<table>
<thead>
<tr>
<th>dsRNA</th>
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<td>241</td>
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<td>31% (74)</td>
<td>63% (39/62)</td>
<td>92% (221)</td>
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<td>odd, bowl, sob</td>
<td>17</td>
<td>0% (0)</td>
<td>47% (8)</td>
<td>77% (13)</td>
<td>82% (14)</td>
</tr>
<tr>
<td>odd, bowl, sob, drm</td>
<td>37</td>
<td>0% (0)</td>
<td>87% (32)</td>
<td>97% (36)</td>
<td>97% (36)</td>
</tr>
<tr>
<td>ab</td>
<td>30</td>
<td>0% (0)</td>
<td>7% (2)</td>
<td>17% (5)</td>
<td>80% (24)</td>
</tr>
<tr>
<td>pdm</td>
<td>62</td>
<td>0% (0)</td>
<td>55% (34)</td>
<td>0% (0)</td>
<td>56% (35)</td>
</tr>
<tr>
<td>total</td>
<td>1067</td>
<td>1%</td>
<td>47%</td>
<td>48%</td>
<td>77%</td>
</tr>
</tbody>
</table>

<sup>a</sup> Some control specimens had single fusions within the tarsus (2/83) or antenna (1/83), spontaneous amputations of a leg (1/83). Similar defects may be seen in unmanipulated beetles.
Figure Legends

Figure 1. Mouthparts of *Tribolium castaneum*. The larval and adult mouthparts differ in size, number of palp segments, and endite composition. (A) The ventral larval head is shown in a scanning electron micrograph (SEM). Dissected larval mouthparts are shown in light micrographs: (B) mandible; (C) maxilla; (D) maxillae and labium. (E) SEM showing a ventral view of adult mouthparts with the mandibles closed and crossed. (F) A light micrograph of isolated maxillae and labium. (G) The adult mandible is a single segment that functions in chewing, with specialized incisor and molar regions. (H) The adult maxilla is a complex structure that functions in manipulation of food items. The medial endites, the lacinia and galea, act to sweep food into the mouth. The maxillary palps contain four segments. (I) The left and right labial appendages fuse along the ventral midline during embryogenesis. The labial palps contain three segments. The endites are fused to form the ligula.

Figure 2. RNA interference effects on adult left mandible dimensions. (A) medial length, (B) lateral length and (C) proximal width. Mean absolute measurements are given by the left axis and white bars, while mean values adjusted to ocular distance are given by the right axis and black bars. Error bars indicate standard deviation. Treatments with a significant difference from *GFP* dsRNA controls are denoted by an asterisk (two-tailed t-test, \( p < 0.05 \)). (D) Ocular distance was used as a proxy for head size. This measurement was affected significantly by knockdown of *Krn* and *Delta*, which both affected eye development. Increase in normalized mandible measurements of *Delta* RNAi specimens appears to result from reduction in ocular width rather than increase in mandible size. These values are omitted from the figure for clarity (medial length: 0.53 ±0.091; lateral length: 0.56 ±0.13; proximal width: 0.43 ±0.12). Numbers of individuals measured in each treatment: unmanipulated (6), *GFP* (8), *hth* (20), *dac* (8), *Dll* (13), *Krn* (11), *Notch* (8), *Ser* (10), *Delta* (10), *odd* (7), *bowl* (5), *sob* (5), *odd, bowl, sob* (8), *odd, bowl, sob, drm* (4), *ab* (9), *pdm* (8).

Figure 3. Development of the adult mandible of *T. castaneum* requires *Krn*. (A) Unmanipulated mandibles. The landmarks used in measurements of mandible size (Fig. 2) are indicated by dotted lines. (B) Mandibles appear unaffected by *Dll* RNAi. (C) *Krn* RNAi reduces the
mandibles and produces an abnormal surface morphology. (D) The eyes are normally the widest points of the head, as in this unmanipulated specimen. The distance between the outermost edges of the eyes (inset) was used to normalize mandible measurements to head size. (E) Krn RNAi causes a drastic reduction in the size of the eyes, and the eye field is recessed into the head capsule.

**Figure 4.** Effects of RNA interference on metamorphosis of the maxilla and labium in *T. castaneum*. Red labels indicate structures showing defects. Red arrows indicate the locations of fusions or deletions. (A) Maxillae and labium from a buffer-injected control specimen. (B) The proximal palp segments are transformed to more distal identity in mildly affected *hth* RNAi specimens. The bases of the maxillae and labium are abnormally shaped. (C) In more strongly affected *hth* RNAi individuals, one segment is missing from each palp. (D) *dac* RNAi results in reduction or deletion of intermediate segments of the maxillary and labial palps. (E) In mild *Dll* knockdowns, the palps are reduced and some fusion of palp segments occurs; a single maxillary endite is present. (F) A scanning electron micrograph of a *Dll* RNAi specimen shows a more severe phenotype, including truncation of the palps, and a single maxillary endite. (G) In *Krn* RNAi specimens the palps are reduced and distal segments are fused. (H-L) Members of the *odd*-related gene family produce similar RNAi phenotypes, which include the reduction and partial fusion of the maxillary palps and endites. The prementum and ligula are also reduced. In more severely affected specimens (K-L), joints fail to form throughout the appendages. (M) *Notch* RNAi disrupts joint formation. (N) *Ser* RNAi also disrupts joint formation, as well as causing fusion of the lacinia and galea. (O) In severe *Ser* knockdown specimens palpal length is greatly reduced. (P) RNAi targeting both *Ser* and *Delta* produced phenotypes resembling *Ser* RNAi phenotypes. (Q-R) *abrupt* RNAi results in fusion of the distal palp segments in the maxilla (Q) or labium (R). (S) The third maxillary palp segment was fused to the fourth or deleted in *pdm* RNAi individuals. Abbreviations: *crd*, cardo; *gal*, galea; *lac*, lacinia; *lig*, ligula; *lp*, labial palp segment; *mnt*, mentum; *mp*, maxillary palp segment; *plf*, palpifer; *pmt*, prementum; *stp*, stipes.

**Figure 5.** Summary of depletion phenotypes in the adult mouthparts of *T. castaneum* (A-C) and comparative data from the *T. castaneum* leg (D) and the maxillary palp (B′), proboscis (C′), and leg (D′) of *D. melanogaster*. For *T. castaneum*, colored bars represent the regions affected by
RNAi, and opacity indicates phenotypic penetrance in a given region. For *D. melanogaster* bars generalize over late larval to pupal stages. Opacity in *D. melanogaster* panels represents relative expression intensity. *ab* has not been described in sufficient detail to map its expression or functional domain. Abbreviations: crd, cardo; cx, coxa; fe, femur; gal, galea; lac, lacinia; lig, ligula; lp1-3, labial palp segments 1-3; mnt, mentum; mp1-4, maxillary palp segments 1-4; pmt, prementum; pt, pretarsus; stp, stipes; t1-5, tarsomeres 1-5; Ti, tibia; tr, trochanter.
References


Greenberg, L., and V. Hatini, 2009 Essential roles for *lines* in mediating leg and antennal proximodistal patterning and generating a stable Notch signaling interface at segment borders. Developmental Biology **330**: 93-104.


