Development 137, 835-844 (2010) doi:10.1242/dev.045948

giant is a bona fide gap gene in the intermediate germband insect, *Oncopeltus fasciatus*

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SUMMARY

Drosophila undergoes a form of development termed long germ segmentation, where all segments are specified nearly simultaneously so that by the blastoderm stage, the entire body plan has been determined. This mode of segmentation is evolutionarily derived. Most insects undergo short or intermediate germ segmentation, where only anterior segments are specified early, and posterior segments are sequentially specified during germband elongation. These embryological differences imply that anterior and posterior segments might rely upon different molecular mechanisms. In *Drosophila*, embryos mutant for *giant* show a gap in the anterior as well fusions of several abdominal segments. In *Tribolium*, a short germ beetle, *giant* is required for segmental identity, but not formation, in gnathal segments and also for segmentation of the entire abdomen. This raises the possibility that *giant* might not act as a gap gene in short and intermediate germ insects. *Oncopeltus fasciatus* is an intermediate germ insect that is an outgroup to the clade containing *Drosophila* and *Tribolium*. We cloned the *Oncopeltus* homolog of *giant* and determined its expression and function during segmentation. We find that *Oncopeltus giant* is a canonical gap gene in the maxillary and labial segments and also plays a gap-like role in the first four abdominal segments. Our results suggest that *giant* was a bona fide gap gene in the ancestor of these insects with this role being lost in the lineage leading towards *Tribolium*. This highlights the conservation of anterior patterning and evolutionary plasticity of the genetic regulation controlling posterior segmentation, even in short and intermediate germ insects.

KEY WORDS: *Giant*, *Oncopeltus fasciatus*, Segmentation, Evolution, Short germ, Intermediate germ

INTRODUCTION

The fruit fly, *Drosophila melanogaster*, undergoes a form of embryogenesis called long germ segmentation where all segments of the entire body are specified during the blastoderm stage so that by gastrulation, the blastoderm fate map represents the entire future body plan in approximately natural proportions (Lohs-Schardin et al., 1979; Hartenstein et al., 1985). Moreover, the entire body is patterned while the embryo is still a syncytium, allowing diffusion of transcription factors between nuclei.

Most insects undergo what is termed short or intermediate germ segmentation, believed to be the ancestral mode of development (Patel et al., 1994). In short and intermediate germ insects, the process of segmentation occurs in a biphasic manner. During the early blastoderm phase, only anterior segments, typically the head and thorax, are specified prior to gastrulation. The remaining posterior regions arise during germband elongation, when the posterior germband lengthens and the remaining posterior segments are specified sequentially, in an anterior to posterior progression. Furthermore, in contrast to the situation in *Drosophila*, cellularization of the blastoderm occurs early in many short germ insects, often as soon as the nuclei reach the periphery and prior to the specification of posterior segments. Thus, free diffusion of transcription factors cannot play as major a role in patterning in short germ insects as they do in *Drosophila*. (As the short and intermediate forms of segmentation are conceptually so similar, for convenience sake we will henceforth refer to both the short and intermediate forms as 'short'.) The differences between these two modes of segmentation at the embryological level imply fundamental differences in patterning at the molecular level.

During *Drosophila* development, the gap class of segmentation genes is responsible for subdividing the embryo into broad regions, each of which will eventually encompass several contiguous segments. Because one essential difference between short and long germ segmentation is how the early blastoderm is initially subdivided (all the body regions in the case of long-germ insects, but only the anterior-most regions in the case of short germ insects), gap genes have been a focus of comparison between long and short germ insects, and understanding how gap genes function is crucial for understanding the evolution of insect segmentation.

The *Drosophila* gap gene *giant* (*Dm'gt*) encodes a basic leucine zipper transcription factor and is required for proper formation of two embryonic domains (Petschek et al., 1987; Petschek and Mahowald, 1990; Kraut and Levine, 1991). In mutant *Drosophila* embryos, the third *engrailed* stripe is missing, resulting in a deletion of part of the labial segment. In addition, anterior compartments of abdominal segments 5-7 are deleted, resulting in a fusion of these segments. *giant* has also been examined in another long germ insect, the parasitic wasp *Nasonia vitripennis*. In *Nasonia*, RNAi knockdown of *giant* causes deletions of the head and thorax and fusion of the sixth and seventh abdominal segments. In contrast to the long germ insects described above, in the short germ beetle *Tribolium castaneum*, RNAi- or morpholino-depletion of *giant* (*Tc'gt*) results in the formation of the maxillary and labial segments, but these segments are homeotically transformed towards thoracic identity. Thus in *Tribolium*, the primary role of anterior *giant* is not to control the formation of segments, but rather to specify their identity. Additionally, *Tc'gt* depletions disrupt segmentation throughout the thorax and abdomen and, in strong cases, up to nine segments are deleted (Bucher and Klingler, 2004). These differences

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Accepted 23 December 2009

seen in the role of *giant* between the three insect species raise the question of the ancestral role of *giant*, particularly its role in other short germ insects.

The milkweed bug, *Oncopeltus fasciatus*, is a Hemipteran intermediate germ insect and thus is a phylogenetic outgroup to the clade that includes the fruit fly, wasp and beetle, making it an ideal comparison to those insects (Savard et al., 2006). Using RNAi to deplete *giant* function, we investigated the role of *Oncopeltus giant* during segmentation. Our analysis shows that in *Oncopeltus*, *giant* plays a role that is more similar to its role in the long germ insects *Drosophila* and *Nasonia*, and fundamentally differs from its role in the short germ *Tribolium*.

MATERIALS AND METHODS

Cloning

Total RNA was isolated from mixed stage *Oncopeltus fasciatus* embryos using Trizol reagent (GibcoBRL/Life Technologies). This RNA was used to synthesize cDNA using the FirstChoice RLM-RACE kit (Ambion). We designed degenerate PCR primers to conserved *giant* and *sloppy-paired* sequences from other arthropod species and used these primers to isolate short fragments of these genes from *Oncopeltus*. For *giant*, a small amount of the initial degenerate PCR was used as a template for a subsequent nested PCR, whereas this nested reaction was not necessary for *sloppy-paired*. We then used the sequence of short fragments to design gene-specific primers for 5′ and 3′ RACE. PCR with the gene-specific primers and the appropriate anchor primer supplied in the FirstChoice RLM-RACE kit yielded longer fragments that were ligated into the pDrive plasmid vector (Qiagen). Multiple independent PCRs were performed and multiple clones were sequenced in order to minimize PCR and sequencing artifacts. The *Oncopeltus giant* and *sloppy-paired* sequences were submitted to GenBank with accession numbers GU123166 and GU123167, respectively.

The degenerate primers used in the primary PCR for *giant* were CCNTTYAARGCNTAYCCNAARGA and TCNARRAANGCNGCNC-KDATNGC, whereas the primers used for the nested PCR were AARGAYGARGCNTAYTGGGARMG and ATNGCDATYTCRTCY-TCYTTNGC. The 5′ *giant* gene-specific primer was ATGGGTTGT-ATGGTGTGATGGTGCC and the 3′ gene-specific primer was AGGCGAAAGAACAATGAGGCAGCCAAG. The degenerate primers used in the PCR for *sloppy-paired* were AAYGCNYTNATHATG-ATGGCNATH and CATCCARTARTTNCCYTTNCCNGG. The 3′ gene-specific RACE primer was AGGCAGAGTCCCGAGAAGAGACTGAC.

Embryo fixation and in situ hybridization

Embryo fixation and probe synthesis were carried out as previously reported (Liu and Kaufman, 2004b). In situ hybridization was carried out as previously reported (Liu and Kaufman, 2005a) but with the following changes: the in situ hybridization buffer used contained 1% SDS, which we found to reduce non-specific background staining; and we also used the BM-Purple alkaline phosphatase substrate (Roche) instead of BCIP/INT during the chromogenic reaction. 5′ and 3′ fragments were used for probe synthesis and gave identical results (Fig. 1), so they were used interchangeably in our analysis.

RNAi

Synthesis of double-stranded RNA for RNAi was carried out as previously reported (Liu and Kaufman, 2005a). Injections for parental RNAi were performed as previously reported (Liu and Kaufman, 2004b).

Microscopy and image capture

Images of hatchling and blastoderm stage embryos were captured using a Zeiss Lumar stereomicroscope with attached Spot Flex camera. Owing to the depth of these samples, a single focal plane was not sufficient to capture all of the detail of the entire embryo. Therefore, several focal planes were taken of each sample and combined into a single composite image using Helicon Focus. Images of germband stage embryos were captured with a Zeiss Axiophot compound microscope with attached Spot Flex camera. When samples were too long to fit within a single frame, multiple images were tiled to produce a single composite image using Adobe Photoshop.

RESULTS

We took a RT-PCR-based approach to isolate the *Oncopeltus* homolog of *giant* (*Of'gt*). First, degenerate primers were designed to conserved regions of *giant* homologs from other arthropod species. PCR using these primers on *Oncopeltus* embryonic cDNA allowed us to recover a short fragment corresponding to the basic region of the predicted protein. We performed multiple independent PCRs using differing cycling conditions and were only able to isolate a single version of *Oncopeltus giant*. If another paralog of *giant* exists in *Oncopeltus*, it is likely to be highly diverged or only minimally expressed. This short initial fragment allowed us to design exact primers for 5′ and 3′ RACE and subsequently isolate both 5′ and 3′ fragments of the gene. We sequenced multiple clones from independent PCRs in order to minimize artifacts. *Of'gt* is predicted to encode a 210 amino acid protein. Regions used for synthesizing in situ hybridization probes and double-stranded RNA for RNAi are shown (Fig. 1).

Oncopeltus giant expression

We synthesized probes targeting either the 5′ or 3′ ends of the *Of'gt* transcript and performed in situ hybridization on fixed staged *Oncopeltus* embryos. The earliest detectable pattern arose during the early blastoderm stage, at around 28 hours after egg lay (AEL). Initially, *Of'gt* transcript accumulates weakly throughout the blastoderm but quickly retreats from the posterior pole, as well as slightly from the anterior pole of the blastoderm, remaining as an anterior-central band of expression (Fig. 2A). By 32 hours AEL, the anterior-central domain splits into two broad stripes of expression and a new region of expression arises de novo in the posterior of the blastoderm (Fig. 2B). At 36 hours AEL, as the germband begins invagination, the two anterior bands of expression further refine and the posterior domain expands to cover the posterior fourth of the blastoderm (Fig. 2C). Double in situs are technically very difficult at the blastoderm stage and we were unable to perform double in situs of *Of'gt* with *Of'eve* or *Of'en*. Therefore, to determine the approximate segmental register of *Of'gt* expression at the blastoderm stage, images of milkweed bug embryos separately stained for *Of'gt* and *Of'en* were juxtaposed (Fig. 2C). The anterior domains roughly span the pregnathal head and mandibular segment and the maxillary and labial segments, whereas the posterior domain covers the second and third thoracic segments. Similar to other segmentation genes that have been examined in *Oncopeltus*, *Of'gt* also shows dorsal-ventral differences in expression during the blastoderm stage, with low or no expression in the dorsal part of the blastoderm, which are probably fated to become extra-embryonic tissues (Liu and Kaufman, 2004b; Liu and Kaufman, 2004a; Liu and Kaufman, 2005a) (data not shown).

During the germband stage, new domains of *Of'gt* expression appear while the earlier blastoderm domains fade. Just after germband invagination, and before abdominal segments become specified, *Of'gt* transcript can be seen in the head, the mandibular segment and the thorax, which are continuations of the domains seen during the late blastoderm stage (Fig. 2D). As the germband elongates, the mandibular and head expression resolves into a punctate pattern similar to the brain expression pattern seen in *Tribolium* (Bucher and Klingler, 2004). Meanwhile, the posterior boundary of the thoracic *Of'gt* domain expands into the second abdominal segment, while the remaining thoracic domain adopts a striped pattern and fades (Fig. 2E,F).

By the time two abdominal *engrailed* stripes have been formed, a new domain of *Of'gt* arises as a broad stripe in the anterior portion of the posterior unpatterned zone (the posterior unpatterned zone, often called the 'growth zone', is the posterior portion of the

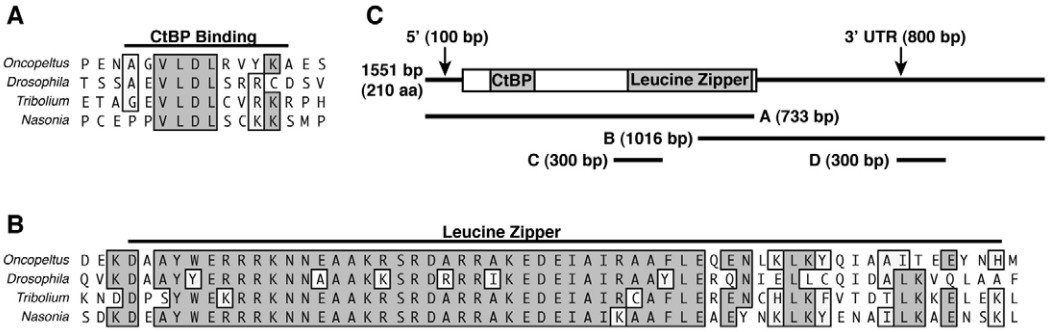

Fig. 1. *Oncopeltus fasciatus giant*. (**A**) Alignment of the binding domain of the co-repressor, CtBP, from the *Oncopeltus*, *Drosophila*, *Tribolium* and *Nasonia* orthologs of Giant. (**B**) The conserved leucine zipper domain of Giant from the four insects. (**C**) Cartoon diagram showing approximate locations of the CtBP binding and leucine zipper domains of *Oncopeltus* Giant. Boxed region is the predicted open reading frame, predicted to encode a 210 amino acid protein. Single lines show approximate relative lengths of the 5′ (100 bp) and 3′ (800 bp) untranslated regions. Lines below the cartoon diagram show approximate lengths and positions of the fragments used to synthesize in situ probes and double-stranded RNA. Fragment A was used for probe synthesis, fragment B to synthesize both probe and double-stranded RNA, fragment C was used only to synthesize double-stranded RNA, and fragment D, located in the 3′ untranslated region, was also only used to synthesize double-stranded RNA. Figure not to scale.

elongating germband that has not yet undergone segmental patterning). This domain is not a continuation of the thoracic expression, but rather represents de novo expression (Fig. 2E,F). *Oncopeltus even-skipped* (*Of'eve*) is expressed continuously throughout the posterior unpatterned zone and in stripes just anterior to it, as segments mature. This expression is maintained throughout the rest of germband elongation, fading about the time the ninth abdominal *Of'en* stripe is formed. Double in situ hybridizations with *Of'eve* show that *Of'gt* expression is maintained in the same relative position within the posterior unpatterned zone and seems to be expressed both between newly formed *Of'eve* stripes as well as co-expressed with the earliest forming *Of'eve* stripes (Fig. 2J-M). We can ascribe no function to this stripe of expression in the posterior unpatterned zone as *Of'gt* RNAi embryos show only localized abdominal defects, rather than defects throughout the entire abdomen (see below), and this stripe has no clear counterpart in either the short germ *Tribolium* or in the long germ insects *Drosophila* and *Nasonia*.

Oncopeltus giant RNAi

To determine the function of *Oncopeltus giant* during segmentation, we used parental RNAi to deplete *Of'gt* activity (Liu and Kaufman, 2004b). We synthesized double-stranded RNA (dsRNA) targeting different non-overlapping regions of the *Of'gt* transcript and injected these dsRNAs both singly and in combination and at different concentrations into several virgin adult females (Fig. 1). These females were raised individually, mated to males and their progeny scored for developmental defects (Table 1). All of the tested dsRNA fragments yielded qualitatively identical phenotypes when injected singly or in combination and we used these embryos interchangeably throughout the remainder of our analysis. *Of'gt* seemed very sensitive to RNAi as almost all of the females injected with 2 μg/μl of dsRNA produced progeny with the strongest phenotype and it was only with injection of 0.2 μg/μl of dsRNA that we saw any embryos with milder phenotypes (Table 1).

We analyzed hatchling stage embryos as well as fixed staged *Of'gt* RNAi embryos. Affected embryos showed segmental deletions in two body regions, and we will describe the defects in these two regions in turn.

Oncopeltus giant function in the gnathal segments

In severely affected hatching stage embryos, it is apparent that at least the labium is deleted but other head structures such as the eyes, antenna and labrum all appear normal (Fig. 3). The mandibular and maxillary segments normally give rise to stylets, which evert during development to eventually lie inside grooves of the labium. Because these stylets are difficult to see if they are not everted, our inability to locate them in RNAi hatchlings does not necessarily indicate a loss of those segments. Therefore, in order to determine if the mandibular and maxillary segments were also deleted, we fixed late germband stage embryos and stained them with a probe targeting *Oncopeltus engrailed* (*Of'en*). The germband stage embryos showed a clear deletion of two gnathal

Table 1. *Of'gt* RNAi results

Fragment injected	[dsRNA] (μg/μl)	Strong (%)	Weak (%)	NS (%)	WT (%)	Total scored
B	2	173 (79)	2 (1)	41 (19)	3 (1)	219
B	4	49 (66)	4 (5)	21 (28)	0 (0)	74
B	0.2	99 (58)	51 (30)	15 (9)	6 (4)	171
C	2	18 (34)	0 (0)	18 (34)	17 (32)	53
D	2	64 (88)	0 (0)	4 (5)	5 (7)	73
C+D	2	51 (59)	18 (21)	11 (13)	6 (7)	86
Totals		454 (67)	75 (11)	110 (16)	37 (5)	676

Fragment injected, the fragments of double-stranded RNA referred to in Fig. 1; Strong, embryos with the full phenotype: loss of the labium and a fully defective anterior abdomen; Weak, embryos where the labium was missing but the anterior abdomen did not display the full defect; NS, non-specific phenotype, including embryos that did not develop; WT, embryos and hatchlings that appeared normal.

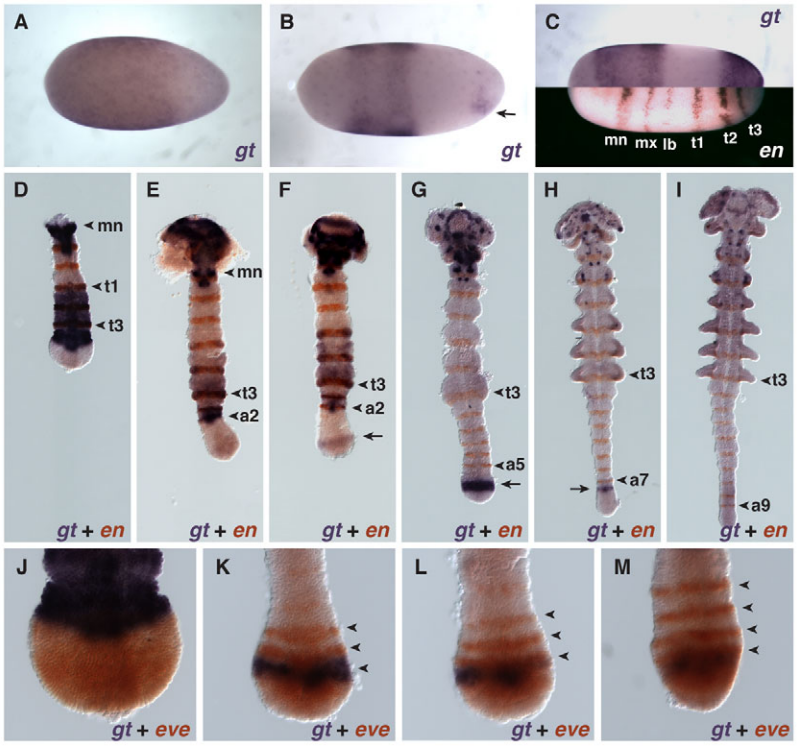

Fig. 2. Wild-type *Of'gt* RNA expression. (**A**) Blastoderm stage embryo at 28-32 hours after egg lay (AEL). *Of'gt* transcript is in the anterior two-thirds of the blastoderm. (**B**) By 32 hours AEL, the anterior band of *Of'gt* has now split into two broad stripes. A new domain of expression has now arisen (arrow). (**C**) Blastoderm stage embryos around 36 hours AEL stained for *Of'gt* (top) and *Of'en* (bottom). By juxtaposing the images of the two embryos, it is possible to gain a rough estimate of the segmental register of *Of'gt* expression. The anterior bands span the head and maxillary segment and the maxillary and labial segments. The posterior domain has now expanded to cover the second and third thoracic segments. (**D-I**) Germband stage embryos stained for *Of'gt* (purple) and *Of'en* (orange). (D) An early germband stage embryo, during germband invagination. The head tissues would have still been on the outside of the yolk ball but have been removed in this preparation. Anterior *Of'gt* expression can be seen in the mandibular, maxillary and part of the labial segments. The posterior expression seen during the blastoderm stage continues as strong thoracic expression. (E) By the time two abdominal *Of'gt* stripes have appeared, gnathal expression has faded, leaving strong head expression and punctate dots in the mandibular and maxillary segments. *Of'gt* expression has also expanded into the second abdominal segment. (F) Anterior expression remains the same but thoracic and anterior abdominal expression forms weak stripes. A stripe of *Of'gt* arises in the growth zone (arrow). (G) By the time five abdominal stripes of *Of'en* have formed, thoracic and anterior abdominal expression has faded. The growth zone stripe is still strong (arrow). (H) An embryo where the seventh abdominal *Of'en* stripe has formed. The growth zone stripe remains. (I) By the time the ninth abdominal *Of'en* stripe has formed, the growth zone stripe of *Of'gt* has faded. (**J-M**) Germband stage embryos doubly stained for *Of'gt* transcript (purple) and *Of'eve* transcript (orange). These images show that the growth zone stripe of *Of'gt* is both co-expressed with the *Of'eve* stripes (M), as well as in the interstripe region (K,L). Panel J shows an early germband stage embryo before the appearance of *Of'eve* stripes. Arrowheads mark the position of *Of'eve* stripes. a2, second abdominal segment; a5, fifth abdominal segment; a7, seventh abdominal segment; a9, ninth abdominal segment; mn, mandibular segment; mx, maxillary segment; t1, first thoracic segment; t3, third thoracic segment.

segments (Fig. 4A,B). In order to confirm the identity of the remaining segments, germband stage RNAi embryos were stained with probes targeting the homeotic genes *Deformed* (*Of'Dfd*), *Sex combs reduced* (*Of'Scr*) and *proboscipedia* (*Of'pb*) (Fig. 5). In uninjected embryos, *Of'Dfd* is expressed in the mandibular and maxillary appendages, *Of'pb* in the labial appendage and *Of'Scr* in the labial appendage and the mesoderm of the legs. In *Of'gt* RNAi germbands, *Of'Dfd* is expressed in a single pair of appendages, indicating a loss of either the mandibular or the maxillary segment.

Unfortunately, the mandibular and maxillary stylets are indistinguishable at the morphological level at the germband stage, and we do not posses any molecular markers to differentiate between the two. Therefore, there are two possibilities for which segments are deleted: a loss of the mandibular and labial segments or a loss of the maxillary and labial segments. We argue the later case for several reasons. First, in *Of'gt* RNAi blastoderms, contiguous stripes of *Oncopeltus even-skipped* and *Oncopeltus sloppy-paired* are deleted (see below). Second, we find no evidence for a discontinuous defect in our examinations of weakly affected *Of'gt* RNAi germbands or in our examinations of strongly affected germbands at the early and mid-germband stage. Lastly, in *Drosophila*, *Tribolium* and *Nasonia*, loss of *giant* activity causes anterior defects in contiguous segments (although the nature and size of the affected region differs). For these reasons, we conclude that *Of'gt* RNAi causes a contiguous deletion of the maxillary and labial segments.

The deletion of the maxillary and labial segments at the late germband stage suggested three possible mechanisms. First, it could be that the deleted segments failed to be specified at the blastoderm stage. Second, those segments might have been initially specified but were not maintained during later development. Lastly, all segments of the blastoderm might have been correctly formed, but their identities homeotically transformed, resulting in only an apparent gap phenotype. To distinguish between these possibilities,

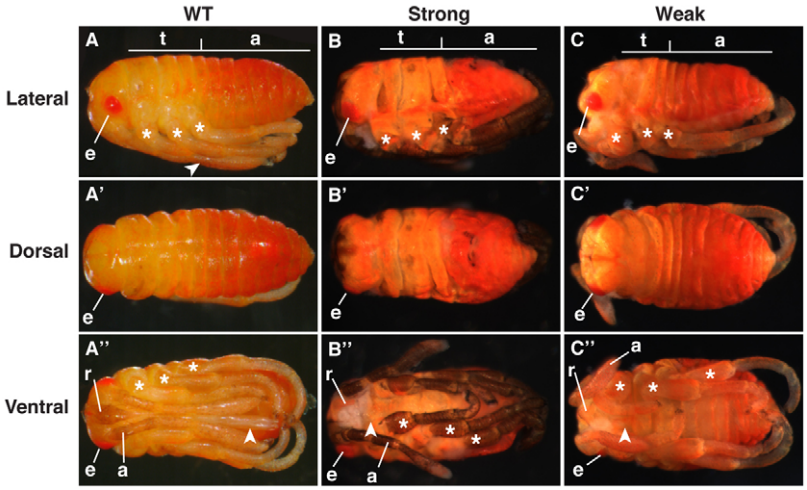

Fig. 3. *Of'gt* RNAi embryos at hatching. (**A-A″**) Uninjected embryo at hatchling stage showing the lateral aspect (A), dorsal aspect (A′) and ventral aspect (A″). The labium is marked with an arrowhead. (**B-B″**) *Of'gt* RNAi embryo with 'strong' phenotype. Note that the abdomen is shortened and the labium is missing (arrowhead). (**C-C″**) *Of'gt* RNAi embryo with 'weak' phenotype. Note that the abdomen is short but less defective than in the strongly affected embryos. Arrowhead marks the loss of the labium. Asterisks mark thoracic legs. a, abdomen; e, eye; r, labrum; t, thorax.

we fixed blastoderm stage embryos and stained them for expression of the homologs of the pair-rule genes *even-skipped* and *sloppy-paired*. We reasoned that if the anterior deletion represented a failure during initial specification, expression of the pair-rule genes would be aberrant.

We therefore examined the expression of a homolog of the primary pair-rule gene *even-skipped* and a homolog of the secondary pair-rule gene, *sloppy-paired*, in *Of'gt* RNAi embryos. In uninjected embryos, *Oncopeltus even-skipped* (*Of'eve*) was expressed in a six-stripe segmental pattern corresponding to the mandibular through third thoracic segments during the late blastoderm stage (Liu and Kaufman, 2005a) (Fig. 6A). In *Of'gt* RNAi embryos, stripes corresponding to the maxillary and labial segments were absent but the remaining *Of'eve* stripes appeared normal (Fig. 6B). We also cloned the *Oncopeltus* homolog of *sloppy-paired* (*Of'slp*) and performed in situ hybridization on blastoderm stage embryos to examine its expression. Like *Of'eve*, *Of'slp* does not have a pair-rule phase of expression, but rather stripes arise in a segmental pattern. By the end of the blastoderm stage, stripes corresponding to the mandibular through third thoracic segments can be seen, in addition to additional stripes in the head (Fig. 6C). *Of'gt* RNAi embryos showed a loss of two stripes with a region of fuzzy expression in their place. The remaining stripes are present and appear normally spaced, although we also detect a loss of some of the head expression (Fig. 6D). That *Of'eve* loses the second and third stripes, whereas *Of'slp* loses the third and fourth stripes, could be because *Of'eve* and *Of'slp* stripes are slightly out of register. Taken together, the loss of the *Of'eve* and *Of'slp* stripes suggest that the anterior gap phenotype in the RNAi embryos is due to a failure in the initial specification of these segments.

Oncopeltus giant function in the anterior abdomen

In addition to the maxillary and labial deletion, depletion of *Of'gt* also caused a gap-like phenotype in the anterior abdomen. Hatchling stage embryos showed defects in the anterior abdomen as well as an overall shortening of the abdomen (Fig. 3A-C″). RNAi embryos fixed at the germband stage and stained for *engrailed* possessed abdomens with only six *engrailed* stripes, indicating a loss of four segments, most likely A1-A4 (Fig. 4A-C). When the activity of the gap gene *hunchback* is depleted in *Oncopeltus* and *Tribolium*, the apparent terminal gap phenotype is actually the combined result of anterior homeotic transformation coupled with compaction of posterior segments (Liu and Kaufman, 2004b; Marques-Souza et al., 2008). In the case of *hunchback*, the effect was detected in examinations of mildly and moderately affected embryos. We found no evidence for a similar mechanism with *Oncopeltus giant* when we examined mildly affected embryos (Fig. 4C). Staining with the abdominal homeotic genes *Ultrabithorax* (*Of'Ubx*), *abdominal-A* (*Of'abd-A*) and *Abdominal-B* (*Of'Abd-B*) were likewise consistent with a loss of the first four abdominal segments (Fig. 5I-N). The phenotypes at the hatchling and germband stages, examinations of intermediate phenotypes and the expression of the Hox genes in RNAi germbands indicate a loss of the anterior four abdominal segments in the *Of'gt* RNAi embryos.

In order to better understand the mechanism causing the RNAi defect in the anterior abdomen, we fixed staged *Of'gt* RNAi embryos that were in the process of specifying the first four abdominal segments and stained them with probes against *engrailed*, *even-skipped* and *sloppy-paired* (*Of'slp*). In normal embryos, the posterior unpatterned zone begins as a fat circular pad of tissue that shrinks as the germband elongates. The newly formed anterior abdomen is narrower than the posterior unpatterned zone and the third thoracic segment. In *Of'gt* RNAi embryos, the putative anterior abdomen appeared wider and more

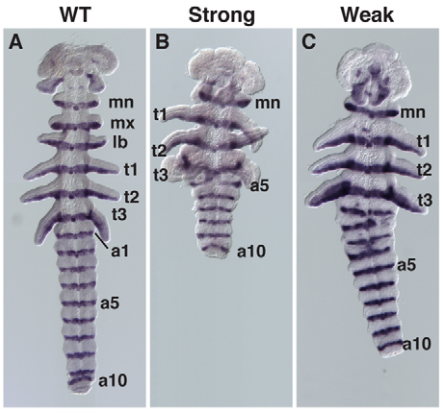

Fig. 4. *Of'gt* RNAi embryos at the germband stage stained for *Of'en*. (**A**) Uninjected late germband stage embryo. (**B**) Late germband stage RNAi embryo with the 'strong' phenotype. Note the loss of the maxillary and labial segments and the loss of the first four abdominal segments. (**C**) Late germband stage RNAi embryo with the 'weak' phenotype. Note the loss of the maxillary and labial segments and the defective *Of'en* stripes in the anterior abdomen. a5, fifth abdominal segment; a10, tenth abdominal segment; lb, labium; mn, mandible; mx, maxillae.

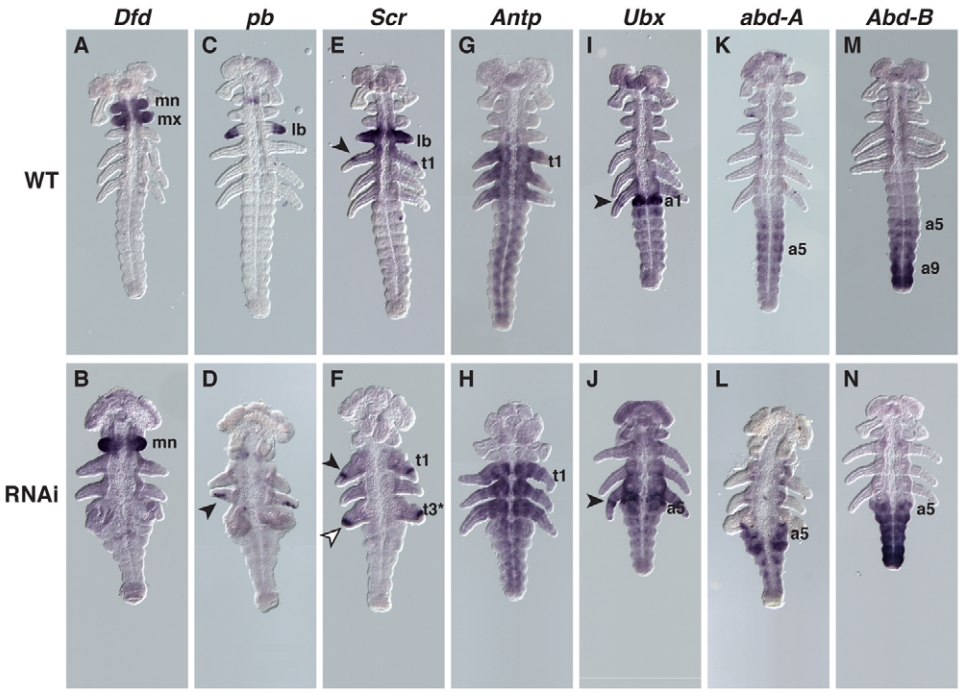

Fig. 5. Hox gene expression in uninjected and *Of'gt* RNAi embryos. (**A**,**C**,**E**,**G**,**I**,**K**,**M**) Uninjected embryos. (**B**,**D**,**F**,**H**,**J**,**L**,**N**) *Of'gt* RNAi embryos. (A) *Of'Dfd* in a normal embryo is expressed in the mandible and maxillae. (B) *Of'gt* RNAi embryo stained for *Of'Dfd.* As the maxillary segment is deleted, only the mandible expresses *Of'Dfd*. (C) *Of'pb* is normally expressed in the labium. (D) In an *Of'gt* RNAi embryo, the labium is deleted. Occasionally, we detect misexpression of *Of'pb* in the second thoracic leg (arrowhead). (E) *Of'Scr* is normally expressed strongly in the labium and weakly in the mesoderm of all the legs. Note the T1 leg patch (arrowhead). (F) *Of'gt* RNAi embryo. Note the labium is deleted and the ectopic expression of *Of'Scr* in a T3 leg patch (white arrowhead). Expression of the T1 leg patch is unaffected (black arrowhead). (G) *Of'Antp* expression is strong in the thorax and extends weakly throughout the abdomen. (H) *Of'Antp* expression in *Of'gt* RNAi embryos is not affected in the remaining segments. (I) *Of'Ubx* expression is strongest in the posterior of the third thoracic segment and the first abdominal segment. *Of'Ubx* is also expressed in a ring in the third thoracic leg (arrowhead). (J) In *Of'gt* RNAi embryos, the strong abdominal expression is missing, reflecting the deletion of that segment. The ring of expression in the third thoracic leg is still present (arrowhead). (K) *Of'abd-A* in normal embryos is expressed in the posterior of the first abdominal segment and strongly in the second through seventh abdominal segments. (L) *Of'gt* RNAi embryos stained for *Of'abd-A*. (M) *Of'Abd-B* expression in a normal embryo. (N) *Of'Abd-B* expression in an *Of'gt* RNAi embryo. a1, first abdominal segment; a5, fifth abdominal segment; a9, ninth abdominal segment; lb, labium, mn, mandible; mx, maxillae; t1, prothorax; t3, metathorax; t3*, transformed metathorax.

compressed than in normal embryos (Fig. 6E-G). *Of'eve* is normally expressed in the posterior unpatterned zone and in two or three stripes just anterior to it in newly formed segments (Fig. 6E). We found that in *Of'gt* RNAi embryos, the expression of *Of'eve* appeared normal in the posterior unpatterned zone, but the stripes were often weaker and reduced in number (Fig. 6F,G). In normal embryos, *Of'slp* is expressed in broad stripes in the posterior of each segment, arising sequentially outside of the posterior unpatterned zone during posterior segmentation and persisting throughout the germband stage (Fig. 6H). In *Of'gt* RNAi embryos, *Of'slp* appeared blotchy in the putative anterior abdomen (Fig. 6I,J). *Of'en* is expressed in the posterior of each segment (Fig. 6K). RNAi embryos showed defective *Of'en* expression in the putative anterior abdomen, with what appeared to be remnants of broken and misaligned stripes (Fig. 6L,M). These morphological defects indicate that *giant* is somehow required for the normal growth or morphogenesis of the anterior abdomen, whereas the abnormal gene expression indicates its importance in patterning. We do not know whether *Of'gt* is directly involved in both growth and patterning or whether one role is primary and the other dependent. Nevertheless, the complete RNAi phenotype is due to a failure of both processes.

giant RNAi causes homeotic transformations of the thorax

In the thorax, *Of'Scr* is normally expressed in a first thoracic leg patch, a dorsal domain and in the mesoderm of all legs (Rogers et al., 1997) (Fig. 7A). The first thoracic leg patch is situated on the inner side of the distal end of the tibia and is required for the normal development of the first thoracic leg comb [the *Oncopeltus* T1 combs, unlike the sex combs of *Drosophila*, are not sex-specific (Hughes and Kaufman, 2000)]. In *Of'gt* RNAi embryos, we noticed that germband stage embryos showed ectopic expression of *Of'Scr* in a similar looking patch on the tibia of the third thoracic leg (Fig. 7A,F). Hatchling stage individuals showed an ectopic set of T1-like combs on the third thoracic legs (Fig. 7H). Occasionally, we also saw ectopic combs on T2 (2 of 18 hatchlings; data not shown). The ectopic *Of'Scr* expression in the T3 tibia and ectopic T1-like combs on the second and third thoracic legs indicate that *Of'gt* is normally required to suppress pro-thoracic identity in the meso- and meta-thorax. In normal embryos, *Of'Ubx* is expressed in rings in the third thoracic leg (Fig. 5I). This expression remained unchanged in *Of'gt* RNAi embryos (Fig. 5J), indicating that the transformation of the third thoracic leg is likely to be incomplete.

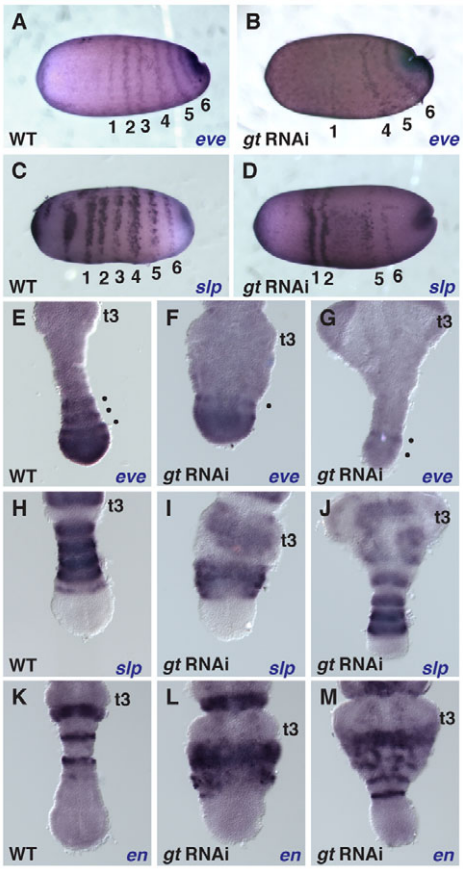

Fig. 6. Expression of segmentation genes in normal and *Of'gt* RNAi embryos. (**A**) Normal late blastoderm stage embryo stained for *Of'eve*. Six stripes *Of'eve* corresponding to the mandibular through third thoracic segment are apparent at this stage. (**B**) *Of'gt* RNAi embryo at the late blastoderm stage. The second and third *Of'eve* stripes are missing. Note that the spacing of the remaining stripes appears normal. (**C**) Normal late blastoderm stage embryo stained for *Of'slp*. Six stripes corresponding to the mandibular through third thoracic segments are labeled. Several head stripes are also visible. (**D**) *Of'gt* RNAi late-stage blastoderm embryo stained for *Of'slp*. The third and fourth *Of'slp* stripes are missing, replaced by a region of fuzzy expression. (**E**) Normal mid-germband stage embryo stained for *Of'eve*. *Of'eve* is normally expressed in the posterior growth zone and in three or four newly formed segments (dots). (**F**) Mid-germband stage *Of'gt* RNAi embryo stained for *Of'eve*. *Of'eve* expression appears normal in the growth zone but only one strong stripe is visible (dot). Also note the putative anterior abdomen appears fatter and shorter than in normal embryos. (**G**) Late germband stage *Of'gt* RNAi embryo stained for *Of'eve*. *Of'eve* expression in the growth zone is fading but the final stripes have formed (dots). (**H**) Normal mid-germband stage embryo stained for *Of'slp*. *Of'slp* is expressed in strong broad bands in the posterior of each segment. (**I**) *Of'gt* RNAi embryo at the mid-germband stage stained for *Of'slp*. Note the region of aberrant *Of'slp* expression in the anterior abdomen. (**J**) *Of'gt* RNAi embryo at the late germband stage stained for *Of'slp*. Note the aberrant *Of'slp* expression in the anterior abdomen and the normal stripes of expression in the posterior abdomen. (**K**) Normal mid-germband stage embryo stained for *Of'en*. *Of'en* is expressed in the posterior of each segment. (**L**) *Of'gt* RNAi embryo at the mid-germband stage stained for *Of'en*. *Of'en* expression is aberrant in the putative anterior abdomen. (**M**) *Of'gt* RNAi embryo at the late germband stage stained for *Of'en*. Defective *Of'en* expression can be seen in the putative anterior abdomen but stripes appear normal in the posterior abdomen. t3, third thoracic segment.

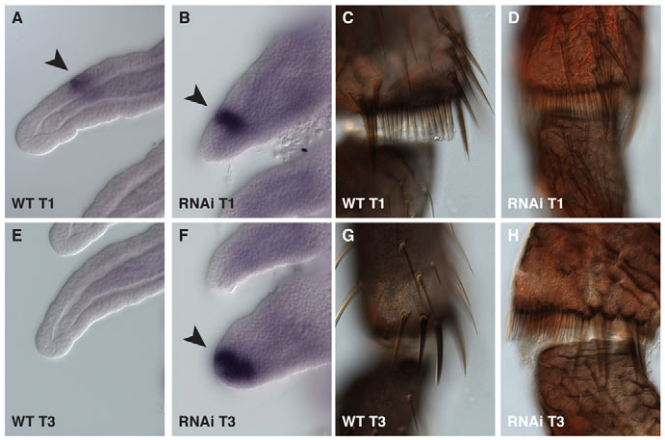

Fig. 7. Homeotic transformation of the third thoracic leg in *Of'gt* RNAi embryos and hatchlings. (**A-H**) A and E show magnified images of the embryo shown in Fig. 5E. B and F show magnified images of the embryo shown in Fig. 5F. (A) T1 leg from a normal germband stage embryo stained for *Of'Scr*. Note the T1 leg patch (arrowhead). (B) *Of'gt* RNAi embryo. *Of'Scr* T1 leg patch appears normal. (C) Magnification of the tibial comb on a T1 leg from a newly hatched normal animal. (D) Magnification of the tibial comb on a T1 leg from an *Of'gt* RNAi embryo at the hatchling stage. The tibial comb is unaffected. (E) T3 leg from a normal germband stage embryo stained for *Of'Scr*. (F) T3 leg from an *Of'gt* RNAi germband stage embryo stained for *Of'Scr*. Note the ectopic patch of expression (arrowhead). (G) Magnification of the T3 distal tibia from a newly hatched normal animal. Note the morphology of distal tibial bristles. (H) Magnification of ectopic T1-like combs on a T3 leg of an *Of'gt* RNAi animal at the hatchling stage. Compare the morphology of the tibial comb to the WT T1 combs in C. RNAi, *Of'gt* RNAi embryo; T1, first thoracic leg; T3, third thoracic leg; WT, normal embryo.

DISCUSSION

Oncopeltus is an intermediate germ insect that undergoes two distinct phases of segmentation, a blastoderm phase during which anterior segments up to the third thoracic segment are specified and a later germband phase when abdominal segments are added sequentially. We cloned and analyzed the expression and function of the *Oncopeltus fasciatus* gap gene homolog, *giant*, and found that it is required for segmentation of two body regions. *Of'gt* acts as a canonical gap gene in the maxillary and labial segments and is also required for correct growth as well as patterning of the first through fourth abdominal segments.

Oncopeltus giant is a bona fide gap gene in the anterior

In *Drosophila*, *giant* mutations cause deletions and fusions of two body regions: an anterior gap encompassing the labial segment and a posterior fusion of abdominal segments 5-7. We have found that in *Oncopeltus*, *giant* RNAi causes an anterior deletion of the maxillary and labial segments as well as a deletion of the first through fourth abdominal segments. We argue that this anterior deletion represents a true gap phenotype for several reasons. First, *Of'gt* is expressed in a broad gap-like pattern spanning the gnathal segments during the blastoderm stage. Second, the terminal phenotype in RNAi hatchlings is a deletion of two contiguous segments (maxillary and labial). Lastly, the gap genes in *Drosophila* are responsible for establishing proper position and expression of the pair-rule genes. In *Oncopeltus*, we have shown that *giant* is required for proper expression of at least two

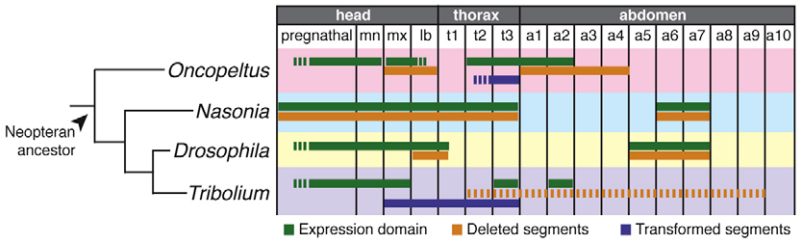

Fig. 8. Summary of *giant* expression and function in *Oncopeltus* and other insects. The evolutionary relationships of the insects are shown in the simplified phylogenetic tree on the left. Arrowhead marks the Neopteran ancestor. The expression domains for the different insects are shown in green. Regions that are deleted or fused in *giant*-depleted embryos are shown in orange and regions of homeosis are shown in blue. The precise boundaries of anterior *Of'gt* could not be determined, and so are shown as broken bars. The second thoracic leg was transformed in only a fraction of *Of'gt* RNAi embryos, denoted by the broken blue bars. In *Tribolium*, *giant* knockdown resulted in defects in a variable number of all of the posterior thoracic and all of the abdominal segments, denoted by the broken orange bars. a1-a10, first through tenth abdominal segments; lb, labium; mn, mandible; mx, maxillae; t1-t3, first through third thoracic segments.

homologs of the pair-rule genes: in RNAi embryos, two stripes of *even-skipped* and *sloppy-paired* expression are missing. This last point does have the caveat that we have not formally proven via functional studies that *sloppy-paired* has a role in segmentation; however, its expression pattern is consistent with such a role. Nevertheless, the dependence of both *even-skipped* and *sloppy-paired* on *giant* and the gap phenotype give us confidence that *Oncopeltus giant* is indeed acting in a canonical gap fashion in the anterior domain.

The anterior gap function of *giant* might be ancestral in the Neoptera

Loss of *giant* function in the long germ insects *Drosophila* and *Nasonia* yield region-specific deletions and segmental fusions. However, in the short germ beetle *Tribolium*, it was reported that *giant* RNAi embryos did not show a canonical gap phenotype, but rather that the maxillary through second thoracic segments were all homeotically transformed (Bucher and Klingler, 2004). The maxilla and labium are transformed to T1 and T2 identity with T1 and T2 being transformed to T3 identity. These phenotypes, and the fact that the head and gnathal *engrailed* stripes appeared normal, were used to argue that the role of *Tribolium giant* is to specify segmental identity but not segment formation. This raised the possibility that the ancestral role of *giant* within the holometabolous insects was primarily for segmental identity and that its segment formation function evolved in the lineages leading to *Nasonia* and *Drosophila*, perhaps in conjunction with the transition to long germ development.

Our results in *Oncopeltus* differ from the *Tribolium* results. We find that in *Of'gt* RNAi blastoderms, two stripes of *even-skipped* and *sloppy-paired* are not formed. This indicates that the anterior gap phenotype is due to a failure in the formation of these segments and not due solely to a shift in segment identity as it was reported in *Tribolium*. Thus, *giant* acts as a canonical gap gene in at least *Drosophila*, *Nasonia* and *Oncopeltus*, suggesting that it had this role in the most recent common ancestor of these insects, which was subsequently modified in the lineage leading to *Tribolium* (Fig. 8).

There is, however, some evidence that *giant* in *Tribolium* does indeed act as a canonical gap gene. Work reported in a doctoral dissertation shows that in *Tribolium* embryos depleted of *giant* activity, the second *hairy* stripe fails to form, implying regulation of the pair-rule genes (Aranda, 2006). However, this interpretation would not explain the larvae with a complete number of total segments. Nevertheless, this raises the possibility that the *Tribolium* terminal phenotype might be produced via a canonical gap deletion underlying the homeotic shift.

Oncopeltus giant has a gap-like role in the anterior abdomen

Of'gt RNAi hatchlings and embryos at the late germband stage show deletions of the first through fourth abdominal segments with adjacent segments appearing intact. For this reason, we have been describing this deletion as a 'gap' phenotype. However, we should be cautious as the specific role of *Of'gt* in patterning the abdominal segments might not use the same mechanism used to pattern the maxillary and labial segments. In the maxillary and labial segments, *Of'gt* acts to specify the formation of the *Of'eve* and *Of'slp* stripes – in the RNAi embryos, these stripes are missing. However, the *Of'eve* and *Of'slp* stripes corresponding to the anterior abdomen are still present, albeit aberrant. This abdominal defect might be similar to the abdominal phenotype in *Drosophila*, where the anterior compartments of the fifth through seventh abdominal segments are deleted, resulting in fusion of the remaining portions of those segments. Nevertheless, in *Oncopeltus*, the terminal phenotype appears very gap-like, whether or not it is due to a loss of pair-rule expression.

We should note that posterior segmentation in short germ insects might not have a direct analogy with segmentation in *Drosophila*. It might be that the sequential nature of abdominal segmentation in short germ insects precludes the type of mechanism found in *Drosophila* gap genes and our *Of'gt* result represents a short germ version of a true gap phenotype. Perhaps knockdowns of other gap gene homologs will manifest the same phenotypes in posterior growth and patterning as we have found for *giant*.

Other roles of *Oncopeltus giant* in the thorax and anterior abdomen

In addition to the role of *giant* as a 'gap' gene for the anterior abdomen, we find several aspects of its posterior function worth pointing out and discussing further. *Oncopeltus giant* is expressed during the late blastoderm and early germband stages in a domain that spans the second thoracic segment to the second abdominal segment. Although this domain of *Of'gt* expression is contiguous and we interpret it as a continuation of a single domain, it seems to have two different functions in the meso- and meta-thorax versus the anterior abdomen. In the thorax, *Of'gt* is required to suppress anterior fate and anterior Hox genes such as *Scr*. This is similar to the 'regionalizing' role that some gap orthologs play in *Oncopeltus* and other short germ insects, where the gap genes do not act to form segments but only to assign identity to those segments (Liu and Kaufman, 2004b; Marques-Souza et al., 2008). In the anterior

abdomen, *Of'gt* is required for segmentation proper as A1-A4 fail to properly form in *Of'gt* RNAi embryos. Thus, although *Of'gt* is expressed in a single contiguous domain, it plays two distinct roles within that domain. Perhaps this is because T2 and T3 are blastoderm-derived, whereas A1-A4 are germband-derived and segmental specification and identification occur in a different developmental context.

Second, we would like to point out that the affected region in RNAi embryos is slightly larger than the domain of *Of'gt* expression. *Of'gt* is expressed from T2-A2, whereas the affected region spans T2-A4. This is not unusual for gap genes, as *Krüppel* in *Drosophila* and *Oncopeltus* affects larger portions of the embryo than the area in which it is expressed (Wieschaus et al., 1984; Hulskamp and Tautz, 1991; Liu and Kaufman, 2004a). *Oncopeltus Krüppel* is expressed from T1-T3 but affects the first four abdominal segments as well. That both *Oncopeltus giant* as well as *Krüppel* affect A1-A4 but not A5 raises the possibility that A5 represents a boundary where another segmentation gene, perhaps *knirps*, plays a role.

Lastly, we have previously speculated that germband elongation during posterior segmentation in short germ insects might occur via cellular rearrangements in the posterior unpatterned zone rather than growth via cell proliferation (Patel et al., 1994; Liu and Kaufman, 2005b). In *Drosophila*, germband extension is due to cell intercalation resulting in convergent extension and is dependent on proper anterior-posterior patterning (Irvine and Wieschaus, 1994). Mutations in any of several segmentation genes, including *giant*, reduce germband extension. It is interesting that in *Of'gt* RNAi embryos at the early germband stage, the morphology of the putative anterior abdomen is defective, appearing more compressed than normal. This might be similar to the *Tribolium giant* RNAi phenotype, where the affected embryos also show abdomens with defective morphology (Bucher and Klingler, 2004). It is possible that in both *Oncopeltus* and *Tribolium*, *giant* is required for the normal cellular rearrangements that occur during abdominal growth and segmentation. In the absence of *giant* function, those cells might fail to undergo their proper movements. However, we do not know whether the role of *giant* in this process would be direct. It might play specific roles in both morphogenesis as well as patterning. Alternatively, one process could be dependent on the other, with the full phenotype a result of an interruption of both.

Conserved and divergent aspects of *giant* expression and function

We have found that *Oncopeltus giant* expression and function shows both conserved and divergent aspects with *giant* in other insect species (summarized in Fig. 8). Although there are slight differences in the precise segmental register, the anterior gap-like expression domain is largely shared with both *Tribolium* and *Drosophila*. A posterior gap-like domain arises later and continues during early germband elongation. This domain is also largely shared with *Drosophila*, *Tribolium* and *Nasonia*, although the expression corresponds to different segments in the different insect species. The function of *giant* as a gap gene in *Oncopeltus* also seems largely conserved, but again with differences in the number and register of segments that it patterns. In *Drosophila*, the abdomen is patterned by several gap genes including *giant*, *hunchback* and *knirps*. The shift in segment number and register of *Oncopeltus giant* function implies that other abdominal gap genes might likewise be responsible for patterning a different set of segments.

In *Tribolium*, *giant* acts on segments a large distance from its posterior expression domain and *giant* RNAi leads to a general breakdown of segmentation in the thorax and entire abdomen. This inability to form posterior segments is also seen in *Tribolium* embryos depleted for *caudal* and *Torso* function (Copf et al., 2004; Schoppmeier and Schroder, 2005). It was pointed out that the long-range effect of *giant* is consistent with the interruption of a 'chain of induction' or oscillatory mechanism similar to that governing vertebrate somitogenesis (Bucher and Klingler, 2004). The gap-like phenotype in the abdomen of *Of'gt* RNAi animals suggests a different underlying mechanism in *Oncopeltus*. In *Oncopeltus*, posterior abdominal segments appear normal, despite the failure of anterior abdominal segmentation, which is similar to the gap-like phenotype seen when another gap gene, *Krüppel*, is depleted (Liu and Kaufman, 2004b). That posterior segmentation is not dependent upon proper anterior patterning argues against both a chain of induction as well as an oscillatory mechanism. The gap-like phenotypes seen with both *Oncopeltus giant* and *Krüppel* seem more *Drosophila*-like and could suggest a system where these genes act to pattern a naive field of cells. It should be noted that the complete failure of posterior segmentation is seen in *Oncopeltus even-skipped* and *hunchback* RNAi embryos, although we do not know whether this is owing to a failure in growth or patterning per se (Liu and Kaufman, 2004b; Liu and Kaufman, 2005a).

Drosophila gap genes are initially expressed during the syncytial blastoderm stage and are therefore able to act as short-range morphogens that position pair-rule stripes. Both the anterior and posterior *Drosophila giant* domains regulate the pair-rule genes in this fashion (Small et al., 1991; Langeland et al., 1994; Wu et al., 1998). *Drosophila giant* is both required for the expression of *eve* stripe 2 as well as the repression of its anterior border. At first glance, this seems similar to the situation in *Oncopeltus*, as the second and third *eve* stripes also fail to form. However, the specific nature of *eve* stripe regulation is likely to be divergent as *eve* stripe 2 in *Drosophila* is a pair-rule stripe and initiates while the blastoderm is still syncytial. In *Oncopeltus*, *eve* stripes arise in a segmental pattern well after cellularization has occurred (Butt, 1947; Liu and Kaufman, 2005a). Nevertheless, normal striped expression of *Of'eve* is dependent on *Of'gt* function, which might indicate that certain elements of the overall genetic logic are conserved between species, but with changes at the molecular and cell biological level.

We must emphasize that we do not yet know whether there are true pair-rule genes in *Oncopeltus*. Neither *Of'eve* nor *Of'slp* have a pair-rule phase of expression, and the only gene with a reported pair-rule-like role in *Oncopeltus* so far is for the nuclear receptor E75A (Erezyilmaz et al., 2009), indicating that the pair-rule network is likely to be very different from *Drosophila*. In *Drosophila*, *giant* acts to both repress and maintain *even-skipped* stripe 2 expression, as *giant* mutants first show expanded expression followed by reduced expression of *eve* stripe 2 (Small et al., 1991; Wu et al., 1998). We do not detect derepression of any of the blastoderm stripes of *Of'eve*, but we do detect loss of two blastoderm stripes, possibly indicating that *Of'gt* might still be involved in *Of'eve* maintenance. *Of'slp*, however, could be repressed by *Of'gt* as the two stripes of *Of'slp* expression that are lost in the RNAi blastoderms are replaced by a region of fuzzy expression spanning the remaining stripes.

The function of *giant* has now been examined in four insects – two long germ (*Drosophila* and *Nasonia*) and two short germ (*Tribolium* and *Oncopeltus*) – and its role has been shown to differ in these insects (Eldon and Pirrotta, 1991; Bucher and Klingler, 2004; Brent et al., 2007) (Fig. 8). In *Drosophila*, growth and patterning of the blastoderm seem more decoupled than in the posterior segments of short germ insects such as *Oncopeltus* and *Tribolium*. Further work in short and intermediate germ insects,

where growth and patterning are still intertwined, will allow us to better understand the ancestral state and therefore how *Drosophila* and *Nasonia* segmentation evolved.

Acknowledgements
We thank the insightful reviewer comments that greatly improved the manuscript and members of the Patel laboratory for entertaining and enlightening discussion during the design, execution and analysis of the experiments and for thoughtful comments on early drafts of the manuscript. P.Z.L. was supported by the Damon Runyon Cancer Research Foundation (DRG-1867-05). N.H.P. is an Investigator of the Howard Hughes Medical Institute. Deposited in PMC for release after 6 months.

Competing interests statement
The authors declare no competing financial interests.

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