*Tc-knirps* plays different roles in the specification of antennal and mandibular parasegment boundaries and is regulated by a pair-rule gene in the beetle *Tribolium castaneum*

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**Tc-knirps** plays different roles in the specification of antennal and mandibular parasegment boundaries and is regulated by a pair-rule gene in the beetle *Tribolium castaneum*

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**Abstract**

**Background:** The *Drosophila* larval head is evolutionarily derived at the genetic and morphological level. In the beetle *Tribolium castaneum*, development of the larval head more closely resembles the ancestral arthropod condition. Unlike in *Drosophila*, a *knirps* homologue (*Tc-kni*) is required for development of the antennae and mandibles. However, published *Tc-kni* data are restricted to cuticle phenotypes and *Tc-even-skipped* and *Tc-wingless* stainings in knockdown embryos. Hence, it has remained unclear whether the entire antennal and mandibular segments depend on *Tc-kni* function, and whether the intervening intercalary segment is formed completely. We address these questions with a detailed examination of *Tc-kni* function.

**Results:** By examining the expression of marker genes in RNAi embryos, we show that *Tc-kni* is required only for the formation of the posterior parts of the antennal and mandibular segments (i.e. the parasegmental boundaries). Moreover, we find that the role of *Tc-kni* is distinct in these segments: *Tc-kni* is required for the initiation of the antennal parasegment boundary, but only for the maintenance of the mandibular parasegmental boundary. Surprisingly, *Tc-kni* controls the timing of expression of the Hox gene *Tc-labial* in the intercalary segment, although this segment does form in the absence of *Tc-kni* function. Unexpectedly, we find that the pair-rule gene *Tc-even-skipped* helps set the posterior boundary of *Tc-kni* expression in the mandible. Using the mutant *antennaless*, a likely regulatory Null mutation at the *Tc-kni* locus, we provide evidence that our RNAi studies represent a Null situation.

**Conclusions:** *Tc-kni* is required for the initiation of the antennal and the maintenance of the mandibular parasegmental boundaries. *Tc-kni* is not required for specification of the anterior regions of these segments, nor the intervening intercalary segment, confirming that *Tc-kni* is not a canonical ‘gap-gene’. Our finding that a gap gene orthologue is regulated by a pair rule gene adds to the view that the segmentation gene hierarchies differ between *Tribolium* and *Drosophila* upstream of the pair rule gene level. In *Tribolium*, as in *Drosophila*, head and trunk segmentation gene networks cooperate to pattern the mandibular segment, albeit involving *Tc-kni* as novel component.

**Keywords:** Knirps, Head gap gene, Tribolium, Antenna, Mandible, Intercalary segment

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Background

The insect head is composed of several segments and a non-segmental anterior region. However, the exact segmental composition of the insect head has long been a matter for debate [1-7]. The posterior gnathocephalon is made up of the mandibular, maxillary and labial segments that each bear a pair of appendages specialized for feeding. The anterior procephalon consists of anterior non-segmental parts and an antennal segment, which is separated from the mandibular segment by an appendage-free segment (the intercalary segment), whose development in insects is significantly delayed, as well as reduced in size.

The genetic mechanisms of head segmentation were first examined in the dipteran fruit fly *Drosophila melanogaster* [3]. Its gnathal segments are patterned by the trunk segmentation gene cascade, involving maternal, gap, pair-rule and segment polarity genes [3,8], while the patterning of the procephalic segments follows a different paradigm [3,9-15]. Whilst segment polarity genes (i.e. en, wg, hh) are involved in establishing these segments, pair-rule genes are not [3,10-15]. Four *Drosophila* head gap genes, orthodenticle (otd), empty spiracles (ems), buttonhead (btd) and sloppy paired are expressed in broad overlapping domains in the developing anterior head [9,16]. Mutation of these genes leads to classic ‘gap phenotypes’ - the loss of all the adjacent segments covered by their domains of expression [9,17]. However, mis-expression studies have shown that only otd affects segment polarity gene expression when expressed in ectopic domains, and only ems, with the help of btd, appears to confer identity to head segments [18-20]. Indeed, second order regulators have been identified that operate at levels in between the head gap genes and segment polarity genes: *i.e. collier* and *cap ‘n’ collar* [11,12,21,22].

*Drosophila* exhibits an evolutionary derived mode of head development, in which the larval head is greatly reduced and undergoes ‘head involution’ during which head regions are folded into the body cavity [3]. This situation is far from typical for insects and moreover, the reduced and experimentally inaccessible *Drosophila* larval head has limited the comprehensive identification and analysis of insect head development genes for technical reasons [1].

In recent years the red flour beetle *Tribolium castaneum* has emerged as a powerful genetic insect model system [23] and offers an opportunity to study the genetic and cellular mechanisms underlying the development of a more insect-typical head [1]. As in *Drosophila*, the *Tribolium* gnathal segments appear to be patterned using similar mechanisms to those operating in the trunk, including a central role for pair-rule gene homologues [24-30]. In the anterior head, second order regulators and the segment polarity genes might be relatively well conserved between *Drosophila* and *Tribolium* [27,28,31-35]. However, clear differences have been identified at the level of the head gap genes, and the maternally provided anterior protein gradients that establish their expression domains [1,36-40]. For example, while the *Tribolium* homologue of *orthodenticle* (Tc-otd) apparently plays a broadly conserved role as a gap gene during head segmentation in *Tribolium*, it appears to be much more involved in axis formation than its *Drosophila* orthologue [36,41,42]. The expression of the *Tribolium* homologues of *empty spiracles and buttonhead* (Tc-ems and Tc-btd) is limited to single segment wide domains instead of large overlapping domains in the blastodermal head anlagen. Tc-ems is required to form parts of the antennal segment only and knockdown of Tc-btd does not lead to a head cuticle phenotype at all [36]. This raised the question of what genes might control development of these head regions in *Tribolium*. Work by Cerny et al. [43] suggests that the answer to this question is, at least in part, the single *Tribolium* homologue of the *Drosophila* genes *knirps* and *knirps-related*.

The *Drosophila* genes *knirps* and *knirps-related* encode steroid hormone receptor-like transcription factors [44-46]. Ancestrally, the insect *knirps* family consisted of two genes, *eagle* and *knirps-related*, while knirps arose via a recent gene duplication of the *knirps-related* gene in the higher Diptera [47]. At the blastodermic stage, *knirps* and *knirps-related* are expressed in almost identical anterior and posterior domains [45,48-50]. *Drosophila* *knirps* acts as a canonical gap gene during trunk segmentation [51-53]. In contrast, the anterior mandibular expression domain is not required for head segmentation, since segment polarity gene (i.e. *engrailed*) expression in the head is not affected in embryos that lack both paralogues [49] while a loss of the stomatogastric nervous system is observed [49].

Cerny et al. [43] have shown that Tc-knirps (Tc-kni), the single *Tribolium* homologue of the *Drosophila* knirps-family paralogues, is also expressed in anterior and posterior domains during early development [43]. However, the Tc-kni posterior domain is shifted anteriorly relative to its position in *Drosophila*, and knockdown of Tc-kni does not lead to a canonical gap phenotype in the trunk, but rather minor defects in the posterior abdomen. The anterior expression domain of Tc-kni is largely conserved. In contrast to *Drosophila*, the anterior domain does play an essential role in head patterning: Knockdown of Tc-kni leads to loss of both antennae and mandibles [43].

Cerny et al. [43] found early loss of Tc-wg expression in the antennal segment in Tc-kni RNAi while the mandibular domain of Tc-wg expression disappeared at a later stage. Further, they showed that Tc-kni is not needed for
correct Tc-wg expression in the intercalary segment. Finally, light abnormalities in the maturation and maintenance of the first pair-rule stripe of Tc-eve expression were observed, where the distance between the first segmental Tc-eve stripe (in the mandibular segment) and the ocular Tc-wg expression domain was reduced in Tc-kni RNAi blastoderm [43]. It has remained unclear, however, whether antennal and mandibular segments are deleted completely and whether the intercalary segment is affected.

In this study we examined the effect of knocking down Tc-kni on a comprehensive set of genes that mark subregions of the antennal, intercalary and/or mandibular segments. We show that Tc-kni is required for correctly specifying only posterior regions of antennal and mandibular head segments (i.e. the parasegmental boundaries). Interestingly, Tc-kni is essential for the initial specification of the antennal parasegmental boundary, while it is required only for the maintenance of the mandibular parasegmental boundary. The intercalary segment does not appear to be affected. Unexpectedly, we find that the trunk pair-rule gene Tc-even-skipped is required to set the posterior boundary of the mandibular Tc-kni expression. Unlike most RNAi studies, we have good evidence that we were investigating a Null situation due to our finding that antennalless, a Tribolium mutant arising from an EMS screen [54], is a likely regulatory Null-mutation of Tc-kni. Taken together, we show that Tc-kni is not a head gap gene, since its mutation does not lead to the complete deletion of several adjacent segments. Further, we provide a model, for how head and trunk patterning mechanisms cooperate to pattern the mandibular segment in Tribolium, and how these interactions differ between Drosophila and Tribolium.

Results and discussion

Early Tc-kni expression prefigures the appearance of the anterior head anlagen

First, we studied in more detail the dynamics of anterior Tc-kni expression because previous studies had focused on posterior aspects [43]. Zygotic Tc-kni expression in the syncytial blastoderm begins as a broad domain covering most of the anterior half of the blastoderm, only absent from a small region around the anterior pole (Figure 1A). Tc-kni expression then retreats from the anterior pole, but much more so on the dorsal side of the egg, clearing from regions that will later become the extra-embryonic serosa (Figure 1B) [55]. Tc-kni expression is maintained in the anterior/ventral regions that will become head tissue [55] (Figure 1C). These early shifts in expression occur before embryonic and extra-embryonic tissue can be distinguished at the level of nuclear morphology (Figure 1A-C and Cerny et al. [43]). When this distinction becomes apparent (Figure 1D-F), the uniform anterior Tc-kni expression domain splits into two anterior lateral domains at the boundary between each head lobe and the abutting extra-embryonic tissue (black arrows), and a more posterior stripe marking the future anterior compartment of the mandibular segment (black arrowheads) (Figure 1D-F). These domains are maintained through subsequent blastoderm (Figure 1G-I) and early germ-band (Figure 1J, K) stages, before fading (Figure 1L, M) and then disappearing completely during mid-germband elongation stages. Hence, the early uniform domain of Tc-kni expression (Figure 1C) encompasses the entire future anterior head anlagen; i.e. everything anterior to the parasegment 0/1 boundary.

Antennalless is likely a regulatory mutation at the Tc-kni locus

In most RNAi experiments outside Drosophila, it remains unclear to what extent a genetic Null-situation is phenocopied. We aimed at determining the Null situation by studying a genetic mutant.

Antennalless is a homozygous lethal mutation that was recovered from an EMS mutagenesis screen [54]. The cuticular phenotypes of antennalless mutant larvae are highly reminiscent of Tc-kni RNAi phenotypes (Tc-kniRNAi) [43]. Larvae of the antennalless mutant lack antennae and mandibles, and occasionally also display minor abdominal defects, very similar to those previously reported for weak Tc-kniRNAi larvae (Figure 1N, O and Cerny et al. [43]). In order to check for defects in dorsal and lateral head tissues which are derived from pre-antennal and intercalary regions, respectively [32,36,56], we scored both sides of 20 antennalless larval cuticles for the head bristle pattern. All bristles were present, albeit shifted somewhat compared to the wildtype condition in these regions (Figure 1N, O). This is consistent with pre-antennal regions and much of the intercalary segment not being affected in the mutant [32,43].

The development of both mandibles and antennae was more severely affected at a higher temperature in antennalless mutants. A larval cuticle was scored as an antennalless mutant if either the mandibles or antennae, or both, were reduced or absent. Most of the antennalless mutant larvae (67%, n = 43) lacked both the antennae and mandibles at 25°C. This increased to 75% (n = 32) at 32°C. In a few cases, rudiments of mandibles were found (5% at 25°C and 6% at 32°C). In other cases, antennae or antennal rudiments were found (28% at 25°C and 19% at 32°C). In contrast, and against the general observation that phenotypes tend to be more penetrant at higher temperatures, the frequency of abdominal defects was found to be higher at 25°C than at 32°C. At 25°C, 90.5% (n = 43) of phenotypic larvae displayed defects within segments A5-A8, compared to only 16% (n = 32) at 32°C. An inverse sensitivity with respect to temperature was also observed for defects in the urogomphi, dorsal outgrowths of the ninth
Figure 1  Embryonic expression of Tc-kni and the Tc-kni head cuticle phenotype. A-M: Expression of Tc-kni during blastoderm (A-H) and early germband stages (I-M). See text for a detailed description. Panels A’-H’ show inverted DAPI images of the embryos in panels A-H respectively. Black arrows in panels D-M mark Tc-kni expression at the anterior border of one or both head lobes. Black arrowheads in panels D-M indicate the stripe of Tc-kni expression marking the anterior compartment of the mandibular segment. White arrowheads in panels E-M mark the posterior domain of Tc-kni expression which first becomes visible within the primitive pit as it forms (E-I) and later resolves into a stripe marking segment A1 (J-M). Anterior is to the left in panels A-H and to the top in panels I-M. Panel B shows a dorso-lateral view; panels D, E and G show ventro-lateral views; panels C and F show lateral views, and panels H-M are all ventral views. N-O: In contrast to wildtype (N), antennules mutant first instar larvae (O) lack antennae (black arrows in panel N) and mandibles (black arrowhead in panel N). All lateral and dorsal head bristles are however present.
segment. At 25°C this structure was defective in 79% of the phenotypic larvae examined (n = 43), compared to 44% at 32°C (n = 32).

In offspring from nine independent pairs of heterozygous parents, the antennalless cuticle phenotype is found in 25% (n = 364) and 27% (n = 267) of larvae at 25°C and 32°C respectively, levels consistent with a highly penetrant homozygous lethal mutation. A significant proportion of mutant larvae were able to hatch despite their lack of antennae and mandibles; 45% at 25°C (n = 91) compared to only 17% at 32°C (n = 72). The vast majority of these larvae died at the L1 stage. Only very rarely did individuals reach the L2 stage. When corrected for a background reduction in hatching rate associated with this shift in temperature (measured from wildtype larvae), these data reveal a 19% increase in sensitivity to temperature with regards to hatching in the antennalless mutant background.

In our Tc-kni RNAi experiments head defects were also found to be more severe at 32°C compared to 25°C (see Additional file 1, compare panel B to panel A). The severity of head defects decreased over time post-injection as expected for parental RNAi experiments [57] (see Additional file 1, panel C). However, as for antennalless, we found inverse temperature sensitivity with respect to defects in segments A5–A8 following Tc-kni RNAi (see Additional file 1, compare panel E to panel D). The frequency of abdominal defects also increased with time post-injection from about 20% (n = 10) at day eight post-injection, to 37% (n = 35) eleven days post-injection, and to 56% (n = 27) thirty-one days post-injection, before dropping again (For eggs at 25°C; see Additional file 1, panel D). This is not in line with the usual observation that in RNAi experiments phenotypic strength decreases over time and indicates a complex relationship between knockdown and phenotype, which we do not fully understand.

Since the antennalless cuticle defects described above are almost identical to the Tc-kni RNAi phenotypes both in terms of physical phenotype and sensitivity to temperature, we suspected Tc-kni as the gene affected by the mutation. We therefore independently sequenced the three Tc-kni exons from the genomic DNA of two first instar larvae that had been identified as homozygous mutant by their cuticle phenotype. We found that the coding sequence of Tc-kni is not altered in mutant beetles (data not shown).

In order to test the hypothesis that antennalless is a regulatory mutation at the Tc-kni locus, we carried out in situ hybridization on embryos from heterozygous mutant parents with a mix of probes targeting Tc-kni and Tc-caudal as positive control. We failed to detect Tc-kni expression in 15% of the offspring examined (n = 100), whereas Tc-caudal was well stained in the same colour reaction in all cases (see Additional file 2). This is consistent with embryos homozygous for the antennalless allele not expressing Tc-kni at levels detectable via in situ hybridization.

Taken together, our results are consistent with the hypothesis that antennalless is a regulatory mutation of Tc-kni, and as such we now refer to it as Tc-kniRNAi. However, we cannot rule out that antennalless is a mutation in a gene that acts rather exclusively upstream of, or is a required interaction partner of, Tc-kni. Further studies will be necessary to confirm our hypothesis by identifying the cis-regulatory regions that are affected by the mutation.

The Tc-kni Null phenotype is revealed by both Tc-kniRNAi and Tc-kniRNAI

The lack of Tc-kni expression in homozygous Tc-kniRNAi embryos implies that Tc-kni function is strongly reduced in the mutant. However, it remains possible that some residual function remains. In order to test whether Tc-kniRNAI constitutes a complete Null phenotype for Tc-kni, we performed Tc-kni RNAi in the Tc-kniRNAI background, assuming that the added effects should not lead to stronger phenotypes if Tc-kniRNAI is a Null mutant. The frequency of the Tc-kni phenotype in the pooled offspring from 30 independent pairs of Tc-kniRNAI heterozygous animals was 11% (n = 149) at 25°C and 17% (n = 82) at 32°C. As expected, after injecting the same females with Tc-kni dsRNA, the frequency of the Tc-kni phenotype strongly increased, to 97% (n = 32; 25°C) and 81% (n = 32; 32°C). Crucially, we did not find evidence for an increase in the severity of the cuticle phenotype: i.e. there were no larvae with phenotypes more severe than those seen in Tc-kniRNAI or Tc-kniRNAI alone. Hence, unlike in most RNAi experiments, we can be rather confident that the most severe RNAI phenotypes we observe fully phenocopy a Null situation. Therefore, we use results from both mutant and RNAI embryos for this work.

Tc-kni is differentially required for specifying antennal, intercalary and mandibular parasegment boundaries

The cuticle phenotypes reported by Cerny et al. [43] raised the question of whether entire segments are deleted in Tc-kni depleted embryos, or only the posterior parts that give rise to the appendages. These depend on parasegmental boundary formation via the segment polarity genes Tc-engrailed (Tc-en), Tc-hedgehog (Tc-hh) and Tc-wingless (Tc-wg) [5,10,13,21,22,27-29]. Therefore, we stained Tc-en and Tc-hh in Tc-kniRNAI embryos as markers for the posterior segment compartment. In the case of Tc-en, embryos were co-stained for Tc-Dfd to help distinguish disrupted mandibular Tc-en expression domains (which are located within the Tc-Dfd domain), from intercalary Tc-en expression domains (arising at
the anterior median boundary of the *Tc-Dfd* domain) (Figure 2J-K, N-O, black asterisks).

We find that both *Tc-en* (Figure 2) and *Tc-hh* (Figure 3) expression is absent in the antennal segment in *Tc-kni*RNAi embryos throughout embryogenesis (marked by white arrowhead in wildtype embryos in Figures 2 and 3). Occasionally, *Tc-en* and *Tc-hh* expression associated with structures at the base of the antenna remains in later germband embryos, despite the clear failure of the antenna to form (marked by a red arrow in panels K, L, M, N and O in Figure 2 and panels F and H in Figure 3).

The mandibular stripe of *Tc-en* expression is initiated in early *Tc-kni*RNAi germband embryos, albeit abnormally; the stripe of expression is broken and in extreme cases only patches of expression are seen (compare panels B and C with panel A in Figure 2; mandibular stripe marked by black arrow). In older *Tc-kni*RNAi germband embryos, *Tc-en* expression associated with the mandibular segment is often missing completely (compare panels F, I and O, with panels D, G and M respectively in Figure 2), suggesting a failure to properly maintain *Tc-en* expression in this segment in strong knockdowns of *Tc-kni* expression. Similarly, *Tc-hh* expression in the mandibular segment is greatly reduced in young *Tc-kni*RNAi embryos, but a thin broken stripe is still detected medially in early germbands (compare black arrows in panels B and D to panels A and C respectively in Figure 3). Later, this *Tc-hh* expression disappears in contrast to wildtype (compare panels F and H to panels E and G respectively in Figure 3). Note that *Tc-en* mandibular expression appears to be more sensitive to the loss of *Tc-kni* expression in lateral regions of the germband (Figure 2E, H, K and N).

*Tc-en* and *Tc-wg* expression in the intercalary segment appears relatively late and at a quite variable time point during mid germband elongation in wildtype embryos. Whilst we cannot completely rule out minor disruptions of intercalary *Tc-en/Tc-hh* expression initiation and/or maintenance in *Tc-kni*RNAi embryos, we do detect wildtype expression of these genes in late elongation (i.e. post 10 *Tc-en* stripe embryos) and early fully elongated *Tc-kni*RNAi embryos (black asterisk in panels K, L, N and O in Figure 2 and black asterisk in panel F in Figure 3).

In conclusion, the disruption of *Tc-kni* function leads to the complete failure to initiate segment polarity gene expression in the antennal segment, failure to properly maintain segment polarity gene expression in the mandibular segment, and most likely has no effect on segment polarity gene expression in the intercalary segment [43].

Molecular markers show that *Tc-kni* is not a canonical head gap gene

Segment polarity genes mark a posterior portion of each segment. Therefore, we also asked whether the anterior parts of the segments are miss-specified and/or missing in *Tc-kni*RNAi embryos by analyzing markers for the anterior regions of head segments. *Tc-goosecoid* (*Tc-gsc*) expression [56] initially partially overlaps the ocular *Tc-wg* domain and extends posterior to it (Figure 4A). Later, this domain widens in lateral regions, forming a wedge shape domain that abuts the antennal *Tc-wg* stripe as it appears (Figure 4C) but later retracts from it (Figure 4E). Thus, *Tc-gsc* is a marker for posterior ocular and anterior antennal regions in early embryos but later predominantly marks ocular tissue. In early *Tc-kni*RNAi germband embryos, *Tc-gsc* expression is down-regulated in posterior-lateral regions of the wedge shaped expression domain (black arrows in Figure 4B, D; compare to Figure 4A, C respectively). In older *Tc-kni*RNAi embryos, the *Tc-gsc* expression domain again closely resembles the wedge shape seen in wild-type embryos at these stages (Figure 4F). This indicates that the ocular parasegment boundary and anterior parts of the antennal segment are not greatly affected by loss of *Tc-kni*, apart from some degree of lateral down-regulation of *Tc-gsc* in early germ bands.

*Tc-lim1* is a similar marker for the posterior of the ocular segment and anterior compartment of the antennal segment [56], albeit shifted slightly posteriorly relative to *Tc-gsc*. In early embryos, expression is partially overlapping the ocular *Tc-wg* domain and extending posterior to it (Figure 4G). In elongating germbands, *Tc-lim1* expression forms a wedge shaped domain that covers all cells between the ocular and antennal *Tc-wg* stripes (Figure 4I). In the *Tc-kni*RNAi background, the posterior boundary of this domain is irregular and the domain is somewhat narrower indicating posterior reduction of the *Tc-lim* marked tissues (Figure 4H). In wild-type elongating germbands, additional *Tc-lim1* segmental expression arises that laterally overlaps the segmental *Tc-wg* domains in gnathal and thoracic segments (Figure 4K). At these stages, the ocular-antennal domain of *Tc-lim1* expression splits into a posterior domain that overlaps the antennal *Tc-wg* stripe, and an anterior domain positioned between the ocular and the antennal *Tc-wg* stripes (Figure 4K; white arrowheads). Expression in the ocular-antennal region remains also in *Tc-kni*RNAi germbands, but the domain is not split as in wildtype (upper red arrowhead in Figure 4L) and appears to be fused with the mandibular domain of lateral *Tc-lim1* expression (lower red arrowhead in Figure 4L). In extreme cases this domain is also fused to the maxillary *Tc-lim1* expression domain (Figure 4L and discussed below). This confirms that anterior antennal tissue is properly specified while the parasegmental boundaries of the antennal and mandibular segments are not formed correctly.

*Tc-empty-spiracles* (*Tc-ems*) is expressed in a segmentally reiterated pattern during *Tribolium* development [36] in mediolaterally-restricted domains that lie anterior
Figure 2 Expression of Tc-en and Tc-Dfd in wildtype and Tc-kni RNAi embryos. The antennal Tc-en stripe (marked by white arrowhead in wildtype panels A, D, G, J, M) is missing in all Tc-kni RNAi embryos. The mandibular Tc-en stripe (marked by black arrow) forms within the Tc-Dfd expression domain, but is broken (panels B, C, E, H, K, L, N) and often missing (panels F, I, O) in Tc-kni RNAi embryos. Tc-en expression in the intercalary segment (black asterisk in panels J-L, N-O; hidden by antenna in panel M) appears at a variable time point in mid-elongation stage embryos at the anterior border of the Tc-Dfd expression domain in both wildtype and Tc-kni RNAi embryos. Tc-en expression associated with structures at the base of the antenna is evident in late Tc-kni RNAi embryos (red arrows in panels K-O). Head lobes appear reduced in size (panels A'-C'), and the Tc-Dfd domain slightly narrower (panels G-O) in Tc-kni RNAi embryos, consistent with a failure to specify and form posterior antennal and mandibular tissue. Black arrowheads in panels D-O mark the maxillary Tc-en stripe. Ventral views, and anterior towards the top, in all panels.
To the Tc-wg stripe in each segment [36]. Tc-ems expression therefore predominantly marks the anterior portion of each segment. In contrast to wild-type expression, the antennal and mandibular Tc-ems segmental domains are fused in Tc-kni RNAi embryos (red arrows in Figure 5B), as seen for Tc-lim1. This is consistent with defects being restricted to the posterior compartment of the antennal segment leading to the fusion of the antennal and mandibular Tc-ems expression domains.

Tc-sloppy-paired-1 (Tc-slp-1) is also expressed in a segmentally reiterated pattern in the developing Tribolium head (Figure 5C, F and [25,32]). Tc-slp-1 expression domains overlap the Tc-wg stripe in each head segment, but also extend further into the anterior compartment,
Figure 4 Expression of Tc-gsc and Tc-lim1 in wildtype and Tc-kni RNAi embryos. All embryos are also co-stained for Tc-wg. Tc-gsc expression is reduced in posterior-lateral regions of its normally wedge-shaped ocular/antennal expression domain in early germband Tc-kni RNAi embryos (black arrows in panels B, D; compare to panels A, C respectively). At slightly later germband stages however, clear differences between the Tc-gsc expression domain in Tc-kni RNAi and wildtype embryos are no longer observed (compare panel F to panel E). Tc-lim1 expression is also irregular and reduced in posterior regions of its wedge-shaped ocular/antennal expression domain in early germband Tc-kni RNAi embryos (black arrowheads in panel H; compare to panel G). At slightly older germband stages clear differences in the Tc-lim1 expression domain are no longer observed between Tc-kni RNAi and wildtype embryos (compare panel J to panel I). In later wildtype germband embryos, the Tc-lim1 ocular/antennal domain splits into two stripes (marked by white arrowheads in panel K). This fails to occur in equivalent stage Tc-kni RNAi embryos, and antennal, mandibular and maxillary expression domains are often fused in lateral regions of the germband (red arrowheads in panel L; compare to panel K). Ventral views, and anterior to the top, in all panels.
as well as a little across the parasegmental boundary into the posterior compartment [25,32]. Tc-slp-1 expression therefore predominantly marks a posterior portion of the anterior segment compartment. Tc-slp-1 is expressed in a stripe in the antennal and each gnathal head segment in both Tc-kniRNAi and Tc-kniatl embryos (Figure 5D, E and G, H), as in wild-type embryos (Figure 5C, F). However, the antennal stripe often appears to broaden (white arrowhead in Figure 5D, E, H), and the distance between the mandibular and the maxillary Tc-slp-1 stripes is often decreased, completely fusing in lateral regions (red arrows in Figure 5D, E and G, H). These data indicate that overall

Figure 5 Expression of Tc-ems and Tc-slp-1 in wildtype, Tc-kni RNAi and antennaless embryos. Panels B, D, E, G and H represent documentation of fluorescent FastRed stainings of the respective gene and are therefore shown in grayscale. Tc-ems expression in the antennal (white arrowhead, panels A, B) and mandibular (black arrow, panels A, B) segments is abnormally fused in Tc-kni RNAi embryos (red arrows in panel B, compare to panel A). A black arrowhead marks the maxillary domain of Tc-ems expression in panels A, B. The antennal Tc-slp-1 expression domain (white arrowhead, panels C-H) is often expanded, while the mandibular (black arrow, panels C-H) and maxillary (black arrowhead, panels C-H) Tc-slp-1 expression domains are fused (red arrows in panels D-E, G-H) in Tc-kni RNAi and antennaless embryos. Ventral views, and anterior to the top, in all panels.
the Tc-slp-1 stripes are not dramatically affected by Tc-kni RNAi. However, the decreased distance between the mandibular and maxillary stripes could indicate incorrectly specified tissue in the intervening posterior compartment of the mandibular segment.

A normal complement of bristles and setae in lateral regions of Tc-kniatl larval heads implies the retention of intercalary segment derived cuticle in Tc-kni knockdown embryos (Figure 1 and [32]). Since Tc-labial (Tc-lab) is expressed during embryogenesis throughout the presumptive intercalary segment [58], and is required for its formation [32], we used it as a molecular marker for the presence/absence of the intercalary segment. In Tc-kni RNAi embryos (Figure 6G-L), Tc-lab expression appears prematurely but with similar dynamics as in wildtype (Figure 6A-F). The premature Tc-lab expression domain first appears medially as a pair of dots on either side of the median mesoderm (Figure 6G; compare to wild-type in Figure 6A). Tc-lab expression then extends into more lateral and medial regions. Expression is not entirely wildtype, as it may form an unusual regular stripe without the typical median expansion (compare Figure 6H, L with D, F), or it may be laterally reduced (Figure 6l, J, K). However, in all instances, the stripe was present indicating that the intercalary segment is present albeit its morphology appears not to be entirely unaffected by adjacent defects.

We also examined the expression of another marker for the intercalary segment in Tc-kniRNAi embryos, the second order regulator Tc-collier (Tc-col), also called Tc-knot. Tc-col acts downstream of Tc-lab and is required for wildtype expression of Tc-en in the intercalary segment [31]. Aside

![Figure 6 Expression of Tc-lab in wildtype and Tc-kni RNAi embryos.](image)

Tc-lab expression appears prematurely in Tc-kni RNAi embryos (panels G, H, I, compare to equivalent stage wildtype embryos in panels A, B, C respectively). In Tc-kni RNAi embryos Tc-lab expression is always present but somewhat disturbed, including lack of median enlargement (H, I, L, compared to wildtype embryos in panels D, F) or lateral reduction (I, J, K, compare to wildtype embryos in panels D, E). See text for further details. The red background staining in each panel is weak FastRed signal for Tc-wg.
Figure 7 Expression of Tc-col and Tc-en in wildtype and Tc-kni RNAi embryos. Abnormalities in Tc-en expression in Tc-kni RNAi embryos are similar to those seen in Figure 2 and described in the text. The appearance of the Tc-col domain in early germband embryos may be slightly delayed following Tc-kni RNAi (compare panels B, C to panel A). Apart from minor irregularities, the Tc-col expression domain is not strongly altered in Tc-kni RNAi embryos (compare wildtype to Tc-kni RNAi embryos in panels D-O). The co-expression of Tc-col and Tc-en in a medial region of the head in mid-to-late germband embryos (black asterisk in panels J-L, N-O, obscured by antennae in M) confirms the identity and presence of intercalary Tc-en expression in a Tc-kni RNAi background. White arrowheads: Tc-en antennal stripe. Black arrows: Tc-en mandibular stripe. Black arrowheads: Tc-en maxillary stripe. Red arrows: Residual Tc-en expression associated with structures at the base of the antennae. Ventral views, and anterior towards the top, in all panels.
from a slight delay in the initial appearance of the Tc-col expression domain in early embryos (Figure 7 compare B, C with A) we found no evidence of strong disruption of Tc-col expression in a Tc-kniRNAi background (Figure 7). Besides, this staining confirmed the identity, and wildtype appearance, of the intercalary spots of Tc-en in late elongating and early fully elongated embryos (black asterisk in panels K, L, N and O in Figure 7).

The Drosophila head gap genes lead to the loss of entire adjacent segments but their Tribolium orthologues do not fit this definition [36]. Similarly, taken together, our data (summarized in Figure 8) show that Tc-kni cannot be considered a canonical head gap gene, because Tc-kni function is only required for the posterior parts of the antennal and mandibular segments (the regions shaded grey in Figure 8A). Moreover, Tc-kni is not required for the formation of the intercalary segment, which lies between the affected antennal and mandibular segments. Intercalary segment polarity gene expression is instead likely dependent on a conserved pathway involving Tc-lab and Tc-col [31,32]. Overall, our data confirm that the regulatory networks underlying the establishment of the anterior head segments have diverged significantly between Tribolium and Drosophila.

Figure 8 Schematic diagrams summarizing the expression of Tribolium head genes in wildtype and Tc-kniRNAi embryos. Changes in marker gene expression following Tc-kni RNAi are consistent with a failure to specify and form posterior antennal and mandibular segmental regions (marked in grey in A, and deleted in B). Deletion of these regions explains the absence of segment polarity gene expression in antennal and mandibular segments; the deletion of posterior regions of the ocular/antennal domains of Tc-gsc and Tc-lim1 at early stages, the reduced width of the Tc-Dfd domain, and the reduction in distance between antennal, mandibular and/or maxillary domains of Tc-lim1, Tc-ems and/or Tc-slp-1. Abnormalities in the expression patterns of the genes bounded by the horizontal doted lines are most apparent in lateral regions of the germband. Additional defects affecting the intercalary segment have to be assumed to explain the fusion of respective Tc-ems and Tc-slp stripes. These could be due to aberrant morphogen signaling of the mandibular parasegment boundary. In Tc-kni RNAi, the antennal parasegment boundary is never established while our model assumes that the posterior of the mandibular segment is lost later due to disturbance of the wg/hh/en regulatory loop. Lightly shaded regions represent early aspects of expression that do not persist to later stages. See Figure 10 and text for details.
involving changing roles for knirps, buttonhead and empty spiracles homologues [36].

The Tc-kni phenotype is due to a failure to correctly specify cell fates
We decided to check whether missing head regions in Tc-kni knockdown and mutant embryos were lost due to a failure to maintain already specified tissue - which might be indicated by high levels of cell death - or through the failure to specify cells to their correct developmental fate. Using TUNEL staining, we did not detect any apoptosis in either the blastoderm (see Additional file 3) or early germband stages (see Additional file 4) in Tc-kniRNAi embryos. In later germband stages there were a few apoptotic cells detectable in the head (as well as the posterior growth zone), but no more than in wild-type embryos, and there was no specific pattern of apoptosis that would indicate the loss of the tissues in question, namely the posterior portions of the antennal and mandibular segments (see Additional files 3 and 4). Thus, the lack of antennae and mandibles in Tc-kniRNAi and Tc-knifl embryos is not due to tissue degeneration, but rather the failure to specify the respective segmental regions.

The posterior border of the anterior Tc-kni expression domain is regulated by Tc-even-skipped
In the Tribolium blastoderm, expression of Tc-kni and the pair rule genes occurs at the same time, opening the possibility Tc-kni could be regulated by pair rule genes. Indeed, in Tc-eveRNAi blastoderm embryos, we found that the anterior domain of Tc-kni expression expanded into more posterior regions (Figure 9: compare panel B to panel A). In contrast, expression defects in this Tc-kni mandibular domain were not seen in Tc-oddRNAi or Tc-runRNAi germ bands (Figure 9: compare panels C and D with A). This is consistent with the first pair-rule and/or segmental stripe of Tc-eve expression being required to set the posterior boundary of mandibular Tc-kni expression.

Head and trunk segmentation gene networks cooperate to establish the mandibular segment in a different way in Tribolium and Drosophila
In Drosophila, the anterior head gene regulatory network (through btd, col and cnc) and the gnathal/trunk regulatory gene network (through eve) cooperate to pattern aspects of the mandibular segment (Figure 10A and [11,12]). In this study we provide evidence that this is also true in Tribolium. Both Tc-eve and Tc-kni RNAi lead to the failure to properly maintain mandibular Tc-en stripe expression and as a result to loss of the mandible itself (this study and [24,43]). In our model (Figure 10B) Tc-kni contributes to the activation of mandibular Tc-wg expression. This interaction could be direct because the respective expression patterns overlap. Tc-eve in turn could be directly involved in activating Tc-hh and Tc-en within its anteriormost expression domain. Subsequently, the interactions of the Wnt and hh pathways ensure maintenance of the parasegmental boundary. In the absence of Tc-kni expression, Tc-eve still partially initiates Tc-en/Tc-hh expression in the posterior compartment, which leads to partial initiation of Tc-wg in the anterior adjacent cells (through Hedgehog signaling). However, absence of Tc-kni leads to failure to maintain Tc-wg expression, and as a result failure to maintain Tc-en expression in posterior adjacent cells (through Wingless signaling). Interestingly, in Drosophila, the maintenance of en and wg expression in the mandibular segment appears more interdependent in dorsal regions [13]. The same might also be true in Tribolium, since both Tc-en and Tc-wg expression is maintained more often in medial (i.e. ventral) regions in Tc-kni mutant and knockdown embryos. Taken together, it appears to be a conserved feature of insects that two systems cooperate in patterning the mandible, but the molecular details of this cooperation have diverged significantly between Drosophila and Tribolium (Figure 10).

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**Figure 9 Tc-kni is regulated by Tc-eve.** The Tc-kni head expression domain, which covers the anterior compartment of the mandibular segment (bounded by black horizontal lines in panels A, C, D) extends posteriorly (arrowhead in panel B), in a Tc-eve RNAi background, but not in Tc-odd RNAi (panel C) or Tc-run RNAi (panel D) embryos. This is consistent with Tc-eve expression in the posterior compartment of the mandibular segment setting the posterior boundary of the Tc-kni head expression domain.
Conclusions

In this study we show that anterior regions of the antennal and mandibular segment, as well as the intervening intercalary segment, are correctly specified in the absence of Tc-kni function. Moreover, we identify key differences in the role of Tc-kni in setting up the non-adjacent antennal and mandibular parasegmental boundaries. We identify the pair-rule gene Tc-even-skipped as a potential positive regulatory input that enables the initial specification (but not maintenance) of the mandibular parasegmental boundary in the absence of Tc-kni function.
of **Tc-kni** function. We also present the first evidence that, as in *Drosophila*, head and trunk gene regulatory networks cooperate to specify the mandibular segment. However, our data, and those of others, point to significant divergence in the molecular interactions involved in mandibular patterning between *Drosophila* and *Tribolium*.

A recent study of the expression and function of head patterning genes in the hemimetabolous insect *Oncopeltus fasciatus* suggests that the developmental gene networks operating in *Tribolium castaneum* more closely resemble the ancestral insect condition, perhaps not surprisingly given the highly derived *Drosophila* larval head [59]. Indeed, recent data from a myriapod suggest that the developmental genetic basis of *Tribolium* larval head development might even closely resemble the ancestral arthropod condition [60,61]. It will be interesting to see whether studies on other arthropods reveal an ancestral role for *knirps*-family homologues in head segmentation, which would imply *ems* and *btd* have usurped the role of *knirps*, and potentially other genes, in the lineage leading to *Drosophila*.

**Methods**

**Antennalless mutant analysis**

The *antennalless* mutant line was maintained as described by Berghammer et al. [62] and Maderspacher et al. [54]. The separation of offspring from single pair crosses was performed after three and six days of egg laying at 32°C and 25°C respectively. Cuticle preparations of mutant larvae were performed after three and six days of egg laying at 32°C and separation of offspring from single pair crosses was established protocols [64]. dsRNA was produced using the T7 and SP6 MEGAscript High Yield Transcription Kits (Ambion). Template DNA was either PCR-amplified using the vector insert flanking primers T7: 5′-TAATACGAC TCATAATTG-3′ and T7-SP6: 5′-TAATACGACTCTATAGGTTAGGTGAACACTATA-3′ or by using a stock of the linearized plasmid. In this case the antisense and sense strands were amplified separately, and later the ssRNA combined in equimolar amounts. A concentration of between 2 and 4.3 μg/μl of *Tc-kni* dsRNA was injected in each experiment, since this concentration range has been previously shown to consistently produce fully penetrant *Tc-kni* RNAi phenotypes [43]. To knockdown *Tc-ene*, *Tc-odd* and *Tc-runt* concentrations of between 2 and 3.5 μg/μl of dsRNA were used.

**Parental RNAi**

Adult or pupal parental RNAi was carried out using established protocols [64]. dsRNA was produced using the T7 and SP6 MEGAscript High Yield Transcription Kits (Ambion). Template DNA was either PCR-amplified using the vector insert flanking primers T7: 5′-TAATACGAC TCATAATTG-3′ and T7-SP6: 5′-TAATACGACTCTATAGGTTAGGTGAACACTATA-3′ or by using a stock of the linearized plasmid. In this case the antisense and sense strands were amplified separately, and later the ssRNA combined in equimolar amounts. A concentration of between 2 and 4.3 μg/μl of *Tc-kni* dsRNA was injected in each experiment, since this concentration range has been previously shown to consistently produce fully penetrant *Tc-kni* RNAi phenotypes [43]. To knockdown *Tc-ene*, *Tc-odd* and *Tc-runt* concentrations of between 2 and 3.5 μg/μl of dsRNA were used.

**TUNEL staining**

Fixed embryos stored in methanol at −20°C were gradually rehydrated by washing in 70% methanol/PBT, then 50% methanol/PBT, then 30% methanol/PBT and finally 100% PBT (PBS + 0.1% (v/v) Tween-20). Embryos were then incubated for six minutes in 1 ml of PBT + 0.5 μl proteinase K (15 mg/ml) and subsequently washed several times in PBT. Embryos were then post-fixed in 1 ml of PBT + 125 μl of formaldehyde (37%) for 20 minutes, before being washed three times in PBT. At this point,
positive control embryos were washed three time in 
DNasel buffer (40 mM Tris–HCl, pH7.5, 0.1 mM 
dithiothreitol, 6 mM MgCl₂), incubated for 30 minutes 
at 37°C in DNasel buffer with 0.06 U DNasel per micro-
litre of buffer, before being washed several times in PBT. 
All embryos were then incubated for 20 minutes in 0.1% 
sodium borohydride. During incubation, the embryos 
were gently shaken several times. The sodium borohyd-
ride was then removed by repeated washing with 
TdT buffer (140 mM cacodylic acid, 1 mM cobalt 
chloride, 30 mM Tris–HCl, pH7.4, 0.1% Tween-20) 
at room temperature, before being incubated at 70°C for 
20 minutes in TBST. Embryos were then washed three 
times for five minutes in PBT (140 mM NaCl, 
2.7 mM KCl, 25 mM Tris–HCl pH7.4, 0.1% Tween-20) 
at temperature in PBT prior to NBT/BCIP staining. The 
staining was stopped by repeated washing in PBT and 
repeated washes in PBT for a 
hour. This was followed by several washes in PBT for a 
three times for five minutes in TBST, before being incubated in 
PBS (PBT with 10 mg/ml bovine albumine (BSA) and 
2% sheep serum) for one hour. Embryos were then incu-
bated in PBS with anti-Dig antibody (1:2000) for one 
hour. Embryos were then incubated for 20 minutes in 
PAS with anti-Dig antibody (1:2000) for one 
hour. This was followed by several washes in PBT for a 
total of two hours. Embryos were stored overnight at 4°C 
in PBT before being washed for 30 minutes at room 
temperature in PBT prior to NBT/BCIP staining. The 
staining was stopped by repeated washing in PBT and 
the embryos stored at 4°C in 1 ml of PBT + 125 μl of for-
maldehyde (37%).

Microscopy
Most in situ hybridization stained preparations were im-
aged on an Axioplan 2 photomicroscope (Carl Zeiss Vi-
sion GmbH, Jena) using a polarized light (DIC) filter 
with low Normaski contrast (ImageProPlus, Version 5.0 
2.9, MediaCybernetics). For clear detection of the fluo-
rescence signal of the Fastred® color reaction the filter set 
No. 43 (Cy3) from Zeiss, and a mercury vapor lamp 
HBO100 as a light source, was used. For the detection of 
Hoechst 33258 and Hoechst 33342 signal the filter set 
No. 49 from Zeiss (DAPI filter), and a mercury vapor lamp 
as the light HBO100 source, was used. A few in situ 
hybridization stained preparations were imaged on a 
Leica MZ16F epifluorescence microscope with a 
DFC300FX digital camera (images in Figures 2 and 7). 
Larval cuticles were imaged using a confocal laser-
scanning microscope (LSM 510, Zeiss) and processed as 
described [65].

Availability of supporting data
The data set supporting the results of this article is in-
cluded within the article (and its additional files).

Additional files

Additional file 1: The sensitivity of Tc-kni RNAi head and abdominal 
larval phenotypes to temperature. (A, B) Head phenotypes are more 
severe at higher temperatures. Note the higher frequency of strong head 
phenotypes (up to ~90% vs. circa 70%) and lower frequency of weak 
head phenotypes (0% at some time points at 32°C) following Tc-kni RNAi 
carried out at 32°C compared to 25°C. Larvae exhibiting strong head 
phenotypes lacked both antennae and mandibles, larvae exhibiting 
medium phenotypes possessed at least one antenna and larvae 
exhibiting weak phenotypes exhibited at least one antenna and 
mandibles. (C) The frequency of larval head phenotypes decreases with 
time post Tc-kni dsRNA injection. (D, E) In contrast to head phenotypes, 
abdominal phenotypes showed an unusual reverse sensitivity with 
respect to temperature and time following Tc-kni RNAi injection. 
Abdominal phenotypes were more common following Tc-kni parental 
RNAi carried out at 25°C compared to 32°C (compare height of blue bars 
in panel D vs. panel E). Abdominal phenotypes showed the unusual 
characteristic of increasing in frequency with time post Tc-kni dsRNA 
injection.

Additional file 2: Tc-kni is not expressed in antennaeless embryos. 
Wildtype and antennaeless blastoderm (panels A-D) and early germband 
(panels E-H) embryos co-stained with a mix of Tc-kni and Tc-cad probes 
detected with the same colour reaction. A probe against Tc-cad was used 
to control against the possibility that the absence of Tc-kni signal in 
antennaeless embryos was due to technical problems. Blastoderm 
embryos were stained with Hoechst 33258 (A’-D’) in order to identify 
similar stage embryos. In antennaeless blastoderm embryos, a block of 
signal (bounded by white lines in panels A, C) corresponding to the 
anterior head Tc-kni expression domain is missing, whereas the posterior 
domain of Tc-cad expression is detected. In antennaeless early germband 
embryos the anterior mandibular stripe of Tc-kni expression (black 
arrowhead in panels E, G) is missing, whereas the posterior growth zone 
domain of Tc-cad expression is detected. Similar experiments using a 
Tc-otd probe as control proved that the posterior Tc-kni expression 
domain is also missing in antennaeless blastoderm and germband 
embryos (data not shown). Panels A-D’: Lateral views, anterior to the left. 
Panels E-H: Ventral views, anterior to the top.

Additional file 3: TUNEL stained wildtype and Tc-kni RNAi 
blastoderm stage embryos. DAPI staining (A’-F) was used to identify 
wildtype and Tc-kni RNAi blastoderm embryos of similar stages. No 
apoptotic nuclei were observed in wildtype or Tc-kni RNAi 
blastoderm embryos.

Additional file 4: TUNEL stained wildtype and Tc-kni RNAi 
germband stage embryos. Apoptotic nuclei were not observed in 
wildtype or Tc-kni RNAi early germband stage embryos (A-F). A few apoptotic 
nuclei (arrowheads in panels G-J) were observed in mid-elongation (G-H), 
late-elongation (I-J) and fully elongated (K-L) wildtype and Tc-kni RNAi 
germband embryos. However, levels of apoptotic nuclei were not higher in 
Tc-kni RNAi germband embryos when compared to controls, and apoptotic 
nuclei were not concentrated in regions within which the anterior and 
mandibular segments should or would develop. Note that in some cases 
(panels C, E, G, I, N) TUNEL reactions were developed for much longer 
needed to detect apoptotic nuclei, leading to background staining. Note that 
apoptotic nuclei can nevertheless be distinguished from background 
(e.g. panels G, I).

Competing interests
The authors declare that they have no competing interests.

Authors’ contributions
AP performed experiments, analyzed the data and wrote the paper, DG, JS, 
FR and GO performed experiments and analyzed the data, JS supervised 
experiments and analyzed the data, AG drafted the paper, MK identified the 
atl mutant, and GB designed the study and wrote the paper. All authors read 
and approved the final manuscript.
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