

# Breakdown of abdominal patterning in the *Tribolium Krüppel* mutant *jaws*

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## Summary

During *Drosophila* segmentation, gap genes function as short-range gradients that determine the boundaries of pair-rule stripes. A classical example is *Drosophila Krüppel* (*Dm'Kr*) which is expressed in the middle of the syncytial blastoderm embryo. Patterning defects in *Dm'Kr* mutants are centred symmetrically around its bell-shaped expression profile. We have analysed the role of *Krüppel* in the short-germ beetle *Tribolium castaneum* where the pair-rule stripes corresponding to the 10 abdominal segments arise during growth stages subsequent to the blastoderm. We show that the previously described mutation *jaws* is an amorphic *Tc'Kr* allele. Pair-rule gene expression in the blastoderm is affected neither in the amorphic mutant nor in *Tc'Kr* RNAi embryos. Only during subsequent growth of the germ band does pair-rule patterning become disrupted. However, only segments arising posterior to the *Tc'Kr* expression domain are affected, i.e. the deletion profile is

asymmetric relative to the expression domain. Moreover, stripe formation does not recover in posterior abdominal segments, i.e. the *Tc'Kr<sup>jaws</sup>* phenotype does not constitute a gap in segment formation but results from a breakdown of segmentation past the 5th *eve* stripe. Alteration of pair-rule gene expression in *Tc'Kr<sup>jaws</sup>* mutants does not suggest a direct role of *Tc'Kr* in defining specific stripe boundaries as in *Drosophila*. Together, these findings show that the segmentation function of *Krüppel* in this short-germ insect is fundamentally different from its role in the long-germ embryo of *Drosophila*. The role of *Tc'Kr* in Hox gene regulation, however, is in better accordance to the *Drosophila* paradigm.

Key words: *Krüppel*, *Giant*, *Even-skipped*, *Dfd*, *Scr*, *Antp*, *Ubx*, Short germ, Long germ, segmentation, Gap gene, Abdomen, *Jaws*, *Tribolium castaneum*, *Drosophila*, Evolution, Parental RNAi

## Introduction

Anteroposterior patterning in *Drosophila* is controlled by regulatory elements that measure the local concentrations of transcription factors and convert them into new expression profiles. In several steps, this machinery translates flat initial gradients spanning most of the egg length into expression domains of increasing detail and precision. At least for the formation of interacting gradients at the maternal and gap gene level, transcription factor diffusion is thought to be essential, which suggests that the *Drosophila* segmentation machinery can work only in a system unimpeded by cell walls, i.e. in a syncytial blastoderm.

How is anteroposterior patterning accomplished in fully cellularized organisms? Somitogenesis in vertebrates has been shown to rely on temporal regulation for the generation of repeating units along the anteroposterior axis, based on a segmentation clock involving components of the *Notch* signalling pathway (Pourquie, 2001). A segmentation clock involving the *Notch* system appears to function in basal arthropods, i.e. spiders (Schoppmeier and Damen, 2005;

Stollewerk et al., 2003) and a clock mechanism may function in centipedes as well (Chipman et al., 2004). Also in these taxa, as in many insects including *Tribolium*, the majority of segments arise by posterior addition of cells to a growing germ band, similar to vertebrate embryos. In contrast to vertebrates, many orthologs of *Drosophila* pair-rule and segment-polarity genes are expressed in stripes also in these short-germ arthropods (Chipman et al., 2004; Damen et al., 2000; Patel et al., 1994; Sommer and Tautz, 1993). It has been suggested, therefore, that the segmentation clock is an ancient mechanism to pattern posteriorly growing embryos, and that pair-rule and segment-polarity genes originally served to transmit the primary clock signal to the growing and differentiating segments (Tautz, 2004). In the evolutionary line leading to *Drosophila*, the regulation of stripe genes then may have come under the control of spatial regulation provided by those genes that, in *Drosophila*, represent the upper levels of the segmentation hierarchy, i.e. gap genes and maternal genes (Peel and Akam, 2003).

In the short-germ beetle *Tribolium*, the embryo elongates by

posterior growth similar to spider and myriapod embryos. However, the *Notch* pathway appears not to be involved in anteroposterior patterning in this insect (Tautz, 2004). Pair-rule genes are expressed and function in double-segmental units in *Tribolium* (Brown and Denell, 1996; Maderspacher et al., 1998), and an analysis of the *Tc'hairy* regulatory region provided evidence for stripe-specific regulation (Eckert et al., 2004). In addition to pair-rule genes, homologues of gap genes are also expressed during germ-band growth in *Tribolium*, and in other short-germ insects (Bucher and Klingler, 2004; Liu and Kaufman, 2004a; Liu and Kaufman, 2004b; Mito et al., 2005; Patel et al., 2001; Schröder et al., 2000; Sommer and Tautz, 1993; Wolff et al., 1995). Functional studies using RNAi in these species have led to diverse interpretations of how similar the role of these short germ gap genes are compared with *Drosophila* gap genes.

One problem with RNAi studies is that the true null phenotype of the genes investigated remains unknown. Unlike many other evo-devo systems, in *Tribolium*, developmental genes can be identified and analysed through the isolation of embryonic lethal mutants. Albeit more laborious, the mutagenesis approach has the potential of providing more defined, and less variable, lack of function situations. In addition, this classical genetics approach allows us to identify short-germ-specific genes that have been lost in long-germ dipteran species, the sequence of which evolves very fast, or which in *Drosophila* are not involved in segmentation. Screens for embryonic lethal genes identified several putative gap and pair-rule mutations (Maderspacher et al., 1998; Sulston and Anderson, 1996). Most of these phenotypes differ substantially from those of known *Drosophila* mutants. In order to determine if any of the segmentation genes already molecularly identified in *Tribolium* is affected in one of these mutants, we tested putative gap gene mutations for linkage to gap gene orthologues.

In this paper, we identify the previously identified *Tribolium* mutant *jaws* (Sulston and Anderson, 1996) as an amorphic *Krüppel* mutant, and provide the first detailed analysis of a gap gene null phenotype in a short-germ embryo. This amorphic *Tc'Kr* phenotype, as well as weaker phenotypes generated by RNAi, clearly differ from those of *Drosophila* *Krüppel* (*Dm'Kr*) mutations (Wieschaus et al., 1984), suggesting a principally different role for this gap gene orthologue in the short-germ embryo of *Tribolium*.

## Materials and methods

### Cloning, RACE and sequence analysis

The *Tc'Kr*-coding region was initially amplified applying 5' and 3' RACE (Gene-Racer, Invitrogen). RACE primers were designed using the *Tc'Kr* zinc-finger fragment available (GenBank Accession Number L01616) (Sommer and Tautz, 1992), using 5' primer GGCCACCTGGACGAACTGC and 3' primer GCAGTTCGTCCA-GGTGGCC. To complete the sequence, additional RACE reactions were performed using 5'RACE primer CAGCCGCATGTGGGTCT-TGAGGTG and 3'RACE primers GTTGATTGGTATGCGAGTTCCTCC and TTGCGGACGGCGAGATGAGGGACC. The presumed ATG is at position 147-149 of the cDNA. One intron between position 182 and 183 of the cDNA separates amino acids 11 and 12. In the *Tribolium* genome sequence (as of March 2005), the *Tc'Kr* cds is covered by the contigs 6872 (bp 1-180) and 5054 (bp 181-1259), whereas the 3' untranslated region is contained in contigs 5054 and

1669. The Accession Number for the *Tc'Kr* cDNA is AF236856. The predicted peptide sequence is given in Fig. S1 in the supplementary material.

### Mapping of *jaws* relative to *Tc'Kr*

Sequence polymorphisms in candidate genes were identified by amplifying and sequencing non-coding fragments (5' UTR, 3' UTR or intronic DNA) from adult beetles of GA-1, SB and Tiw-1 wild-type strains. Identified sequence polymorphisms could either be scored directly as PCR fragments on an agarose gel or were converted into RLFPs. For *Tc'Kr*, a polymorphism in the 3'UTR was identified. This polymorphism was amplified as a 205 bp fragment by primer sequences ACGACTTGGCGGTTAATG and TACGAAAGTAGG-CACACAAC. In Tiw-1, but not in SB, this fragment is cleaved by *AseI* into subfragments of 141 and 64 bp (Fig. 2) that were visualized on a 2.5% NuSieve Agarose gel (Cambrex Bio Science). For mapping, DNA was isolated from single beetles that had been identified as mutant carriers by scoring the offspring from single matings for presence of mutant larvae. Detailed protocols concerning our general mapping strategy, and DNA extraction from beetles and larvae can be provided on request.

### Parental RNAi

Parental RNAi was performed as described (Bucher et al., 2002). As template for in vitro transcription, PCR-products with T7 sequences at both ends were amplified from cDNA plasmids or genomic *Tribolium* DNA. For injection, dsRNA was used at a concentration of 1-4 µg/µl.

### Harvest of mutant *jaws* embryos

In order to obtain *jaws* mutant embryos in large numbers, offspring from 40 identified *jaws*/+ parents was sexed as pupae, and virgin females were crossed to their fathers. One-sixth of the eggs produced by this father/daughter population will be homozygous for the mutants. Similarly, to obtain the *Tc'gt/jaws* 'double mutant' phenotype, *Tc'gt* dsRNA was injected into the same offspring pupae and eclosed females then were crossed to identified *jaws* carrier males.

### Confocal images

First instar larvae were cleared in lactic acid/10% ethanol overnight at 60°C. After washing with lactic acid, cuticles were mounted on a slide under a cover-slip that was supported with rubber gum. This allowed manual positioning to a ventral-up orientation. Cuticular autofluorescence in the 520 to 660 nm range was detected on a Leica confocal microscope by excitation at 488 nm and maximum projection images were generated from image stacks.

### Expression analysis

Single (Tautz and Pfeifle, 1989) and double label (Prpic et al., 2001) whole-mount in situ hybridisations were carried out as described. *Tc'Kr*-RNAi germband stage embryos are particularly fragile and were manually devitellinized on double sticky tape: 12- to 18-hour-old embryos were transferred to ethanol and then gently attached to a double-sided sticky tape. After replacing ethanol with water, the vitelline membrane tightly adheres to the tape and embryos can be manually devitellinized using diminutive insect needles. In order to avoid RNA degradation, devitellinized embryos were promptly transferred to methanol and stored at -20°C.

## Results

### Reanalysis of *Tc'Kr* expression

A fragment from the *Tc'Kr*-coding sequence had been identified previously and used for expression analysis (Sommer and Tautz, 1993). We extended the molecular analysis of *Tc'Kr* in order to identify non-coding sequences carrying

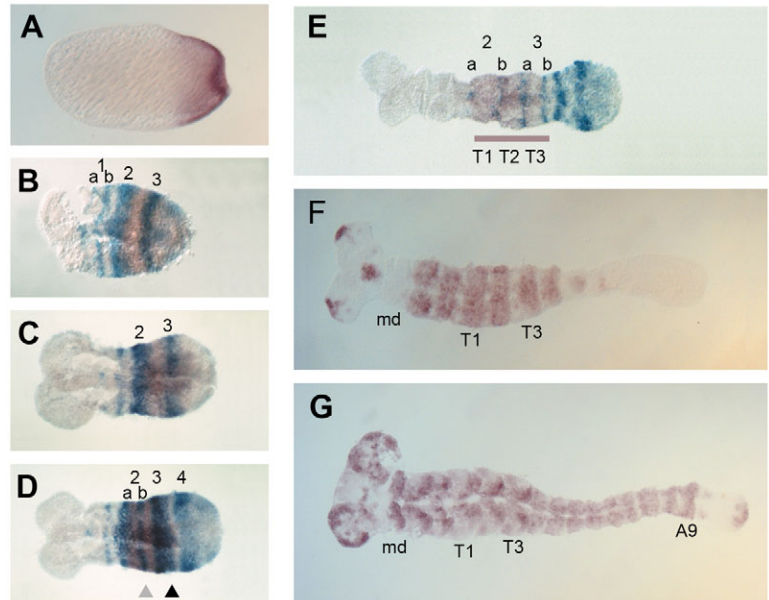
polymorphisms useful for mapping, and to obtain a complete cDNA suitable for more comprehensive RNAi knock down as well as more sensitive in situ hybridization (see Materials and methods).

While *Dm'Kr* is expressed in the centre of the blastoderm embryo, in the *Tribolium* blastoderm this domain appears at the posterior pole (Fig. 1A). Relative to the segment primordia, however, this position is roughly conserved, as *Tribolium* is a short-germ embryo (Sommer and Tautz, 1993). We used *Tc'even-skipped* (*Tc'eve*) as an additional marker to map the position of the gap domain precisely (Fig. 1B-E). During germ rudiment formation, *Tc'Kr* remains expressed in a broad central domain. In early germ band stages (Fig. 1B,C), the anterior border of *Tc'Kr* lies within the 2nd stripe of *Tc'eve* ('eve2'). When *eve2* splits into segmental stripes, *eve2a* and *eve2b* [corresponding to labial and first thoracic segments (Patel et al., 1994)], the *Tc'Kr* domain abuts the posterior border of *eve2a* (1D). At this time, *Tc'Kr* also fades from the growth zone and a posterior border forms just anterior to the *eve4* stripe as it arises near the growth zone (Fig. 1C,D). As the segmental stripes *eve3a* and *eve3b* form, the posterior boundary of the *Tc'Kr* gap domain coincides with *eve3b* (Fig. 1E). Accordingly, in germ band embryos the *Tc'Kr* gap domain overlaps very precisely the three thoracic segments – which is more anterior than in *Drosophila*, where the *Tc'Kr* domain is centered over the primordia of segments T2 to A2 (Myasnikova et al., 2001).

During later stages of development, the gap-domain of *Tc'Kr* disappears. A second phase of rather homogenous expression emerges in all segments, excluding recently formed segments close to the growth zone (Fig. 1F,G). This signal later intensifies in the appendages and extends to anterior and posterior gut primordia. Additionally, a dynamically changing pattern of *Tc'Kr* expression is observed in the head region. These late expression aspects probably relate to possible functions during mesoderm development, gut development and neurogenesis as described for other *Krüppel* orthologues (Gaul et al., 1987; Liu and Kaufman, 2004a).

### *jaws* is closely linked to the *Tc'Kr* locus

The *jaws* mutation was originally induced in a GA-1 background (Sulston and Anderson, 1996). Preliminary experiments suggested that this mutation had been induced in a chromosome carrying a RFLP polymorphism in the *Tc'Kr* gene ('Tiw-1 specific polymorphism') that differs from the corresponding sequence in the SB wild-type strain ('SB specific polymorphism'). In order to test for close linkage between *jaws* and *Tc'Kr*, we made use of the fact that a *jaws* mutant strain had been kept in our laboratory by recurrent outcrossing to SB females for over six generations [for stock-keeping of embryonic lethal mutations see Berghammer et al. (Berghammer et al., 1999)]. Therefore, in our stock collection, most of the genome in the *jaws* strain must have been replaced by SB-specific alleles. Only loci very close to *jaws* are likely to still be represented by GA-1-specific alleles, because presence of the *jaws* mutant had been selected for in every



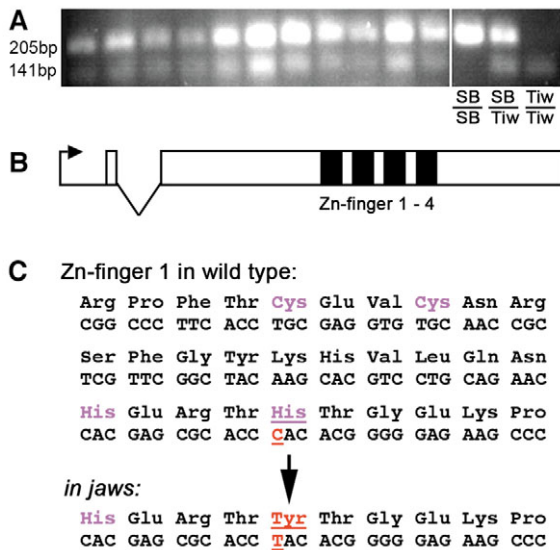
**Fig. 1.** Segmental position of the *Tc'Kr* expression domain. Wild-type embryos stained for *Tc'Kr* (brown, A-G) and *Tc'eve* (blue, B-E). As in the remaining figures, all embryos are oriented anterior towards the left. The gap domain of *Tc'Kr* arises at the posterior pole of the blastoderm (A, posterior pit stage). In early germ rudiments (B,C) *Tc'Kr* expression extends from the 2nd *Tc'eve* stripe to beyond the 3rd *Tc'eve* stripe. When the 2nd (D) and 3rd (E) *Tc'eve* stripes split into segmental stripes 2a, 2b, 3a and 3b, the *Tc'Kr* gap domain (brown bar) is demarcated by 2a and 3b, i.e. it covers the three thoracic segments (E). At later stages, secondary expression domains arise in the head and eventually in all segments of the extended germ band (F,G). md mandible; T1 to T3, thoracic segments 1 to 3; A9, 9th abdominal segment.

generation. When we scored 80 adult beetles from our stock collection that carried one copy of the *jaws* mutation, we found that every one of these animals was heterozygous for both polymorphisms at the *Tc'Kr* locus (Fig. 2A). This shows very close linkage between the *jaws* mutation and the *Tc'Kr* gene and suggested that *jaws* is a mutation in the *Tc'Kr* gene. As a control, we also tested 20 of these animals for polymorphisms in the *Tc'eve* gene and found, as expected for a locus not linked to *jaws*, that they all were homozygous for a SB-specific *Tc'eve* polymorphism.

### The first zinc finger of *Tc'Kr* is altered in *jaws*

To confirm the identity of the *jaws* and *Tc'Kr* loci, we isolated genomic DNA from homozygous *jaws*-mutant larvae and PCR-amplified three fragments from the *Tc'Kr* locus that cover both exons. Sequence comparison with control amplicates from the SB and GA-1 strains revealed an amino acid replacement in the *Tc'Kr*-coding sequence of mutant animals. This transition changes the second histidine of the first zinc finger to a tyrosine (Fig. 2B,C). As the Cys-Cys-His-His Zn-finger motive is essential for the correct structure of the DNA-binding domain, a missense mutation in such a key amino acid is likely to inactivate the *Tc'Kr* gene. In this respect, the *jaws* mutation, now to be termed *Tc'Kr<sup>jaws</sup>*, is similar to an amorphic *Krüppel* mutation identified in *Drosophila*: in the *Dm'Kr<sup>9</sup>* allele, one of the crucial Zn-finger cysteines is converted to serine, completely abolishing *Dm'Kr* function (Redemann et





**Fig. 2.** *jaws* is a mutation in the first Zn finger of *Tc'Kr*. (A) After repeated outcrossing of the *jaws* mutation (induced in a GA-1 background) with the SB wild-type strain, all adult beetles that were *jaws* mutant carriers are also heterozygous for polymorphisms at the *Tc'Kr* locus, indicating close linkage between the mutation and the polymorphism. The polymorphism 'SB' represents a *Tc'Kr* 3'UTR sequence specific to the SB strain that can be detected as a 205 bp RFLP band. The polymorphism 'Tiw' is specific to the *Tc'Kr* copy of the *jaws*-carrying chromosome and results in a 141 bp RFLP (this polymorphism originally had been identified in the Tiw-1 wild-type strain). Depicted is an agarose gel with 11 samples (of 80 total). As controls, *AseI*-digested DNA amplified from a SB animal (SB/SB), a Tiw-1 animal (Tiw/Tiw) and an animal that resulted from a cross between Tiw-1 and SB parents (SB/Tiw) are shown. (B) *Tc'Kr* gene structure; the four Zn fingers are shown as black boxes. (C) Sequence of the first Zn finger of *Tc'Kr*. In the *jaws* mutant, the 2nd histidine is altered to a tyrosine.

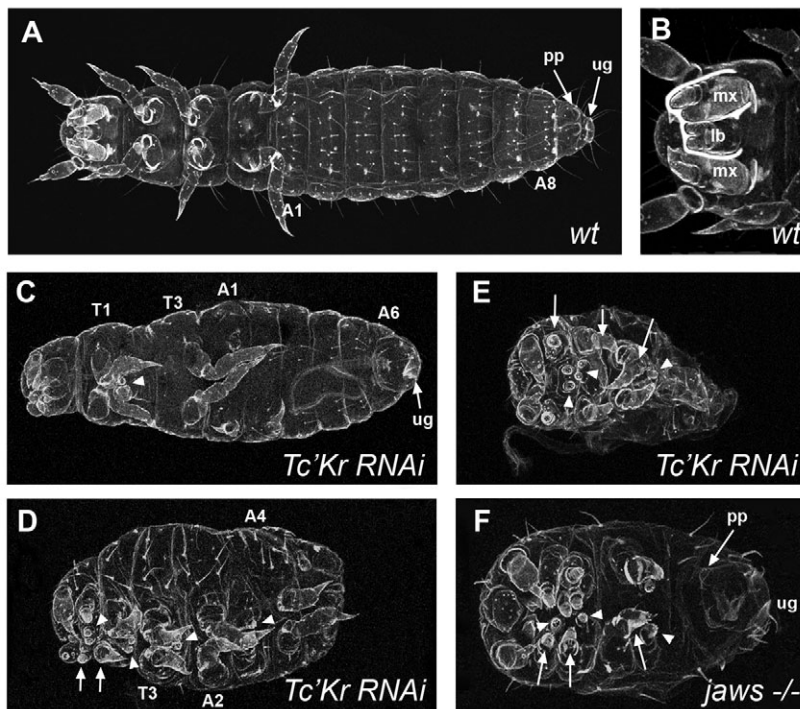
al., 1988). Below, we provide additional evidence that *Tc'Kr<sup>jaws</sup>* indeed does fully inactivate the *Tc'Kr* locus.

### Phenotypic series caused by *Tc'Kr* loss or depletion

The identification of *jaws* as a *Tc'Kr* allele is also supported by RNAi evidence. Injecting dsRNA representing *Tc'Kr* cDNA or genomic sequences (see Materials and methods) resulted in various homeotic and segmentation phenotypes (Fig. 3C-E) which – in some injection experiments – included phenotypes very similar or identical to *Tc'Kr<sup>jaws</sup>*.

In *Tc'Kr<sup>jaws</sup>* embryos (Fig. 3F), the head is differentiated as in wild type. The next four segments (thoracic and 1st abdominal) develop gnathal structures such that the regular maxillary (mx) and labial (lb) segments are followed by two additional pairs of maxillary and labial segments. Including the normally developed mandible (md), this results in a total of seven gnathal segments (md-mx-lb-mx-lb-mx-lb). Posteriorly, these gnathal segments are followed by one segment of abdominal morphology, and the posterior end of the embryo is formed by terminal structures similar to wild type, including urogomphi and pygopodes, the derivatives of the 9th and 10th abdominal segments. Hence, the total of gnathal, thoracic and abdominal segments in *Tc'Kr<sup>jaws</sup>* embryos is 10 compared with 16 in wild type, i.e. six segments are deleted, while four segments are homeotically transformed (Sulston and Anderson, 1996). This phenotype differs from that of strong *Dm'Kr* mutants where the thoracic and the first four abdominal segments are deleted and no homeotic transformations are evident in differentiated mutant larvae. The ectopic maxillary structures of *Tc'Kr<sup>jaws</sup>* mutant embryos deviate somewhat from normal maxillae in that they lack endites (the mala) and sometimes possess distal claws rather than the sensory structures characteristic of maxillary palps (this is especially the case for the most posterior pair of maxillae). In addition, the ectopic labia (as well as the endogenous labium) are abnormal in that they usually do not fuse ventrally. Weaker phenotypes obtained by RNAi support the interpretation that these imperfect gnathal segments in fact are of mixed gnathal and thoracic character (Fig. 3D,E).

In intermediate strength and weak RNAi phenotypes (Fig. 3C,D), more abdominal segments remain and the transformation of thoracic segments towards gnathal fate is less



**Fig. 3.** Phenotypic series for *Tc'Kr*: ventral views of first instar larvae (confocal image projections based on cuticle autofluorescence). (A,B) Wild-type first instar larva (A). (B) Enlarged view of the ventral head, with left maxilla and labium outlined in white. (C-E) *Tc'Kr* RNAi embryos of increasing phenotypic strength. (F) *Tc'Kr<sup>jaws</sup>* mutant embryo. Appendages resembling maxilla are labelled with arrows, those resembling labial palps with arrowheads. In some embryos, the urogomphi and pygopodes, corresponding to the 9th and 10th abdominal segments, respectively, are also indicated. For detailed phenotypic description see Results. The embryo in F is at a higher magnification than those in A-E. mx maxilla; lb labium; T1 to T3, thoracic segments 1 to 3; A1 to A8, abdominal segments 1 to 8; pp, pygopod; ug, urogomphi.

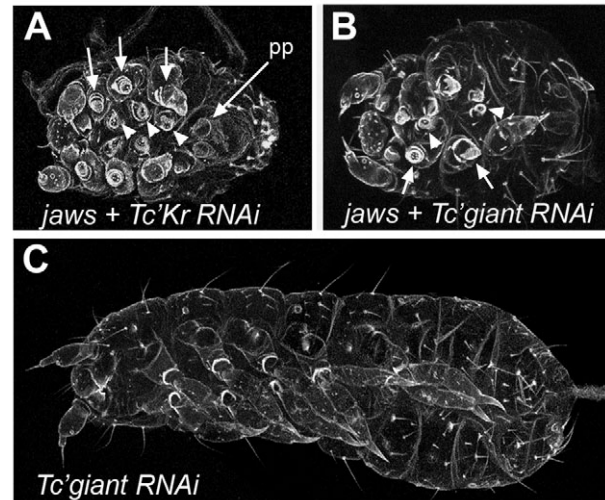
pronounced. Frequently, the first and third thoracic segments still differentiate legs in embryos whose second thoracic segment already is transformed into labium. This indicates that higher levels of *Tc'Kr* activity are required for inhibiting labial fate than for repressing maxillary fates. In addition, the additionally present abdominal segments in these embryos usually display homeotic transformations towards a more anterior, i.e. thoracic or gnathal, fate (Fig. 3D). In these abdominal segments, there is also a tendency for alternating maxillary and labial fates, and small irregular appendages can sometimes be observed. In conclusion, the weak *Tc'Kr* RNAi phenotypes also differ significantly from those of weak *Dm'Kr* mutants, displaying additional homeotic transformations of abdominal segments towards more anterior fates.

### Is *Tc'Kr<sup>jaws</sup>* a null-allele?

The sequence alteration in *Tc'Kr<sup>jaws</sup>* is no definite proof that *Tc'Kr* activity is entirely abolished in mutant embryos. In *Drosophila*, a mutation is regarded as 'amorphic' if the mutant allele (mut) in trans over a deficiency (Df) for the locus displays the same phenotype as in the homozygous condition. The rationale behind this test is that if some gene activity remains in the mutation, then mut/Df embryos would possess only half as much activity for the gene in question than mut/mut embryos, and therefore should display a discernibly stronger phenotype (Muller, 1932). No deletion for the *Tc'Kr* locus is available in *Tribolium*. However, by combining the mutation with RNAi knockdown allows for a similar test: if the phenotype of larvae homozygous for *Tc'Kr<sup>jaws</sup>* is the same as that of larvae with additional RNAi induced depletion of the mutant *Tc'Kr* transcript, then we can conclude that *Tc'Kr<sup>jaws</sup>* represents the strongest possible loss-of-function phenotype. We performed *Tc'Kr* RNAi knock-down in a *Tc'Kr<sup>jaws</sup>*-mutant background using a moderate concentration of dsRNA such that we could distinguish mutant and RNAi embryos. Beside intermediate strength *Tc'Kr* RNAi phenocopies, this experiment also yielded *Tc'Kr<sup>jaws</sup>* larvae. These homozygously mutant larvae did not show a stronger phenotype than *Tc'Kr<sup>jaws</sup>* larvae (Fig. 3F; Fig. 4A). From this experiment, we conclude that *Tc'Kr<sup>jaws</sup>* is an amorphic *Tc'Kr* allele that is functionally equivalent to a null mutation. In order to understand the homeotic and segmentation phenotypes of this mutant, we analysed the expression of potential target genes in *Tc'Kr<sup>jaws</sup>*.

### The homeotic effect of *Tc'Kr<sup>jaws</sup>* is epistatic over that of *Tc'gt* RNAi

Interestingly, RNAi knock-down of the *Tc'giant* gene (*Tc'gt*) leads to a homeotic phenotype opposite to that caused by *Tc'Kr* inactivation. In *Tc'gt* RNAi embryos, the maxillary and labial segments are transformed towards thoracic identity (Bucher and Klingler, 2004) (see also Fig. 4C). We wondered which of these transformations would prevail in a 'double-mutant' situation. To this end, we performed *Tc'gt* RNAi knock-down in a *Tc'Kr<sup>jaws</sup>* mutant background (see Materials and methods). In this experiment, we obtained *Tc'gt* knock-down phenotypes in the majority of embryos while a fraction corresponding to *Tc'Kr<sup>jaws</sup>* homozygous animals showed a phenotype very similar to that of *Tc'Kr<sup>jaws</sup>* alone (Fig. 4B). They differed only from the normal *Tc'Kr<sup>jaws</sup>* phenotype in that they lacked one or two additional segments. This is to be expected, because in *Tc'gt* RNAi embryos, thoracic and abdominal segments can be



**Fig. 4.** *Tc'Kr<sup>jaws</sup>* is an amorphic mutation, and the homeotic transformations in *jaws* are epistatic over those in *Tc'gt* RNAi embryos. (A) *Tc'Kr<sup>jaws</sup>* mutant embryo in which the defective *Tc'Kr* mRNA additionally has been depleted by RNAi. The phenotype of this embryo is not stronger than in *Tc'Kr<sup>jaws</sup>* alone, indicating that the *Tc'Kr<sup>jaws</sup>* mutant itself fully inactivates the gene. (B) *Tc'Kr<sup>jaws</sup>* mutant embryo in which the *Tc'gt* gene has been inactivated by RNAi. The normally present maxilla and labium are fully developed, followed by one ectopic maxillary and one labial segment. Thus, the homeotic effect of *Tc'Kr* inactivation is epistatic over that observed in *Tc'gt* RNAi larvae. Because *Tc'gt* also results in segmentation defects, the 2nd ectopic pair of maxillary and labial segments are incomplete or missing. Arrows indicate maxillary structures; arrowheads labial appendages; pp, pygopode. (C) Phenotype of a *Tc'gt* RNAi larva. In addition to abdominal segmentation defects, maxillary and labial segments are transformed into thoracic segments.

deleted that are not affected in *Tc'Kr<sup>jaws</sup>*, i.e. the segmentation phenotypes of these experimental larvae corresponds to a superposition of *Tc'gt* RNAi and *Tc'Kr<sup>jaws</sup>*. However, the homeotic transformations caused by *Tc'Kr<sup>jaws</sup>* are clearly epistatic over those produced by *Tc'gt* RNAi knock-down. This suggests that the homeotic transformation of gnathal segments into thorax in *Tc'gt* RNAi embryos is an indirect effect (see Discussion).

### Expression of homeotic genes in *Tc'Kr<sup>jaws</sup>* and *Tc'gt* RNAi embryos

The striking homeotic transformations in *Tc'Kr<sup>jaws</sup>* larvae could either be due to misregulation of homeotic genes, or could indicate a direct role of *Tc'Kr* in specifying segmental fates. Previous work already has shown that the Hox gene *proboscipedia* (*Tc'pb*) is ectopically expressed in *Tc'Kr<sup>jaws</sup>* mutant embryos (Sulston and Anderson, 1998). However, *Tc'pb* becomes active relatively late during development, and only in the maxillary and labial palps, not in complete segments. Thus, we asked how the expression of Hox genes early active in the maxillary, labial and thoracic segments would relate to the *Tc'Kr<sup>jaws</sup>* phenotype.

The *Deformed* (*Tc'Dfd*) gene is expressed in the mandibular and maxillary segments (Brown et al., 1999). In *Tc'Kr<sup>jaws</sup>* embryos, two strong and one weak additional *Tc'Dfd* domains



are observed that are separated from each other by gaps approximately one segment wide (Fig. 5A-D). The two strongly expressing ectopic domains correspond to the first and third thoracic segments that, in *Tc'Kr<sup>jaw</sup>* mutant larvae, develop maxillary characteristics. The *Sex combs reduced* (*Tc'Scr*) gene is active in the ectoderm of the second parasegment in *Tribolium* (Curtis et al., 2001), which largely corresponds to the labial segment (Fig. 5E-G; *Tc'Scr* expression is also present in the mesoderm of additional segments). In *Tc'Kr<sup>jaw</sup>* embryos (Fig. 5H-J), ectopic activity of *Tc'Scr* is present in the primordia that correspond to the second thoracic and first abdominal segments of wild-type animals, i.e. in those segments that differentiate labial palps in mutant larvae. Therefore, the gnathal Hox genes *Tc'Dfd* and *Tc'Scr* are active in complementary double-segmental frames in *Tc'Kr<sup>jaw</sup>* mutant embryos, which is consistent with the phenotype of differentiated mutant larvae.

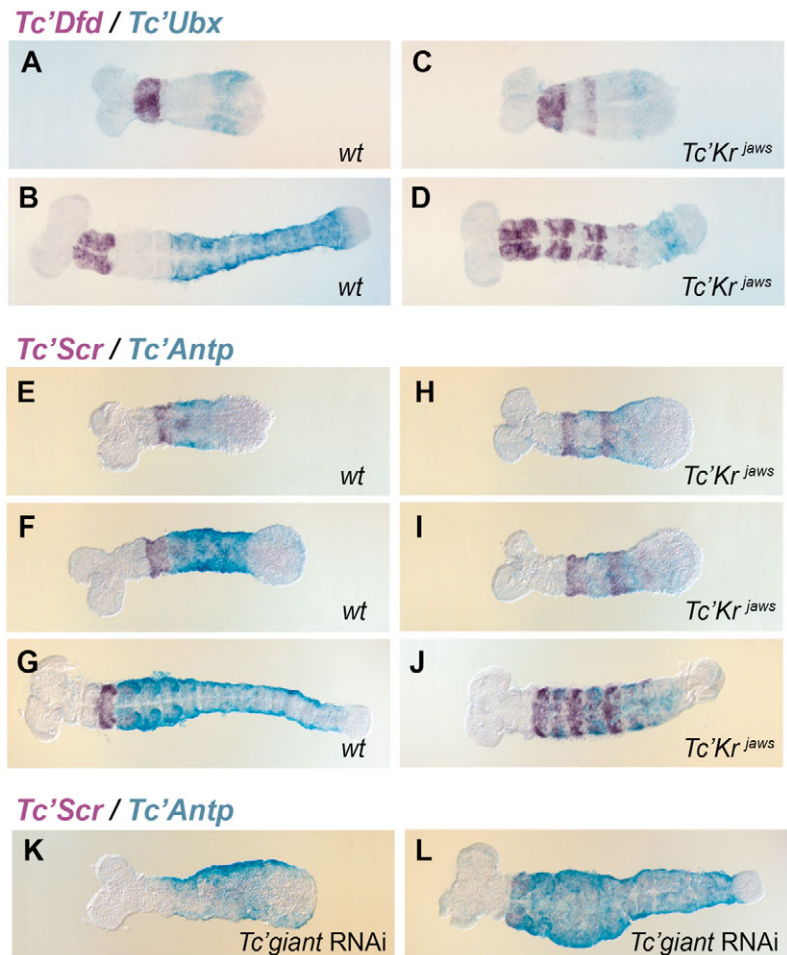
Concomitant with the expanded expression of gnathal Hox genes, the *Tc'Ubx* gene, the anterior expression boundary of which lies in the thorax, is shifted posteriorly in *Tc'Kr<sup>jaw</sup>* mutants (Fig. 5C,D). This may explain, at least in part, why in weak *Tc'Kr* RNAi phenocopies anterior abdominal segments are transformed towards thorax (Lewis et al., 2000). The anterior boundary of *Tc'Antp*, however, is similar as in wild type (Fig. 5H-J). This is consistent with our interpretation that the ectopic maxillary structures in *Tc'Kr<sup>jaw</sup>* are incompletely transformed and retain some thoracic characteristics. We also investigated the expression of *Tc'Scr* and *Tc'Antp* in *Tc'gt* RNAi embryos (Fig. 5K,L) as they display homeotic transformations opposite to those in *Tc'Kr<sup>jaw</sup>* mutants. Indeed we find that *Tc'Antp* expands towards anterior by two segments whereas *Tc'Scr* expression is largely abolished in these embryos (which lack maxillary and labial differentiation).

Together, these data show that the *Tc'Kr<sup>jaw</sup>* homeotic phenotype can be explained by defective Hox gene regulation, and they suggest inhibition of *Tc'Dfd* and *Tc'Scr* by *Tc'Kr*, whereas *Tc'Ubx* positively depends on Kr activity. In addition, the double-segmental appearance of ectopic gnathal Hox expression domains suggests that the Hox genes *Tc'Dfd* and *Tc'Scr* also are under strict pair-rule control.

### Function of *Tc'Kr* in regulating segmentation genes

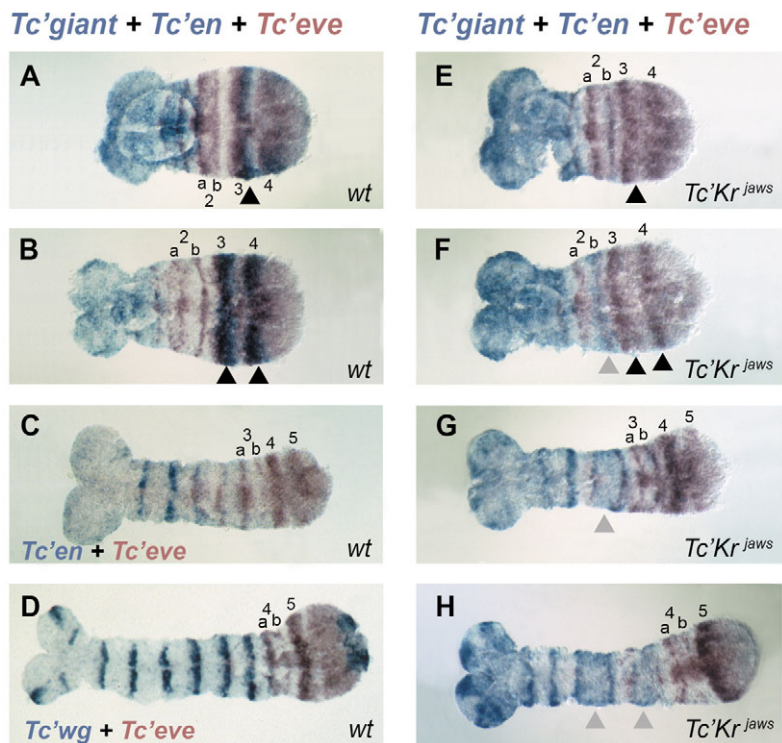
Previous work has already revealed that the pattern of the segment-polarity gene *engrailed* (*Tc'en*) and the pair-rule genes *Tc'eve* and *Tc'runt* are altered in *Tc'Kr<sup>jaw</sup>* (Sulston and Anderson, 1998). We repeated and extended this work in order to relate the defects observed with what we now know about the spatial expression of the gene that is inactivated in this mutant.

We first attempted to identify which stripes of *Tc'eve* exactly



**Fig. 5.** Hox gene expression in wild-type, *Tc'Kr<sup>jaw</sup>* and *Tc'gt* RNAi embryos. (A-D) *Tc'Dfd* (purple) and *Tc'Ubx* (blue) in situ double staining in stage-matched wild-type (A,B) and *Tc'Kr<sup>jaw</sup>* mutant (C,D) embryos. In the mutant (D), the anterior boundary of the *Tc'Ubx* domain recedes towards the posterior. *Tc'Dfd* is expressed in three strong and one weak domain of double segmental periodicity. The two ectopic domains with stronger expression correspond to the ectopic maxillary segments in *Tc'Kr<sup>jaw</sup>*. (E-J) *Tc'Scr* (purple) and *Tc'Antp* (blue) staining of wild-type (E-G) and *Tc'Kr<sup>jaw</sup>* mutant (H-J) embryos. *Tc'Scr* is also strongly expressed in two ectopic domains with double-segmental periodicity, corresponding to the two ectopic labial segments (J). In contrast to *Tc'Ubx*, *Tc'Antp* expression is not shifted in *Tc'Kr<sup>jaw</sup>* germbands (H-J). (K,L) *Tc'Scr* (brown) and *Tc'Antp* (blue) in *Tc'gt* RNAi embryos. *Tc'Antp* expands towards anterior by two segments, which correlates with the fact that the maxillary and labial segments attain thoracic appearance in *Tc'gt* RNAi embryos. High-level expression of *Tc'Scr* in the labial segment is repressed in these embryos. Weak *Tc'Scr* expression in the maxillary segment of older embryos (L) probably corresponds to the weak expression seen in the prothoracic segment in wild type (G).

are affected by the *Tc'Kr<sup>jaw</sup>* mutation. To distinguish pair-rule stripes arising in the growing germ band, we performed double staining with segment polarity genes, and to identify *Tc'Kr<sup>jaw</sup>* mutant embryos at stages before morphological differences to wild types become evident, *Tc'gt* was included as an additional marker in some experiments (in *Tc'Kr<sup>jaw</sup>* embryos, the posterior domain of *Tc'gt* is absent, while an additional stripe of expression appears; A.C., unpublished). We find that



**Fig. 6.** The first five *Tc'eve* stripes are formed normally in *Tc'Kr<sup>jaws</sup>*. Ventral views of stage-matched wild-type (A-D) and *Tc'Kr<sup>jaws</sup>* (E-H) embryos. The youngest wild-type (A,B) and all *Tc'Kr<sup>jaws</sup>* (E-H) embryos have been triply labelled for *Tc'eve* (brown), *Tc'en* and *Tc'gt* mRNA (both blue); in the older wild-type embryos (C,D), only *Tc'eve* (brown) and *Tc'en* or *Tc'wg* (blue) are labelled. *Tc'gt* expression was used to identify mutant embryos: the posterior *giant* domain(s) present in wild-type germbands (A,B) are missing in *Tc'Kr<sup>jaws</sup>* (E,F); black arrowheads indicate the position of these domains. In older *Tc'Kr<sup>jaws</sup>* embryos, two ectopic *Tc'gt* domains appear in the first and third thoracic segments (grey arrowheads in F-H). In *Tc'Kr<sup>jaws</sup>* mutant germbands (E,F), the primary *eve* stripes 3 and 4, and the secondary segmental stripes of *eve2* form in a similar way to wild type (A,B) (stripes *eve1a* and *eve1b* are partially obscured by *en* stripes and the maxillary *Tc'gt* expression). Later on, *eve3* splits into segmental stripes, while *eve5* becomes detectable (C,G). The segmental stripes 4a and 4b remain imperfect in *Tc'Kr<sup>jaws</sup>*, while *eve5* forms a sharp anterior boundary (H).

the first three *Tc'eve* stripes arise and split into segmental stripes in *Tc'Kr<sup>jaws</sup>* mutant embryos exactly as in wild type (Fig. 6A-C,E-G). In addition, a stripe of *eve4* is formed in the growth zone as a distinct band with sharp boundaries. Although this stripe arises just posterior to the *Tc'Kr* domain, *Tc'Kr* apparently has no role in defining its anterior boundary. However, segmentation defects become evident at subsequent stages: while *eve4* does split into segmental stripes 4a and 4b, these segmental stripes (particularly *eve4b*) appear somewhat irregular. The anterior boundary of *eve5* also forms perfectly in *Tc'Kr<sup>jaws</sup>* (Fig. 6G,H), very similar to wild type. However, this stripe never progresses into segmental stripes 5a and 5b (Fig. 7A-C); instead, its expression becomes irregular in shape and then decreases in strength and fades away (Fig. 7G-I). The defects of *Tc'eve* patterning observed in *Tc'Kr<sup>jaws</sup>* differ strongly from the situation in *Drosophila* gap gene mutants, where negative regulation of stripe-specific elements results in widened stripes.

We did not observe re-establishment of *Tc'eve* stripes at later stages, i.e. posterior of a gap-like deletion zone. At the time when *eve7* and *eve8* form in wild-type embryos (Fig. 7D-F), *Tc'eve* expression in *Tc'Kr<sup>jaws</sup>* already has ceased (Fig. 7J-L). The pattern of the segmental marker *Tc'wg* confirms that the gnathal and thoracic segments form normally in the *Tc'Kr<sup>jaws</sup>* mutant (Fig. 7G-L) (Sulston and Anderson, 1996). In contrast to this earlier analysis, however, in the pattern of a segment-polarity gene we also find no evidence for re-establishment of stripe formation. Using the dynamic *Tc'wg* head expression as marker for developmental time, we find that after six or seven normally formed gnathal and thoracic *Tc'wg* stripes, the pattern becomes irregular in *Tc'Kr<sup>jaws</sup>* embryos. Several more posterior *Tc'wg* stripes arise but are fragmentary, weakly expressed or only present on one side of the embryo (Fig. 7I-K). As with *Tc'eve* stripes, no additional stripes re-emerge at

later stages in *Tc'wg*. Instead, the initially irregular and fragmentary stripes reorganize themselves later on into a more orderly pattern, such that older embryos can display a very regular pattern of typically 10 gnathal, thoracic and abdominal *Tc'wg* stripes, corresponding to the number of segments differentiated in mutant larvae (Fig. 7L). Such pattern repair phenomena also are observed in other *Tribolium* segmentation mutants and RNAi embryos (Bucher and Klingler, 2004; Maderspacher et al., 1998).

## Discussion

Our description of *Tc'Kr* phenotypes represents the first definite functional analysis of an insect gap gene orthologue outside the diptera. This was possible by combining the complementary advantages of RNAi and a chemically induced mutation (Sulston and Anderson, 1996) that appears to represent a null situation given that its phenotype is not further enhanced by parental RNAi (Fig. 4A).

### Regulation of homeotic genes by *Tc'Kr*

The most obvious difference between the phenotypes of *Krüppel* in *Tribolium* and *Drosophila* are the homeotic transformations in *Tc'Kr<sup>jaws</sup>* and *Tc'Kr* RNAi larvae that are not evident in *Dm'Kr* mutants. Such transformations are not entirely unexpected given that in *Drosophila* the expression boundaries of Hox genes are also set by gap genes, including *Dm'Kr*. However, in *Drosophila* gap mutants all segments that would be transformed because of misregulation of homeotic genes usually also suffer segmentation defects and fail to develop. By contrast, *Tribolium* segment primordia anterior of, and within, the *Krüppel* expression domain do differentiate, such that homeotic transformations can manifest themselves in the differentiated larva.



The expression of homeotic genes in *Tc'Kr<sup>jaws</sup>* embryos is consistent with the morphological transformations observed (Fig. 3F, Fig. 5). Our results with *Tc'Dfd*, *Tc'Scr*, *Tc'Antp* and *Tc'Ubx* confirm and extend earlier findings for *Tc'pb* and *Tc'UBX/Tc'ABD-A* expression (Sulston and Anderson, 1998). Notably, the complementary double-segmental expression of *Dfd* and *Scr* in *Tc'Kr<sup>jaws</sup>* embryos explains the phenotype of alternating maxillary and labial segments. As summarized in Fig. 8, these expression patterns indicate that

the posterior limit of *Tc'Dfd* and *Tc'Scr* domains is set through inhibition by *Tc'Kr*. In this respect, *Tc'Kr* fulfils a function similar to *Drosophila* gap genes.

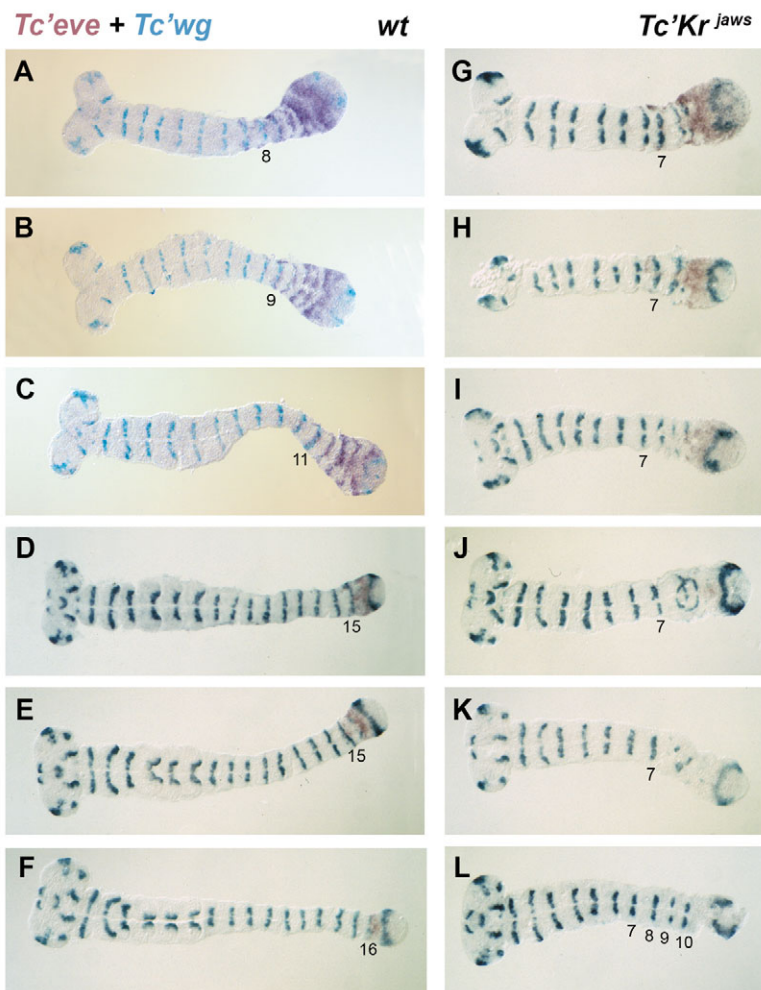
The homeotic phenotype of *Tc'gt* RNAi embryos (Bucher and Klingler, 2004) could suggest a similar function in Hox regulation for *Tc'gt*. Indeed we find *Tc'Antp* anteriorly expanded and gnathal Hox genes (*Tc'Scr*) repressed in *Tc'gt* RNAi embryos, consistent with the expansion of thoracic fates found in differentiated *Tc'gt* RNAi larvae. These transformations are just opposite to those of *Tc'Kr<sup>jaws</sup>* larvae. Interestingly, in embryos that lack *Tc'Kr* and at the same time have reduced *Tc'gt* activity, the homeotic effect of *Tc'Kr<sup>jaws</sup>* clearly is epistatic (Fig. 4B). This shows that the ectopic *Tc'gt* stripes in the *Tc'Kr* mutant do not contribute to the *Tc'Kr* phenotype. However, this experiment suggests that the homeotic transformation of gnathal segments into thorax in *Tc'gt* RNAi embryos is indeed an indirect effect and comes about through misregulation of *Tc'Kr* in these embryos. This interpretation is supported by our finding that the *Tc'Kr* expression domain expands anteriorly in *Tc'gt* RNAi embryos (A.C.C. and M.K., unpublished). Evidently, it is expansion of *Tc'Kr* that results in repression of gnathal Hox genes in maxilla and labium of *Tc'gt* RNAi embryos, not loss of gnathal Hox gene activation. Similarly, expansion of *Tc'Antp* in *Tc'gt* RNAi larvae could be due to activation by anteriorly expanded *Tc'Kr*. However, as *Antp* is not significantly reduced in *Tc'Kr<sup>jaws</sup>*, it seems more likely that *Tc'gt* acts directly to define the anterior boundary of the *Tc'Antp* domain (stippled arrow in Fig. 8).

In addition to gap gene input, *Drosophila* Hox genes also receive input from pair-rule genes. The near-pair-rule pattern of *Tc'Dfd* and *Tc'Scr* in *Tc'Kr<sup>jaws</sup>* embryos reveals an important role of pair-rule genes also in defining *Tribolium* Hox domain boundaries. It seems likely that regulation of *Tc'Dfd* and *Tc'Scr* by pair-rule genes is responsible for the precision of their expression boundaries in wild-type *Tribolium* embryos, while input from gap genes defines the broad region where a particular Hox gene can become active (Fig. 8).

### ***Tc'Kr* does not function as a canonical gap gene during segmentation**

In *Drosophila*, *Krüppel* is expressed in a bell-shaped profile centered over the primordia of segments T2 to A3 (Gaul and Jäckle, 1987; Myasnikova et al., 2001). In the *Tribolium* blastoderm, only one such gradient is present as the *Tc'Kr* domain covers the posterior pole (Sommer and Tautz, 1993). When the germ rudiment has formed, the *Tc'Kr* domain retracts from the posterior end and forms a distinct domain overlapping the three thoracic segment primordia (Fig. 1). At this stage, therefore, the *Tc'Kr* domain covers more anterior segment primordia (and more anterior pair-rule stripes) than does its *Drosophila* counterpart.

Both boundaries of the *Dm'Kr* expression domain



**Fig. 7.** Segment formation in *Tc'Kr<sup>jaws</sup>* is not re-established during later stages of development. Embryos are doubly labelled for *Tc'Kr* (brown) and *Tc'wg* RNA (blue). Numbers relate to canonical *Tc'wg* stripes, i.e. starting with the mandibular segment. (A-F) Wild-type embryos of increasing developmental age, stage-matched to *Tc'Kr<sup>jaws</sup>* embryos (G-L; staging is based on the dynamic *Tc'wg* head expression). Compared with wild type (A-C), *Tc'Kr* expression becomes irregular and prematurely fades in *Tc'Kr<sup>jaws</sup>* embryos (G-I). Initially, only the seven anteriormost *wg* stripes (md to A1) are properly formed in *Tc'Kr<sup>jaws</sup>*. Posterior to A1, several irregular or fragmentary *wg* stripes form (G-K), which then reorganize such that in more mature embryos (L) normal *Tc'wg* stripes 8-10 are frequently observed, consistent with the 10 differentiated segments in *Tc'Kr<sup>jaws</sup>* larvae. No new *Tc'Kr* or *Tc'wg* activity is evident at later stages, when in wild-type embryos the last *Tc'wg* stripes form (15 and 16 in our notation, which correspond to abdominal segments A9 and A10) (D-F). A non-segmental *Tc'wg* domain is present in the growth zone throughout development. This domain represents terminal fates and eventually becomes part of the proctodeum.





regulate Hox genes, but not to directly regulate pair-rule genes (G. Bucher, PhD thesis, Ludwig-Maximilians-Universität, München, 2002) (Liu and Kaufman, 2004a). In *Tribolium*, however, some blastoderm pair-rule stripes are affected by gap gene orthologues other than *Kr* (A.C.C. and M.K., in preparation), and there is good evidence for stripe-specific elements driving at least the first two *Tc'hairy* stripes (Eckert et al., 2004).

Our results for *Tc'Kr* deviate from those obtained for *Krüppel* in *Oncopeltus fasciatus* (Liu and Kaufman, 2004b). In this short-germ insect, knock-down of *Kr* also results in mis-expression of Hox genes, although the effects are more limited as only one ectopic *Of'Dfd* domain is detected. Interestingly, expression of *Of'en* in such embryos seems to indicate a clear gap phenotype, i.e. perfect segmental stripes reappear posterior to a region of segmental disruption. Incomplete inactivation of *Of'Kr* could be responsible for this difference; we note, however, that weak *Tc'Kr* RNAi situations do not result in obvious gap phenotypes (see Fig. S2 in the supplementary material). Rather, in such embryos the segmentation process simply breaks down somewhat later than in *Tc'Kr<sup>jaw6</sup>*, i.e. the additional segments present in weak *Tc'Kr* RNAi embryos appear to represent anterior abdominal rather than posterior (post-gap) abdominal segments. *Oncopeltus* is sometimes denoted an intermediate-germ insect, because a few more segments are formed already in the blastoderm than, for example, in *Tribolium*. It will be interesting to see if the 'next posterior' gap gene in *Oncopeltus* will also display a 'gap' phenotype, and to find out whether pair-rule gene expression in *Of'Kr* RNAi embryos indicates a role in the regulation of specific stripes boundaries.

If our interpretation is correct that *Tc'Kr* does not directly specify pair-rule stripes during abdomen formation, what could its function be in this process? All abdominal cells derive from progenitors that expressed *Tc'Kr* at the blastoderm stage. Therefore, regulation of later-acting abdominal expression domains (e.g. the posterior domains of *Tc'gt* and *Tc'hb*), may depend on *Tc'Kr* activity in the blastoderm, rather than on its activity at later stages when its domain forms a distinct posterior boundary. In this way, the long-ranging action of *Tc'Kr* could be explained through a temporal persistence rather than a spatial diffusion mechanism. Later acting genes depending on *Tc'Kr* activity then could have a role in regulating pair-rule genes.

However, the discovery that a segmentation clock appears to pattern lower arthropods (Chipman et al., 2004; Stollewerk et al., 2003) raises the issue of when in the evolutionary line leading to the diptera this clock was replaced by the hierarchical mode of *Drosophila* segmentation. Although at present no evidence is available for a segmentation clock functioning in *Tribolium*, it is conceivable that a modified clock is installed at the posterior end of the blastoderm embryo. *Tc'Kr* could have a role in initiation of this clock machinery. Alternatively, it could be required for its continued function. Because the number of abdominal segments is constant in insects, some type of counting principle would be required to stop the clock once the last segment has formed. Such a counting mechanism could be provided, for example, by a series of abdominal 'gap gene' activities (including the posterior domains of *Tc'gt* and *Tc'hb*), the last of which would shut off the clock. In this view, abdominal 'gap genes' would

have a permissive rather than a positionally instructive function during abdominal segmentation of short germ embryos.

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### Supplementary material

Supplementary material for this article is available at <http://dev.biologists.org/cgi/content/full/132/24/5353/DC1>

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