even-skipped is not a pair-rule gene but has segmental and gap-like functions in *Oncopeltus fasciatus*, an intermediate germband insect

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Accepted 1 March 2005

Development 132, 2081-2092
Published by The Company of Biologists 2005
doi:10.1242/dev.01807

Summary

The pair-rule gene *even-skipped* is required for the initiation of metameric pattern in *Drosophila*. But *Drosophila* segmentation is evolutionarily derived and is not representative of most insects. Therefore, in order to shed light on the evolution of insect segmentation, homologs of the pair-rule gene *even-skipped* have been studied in several insect taxa. However, most of these studies have reported the expression *eve* but not its function. We report the isolation, expression and function of the homolog of *Drosophila even-skipped* from the intermediate germband insect *Oncopeltus fasciatus*. We find that in *Oncopeltus*, *even-skipped* striped expression initiates in a segmental and not pair-rule pattern. Weak RNAi suppression of *Oncopeltus even-skipped* shows no apparent pair-rule like phenotype, while stronger RNAi suppression shows deletion of nearly the entire body. These results suggest that in *Oncopeltus*, *even-skipped* is not acting as a pair-rule gene. In almost all insects, prior to its striped expression, *even-skipped* is expressed in a conserved broad gap-like domain but its function has been largely ignored. We find that this early broad domain is required for activation of the gap genes *hunchback* and *Krüppel*. Given the large RNAi deletion phenotype and its regulation of *hunchback* and *Krüppel*, *even-skipped* seems to act as an über-gap gene in *Oncopeltus*, indicating that it may have both upstream and downstream roles in segmentation.

Key words: *even-skipped*, *Oncopeltus*, Milkweed bug, RNAi, Segmentation, Growth zone, Convergent extension, *hunchback*, *Krüppel*, Pair-rule, Evolution, Short germband

Introduction

All adult insect bodies are composed of repeated metameric units called segments. The fruit fly *Drosophila melanogaster* undergoes what is termed 'long germband' segmentation, where the entire set of body segments are specified almost simultaneously during early embryogenesis. The action of the segmentation gene cascade serves to subdivide the embryo into finer and finer domains. The upstream maternal and gap genes first allocate the early blastoderm into broad regions, each of which will eventually correspond to several body segments (reviewed by Hulskamp and Tautz, 1991; Pankratz and Jackle, 1993; St Johnston and Nusslein-Volhard, 1992). The downstream pair-rule genes then subdivide these initial broad domains into repeated units that will form the segments. Reflecting this role, the pair-rule genes are expressed in a two-segment periodicity and as such, represent the first periodic gene expression in the segmentation cascade. Thus, the *Drosophila* pair-rule genes occupy an important position in this cascade, translating the broad gradients of the upstream genes into the periodic patterns of the segmented insect body plan.

The *Drosophila* mode of segmentation is not representative of all insects and is actually evolutionarily derived. Evolutionarily basal insects undergo what is termed 'short' or 'intermediate' germband development where only the anterior segments are initially specified with the posterior body regions arising later in a sequential anterior to posterior progression (Davis and Patel, 2002; Krause, 1939). The sequential nature of posterior segmentation in short and intermediate germband insects implies that the underlying mechanisms that govern the production of posterior segments potentially differ from *Drosophila*. Given the importance of pair-rule genes in producing the first periodic gene expression patterns in *Drosophila*, they make a logical choice for understanding posterior segmentation in short and intermediate germ insects. Here, we focus our attention on the pair-rule gene *even-skipped*.

even-skipped (*eve*) was originally identified in *Drosophila* as a member of the pair-rule class of segmentation genes because hypomorphic alleles produced embryos that lacked the denticle band and adjacent cuticle from even-numbered segments (odd-numbered parasegments) – a canonical 'pair-rule' phenotype (Nusslein-Volhard et al., 1984). The gene encodes a homeodomain-containing transcription factor and acts as a transcriptional repressor (Biggin and Tjian, 1989; Macdonald et al., 1986). *even-skipped* is initially expressed in a broad blastoderm domain that first resolves into a striped primary pair-rule pattern with a two segment periodicity (a pattern that correlates well with the hypomorphic *eve* phenotype). This primary pair-rule pattern then matures into a secondary segmental one (Frasch et al., 1987; Macdonald et al., 1986).

Previous studies of *eve* in other insects have found its expression to be variable, with patterns similar to *Drosophila*, or having only the pair-rule or segmental phases, implying that the role of *even-skipped* may be highly plastic during insect evolution (Binner and Sander, 1997; Grbic et al., 1996; Grbic

and Strand, 1998; Kraft and Jackle, 1994; Miyawaki et al., 2004; Patel et al., 1992; 1994; Rohr et al., 1999; Xu et al., 1994). However, the vast majority of these studies have examined only the expression but not function of the gene. Outside of *Drosophila*, *eve* function has only been examined in the beetle *Tribolium castaneum* where its pair-rule function is consistent with its pair-rule expression pattern (Schroder et al., 1999). Thus, the evolution of *even-skipped* function within the insects, especially among more basal groups, is not at all clear.

In order to gain insight into the evolution of *even-skipped* function in the insects, we have examined the expression and function of *even-skipped* in the milkweed bug, *Oncopeltus fasciatus* (Hemiptera:Lygaeidae) an intermediate germband insect. We find that in this insect, *even-skipped* does not act as a pair-rule gene, but rather is expressed in a segmental pattern and is required for proper growth and patterning of nearly all segments.

Moreover, all previous studies have only examined the role of *striped eve* expression, ignoring the earlier broad domain seen in almost all insects. The *Drosophila eve* phenotype does not suggest any function for this early domain, and has therefore been largely ignored. Here, we present the first report that this earlier domain actually has an important function during segmentation. Our results indicate that in *Oncopeltus*, this early domain is required for proper expression of the gap genes *hunchback* and *Krüppel*.

Materials and methods

Cloning

Embryonic total RNA was isolated from mixed stage *Oncopeltus fasciatus* embryos using the Trizol reagent (GibcoBRL/Life Technologies). Poly(A$^+$) RNA was isolated using the Oligotex mRNA minikit (Qiagen). cDNA was synthesized using the FirstChoice RLM-RACE kit (Ambion). We first performed PCRs with degenerate primers designed to conserved *even-skipped* sequences from other arthropod species. This was followed by 5′ and 3′ RACE for isolation of the remainder of the transcript. For the degenerate PCR, the primer pairs were as previously reported (Patel et al., 1992). 5′ RACE PCR was performed using the gene-specific primer TCCATGTAGGGCTGAAAGAGGCGCTTCT, while 3′ RACE required nested PCRs with gene-specific primers ACTACGTTTCACGACCAAGGCGTTGCGAGC and TGGCAGCTCAACTGGGTCTTC, along with the anchor primers supplied in the FirstChoice RLM-RACE kit. All PCRs were performed using the Advantage2 polymerase mix (BD Biosciences). After separation on an agarose gel, candidate PCR products were gel-extracted if necessary (Qiagen), and cloned using either the PCR-Script Amp Cloning kit (Stratagene) or the TOPO-TA for Sequencing kit (Invitrogen). At least three independent PCRs were performed and several clones sequenced in order to minimize PCR and sequencing artifacts. The *Of'eve* cDNA sequence has been submitted to GenBank with Accession Number AY870400.

Embryo fixation, in situ hybridization and antibody staining

Embryo fixation, probe synthesis and in situ hybridization were carried out as previously reported (Liu and Kaufman, 2004a). The final color development step was carried out essentially as described by Liu and Kaufman (Liu and Kaufman, 2004a), except for two-color in situs using BCIP/NBT and BCIP/INT where the first AP antibody was inactivated by heating to 70°C for 30 minutes in Tris-EDTA followed by additional fixation for 2 hours before continuing with the second AP antibody.

RNAi

Double-stranded RNA used in parental and embryonic RNAi was in vitro transcribed from template prepared one of two ways. Plasmid containing the insert of interest was linearized by restriction digest, or template was prepared from a PCR where T3 and T7 phage promoter sequences were added to the primers. Sense and antisense RNA was synthesized in two separate reactions using the MEGAscript kit (Ambion). Following in vitro transcription and DNase treatment, the transcription reactions were immediately mixed in a single tube and annealed. The RNA was annealed in a PCR machine set to incubate at 94°C for 3 minutes, then set to quickly reach 85°C followed by a slow cooling to 25°C over the course of 1 hour. We removed unannealed single-stranded RNA by digestion with RNase A (Ambion) for 15 minutes. A small amount of annealed RNA was analyzed on an agarose gel to confirm successful annealing and digestion and we found that the RNase A treatment resulted in much less smearing of the dsRNA on the gel when compared with previous methods. Injections for embryonic and parental RNAi was performed as previously reported (Liu and Kaufman, 2004a).

Image capture and processing

Images of blastoderms and RNAi embryos were captured using a Nikon SMZ1500 stereomicroscope with attached Nikon DXM1200 digital camera. As these samples were relatively large, a single focal plane was not sufficient to capture all the detail of the entire embryo. Therefore several focal planes were taken for each sample and were combined into a single composite image in Photoshop (Adobe). Images of germband-stage embryos were captured on a Zeiss Axiophot microscope with attached Nikon DXM1200 digital camera.

Results

Isolation of *Oncopeltus even-skipped*

We took an RT-PCR approach in order to clone the *Oncopeltus fasciatus* homolog of *even-skipped*. First, degenerate primers designed to the conserved *even-skipped* homeodomain from other insect species was used to PCR a short initial fragment. Sequence of this fragment allowed us to design exact primers for subsequent 5′ and 3′ RACE reactions. We sequenced several clones from independent PCRs and found no evidence for additional copies of *Of'eve*.

Using this strategy, we were able to isolate a 1038 bp fragment of the *Of'eve* transcript encoding a polypeptide of at least 236 amino acids. As there is no in frame stop codon in the 5′ sequence prior to the first methionine codon, it is possible that this fragment does not include the entire open reading frame. However, alignments of the predicted *Oncopeltus* polypeptide with other insect *eve* sequences show very strong conservation at the N terminus, suggesting that our clones represent most of, if not the entire, open reading frame. Alignments with other insect *eve* sequences show sequences similar to the homeodomain, the Groucho co-repressor interaction domain, and an additional region of similarity at the N terminus (Fig. 1).

Oncopeltus even-skipped expression in the blastoderm

In order to gain insight to the potential function of *Of'eve* in milkweed bug segmentation, we used in situ hybridization to determine its pattern of expression during embryonic development. Probes for in situ hybridization were synthesized to two non-overlapping regions of the *Of'eve* cDNA, an ~600 bp 3′ fragment that contained a region of the homeodomain and

an approximately 260 bp 5′ fragment that did not include homeodomain sequences (Fig. 1A). Both probes gave identical results.

Oncopeltus even-skipped transcript first appears during the blastoderm stage ~20-24 hours after egg lay (AEL). At this stage, *Of'eve* transcript accumulates as a broad band covering the posterior two thirds of the blastoderm (Fig. 2A) and is reminiscent of early *eve* expression in *Drosophila*, where it also first accumulates in all nuclei before the appearance of its later striped expression (Frasch et al., 1987; Macdonald et al., 1986). Shortly thereafter, at 24-28 hours AEL, the *Oncopeltus* pattern becomes weaker on the ventral surface (not shown). This is not likely to reflect a role in determining the dorsal/ventral axis, but probably reflects the distribution of embryonic and extra-embryonic cells. Indeed expression of *hunchback*, *Krüppel*, and *Deformed* are also weaker on the ventral blastoderm surface (Liu and Kaufman, 2004a; Liu and Kaufman, 2004b) (P.Z.L., unpublished). Given the extreme embryonic movements during development, the embryo actually rotates twice during embryogenesis relative to the eggshell. In the interest of consistency, we orient all blastoderm images as if the egg is held constant and the embryo moves within it. A consequence of this is that blastoderm cells that are near the dorsal surface of the egg are actually fated to become ventral in the embryo.

The anterior boundary of this initial broad domain then sharpens so that by 32-36 hours AEL, a strong stripe circumscribing the early blastoderm can be seen superimposed on the early broad domain (Fig. 2B). In embryos 36-40 hours AEL, the expression pattern then changes in two ways. The broad diffuse domain fades from most of the blastoderm, but remains in a small patch in the very posterior. Interestingly, this maintenance *Of'eve* expression corresponds well with the concomitant clearing of *Oncopeltus Krüppel* expression in late blastoderms (Fig. 2F,I). Additionally, as the initial broad domain fades, *Of'eve* stripes appear in its place and seem to arise in a slight anterior to posterior progression (Fig. 2A-F). Thus, by 40 hours AEL, this expression dynamic results in a total of six vertical *even-skipped* stripes spaced on the blastoderm surface (Fig. 2F). As *Oncopeltus* is a short-germ insect, the number and positions of these stripes do not correspond to the same segments as they would on a long-germ insect, such as *Drosophila*. In milkweed bugs, only the mandibular through third thoracic segments are specified during the blastoderm stage as can be seen by *Oncopeltus engrailed* (*Of'en*) expression (Fig. 2G) (Butt, 1947; Liu and Kaufman, 2004a). The six *Of'eve* stripes appear very similar to the *Of'en* expression and thus probably also correspond to these same segments.

In *Drosophila*, gap genes such as *hunchback* and *Krüppel* regulate the position and spacing of the primary *even-skipped* stripes (Clyde et al., 2003; Frasch and Levine, 1987; Small et al., 1992). We wished to know if expression of these same genes correlated with the *even-skipped* stripes on the *Oncopeltus* blastoderm. To this end, we performed double in situ hybridizations for *Oncopeltus even-skipped* with *hunchback* (*Of'hb*) and *Krüppel* (*Of'Kr*). *Of'hb* is expressed in two broad domains in the blastoderm, a weaker anterior band which is anterior to the mandibular segment and corresponds to the anterior head, and a stronger central one corresponding to the posterior of the mandibular through labial segments (Liu and Kaufman, 2004a). *Of'Kr* is expressed in a broad posterior domain in the blastoderm, corresponding to the thoracic segments (Liu and Kaufman, 2004b). *Of'eve* stripes 1-3 (mandibular through labial) coincide with the strong central domain of *hb*, while stripes 4-6 (thoracic) underlie the *Kr* domain (Fig. 2H,I). This is in contrast with *Drosophila*, where *hb* and *Kr* only span two stripes each.

Oncopeltus even-skipped expression in the germband

Oncopeltus embryos undergo 'germband invagination' during which cells of the late blastoderm migrate to the posterior pole of the egg and dive into the interior of the yolk mass to contribute to the formation of the germband (Butt, 1947; Liu and Kaufman, 2004a). This process results in cells that originally occupied the posterior tip of the blastoderm ending up as part of the posterior growth zone of the early germband.

During germband invagination and throughout the remainder of germband growth and segmentation, *Oncopeltus even-skipped* is continuously expressed in both the mesoderm and ectoderm of the posterior growth zone (Fig. 2J-K; Fig. 3), reminiscent of *even-skipped* expression in the grasshopper *Schistocerca* (Patel et al., 1992). Additionally, there are a few stripes of expression directly anterior to this growth zone domain. As *Oncopeltus* is an intermediate-germ insect and posterior segments are specified sequentially in an anterior-to-posterior progression, these *even-skipped* stripes do not correspond to any particular segments but rather are always expressed in the chronologically youngest (most posterior) ones.

We next wished to determine the segmental register of the

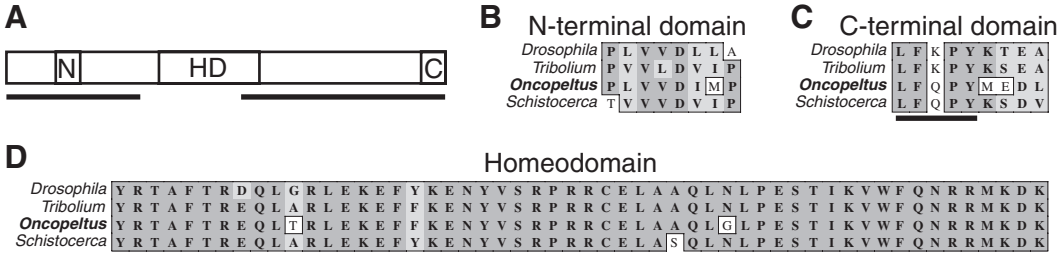

Fig. 1. Predicted structure and sequence analysis of *Oncopeltus even-skipped*. (A) Cartoon of predicted *Of'eve* protein structure with conserved N-terminal domain (N), homeodomain (HD) and C-terminal domain (C). Underlined are the regions used for synthesizing dsRNA and in situ probes. The 3′ fragment is approximately 600 bp long and spans a small region of the homeodomain. The 5′ fragment is approximately 260 bp long and does not include any of the homeodomain. (B) Conserved N-terminal domain of *even-skipped* from *Drosophila melanogaster*, *Tribolium castaneum*, *Oncopeltus fasciatus* and *Schistocerca americana.* (C) Conserved C-terminal domain from the same insect species. The Groucho co-repressor interaction domain is underlined. (D) Conserved homeodomain region from the four insect species.

striped *Of'eve* expression. As the stripes fade before segmental grooves form, we used expression of *Oncopeltus engrailed* as a segmental marker. *Of'en* is eventually expressed in all body segments but during germband growth, *Of'en* stripes initiate anterior to the growth zone, just as the stripes of *Of'eve* expression are fading. As abdominal segmentation proceeds in an anterior to posterior progression, expression of these *Of'eve* stripes slightly precedes initiation of the *Of'en* stripes. However, there is some overlap of both genes. For example, Fig. 3C2 shows three young segments (labeled as A3-A5), each of which shows some expression of both *Of'eve* and *Of'en*. This co-expression in the germband shows that *even-skipped* and *engrailed* stripes have a one-to-one correspondence. For at least the stripes that are far outside the growth zone then, it seems that *Of'eve* is expressed in a segmental rather than pair-rule pattern.

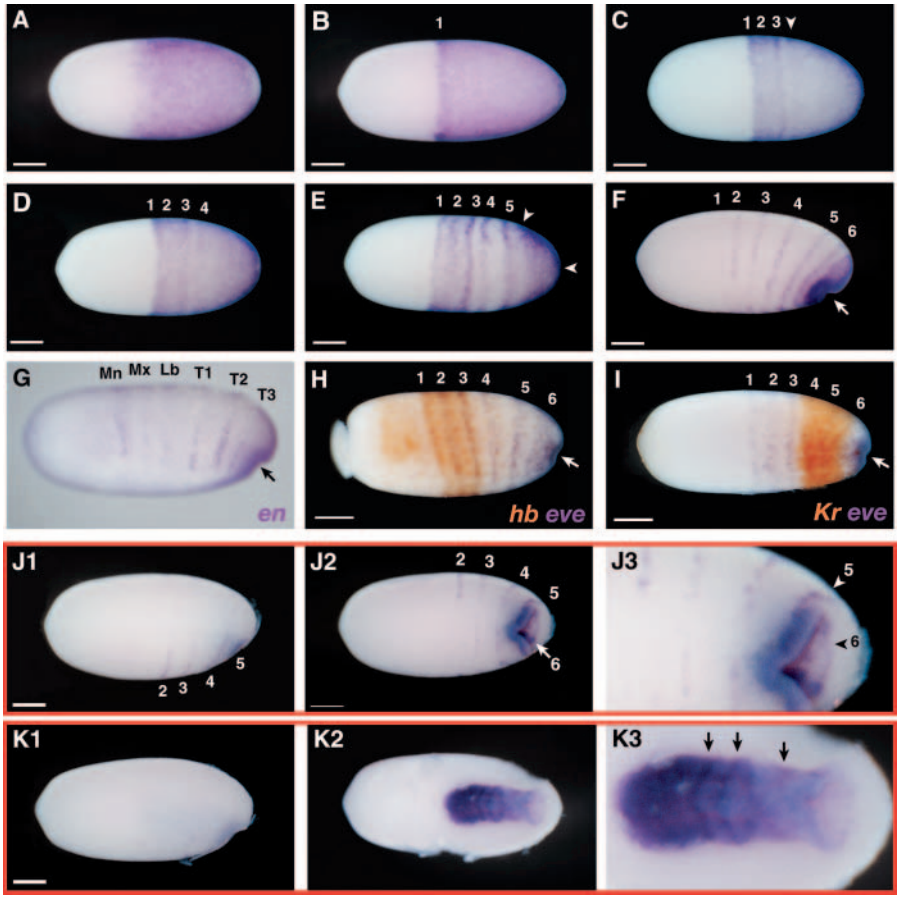

Fig. 2. *Oncopeltus even-skipped* blastoderm expression. (A-F,J1-K3) stained with probe made to *Of'eve*. (A) Blastoderm at 20-24 hours. *Of'eve* transcript appears on the posterior two-thirds of the blastoderm surface. At this stage, transcript is evenly distributed around the blastoderm, without dorsal or ventral differences. (B) Blastoderm at 32-36 hours; dorsal aspect. The anterior boundary has refined to become the first stripe. (C) Embryo at a slightly later stage than in B; dorsal aspect. The first three stripes of *Of'eve* have already formed with the fourth stripe just becoming visible (arrowhead). (D) Embryo with four stripes visible. (E) Embryo with five stripes visible. Arrowheads bracket the remainder of the initial broad domain. (F) Lateral view of 36- to 40-hour-old embryo with all six *Of'eve* stripes visible on blastoderm surface. Posterior patch of transcript remains during germband invagination. Arrow indicates invagination site. (G) Blastoderm at 36-40 hour stained for *Oncopeltus engrailed*. Six vertical stripes of *engrailed*, corresponding to the mandibular (Mn) through third thoracic segments (T3) are present on blastoderm surface. Arrow indicates site of germband invagination. (H) Embryo at 36-40 hours stained for both *hunchback* (orange) and *even-skipped* (purple). Central band of *Of'hb* expression spans first three *Of'eve* stripes. Arrow indicates germband invagination. (I) Embryo at 36-40 hours stained for both *Krüppel* (orange) and *even-skipped* (purple). *Of'Kr* expression spans *Of'eve* stripes 4-6. Arrow indicates germband invagination. (J1-J3) The same 40-44 hour embryo as in I undergoing germband invagination stained for *eve*. (J1) Lateral aspect showing that first *Of'eve* stripe has faded, while stripe 6 is no longer visible on blastoderm surface. (J2) Ventral aspect. Stripe 6 in process of migrating to contribute to the germband. (J3) Higher magnification of embryo shown in J2. (K1-K3) Embryo during germband invagination, at a later stage than in J. (K1) Lateral aspect of blastoderm surface, showing that all *Of'eve* stripes on the blastoderm have faded. (K2) Embryo with ventral region of yolk removed to show underlying early germband. (K3) Higher magnification of same embryo. Expression of *Of'eve* can be seen in early germband and in three stripes (arrows). Scale bars: 200 μm.

Of'eve stripes also seem to be generated from the growth zone in a segmental fashion. In *Drosophila* the secondary segmental stripes arise de novo after the primary stripes are refined, while in other insects with both primary pair-rule and secondary segmental patterns, the secondary stripes are generated from 'splitting' of the broader primary stripes (Binner and Sander, 1997; Macdonald et al., 1986; Patel et al., 1994). Thus, the pair-rule nature of expression is revealed by the dynamics of stripe formation – a broad primary stripe of expression followed by narrower segmental secondary ones. In *Oncopeltus*, early stripes close to the growth zone often have a characteristic 'V' shape at the midline where they remain contiguous with the growth zone (Fig. 3B,E). These stripes seem to 'peel' off of the growth zone in a segmental register as they maintain their width as they mature (compare chronologically younger and older stripes in Fig. 3B,C2) and do not 'split' to form secondary stripes.

Moreover, early growth zone expression often shows three or four stripes of *Of'eve* within the unelongated growth zone (Fig. 3A2). These stripes may correspond to anterior abdominal stripes that migrate into the rest of the germband as the germband elongates. If this is the case, then the growth zone may become patterned before actual elongation. At any rate, these stripes also do not appear to be any broader than the abdominal stripes to which they then give rise. Thus, the dynamics of *Of'eve* stripe formation reveal no obvious pair-rule phase of expression.

Oncopeltus even-skipped RNAi

With *Of'eve* expression suggesting roles in segmentation and growth zone function, we wished to *functionally* test its developmental role. We therefore used RNAi to specifically knockdown *even-skipped* function in *Oncopeltus* in order to gain insight into its role in milkweed bug

Table 1. *Of'eve* RNAi results

dsRNA	[dsRNA] (μg/ul)	Nonspecific [*n* (%)]	Wild type [*n* (%)]	Class III [*n* (%)]	Class II [*n* (%)]	Class I [*n* (%)]	Totals
3′ eRNAi		192 (74.4)	2 (0.8)	0 (0)	0 (0)	64 (24.8)	258
5′ pRNAi	2.0	0 (0)	0 (0)	0 (0)	0 (0)	182 (100)	182
3′ pRNAi	2.0	1 (0.6)	0 (0)	0 (0)	0 (0)	171 (99.4)	172
	0.2	8 (1.5)	0 (0)	0 (0)	0 (0)	540 (0)	548
	0.02	0 (0)	0 (0)	0 (0)	0 (0)	150 (100)	150
	0.002	2 (0.5)	169 (44.8)	21 (5.6)	26 (6.9)	159 (42.2)	377
pRNAi totals		11 (0.8)	169 (11.8)	21 (1.5)	26 (1.8)	1202 (84.1)	1429

The nonspecific class included embryos which underwent at least some development, but whose final morphology was uninterpretable.
Percentages may not add up to 100 due to rounding.

Table 2. *Of'eve* RNAi suppression fades over subsequent egg clutches

Clutch number	Nonspecific [*n* (%)]	Wild type [*n* (%)]	Class III [*n* (%)]	Class II [*n* (%)]	Class I [*n* (%)]	Clutch totals*
1	0 (0)	0 (0)	0 (0)	0 (0)	38 (100)	38
2	0 (0)	0 (0)	0 (0)	0 (0)	6 (100)	6
3	0 (0)	6 (17.6)	7 (20.6)	6 (17.6)	15 (44.1)	34
4	0 (0)	20 (100)	0	0	0	20
5	0 (0)	28 (100)	0	0	0	28
6	0 (0)	12 (100)	0	0	0	12

All egg clutches from a single female injected with 0.002 μg/ul of *Of'eve* dsRNA were collected and scored.
*Total number of eggs scored from that clutch.
Percentages may not add up to 100 due to rounding.

embryogenesis. We directly injected double-stranded RNA into early *Oncopeltus* embryos (termed embryonic RNAi, eRNAi) (Hughes and Kaufman, 2000), and also injected double-stranded RNA into the abdomens of adult females (termed parental RNAi, pRNAi) (Liu and Kaufman, 2004a), and both yielded equivalent knockdown phenotypes. We found that occasionally, the first clutch from a given injected female would contain wild-type embryos, while later clutches would then show the *even-skipped* phenotype. This is probably because in the developing oocytes that give rise to these early broods, the egg chorions were likely already deposited, preventing the entry of the dsRNA. We therefore excluded these clutches from our analysis.

We also injected two different dsRNAs corresponding to two non-overlapping regions of the *Of'eve* transcript and both regions produced identical knockdown phenotypes (Table 1). RNAi of other genes in *Oncopeltus* results in phenotypes that range in severity and the resulting hypomorphic series often aids in the interpretation of the phenotype (Angelini and Kaufman, 2004; Liu and Kaufman, 2004a; Liu and Kaufman, 2004b). We took advantage of this and injected dsRNA to *Of'eve* in a range of concentrations (Table 1). Based on their phenotypic severity, the RNAi embryos were categorized into three classes, ranging from the strongest (class I) to the mildest (class III).

Milkweed bug embryogenesis seems to be very sensitive to *even-skipped* RNAi as injections with concentrations as low as 0.002 μg/μl (one thousandth of the concentration that we typically use for pRNAi) still yielded the most severe class I embryos and it was only at this low concentration that the milder and moderate phenotypic classes were produced. As reported previously, for a given injected female, the pRNAi suppression effect eventually fades over the course of several clutches – later clutches show gradually weaker phenotypes (Liu and Kaufman, 2004a). Moreover, when an individual female did produce moderate and weak phenotypes, we found that there was a very rapid transition from severely affected progeny, to weakly affected, and finally to wild type, often within the span of a single clutch. For example, we tracked a single injected female and scored each of her successive clutches of progeny (Table 2). For this individual, the first and second clutches were composed only of strongly affected progeny. The third clutch contained a mixture of class I, II and III progeny, and in the next clutch, all the progeny were wild type. This rapid transition between clutches of severely affected animals to clutches with only wild-type embryos along with the extremely low *Of'eve* dsRNA concentrations needed to produce the phenotypes suggest that *Oncopeltus* development is very sensitive to *even-skipped* function.

even-skipped RNAi phenotype

Suppression of *Oncopeltus even-skipped* results in defects in germband growth and segmentation of almost the entire body, including both growth-zone and blastoderm-derived segments. The more weakly affected RNAi embryos were rare, but nonetheless informative in understanding the phenotype. The hypomorphic series shows that the abdomen is most sensitive to RNAi depletion and as the depletion increases in severity, the thorax and eventually the gnathal segments also become affected.

The mild class III embryos constituted only 1.7% of the total affected pRNAi embryos and as noted were produced only from injection of low concentrations of *Of'eve* dsRNA (Table 1). Several abdominal segments were formed, but appeared much smaller than normal (Fig. 4B1-B3). The mandibular and maxillary stylets were present, although not extended (not unexpected because the uncoiling of the internal stylets usually occurs at hatching). Segmental grooves of the thorax were occasionally less prominent giving the thorax a smoother appearance. The thoracic legs appeared slightly deformed, although this may be due to steric deformation within the confines of the eggshell rather than reflecting defects in

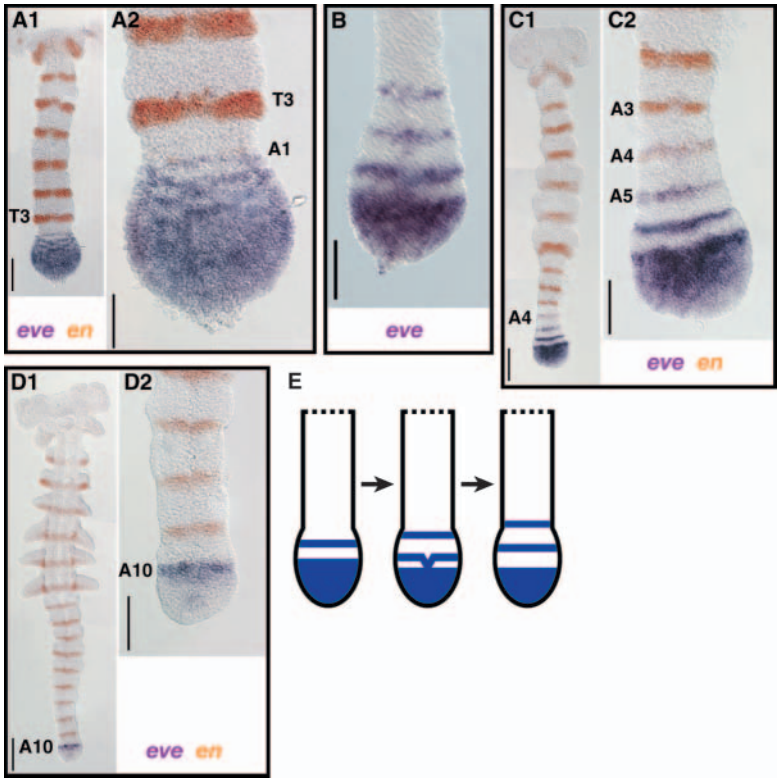

Fig. 3. *even-skipped* expression in the posterior growth zone and germband. Germband stage embryos are stained for *even-skipped* (purple) and *engrailed* (orange). (A1) Early germband with *Of'eve* accumulation in growth zone. Third thoracic segment is marked (T3). The growth zone is large. (A2) Higher magnification of embryo shown in A1, showing appearance of first abdominal *Of'en* stripe, coincident with a stripe of *Of'eve*. (B) Mid-germband stage embryo stained only for *Of'eve*. The midline region of the early stripe is still contiguous with the growth zone patch, indicating that this stripe is still in the process of leaving the growth zone. (C1) Mid-germband stage embryo. *Of'eve* expression is maintained in the growth zone and in three stripes just anterior to growth zone expression. Growth zone is now smaller than in A1. (C2) Higher magnification of embryo shown in B1, showing coincident expression of *Of'eve* and *Of'en* in three early segments. *Of'eve* is most strongly expressed in the younger (more posterior) segments, while *Of'en* is expressed more strongly in older (more anterior) segments. Thus, *Of'eve* expression is fading just as *Of'en* expression initiates. (D1) Embryo at end of germband elongation. Growth zone expression of *Of'eve* fades. (D2) Higher magnification of embryo shown in D1. Final *Of'eve* stripe is coincident with tenth abdominal *Of'en* stripe. Remaining posterior dot of *Of'eve* may represent anal ring expression. (E) Cartoon showing birth and progressive maturation of *Of'eve* germband stripes. Early stripes seem to 'peel' away and are often contiguous with growth zone patch. Scale bars: 200 μm for A1,C1,D1; 100 μm for A2,B,C2,D2.

patterning. Importantly, any weak disruptions in the thorax affected all segments. Any bias in sensitivity seemed graded, increasing towards the posterior, with no evidence of any skipping of segments. In order to more clearly examine the phenotype, we used *Oncopeltus engrailed* expression as a convenient segmental marker in germband stage RNAi embryos. Fig. 4D1,D2 shows late-stage class III germband stained for *engrailed* and shows that the abdomen is reduced with highly disorganized segmentation (compare Fig. 4D1 and D2 with Fig. 3D1) while the head and thoracic segments appear normal. Thus, class III embryos have defective abdomens but relatively normal heads and thoracic segments.

We should note that in *Drosophila*, *even-skipped* is required for expression of *engrailed*, raising the issue of why *engrailed* expression is detected at all in the RNAi germbands. In *Drosophila*, after the segment polarity genes *wingless* and *engrailed* are initiated, they maintain each other's expression (reviewed by Perrimon, 1994) and a similar process of mutual reinforcement may be occurring here.

The moderate class II embryos were also rare, making up only 2.1% of the RNAi embryos and were also only produced at lower dsRNA concentrations (Table 1). When compared with the milder class III embryos, these embryos show stronger abdominal defects as well as defects in the thorax – the abdomen is severely reduced and thoracic segmentation is defective (Fig. 4E1,E2). In these embryos, posterior thoracic legs are either reduced or missing but anterior structures are left relatively unaffected. Germband stage embryos corresponding to this phenotypic class stained for *engrailed* reveal strong disruption of posterior growth and patterning but relatively normal anterior segmentation (Fig. 4F). As with class III, these embryos also seem to show a gradient of defect, stronger in the posterior and weaker in the anterior, without any pair-rule like defects.

Class I constituted 81.8% of the RNAi embryos (Table 1). These embryos are characterized by a very large deletion of almost the entire body (Fig. 4G-H2). Given this severity, it is difficult to capture all aspects of the phenotype photographically, so we will describe their morphology based on observations of several class I embryos. These embryos lack most of the body, with no apparent gnathal, thoracic or abdominal segments (as the intercalary segment is so small, we cannot determine its presence or absence). Antenna, eyes and a labrum can still be found and seem morphologically normal, albeit smaller in size (Fig. 4G). Mandibular and maxillary stylets are missing, suggesting that the deleted region spans these segments as well. Late-stage RNAi germbands stained with *engrailed* probe show that although anterior head elements do form, the mandibular through abdominal regions are entirely missing (Fig. 4H1,H2), consistent with what is seen in hatching-staged embryos. Thus, strong suppression of *Of'eve* results in loss of almost the entire body, from the mandibular segment to the posterior of the animal.

even-skipped RNAi results in gap gene misexpression

The severe *Of'eve* phenotype, complete loss of the mandibular through abdominal segments, was much stronger than we had expected. Although loss of abdominal segments may be explained by disruption of patterning in the growth zone, we noticed that loss of the gnathal and thoracic segments correlated well with the early broad blastoderm domain of expression (mandibular to the posterior of the blastoderm). We reasoned that as the head and thorax are normally specified

during the blastoderm stage, blastoderms that are depleted for *Of'eve* function might become repatterned to reflect loss of the deleted regions.

We first noticed that RNAi embryos showed defects in germband invagination. In normal 48-52 hour embryos, germband invagination is nearly complete with the site of invagination at the posterior pole of the blastoderm (arrow in Fig. 5A). *Of'eve* RNAi embryos at a similar stage show a failure of germband invagination with a mislocalization of the invagination site to a more ventral position on the blastoderm (arrow in Fig. 5B). When dissected, these embryos do not have an elongating germband in the yolk (not shown), which is consistent with loss of the entire body in the class I animals. These defects suggest that depletion of *Of'eve* function may repattern the blastoderm.

We further reasoned that blastoderm repatterning might be reflected in abnormal gap gene expression. As noted earlier, *Of'hb* is normally expressed in two broad blastoderm domains, a weaker anterior domain corresponding to the head and a stronger central domain corresponding to the maxillary through first thoracic segments (Fig. 5C) (Liu and Kaufman, 2004a). We found that when *Of'eve* function is reduced, we detect only a single *Of'hb* domain spanning the posterior half of the blastoderm (Fig. 5D1,D2). In uninjected animals, the two *Of'hb* bands are clearly distinct on the blastoderm but in the RNAi embryos, this remaining band of expression appears uniform throughout its domain. We interpret the remaining single band as probably representing a loss of the stronger central gnathal *Of'hb* domain, with a concomitant expansion and posterior displacement of the remaining head domain for two reasons. First, the severe *Of'eve* RNAi phenotype is a loss of all segments except the anterior head, a region that spans the central, but not anterior head, domain of *Of'hb* expression. Second, the broad domain of *Of'eve* expression spans the central gnathal, but not anterior head, domains of *Of'hb* expression (compare Fig. 2B with Fig. 5C), indicating that *Of'eve* is spatially positioned to activate the central gnathal *Of'hb* domain. These results suggest that *Of'eve* is required for activation of *Of'hb* in the mandibular to first thoracic segments; when *Of'eve* function is reduced, the remaining head domain of *Of'hb* expands to fill the posterior of the blastoderm.

In uninjected animals, *Of'Kr* is expressed in the posterior third of the blastoderm, corresponding to the thoracic segments (Fig. 5E) (Liu and Kaufman, 2004b). *Of'Kr* expression in

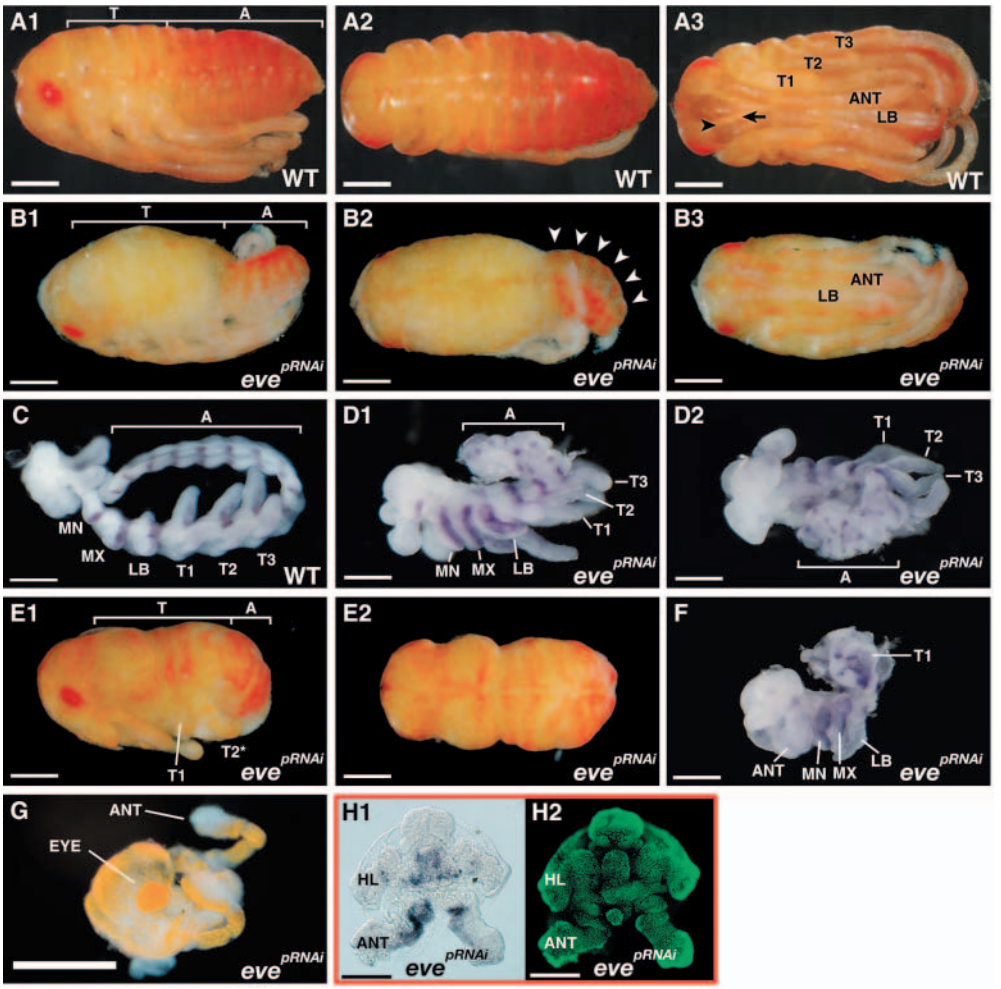

Fig. 4. *Oncopeltus even-skipped* RNAi phenotype. (A1-A3) Uninjected hatching-staged animals. (A1) Lateral view. Thoracic and abdominal regions are marked. (A2) Dorsal aspect. (A3) Ventral view. (B1-B3) Class III (weak) phenotype. (B1) Lateral view of class III animal. Thorax weakly affected, all legs are present. The abdominal region is shortened. (B2) Dorsal view. Abdominal segments marked by arrowheads. (B3) Ventral view. (C) Fully formed germband of uninjected embryo, stained for *engrailed*. (D1,D2) Putative class III germband stained for *engrailed*. (D1) Lateral view. Head and thorax are at most weakly affected, but abdominal region is shortened and shows strong segmental defects. As the germbands were extremely curved and ruffled, and therefore impossible to mount under a cover slip, they were photographed unmounted. (D2) Dorsal aspect, showing segmentation defects in abdomen. (E1,E2) Class II (moderate) hatching stage embryo. (E1) Lateral view showing presence of first thoracic leg. Second thoracic leg is also present but difficult to see in this photograph. Third thoracic leg is missing. Abdomen is now severely shortened. (E2) Dorsal aspect. There are segmental defects in head and thorax. (F) Putative class II germband stained for *engrailed*. Body now highly reduced, and second and third thoracic legs are missing. Gnathal and first thoracic appendages still present. (G) Class I (strong) embryo. Only anterior structures such as labrum, eye and antenna are formed. (H1-H2) Class I embryo stained for *engrailed* (H1) and DNA (H2). All segments posterior of the head are missing. ANT, antenna; MN, mandible; MX, maxillary; LB, labium; T1-T3, thoracic segments; A, abdomen. Scale bars: 200 μm in A1-G; 100 μm in H1 and H2.

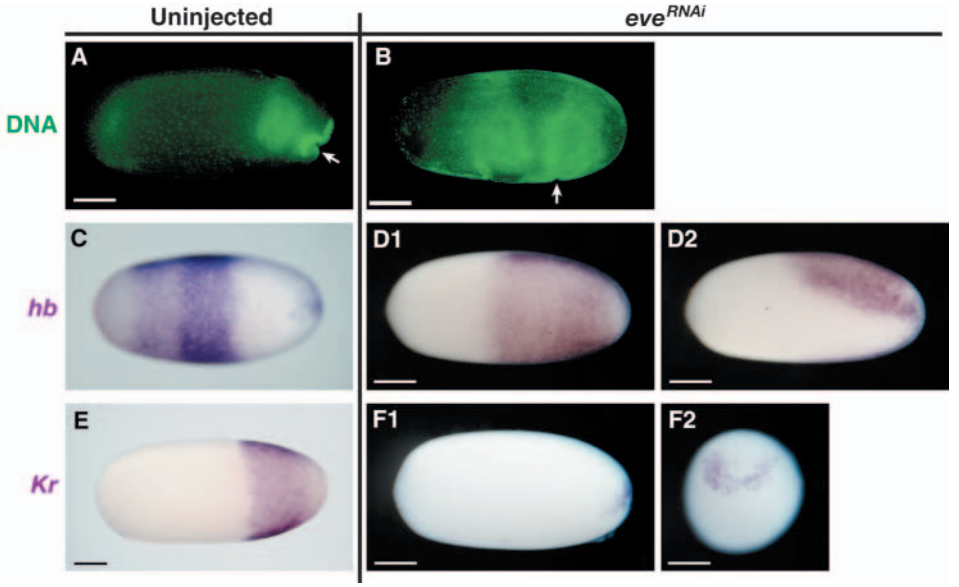

Fig. 5. *hunchback* and *Krüppel* expression in *Of'eve* RNAi blastoderms. (A,C,E) Uninjected animals. (B,D1-2,F1-2) *Of'eve* RNAi animals. (A) Lateral view of uninjected 44-48 hour embryo stained for DNA. Germband has fully invaginated, leaving behind head lobes on outside posterior of yolk ball (densely staining region). Arrow indicates site of invagination. (B) Lateral view of 44-48 hour *Of'eve* RNAi embryo stained for DNA. Odd wrinkles and putatively mislocalized invagination site (arrow) can be seen. (C) Dorsal aspect of uninjected 36-40 hour blastoderm stained for *Oncopeltus hunchback* showing that *Of'hb* normally accumulates in two broad bands. Weaker anterior band corresponds to anterior head, while central band corresponds to posterior mandible through anterior first thoracic segments. (D1) Dorsal view of 36-40 hour *Of'eve* RNAi blastoderm, showing that *Of'hb* is now expressed in a single uniform domain in the posterior half of the blastoderm. (D2) Same embryo as in (D1), but rotated to show lateral view. *Of'hb* expression is restricted to the dorsal part of the blastoderm and is typical in late stage blastoderms. (E) Dorsal view of uninjected 36-40 hour embryo stained for *Oncopeltus Krüppel*. *Of'Kr* normally accumulates in posterior one-third of blastoderm. (F1) *Of'eve* RNAi blastoderm at 36-40 hour. *Of'Kr* expression is largely absent, with only a very small posterior patch remaining. (F2) Same embryo as in F1, end-on view of posterior tip of blastoderm, showing remaining *Of'Kr* patch. Scale bars: 200 μm.

Of'eve RNAi blastoderms is highly reduced, so that only a tiny patch of expression remains at the posterior tip of the blastoderm (Fig. 5F1,F2). This suggests that *Of'eve* is also required for proper expression of *Of'Kr* in the blastoderm.

Of'eve is expressed in a dynamic pattern during the blastoderm stage, with an early broad domain covering the posterior two thirds of the blastoderm and a later striped phase of expression. Temporally and spatially, it seems most likely that it is this early broad blastoderm domain (rather than the later striped expression) that is responsible for these gap gene-regulating functions. Thus, in the absence of *Of'eve* function, the early blastoderm becomes re-allocated to represent only the anterior head.

Discussion

We have isolated the *Oncopeltus fasciatus* homolog of the *Drosophila* pair-rule gene, *even-skipped.* In order to determine the role of *even-skipped* during *Oncopeltus* development and its evolution within the insects, we examined *Of'eve* expression using in situ hybridization and its developmental function using RNAi. There are two major findings of our work: first, that *even-skipped* in *Oncopeltus* does not act as a pair-rule gene; and second, that *even-skipped* acts as a gap gene. We discuss these two findings and their implications in turn.

even-skipped does not act as a pair-rule gene in *Oncopeltus*

One of the characteristics of *Drosophila even-skipped* and most of the other pair-rule genes is that they are expressed in the blastoderm in a series of seven transverse stripes with a two-segment periodicity (Carroll et al., 1988; Carroll and Scott, 1986; Gergen and Butler, 1988; Grossniklaus et al., 1992; Hafen et al., 1984; Harding et al., 1986; Macdonald et al., 1986). *even-skipped* expression has been examined in a number of insects, and in many species, a primary pair-rule pattern is followed by a later segmental one. For example, in *Drosophila*, *eve* primary stripe expression is in odd numbered parasegments, and then later minor stripes arise de novo in the even numbered parasegments (Frasch et al., 1987; Macdonald et al., 1986). In both the long germ honeybee *Apis mellifera* and short germ beetle *Tribolium*, secondary stripes appear through 'splitting' of the primary pair-rule stripes (Binner and Sander, 1997; Brown et al., 1997; Patel et al., 1994). The *Oncopeltus eve* expression dynamic shows none of these pair-rule patterns, instead initiating in a segmental manner.

Function of *even-skipped* had previously only been examined in *Drosophila* and *Tribolium*, and was found to have a pair-rule requirement, reflecting the pair-rule expression pattern in both of these insects (Nusslein-Volhard et al., 1984; Schroder et al., 1999). In *Oncopeltus,* there is no apparent pair-rule phenotype. Instead, there seems to a gradient of sensitivity, with posterior segments being more sensitive to RNAi depletion. We should note that as *Of'eve* is expressed in a broad blastoderm domain, in the growth zone and in segmental stripes, teasing apart the functions of each of these individual domains is difficult without more sophisticated genetic techniques. Because we can assay for a pair-rule phenotype only in the thorax, we cannot rule out a hidden pair-rule function in the abdominal segments. Nevertheless, as neither the expression nor functional analyses reveal an apparent pair-rule role, *even-skipped* is probably not acting as a pair-rule gene in this insect.

Divergent regulation of the segmentation genes in *Oncopeltus*

The non-pair-rule role of *Oncopeltus even-skipped* suggests

that in this insect, the genetic paradigm regulating the segmentation gene cascade must differ from *Drosophila* in several respects.

First, regulation of striped expression of *Of'eve* by the upstream gap genes must be divergent. In *Drosophila*, *even-skipped* is directly regulated by gradients of gap gene proteins that bind to stripe-specific enhancers in the *eve* promoter (Clyde et al., 2003; Frasch and Levine, 1987; Small et al., 1992). The anterior *Drosophila hunchback* domain covers *eve* stripes 1 and 2, while the *Krüppel* domain covers stripes 3 and 4. This is in contrast to *Oncopeltus*, where during the blastoderm stage, *Of'hb* spans the first three *Of'eve* stripes and *Of'Kr* spans stripes 4, 5 and 6. Moreover, the stripes in *Drosophila* are pair-rule but in *Oncopeltus*, are segmental in register. Although *Of'eve* stripes are expressed in a manner consistent with their potential regulation by the gap genes, the precise mechanism governing this regulation is likely to be fundamentally different from that in *Drosophila*. Moreover, during germband elongation, *Of'eve* stripes are generated sequentially out of the growth zone, a dynamic very different from *Drosophila*. Given these differences, the cis-regulatory elements that govern *Of'eve* regulation should prove to be very interesting.

Second, the overall pair-rule mechanism is likely to show fundamental differences in *Oncopeltus*. In *Drosophila*, the primary *eve* stripes act within the context of a pair-rule network (Carroll and Vavra, 1989). But given the segmental register of *Of'eve*, this network of cross-regulation is likely to be significantly different. Moreover in *Drosophila*, striped *eve* expression initiates in the pre-cellular blastoderm, where these early primary stripes each act as morphogenetic gradients to regulate the other pair-rule genes (Fujioka et al., 1995). In *Oncopeltus*, cellularization of the blastoderm nuclei occurs at around 17 hours AEL (Butt, 1947), well before the initiation of *Of'eve* striped expression at around 32 hours. The lack of a prolonged syncytial blastoderm stage in *Oncopeltus* suggest that morphogenetic gradients are not involved in the same fashion in the regulation of *Of'eve* or in its regulation of other pair-rule genes.

The third aspect which may differ between *Drosophila* and *Oncopeltus* is the regulation of *engrailed* by *even-skipped*. The expression dynamics of these two genes strongly suggest that *Of'eve* regulates *Of'en*. *Of'eve* expression slightly precedes and becomes coincident with expression of *Of'en* during both the blastoderm and germband stages. Thus, *Oncopeltus even-skipped* is temporally and spatially poised to regulate *engrailed*. However, the details are again likely to differ between *Oncopeltus* and *Drosophila*. In *Drosophila*, both the odd- and even-numbered *engrailed* stripes are initiated solely through action of the primary *eve* stripes, while the role of the minor stripes is unclear (Fujioka et al., 1995; Jaynes and Fujioka, 2004). It may be that *engrailed* activation in *Oncopeltus* is more similar to the activation of either the odd- or even-numbered *engrailed* stripes in *Drosophila* and that all *engrailed* stripes are generated using the same mechanism. It is interesting that the *Of'eve* pattern has more affinity to the late *Drosophila* (14 stripe) pattern, and may indicate that the minor stripes in *Drosophila* are an evolutionary vestige of a previous function.

eve expression has been found to be surprisingly variable in several insects, with some insects showing pair-rule only, segmental only, both, or neither patterns (Fig. 6A) (Binner and Sander, 1997; Grbic et al., 1996; Grbic and Strand, 1998; Kraft and Jackle, 1994; Miyawaki et al., 2004; Patel et al., 1992; Patel et al., 1994; Rohr et al., 1999; Xu et al., 1994) (S. Noji, personal communication). Additionally, *eve* expression has been examined in a crustacean and found to have no obvious pair-rule pattern (Davis and Patel, 2003). Thus, it is not clear what the ancestral state was in insects. Perhaps what this variability in striped expression is telling us is that we should be focusing on what inherent architectural features in the pair-rule network are allowing such easy change. This will require in-depth functional analysis of multiple pair-rule genes in an insect such as *Oncopeltus*, as well as other arthropods.

Oncopeltus even-skipped acts like a gap gene

One of the unexpected findings of this work is that *Oncopeltus even-skipped* RNAi embryos lack such a large region of the body. This complete deletion of the mandibular through abdominal segments is much more severe than the null phenotype in *Drosophila*. On one hand, a complete loss of the abdomen may be explained as an interruption of progressive segmentation in the posterior, as abdominal segments are specified during germband elongation. By preventing proper segmentation of the first abdominal segment, posterior metameres may never be specified. However, this does not explain why the mandibular through thoracic segments are also lacking, as these segments are specified during the blastoderm stage. We found that the early broad blastoderm domain of *Of'eve*, which spans the mandibular through thoracic segments, matches well with the deletion phenotype. Knockdown of *Oncopeltus even-skipped* in blastoderms shows a loss of the central *hunchback* domain with a concomitant expansion of the anterior head domain coupled with a near-complete loss of *Krüppel* expression, indicating that *Of'eve* is required for proper activation and positioning of *Of'hb* and *Of'Kr*. As *Of'eve* regulates the gap genes and also gives a deletion phenotype spanning several contiguous segments (two characteristics usually associated with gap genes), *Of'eve* in some sense also acts as a gap gene.

That a supposedly downstream pair-rule gene regulates supposedly upstream gap genes is not entirely without precedent. The *Drosophila* pair-rule gene *runt* is also required for proper expression of some of the gap genes (Tsai and Gergen, 1994). *Drosophila runt* is initially expressed in an early broad blastoderm domain, before the appearance of the characteristic pair-rule stripes and it is this broad initial domain that is responsible for proper gap gene expression. In *Oncopeltus*, the initial broad blastoderm expression of *Of'eve* may serve a similar function. It may therefore be the case that the early broad blastoderm domain regulates the gap genes, while the later striped expression is in turn regulated by them. This would mean that *Of'eve* occupies both upstream and downstream positions in the segmentation gene hierarchy (Fig. 6B).

Speculations on non-striped *even-skipped* function

Given that the gap-like function of *Of'eve* is novel and has not been reported for other insects, we have much less context in which to discuss its implications. We therefore offer some speculation that we feel is important to discuss explicitly.

First, *eve* in several other insects is also expressed in a

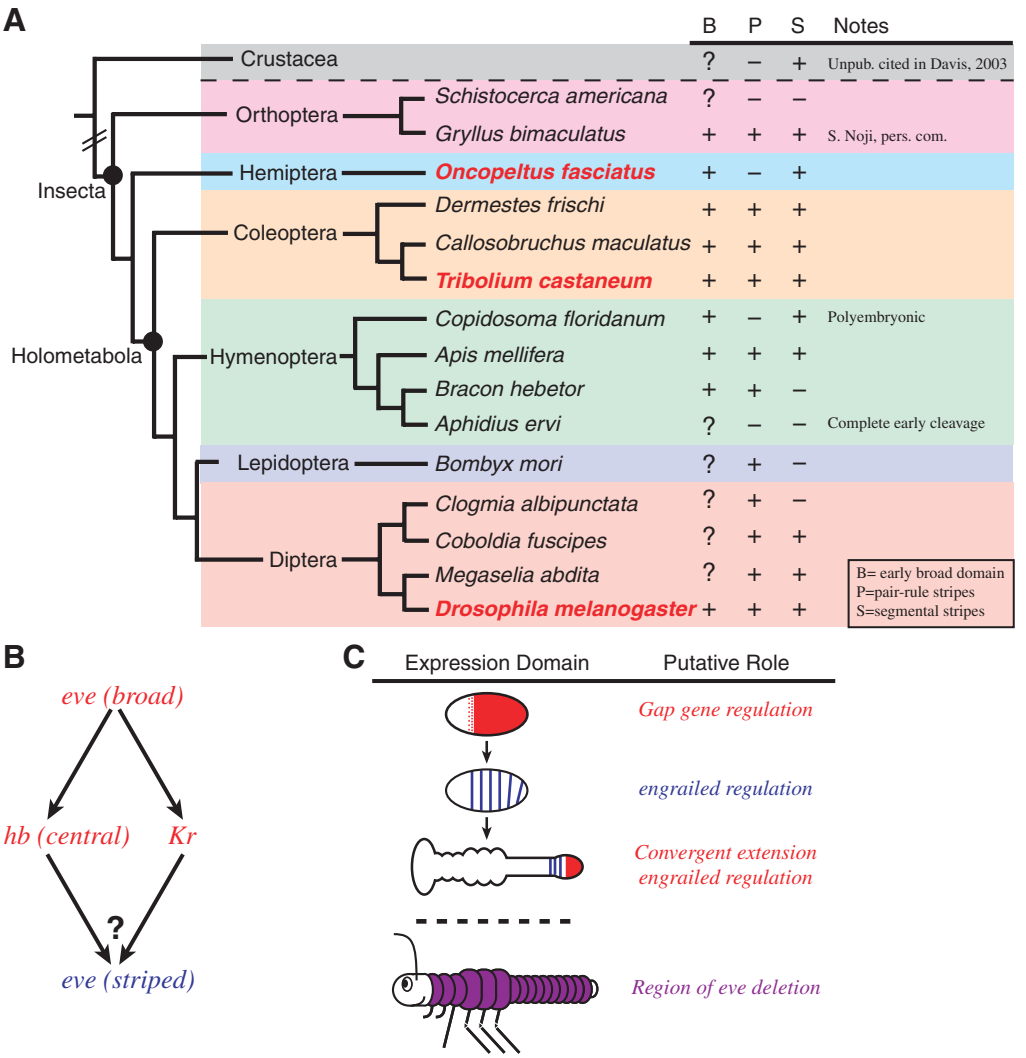

Fig. 6. Summary of *Oncopeltus even-skipped* evolution, expression and function. (A) Summary of *even-skipped* expression within the insects. Species where functional data for *eve* is known are listed in red. Some species with derived modes of embryogenesis such as polyembryony or complete early cleavage, have altered *eve* expression and may therefore represent secondarily derived conditions. The early broad domain of *eve* expression has been reported in several insects, but no function has ever been attributed to it. +, expression present in this species; –, expression not present in species; ?, neither presence nor absence of expression was reported. Coleopteran, hymenopteran and dipteran phylogenies based on previous work (Lawrence, 1982; Dowton, 1994; Yeates, 1999). (B) *Oncopeltus eve* may occupy both upstream and downstream positions in the segmentation hierarchy. The early broad blastoderm expression (red) is required for activation of the blastoderm expression of the central *hunchback* and *Krüppel* domains. These gap genes may then in turn regulate striped *Of'eve* expression. (C) Summary of putative roles for *Of'eve*. The early broad blastoderm domain (red) is most likely responsible for regulation of *hunchback* and *Krüppel*. The *eve* stripes (blue) seen on blastoderms and germbands may regulate *engrailed*. The growth zone expression (red) may be required for proper growth of the posterior. The deleted region in *Of'eve* RNAi embryos is shown in purple.

similar initial broad domain, but as this domain has no apparent function in *Drosophila*, its potential role in segmentation has been previously largely ignored (Fig. 6A) (Binner and Sander, 1997; Grbic et al., 1996; Grbic and Strand, 1998; Patel et al., 1994). In light of our results, this assumption may need to be re-examined. It may be that ancestrally, *eve* had an important gap-like function that was subsequently lost in the lineage leading to *Drosophila*.

Second, function of the early broad domain may provide clues to function of the later growth zone domain. Both can be viewed as different manifestations of a similar underlying pattern. Recall that *Of'eve* expression does not fade from the blastoderm completely, but is maintained as a posterior patch in the blastoderm at the outset of germband invagination and as the germband invaginates, eventually contributes to the posterior growth zone. Therefore, the growth zone domain is a direct continuation of the early broad blastoderm expression. Moreover, the early broad domain fades from the blastoderm surface in an anterior to posterior direction, leaving behind segmental stripes of expression. The growth zone expression can also be thought of as following the same dynamic: the posterior growth zone maintains expression of *Of'eve* but as it extends in an anterior to posterior direction during germband growth, expression of segmental stripes seem to be left behind. This potentially equates the function of the early broad domain with function in the growth zone. As the segmentation hierarchy proceeds through gap, pair-rule and segment polarity levels, it is possible that this expression in the growth zone indicates that it is being held at a 'higher' or 'earlier' state, much as the early gap-like domain precedes the later striped expression.

Third, in addition to the role of *eve* in patterning the growth zone as discussed above, it is also possible that *Of'eve* is required for its growth. The abdomen of short and intermediate germ insects dramatically elongate during embryogenesis. As the *Oncopeltus* growth zone narrows as the germband elongates, it may be that cell rearrangements contribute to germband growth (Fig. 3A2,C2,D2). In *Drosophila*, germband extension is chiefly due to cell rearrangements that are termed 'convergent extension' (Edgar et al., 1989; Hartenstein and

Campos-Ortega, 1985). It turns out that several segmentation genes, including *even-skipped* and *hunchback*, play important roles in convergent extension (Irvine and Wieschaus, 1994). Thus, it is intriguing that both *even-skipped* and *hunchback* are so strongly expressed in the *Oncopeltus* growth zone and that RNAi of these genes results in a failure of posterior growth (Liu and Kaufman, 2004a). This raises the possibility that in addition to posterior patterning, *Of'eve* and *Of'hb* may also be required for a process similar to convergent extension in *Oncopeltus*. However, it is also possible that elongation occurs through increased cell proliferation. Although no convincing increase of mitotic activity has been in found in the growth zones of several insects, including *Oncopeltus*, we cannot rule out cell proliferation as a source of germband elongation (Brown et al., 1994) (P.Z.L., unpublished; N. Patel, unpublished). But the growth zone shape changes during abdominal growth suggest that cellular rearrangments may at least be one component to posterior elongation

Interestingly, it has been recently shown that RNAi of the posterior gene *caudal* in the short germband beetle *Tribolium castaneum*, the intermediate germband cricket *Gryllus bimaculatus*, as well as in the crustacean *Artemia franciscana* results in a loss of posterior growth and segmentation (Copf et al., 2004; Shinmyo et al., 2005). In strongly affected *Tribolium* embryos, only the pregnathal head was formed, a phenotype very similar to the *Oncopeltus even-skipped* phenotype. In all three organisms, *caudal* RNAi leads to weakened, abnormal expression of *even-skipped*. Additionally, *caudal* RNAi in the cricket also leads to loss of *hunchback* and *Krüppel* expression, again similar to the situation to *Oncopeltus even-skipped*. Taken together, this suggests that both *caudal* and *even-skipped* are involved in similar functions in posterior growth and patterning, and that possibly, some functions of *caudal* may be mediated via *even-skipped*.

We thank Cynthia L. Hughes for critical reading of the manuscript. Equipment and facilities used in this work were partially supplied by INGEN funding. P.Z.L. was supported by NIH Genetics Training Grant GMO7757, and NSF Integrative Graduate Education and Research Traineeship (IGERT) 2039L1A-A1.

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