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Notch/Delta signalling is not required for segment generation in the basally branching insect *Gryllus bimaculatus*

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SUMMARY

Arthropods and vertebrates display a segmental body organisation along all or part of the anterior-posterior axis. Whether this reflects a shared, ancestral developmental genetic mechanism for segmentation is uncertain. In vertebrates, segments are formed sequentially by a segmentation 'clock' of oscillating gene expression involving Notch pathway components. Recent studies in spiders and basal insects have suggested that segmentation in these arthropods also involves Notch-based signalling. These observations have been interpreted as evidence for a shared, ancestral gene network for insect, arthropod and bilaterian segmentation. However, because this pathway can play multiple roles in development, elucidating the specific requirements for Notch signalling is important for understanding the ancestry of segmentation. Here we show that *Delta*, a ligand of the Notch pathway, is not required for segment formation in the cricket *Gryllus bimaculatus*, which retains ancestral characteristics of arthropod embryogenesis. Segment patterning genes are expressed before *Delta* in abdominal segments, and *Delta* expression does not oscillate in the pre-segmental region or in formed segments. Instead, *Delta* is required for neuroectoderm and mesectoderm formation; embryos missing these tissues are developmentally delayed and show defects in segment morphology but normal segment number. Thus, what initially appear to be 'segmentation phenotypes' can in fact be due to developmental delays and cell specification errors. Our data do not support an essential or ancestral role of Notch signalling in segment generation across the arthropods, and show that the pleiotropy of the Notch pathway can confound speculation on possible segmentation mechanisms in the last common bilaterian ancestor.

KEY WORDS: Arthropod, Segmentation clock, Evolution, Neurogenic phenotype, *Gryllus bimaculatus*

INTRODUCTION

Segmented body plans or body regions are characteristic features of many animals, including arthropods and vertebrates. Whether the last common ancestor of arthropods and vertebrates was segmented, and how it might have achieved segmentation, are a matter of intense debate. A related question concerns the mechanism of segmentation that operated in the last common ancestor of each of these groups. In vertebrates, somites are formed by a mechanism involving oscillatory waves of gene expression (Dequéant and Pourquie, 2008), and the Notch signalling pathway is a crucial component of this mechanism. In arthropods, however, the situation is more complex. The molecular mechanisms underlying *Drosophila* segmentation do not involve the Notch pathway; instead, segments are formed near simultaneously (long-germ segmentation) via progressive spatial delimitation by a transcription factor cascade (Peel et al., 2005). However, most arthropods generate segments sequentially from anterior to posterior (called short-germ segmentation in insects), by elongation of a subterminal region of the embryo termed the 'growth zone'. This process morphologically resembles vertebrate somite formation, and is considered to be ancestral in arthropods. In these animals, creating segments thus involves the distinct, but linked, processes of posterior elongation, which creates apparently naïve tissue, and segment patterning and morphogenesis, in which groups of cells differentiate into segments (Minelli, 2005; Dequéant and Pourquie, 2008; Aulehla and Pourquie, 2009).

The first suggestion that the Notch pathway was involved in arthropod segmentation came from functional studies in the spider *Cupiennius salei* (Stollewerk et al., 2003; Schoppmeier and Damen, 2005), and then from the cockroach *Periplaneta americana* (Pueyo et al., 2008), which both make segments sequentially. These observations, as well gene expression pattern data from a centipede (Chipman and Akam, 2008), have been interpreted as revealing an ancestral Notch-based mechanism of arthropod segmentation. Further, they have been proposed to support the hypothesis of a common origin of segmentation across the Bilateria (Stollewerk et al., 2003; Schoppmeier and Damen, 2005; De Robertis, 2008; Pueyo et al., 2008). Recent data from the cricket *Gryllus bimaculatus* also suggest that Notch signalling plays some role in segmentation (Mito et al., 2011).

However, the Notch pathway plays multiple roles in metazoan development (Artavanis-Tsakonas et al., 1999), and several complex developmental processes take place simultaneously with segment patterning in short-germ arthropods, including neurogenesis, axis elongation and apoptosis. Expression of Notch pathway genes in the short-germ insects *Tribolium castaneum* (beetle) and *Schistocerca gregaria* (locust) do not suggest roles in early segment generation (Dearden and Akam, 2000; Aranda et al., 2008). Moreover, in *G. bimaculatus*, Notch signalling is maternally

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required for growth zone maintenance and posterior elongation (Mito et al., 2011). It is thus unclear whether the zygotic processes of segment patterning and morphogenesis also specifically require Notch signalling.

We have investigated whether putative 'segmentation phenotypes' caused by Notch pathway disruption in short-germ arthropods could be due to defects in embryogenesis unconnected with a role in segment patterning per se. In orthopterans (crickets, grasshoppers and locusts), the Notch ligand *Delta* has a complex expression pattern throughout embryogenesis (Dong and Friedrich, 2005), and maternal knockdowns result in apparent segmentation disruption (Mito et al., 2011). However, the causes of segment malformation remain unknown, and zygotic requirements for *Delta* have not yet been examined. We therefore investigated the role of zygotic Notch signalling during segmentation in *G. bimaculatus*, a short-germ insect of the orthopteroid insect orders that branch basal to *Drosophila* and all other higher insects (supplementary material Movie 1).

MATERIALS AND METHODS

Gene cloning and phylogenetic analysis

A *Delta* sequence was recovered from *G. bimaculatus* cDNA by degenerate PCR and RACE. Identity was confirmed through Bayesian analysis using MrBayes 3.1.2 MPI (Huelsenbeck and Ronquist, 2001; Altekar et al., 2004) with mixed amino acid fixed-rate models, two independent searches, four chains, 25% burn-in of trees and 1000 generation sample frequency. The dataset reached convergence within 2×10^6 generations with standard deviation of split frequencies below 0.01 for the two independent searches. The final consensus tree was visualised in Dendroscope 2.3 (Huson et al., 2007) and edited in Illustrator CS3 (Adobe). Sequence data from this study have been submitted to GenBank under accessions JF339970 and JF339971.

G. bimaculatus culture

G. bimaculatus laboratory culture was established with animals from Livefoods Direct (Sheffield, UK). Species identity was confirmed by analysis of Cytochrome B and 16s rRNA sequences (F. Kainz, PhD thesis, University of Cambridge, 2009). Crickets were reared at 28°C with a 12 hour light/12 hour dark cycle and fed with dry cat food (Purina Kitten Chow), whole grain cereals and Cricket Quencher water gel (Fluker Farms).

Embryo collection, dissection and fixation

Eggs were collected in moist cotton wool or sand, freed in tap water and incubated on filter paper at 28°C. Embryos were hand-dissected in 1× PBS pH 7.4, fixed in 3.7% formaldehyde in 1× PBS at 4°C overnight (in situ) or for 30-120 minutes at room temperature (immunostaining), and stored in 100% methanol at –20°C (in situ) or in 1× PBS (immunostaining). For split germ band experiments, embryos were bisected with a microscalpel after fixation.

In situ hybridisation

Whole-mount in situ hybridisation was carried out according to standard protocols.

Zygotic RNAi

Double-stranded (ds) RNA was prepared for 526 bp of the *Gb-Dl* coding region and for 678 bp of the *DsRed* coding region by amplifying a PCR product from cDNA plasmids with the T7 promoter sequence at both ends (supplementary material Table S1), purifying with the Qiagen PCR Purification Kit (28104), and using 300-500 ng as template for in vitro transcription (Ambion T7 MEGAscript Kit, AM1334). dsRNA concentration was measured by NanoDrop (Thermo Scientific) and gel electrophoresis. dsRNA was resuspended in saline solution (5 mM KCl, 10 mM NaH_2PO_4) (Spradling, 1986) containing 5% Alexa 488-coupled Dextran (Invitrogen D22910) to 5-6 μg/μl for injection.

Quantitative PCR

Injected (four biological replicates) and uninjected (three biological replicates) eggs from the same collection were aged 48-55 hours post-injection, flash frozen in liquid N_2, and stored at –80°C for 3 days prior to RNA isolation. RNA was isolated from whole eggs with Trizol (Invitrogen 15596-026) using standard protocols and 2 μl 25 mg/ml GenElute LPA (Sigma 56575) for precipitation, resuspended in DEPC-treated H_2O, and treated with TURBO DNase (Ambion AM2238) at 37°C for 30 minutes. After inactivation at 95°C for 15 minutes, RNA was extracted with phenol/chloroform and resuspended in RNase-free H_2O. cDNA was synthesised with qScript cDNA SuperMix (Quanta Biosciences 95048). Quantitative PCR was carried out using a Stratagene MX3005P with PerfeCTa SYBR Green Super Mix, UNG, Low ROX (Quanta Biosciences 95070) and the primers shown in supplementary material Table S1. Ct values were obtained with MxPro version 4.10 (Agilent). Ct values [normalised and standardised, calculated as described by Livak and Schmittgen (Livak and Schmittgen, 2001)] for *dsRed* dsRNA-injected and uninjected samples were not significantly different for any genes tested ($P<0.05$, independent one-sample *t*-test). *Dl* dsRNA-injected and uninjected samples showed significant differences ($P<0.05$, unequal variance two-tailed *t*-test) between *Dl* RNAi embryos and controls for *Dl* but not for *tubulin*.

Immunostaining

Immunohistochemistry was according to standard protocols and used the following primary antibodies: rabbit anti-β-catenin (Sigma C2206) 1:100; mouse anti-acetylated tubulin (Sigma T6793) 1:100; rabbit anti-cleaved caspase 3 (Cell Signaling 9661) 1:100; mouse anti-Pax3/7 (DP311; a gift from Greg Davis, Bryn Mawr College, PA, USA) 1:20; and anti-HRP (a gift from Sam Kunes, Harvard University, MA, USA) 1:50; secondary antibodies were goat anti-rabbit and goat anti-mouse coupled to Alexa 488, 555, 568, 633 or 647 (Invitrogen) at 1:1000.

Imaging and image analysis

In situ hybridisation images were captured with AxioVision version 4.8 (Zeiss) driving a Zeiss Stereo Lumar microscope with an AxioCam MRc camera, and a Zeiss AxioImager microscope with an AxioCam MRm camera and Apotome. Confocal microscopy was performed with a Zeiss LSM 710 microscope using comparable gain, offset and averaging parameters for all samples. Image analyses and assembly were performed with AxioVision version 4.8, Zen 2009 (Zeiss), Helicon Focus Pro version 4.1.1 (HeliconSoft), Photoshop, Illustrator or AfterEffects CS4 (Adobe).

RESULTS

Identification of a *G. bimaculatus Delta* homologue (*Gb-Dl*)

A *G. bimaculatus Delta* orthologue was recently identified (Mito et al., 2011). We independently identified this gene (*Gb-Dl*), and confirmed its identity by phylogenetic analysis (supplementary material Fig. S1A). Deep sequencing of a ~1.5×10^9 bp *G. bimaculatus* developmental transcriptome representing over 16,000 unique protein-coding genes did not reveal alternative *Dl* homologues (V. Zeng and C.G.E., unpublished observations), and other annotated arthropod genomes contain a single *Delta* homologue (supplementary material Table S2). We therefore believe that this gene represents the single *G. bimaculatus Delta* orthologue.

Abdominal expression of *Gb-Dl* does not oscillate

A recent description of *Gb-Dl* expression during abdominal segmentation focused largely on the very early and very late stages of segment patterning (Mito et al., 2011). To investigate the possibility of a Notch-based 'segmentation clock' in the cricket, we asked whether *Gb-Dl* expression oscillated in abdominal segments throughout the duration of segment patterning (supplementary material Movie 1; Fig. 1A). We collected embryos at short intervals

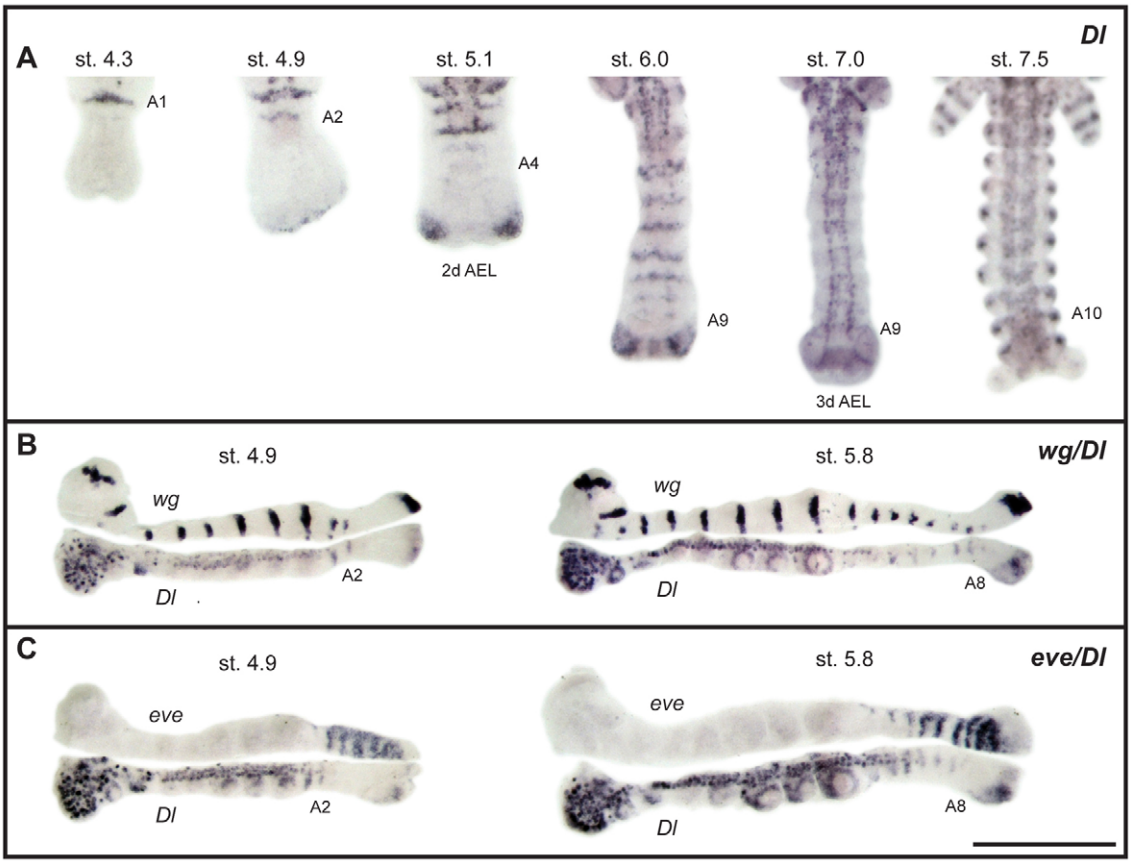

Fig. 1. *Delta* expression during abdominal segment patterning in *G. bimaculatus*. (**A**) Before abdominal segment patterning begins (st. 4.3), *Dl* is expressed in the nervous system, but not in the abdomen. During abdominal segmentation (st. 4.9-7.5), *Dl* expression arises in stripes corresponding to each abdominal segment. (**B**) In situ hybridisation for *Dl* (bottom) and *wg* (top) on different halves of the same embryo. At early (st. 4.9) and late (st. 5.8) stages of abdominal segmentation, *Dl* expression does not precede *wg* expression. (**C**) In situ hybridisation for *Dl* (bottom) and *eve* (top) on different halves of the same embryo. At early (st. 4.9) and late (st. 5.8) stages of abdominal segmentation, *eve* is expressed before *Dl* in abdominal segments. The most recently patterned (posteriormost) abdominal segment is labelled in each panel. Anterior is up in A and to the left in B,C. See also supplementary material Fig. S2, Movie 1. A1, A2, A4, A8, A9, A10, abdominal segments. Scale bar: 500 μm.

(1-6 hours) and determined embryonic stages by comparing morphology and length of the post-thoracic region. Consistent with what has been described for other short-germ insects (Aranda et al., 2008; Pueyo et al., 2008), expression of *Gb-Dl* in abdominal segments precedes the formation of the segmental furrows that define segments morphologically [Fig. 1A, stage (st.) 6.0]. However, in contrast to reports on a cockroach (Pueyo et al., 2008), *Gb-Dl* expression in abdominal segments does not precede the formation of mesoderm (supplementary material Movie 2). As in other short-germ insects (Roonwal, 1936; Handel et al., 2005), gastrulation and mesoderm formation take place several hours before the stage at which abdominal *Gb-Dl* is first expressed (Fig. 1A; supplementary material Movie 2).

Gb-Dl expression does not fade from anterior segments as posterior segments are added; once *Gb-Dl* expression is established in a given abdominal stripe, it remains through to the end of abdominal segment patterning without oscillating (Fig. 1A, st. 6.0). Although *Gb-Dl* is transiently expressed in the posterior growth zone prior to abdominal segmentation (Mito et al., 2011), in contrast to what was reported for a centipede (Chipman and Akam, 2008), no waves of expression appeared to emanate from that domain or in the posterior region (Fig. 1A), and this expression domain disappears before the appearance of the first abdominal stripe (Mito et al., 2011). We did not observe variations in the expression pattern in embryos of the same developmental stage. The posterior expression of *Gb-Dl* arising 2 days (d) after egg-laying (AEL) corresponds to the cerci primordia (Fig. 1A, st. 5.1), and persists until just before the end of abdominal segmentation (Fig. 1A, st. 7.0). In summary, abdominal *Gb-Dl* expression did not appear to be dynamic or cyclic, but rather was detected in stable domains at all stages examined.

Early segment patterning takes place before *Gb-Dl* is expressed in the abdomen

We then compared *Gb-Dl* expression with that of known segment patterning genes (Fig. 1B,C). *wingless* (*Gb-wg*) is expressed in all segments in *G. bimaculatus* before the formation of morphological segment boundaries (Niwa et al., 2000), and has a conserved segment polarity function in both long-germ and short-germ insects (Oppenheimer et al., 1999; Miyawaki et al., 2004). A recent study performed colourimetric double in situ hybridisations for *Gb-wg* and *Gb-Dl* and concluded that *Gb-wg* expression preceded and overlapped with *Gb-Dl* expression in each abdominal segment (Mito et al., 2011). However, we have found that overlapping expression patterns make clear interpretation of colourimetric double stainings difficult, especially at low levels of expression

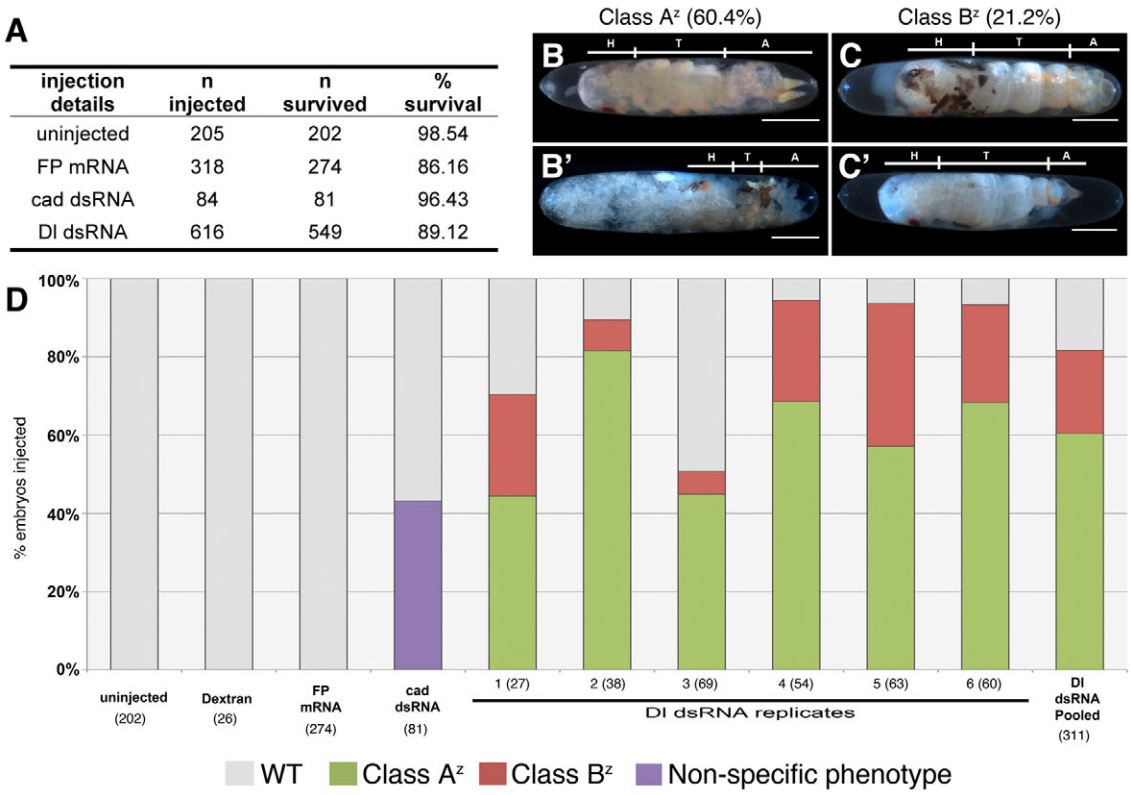

Fig. 2. Zygotic *Dl* RNAi yields reproducible embryonic phenotypes. (**A**) Embryos injected with *Dl* dsRNA show a survival rate of 89.12%, which is comparable to uninjected controls and to embryos injected with nonspecific RNAs [FP, fluorescent protein mRNA: mRNAs for DsRed (Baird et al., 2000) (*n*=110), Dendra (Gurskaya et al., 2006) (*n*=187) or GFP (Shimomura et al., 1962) (*n*=21)] or dsRNA for *G. bimaculatus caudal* (*cad*). (**B**,**C**) Zygotic *Dl* RNAi phenotypic classes. Two examples each are shown of class A^z (B,B′) and class B^z (C,C′) phenotypes. Anterior is to the left. H, head; T, thorax; A, abdomen. Scale bars: 500 μm. (**D**) Phenotypic class frequencies of embryos injected with *Dl* dsRNA. *Dl* phenotypes are specific to injection of *Dl* dsRNA. Numbers in parentheses indicate the number of embryos that survived and were scored.

(not shown). We therefore analysed the expression of *Gb-Dl* and *Gb-wg* in the same embryo bisected along the anterior-posterior axis and subject to in situ hybridisation for different genes on each half (Fig. 1B,C). *Gb-Dl* was expressed at the same time as, but not before, *Gb-wg* in abdominal stripes at both early (Fig. 1B, left) and late (Fig. 1B, right) stages of abdominal segmentation (*n*=25). Overstaining embryos did not reveal the presence of *Gb-Dl* stripes prior (posterior) to *Gb-wg* stripes (not shown). The strong staining consistently seen in the central nervous system (Fig. 1B) and detection of expression of other genes in the *Gb-Dl*-negative abdominal region at these stages [*even-skipped* (Fig. 1C) and *caudal* (F. Kainz, PhD thesis, University of Cambridge, 2009)], confirmed that the lack of staining in the posterior abdomen was not due to technical problems with the in situ probe, nor to unusual characteristics of that region of the embryo. The absence of segmentally patterned *Gb-Dl* expression that precedes *Gb-wg* expression strongly suggests that *Gb-Dl* does not play a role in early segmentation.

To further explore whether *Gb-Dl* might be involved in establishing segments, we also compared expression of *Gb-Dl* with that of *even-skipped* (*eve*) using the split embryo approach described above. In all arthropods examined to date (including *G. bimaculatus*), *eve* expression is required for, and/or precedes, expression of segment polarity genes including *wg* (Patel et al., 1992; Mito et al., 2007), and loss of *eve* function results in loss of segments. *Gb-eve* was expressed before *Gb-Dl* in all abdominal segments (*n*=13; Fig. 1C). For example, at st. 4.9 (just before 2d AEL), when only two *Gb-Dl* stripes are present, the domain posterior to the second *Gb-Dl* stripe is already patterned by six stripes of *Gb-eve* (Fig. 1C, left). Similarly, at st. 5.8 (2.5d AEL), when *Gb-Dl* expression is present in abdominal segments A1-A8, all eight of these stripes also express *Gb-eve*, but *Gb-eve* has additionally patterned A9 and A10, which do not yet express *Gb-Dl* (Fig. 1C, right). We confirmed that *Gb-eve* segmental expression precedes *Gb-Dl* expression at all stages of abdominal segmentation (supplementary material Fig. S2). Overall, these expression data indicate that early abdominal segment patterning is likely to involve *eve* and *wg*, but not Notch/Delta signalling.

Zygotic *Gb-Dl* function is not required for segment formation

A recent study reported that mothers injected with *Gb-Dl* dsRNA laid a high proportion of apparently undeveloped embryos, and a minority of embryos displayed a loss of terminal structures and abdominal segments and exhibited posterior elongation defects (Mito et al., 2011). This suggests that a maternal requirement in posterior growth zone maintenance might be responsible for the apparent defects in abdominal segment patterning. We detected *Gb-Dl* expression ubiquitously in oocytes (not shown), consistent with a maternal provision of *Delta* that might participate in early growth zone function. If this is the case, it might obscure a later zygotic role for *Delta* specifically in segment patterning, as distinct from

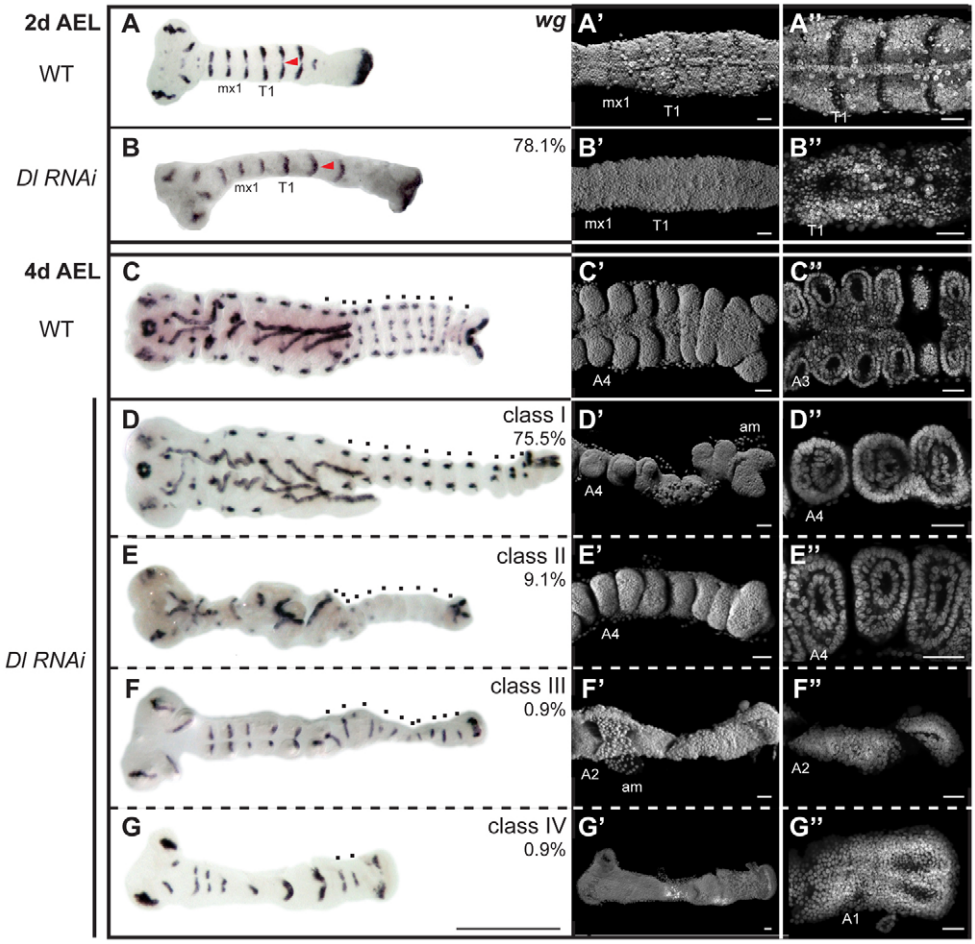

Fig. 3. Zygotic *Dl*^{RNAi} *G. bimaculatus* embryos develop all abdominal segments. (**A-G″**) The effect of zygotic *Dl* RNAi on abdominal segmentation assessed by in situ hybridisation for *wg* (A-G) and examination of external (A′-G′) and internal (A″-G″) segment morphology at 2 days (d) (A,B) and 4d (C-G) after egg laying (AEL). The three images in each row show *wg* expression (left), a 3D reconstruction of the thorax (A,B) or abdomen (C-G) (middle), and a 2D projection of multiple (A″,B″,F″,G″) or a single (C″,D″,E″) optical section of Hoechst-stained embryos (right). All images in a given row are of the same embryo, except for rows C and D, where C′,C″ and D-D″ are images of the same embryo, and C and D are different embryos from the same phenotypic class. Black dots in C-G indicate abdominal segments. am, amnion (extra-embryonic membrane); mx1, first maxillary segment; T1, first thoracic segment. WT, wild type. The anteriormost abdominal segment is labelled in C″-G″. Anterior is to the left in all panels. Scale bars: 50 μm, except 500 μm in A-G.

posterior elongation. Moreover, although maternal RNAi can sometimes interfere with zygotic gene function in insects (e.g. Panfilio et al., 2006), we have found that for some *G. bimaculatus* genes, maternal RNAi achieves effective knockdown of transcript levels in early embryos but that normal transcript levels are recovered between 2d and 4d AEL (B.E.-C. and C.G.E., unpublished), which are the critical stages for abdominal segment patterning. We therefore performed zygotic RNAi (Miyawaki et al., 2004) by injecting embryos at 3-5 hours AEL with dsRNA targeting *Gb-Dl* (Fig. 2A).

We first scored embryos by visualisation through the transparent eggshell shortly before hatching, and observed two classes of phenotypes (Fig. 2B,C). Class A^{z} embryos (60.4%) developed abdominal regions that were shortened (Fig. 2B) or reduced in width (Fig. 2B′). Class B^{z} embryos (21.2%) showed segments that were morphologically distinct but abnormally shaped, and the whole embryo was reduced in length (Fig. 2C,C′). These phenotypes were reproducible (n=311 across six technical replicates), specific to *Gb-Dl* (Fig. 2A), and correlated with absent or severely reduced levels of *Gb-Dl* transcript at all developmental stages examined (supplementary material Fig. S3), confirming that we had achieved zygotic knockdown of *Gb-Dl*.

To determine the role of *Gb-Dl* during the process of abdominal segmentation, we examined segment morphology and *wg* expression in *Dl*^{RNAi} embryos (Fig. 3). At early stages of abdominal segmentation (2d AEL, one abdominal *wg* stripe), all segments of *Dl*^{RNAi} embryos still expressed *wg* (n=57; Fig. 3A,B). However, the gap in *wg* expression at the ventral midline seen in wild-type embryos was missing in *Dl*^{RNAi} embryos, suggesting loss of ventral tissue. A maternal RNAi study had also suggested loss of ventral tissue, but did not determine the nature of the tissue lost (Mito et al., 2011). We performed cellular-resolution analysis of *Dl*^{RNAi} embryos and detected loss of a ventral strip of cells, 3-4 cells wide (Fig. 3B′,B″; supplementary material Movie 2). This tissue is analogous to the mesectoderm in *Drosophila*, a ventral midline tissue that requires Notch signalling for specification (Menne and Klambt, 1994; Martin-Bermudo et al., 1995; Morel and Schweisguth, 2000).

At later stages of abdominal segmentation (4d AEL, ten abdominal *wg* stripes), *Dl*^{RNAi} embryos had formed all ten abdominal segments (this count of ten excludes the terminal cerci-bearing segment; n=110; Fig. 3C-F). Embryos fell into four *Dl*-specific phenotypic classes (Fig. 3D-G). Class I embryos (75.5%; Fig. 3D) had all ten abdominal segments, but showed the same loss of ventral tissue observed at earlier stages (Fig. 3C). Abdominal segments of wild-type embryos at this stage have two lateral mesodermal coelomic pouches separated by the ventral neuroectoderm (Fig. 3C′,C″). By contrast, *Dl*^{RNAi} class I embryos have both coelomic pouches fused into a single pouch and lack ventral neuroectoderm (Fig. 3D′,D″). Class II embryos (9.1%; Fig. 3E,E′) had all ten abdominal segments, which were morphologically distinct but had little or no *wg* expression. These embryos displayed the same coelomic pouch fusion seen

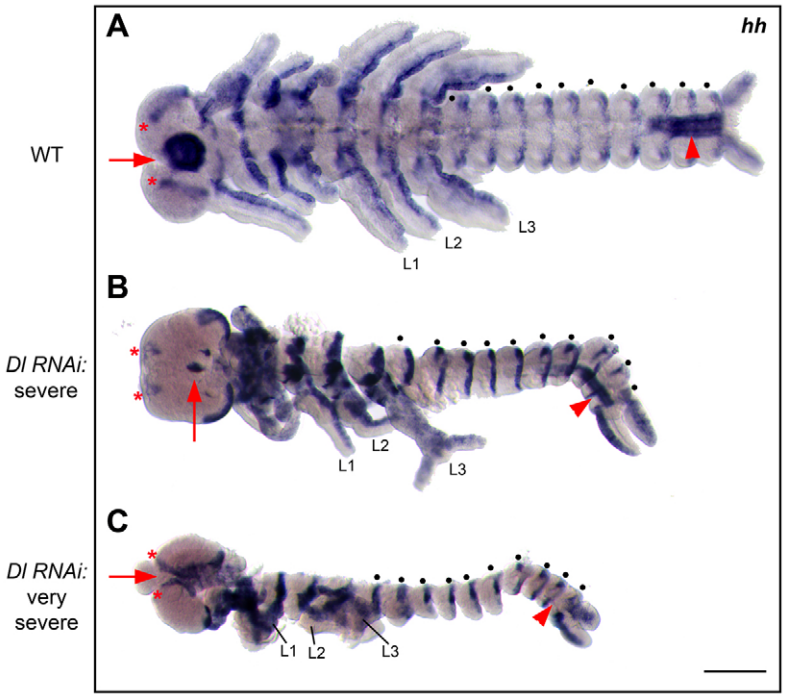

Fig. 4. *hedgehog* expression in Dl^{RNAi} *G. bimaculatus* embryos at 4d AEL. (**A**) Wild-type in situ hybridisation for *hedgehog* (*hh*). (**B**) Dl^{RNAi} embryo with a severe phenotype retains *hh* expression in all abdominal segments and in the hindgut (arrowhead). Arrow indicates the stomodaeal expression domain; asterisks indicate optic lobe expression domains. (**C**) A Dl^{RNAi} embryo with a stronger phenotype than that shown in B still retains *hh* expression in all abdominal segments, but lacks the hindgut (arrowhead). The stomodaeal expression domain is absent (arrow), and optic lobe expression domains (asterisks) are adjacent. Anterior is to the left. L1, L2 and L3, first, second and third thoracic legs. Scale bar: 200 μm.

in class I embryos (Fig. 3E,E′). One out of 110 embryos (class III, 0.9%) had ten abdominal *wg* stripes (Fig. 3F), indicating that abdominal segment patterning had proceeded normally, but lacked the morphological distinction of segments seen in class I and II embryos (Fig. 3F′,F″). The thoracic and head morphologies of class III embryos indicated that they were developmentally delayed compared with over 85% of embryos examined (compare Fig. 3F with 3C-E). Finally, a single embryo out of 110 (class IV, 0.9%) possessed only two abdominal *wg* stripes (Fig. 3G) and no morphological segment distinction (Fig. 3G′,G″). This degree of abdominal segmentation, as well as the head and thoracic morphologies of this embryo, are equivalent to those of a wild-type embryo at ~2d AEL (compare Fig. 3G with 3A), indicating a developmental delay in all embryonic processes rather than a defective abdominal segmentation programme.

These data indicate that generic developmental delays, rather than specific segment patterning roles for Notch/Delta, may contribute to the reported loss of segments caused by maternal RNAi for *Notch* and *Delta* (Mito et al., 2011). Class III and IV phenotypes appeared in fewer than 2% of embryos, and as such are clearly not representative of the *Gb-Dl* loss-of-function phenotype. However, we present them here because they illustrate that a low frequency of overall developmental delay phenotypes can appear very similar to the segmentation phenotypes that we sought to investigate in this study. In summary, in contrast to embryos depleted of maternal *Gb-Dl*, the vast majority of zygotic Dl^{RNAi} embryos do not exhibit loss of terminal structures, abdominal or thoracic segments, and the small minority of embryos with fewer *Gb-wg* stripes than controls are severely developmentally delayed.

We also examined expression of the segmental marker *hedgehog* (*Gb-hh*) in Dl^{RNAi} embryos (Fig. 4A). As for *Gb-wg*, ten abdominal stripes of *Gb-hh* were present in Dl^{RNAi} embryos, corresponding to ten morphologically defined segments (Fig. 4B,C). Our analysis of knockdown phenotypes over developmental time thus reveals that Notch/Delta signalling is not required for early abdominal segment patterning, nor for the formation of morphological segment boundaries, in *G. bimaculatus*.

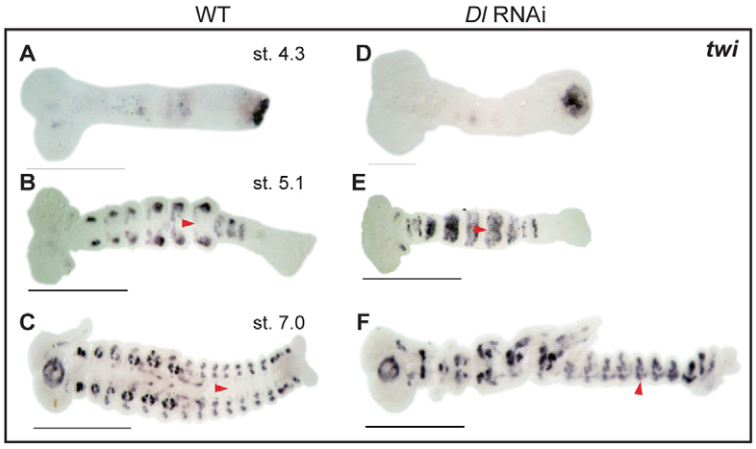

Fig. 5. *Dl* is not required for mesodermal specification. In situ hybridisation for *twist* (*twi*) in wild-type (**A-C**) and Dl^{RNAi} (**D-F**) *G. bimaculatus* embryos. All segments of Dl^{RNAi} embryos specify mesoderm, but lack the central *twist*-free domain along the ventral midline that is present in wild-type embryos (arrowheads). Anterior is to the left in all panels. Scale bars: 500 μm.

Gb-Dl knockdown causes loss of mesectoderm and excess caspase-dependent apoptosis in the nervous system

We noted that in Dl^{RNAi} embryos, although all abdominal segments were formed, segment morphologies were abnormal (Fig. 3D′-G′; Fig. 4B,C). Specifically, we observed that ventral domains of the abdomen were more affected by loss of *Gb-Dl* than lateral or dorsal domains. Recent maternal RNAi experiments reported similar phenotypes, but did not determine the causes of these segment malformations (Mito et al., 2011). To investigate the origin of these abdominal abnormalities and determine the role of *Dl* in wild-type *Gryllus* development, we examined cell fate allocation and morphogenesis of specific cell types in Dl^{RNAi} embryos.

As recently reported (Mito et al., 2011), we observed that the posterior region of Dl^{RNAi} embryos retained expression of the posterior marker *caudal* (not shown), consistent with a correctly established posterior growth zone. Formation and identity of mesoderm was also normal in Dl^{RNAi} embryos (Fig. 5). However, as noted above, we observed that at 2d AEL the ventral mesectoderm was absent (Fig. 6B,B′,F; supplementary material Movie 2). This absence appeared to be due to a lack of specification rather than cell death, as apoptosis levels throughout the germ band were only slightly higher than in controls at 2d AEL (not shown). By contrast, by 4d AEL, whereas the single fused abdominal coelomic pouches retained mesodermal identity (Fig. 5F), nearly all nuclei of ventral cells lying between the coelomic pouches and the overlying ectoderm were pycnotic (Fig. 6H) and undergoing caspase-mediated apoptosis (Fig. 7B′-D′). In Dl^{RNAi} embryos, this apoptosis sets in after the generation and morphological definition of abdominal segments (Fig. 7). This phenotype is not confined to the abdomen, but occurs throughout the embryo, including the head, trunk and thoracic appendages (supplementary material Figs S4-S6). This apoptosis results in massive loss of abdominal ventral tissue and subsequent collapse of the ventral midline, leading to the observed coelomic pouch fusion (Fig. 3D,E; Fig. 6H). In severely affected embryos, the invaginating hindgut (a medial structure) is also absent (Fig. 4C, arrowhead). Also consistent with the loss of midline tissue is the reduction or loss of the stomodaeum (Fig. 4C,D, arrows) and the spacing of the optic lobe *Gb-hh* expression domains, which are closer together in Dl^{RNAi} embryos than in controls (Fig. 4C,D, asterisks).

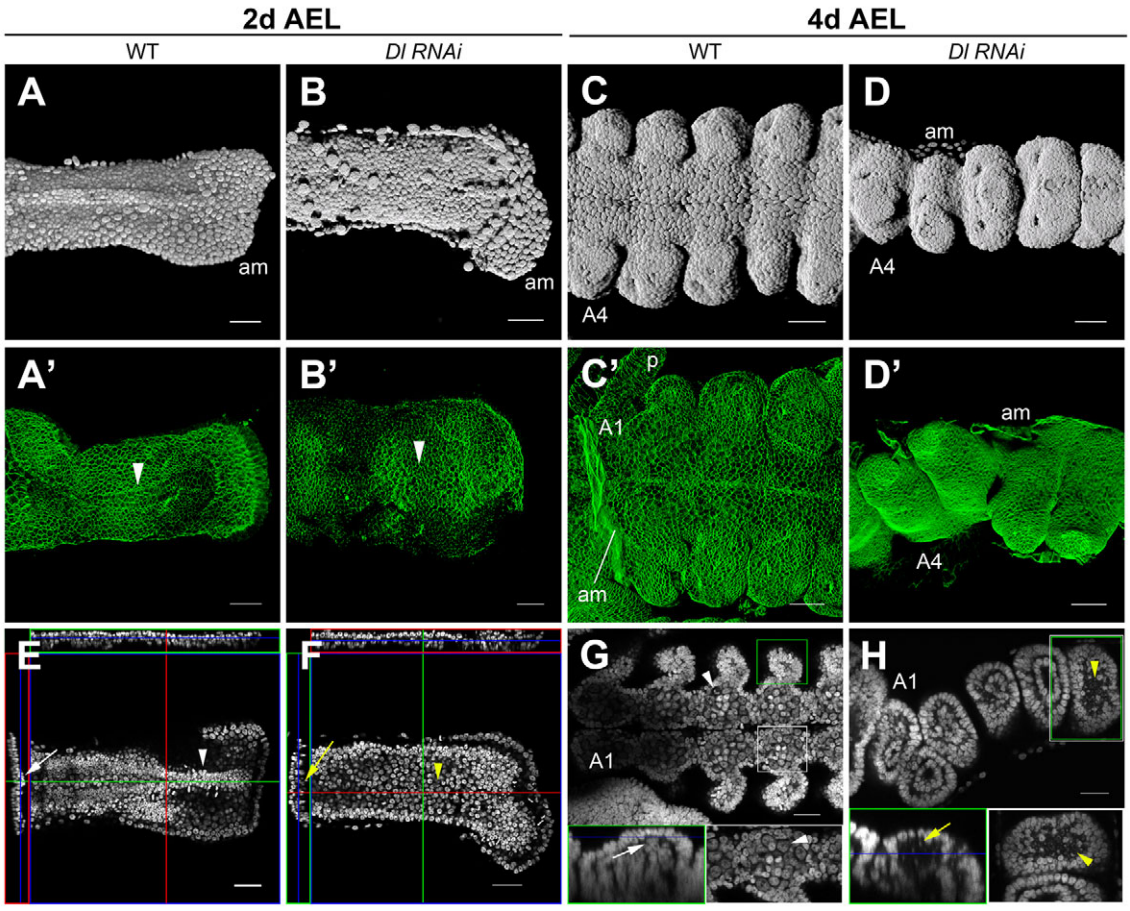

Fig. 6. *Dl* RNAi results in specific loss of midline tissue during abdominal segmentation. Wild-type or Dl^{RNAi} *G. bimaculatus* embryos were analysed at 2d or 4d AEL. (**A-D**) Three-dimensional reconstructions of embryos. (**A′-D′**) β-Catenin immunostaining (green) of embryonic abdominal segments. Distinct cell shapes of ventral midline central nervous system tissue are missing in Dl^{RNAi} embryos (arrowheads). (**E-H**) Single optical sections of Hoechst-stained embryos. (E,F) Green and red lines indicate orthogonal section planes of the central image; left and top boxes show the orthogonal section outlined in the corresponding colour. Blue line indicates plane of central image in each orthogonal section image. Arrowheads indicate that central nervous system tissue along the ventral midline (arrows) is missing in Dl^{RNAi} embryos. (G,H) Boxed regions are shown at higher magnification beneath. Neuroectoderm (white inset, arrowheads) visible in orthogonal section (green inset, arrows) has pycnotic nuclei in Dl^{RNAi} embryos. The anteriormost abdominal segment is labelled in each panel. Anterior is to the left in all panels. am, amnion; p, pleuropodium. Scale bars: 50 μm.

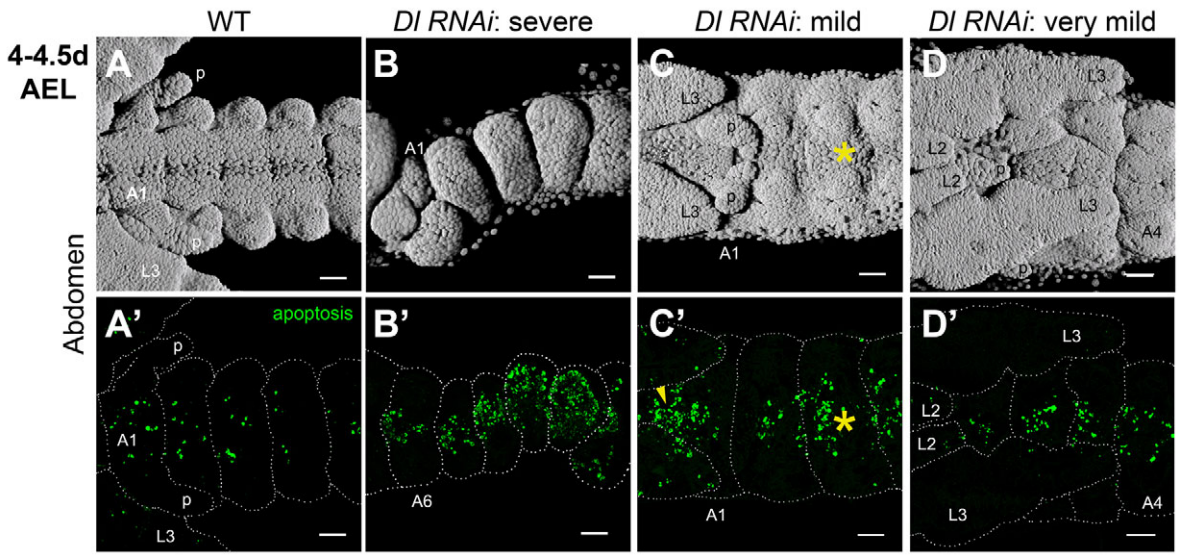

Fig. 7. *Dl* RNAi upregulates caspase-mediated apoptosis of midline tissue. (**A-D**) Three-dimensional reconstructions of *G. bimaculatus* embryos showing Dl^{RNAi} phenotypes ranging from severe to very mild. (**A′-D′**) Immunostaining for anti-cleaved caspase 3 on the same embryos shown in A-D, except for B, which shows a different embryo of the same phenotypic class. White dotted outlines indicate segment boundaries and lateral embryo borders. (C,C′) Embryos with mild Dl^{RNAi} phenotypes show high levels of apoptosis both in midline neuroectoderm tissue with pycnotic nuclei (arrowhead) and in midline neuroectoderm tissue that still appears superficially wild-type (asterisks). (D,D′) Even apparently mild phenotypes show high levels of midline apoptosis compared with controls (A′). The pleuropodium marks the first abdominal segment in A,C,D; additional abdominal segments (A1, A4, A6) are labelled for clarity. L2 and L3, second and third thoracic legs; p, pleuropodium. Anterior is to the left. Scale bars: 50 μm.

Loss of *Gb-Dl* function results in early neurogenic phenotypes and later hypoectodermal phenotypes

We examined whether the observed apoptosis was due to an overall, nonspecific abdominal growth disruption, or reflected a specific role for Notch/Delta signalling in cell or tissue differentiation. Consistent with the latter hypothesis, as noted above, we observed that the tissue lost in Dl^{RNAi} embryos is the neuroectoderm (Fig. 3B, Fig. 6B), in agreement with a conserved role of the Notch pathway in neurogenesis (Lehmann et al., 1981; Lehmann et al., 1983; Artavanis-Tsakonas et al., 1999). In maternal *Gb-Dl* knockdowns, embryos were reported to show loss, hyperplasia and disorganisation of the nervous system (Mito et al., 2011), but how all of these phenotypes can manifest at a single developmental stage, or how they relate to segmentation, is unclear. These data were particularly difficult to interpret as the *Gb-Dl* knockdown embryos shown were clearly at a different developmental stage than control embryos (Mito et al., 2011), and the organisation of the nervous system changes radically over developmental time (Miyamoto, 1983; Miyamoto and Shimozawa, 1983). We therefore compared the progression of neuronal specification and patterning in zygotic Dl^{RNAi} embryos and wild-type embryos at each segmentation stage (Fig. 8).

At 2d AEL (Fig. 8B,B′) and early 3d AEL (Fig. 8D,D′), Dl^{RNAi} embryos show a classic neurogenic phenotype (Lehmann et al., 1983) of neuronal overproliferation in the thorax (Fig. 8B,B′), abdomen (Fig. 8D,D′) and head (not shown). This indicates that loss of *Gb-Dl* function results specifically in excess neuroblast specification at early germ band stages, and not in a generic loss of germ band organisation. By late 3d AEL, neurons were significantly reduced in number due to apoptosis (Fig. 8E,E′), and were almost entirely absent by 4d AEL (Fig. 8G′,H′; supplementary material Movie 3). The ventralmost neurons were the most vulnerable to *Gb-Dl* knockdown, and progressively lateral neurons were eliminated in more severe phenotypes (Fig. 8H,H′). These phenotypes were not confined to the abdominal segments, but were consistent throughout the anterior and thoracic nervous system (not shown). This demonstrates that the loss of ventral tissue that is responsible for segment malformations is the result of an early neurogenic effect of loss of zygotic *Gb-Dl* function, followed by apoptotic death of the ectopic tissue and a hypoectodermal condition at the ventral midline.

DISCUSSION

The evolution of animal segmentation is a complex problem that has been heavily debated in the literature (Davis and Patel, 1999; Seaver, 2003; Peel, 2004; Tautz, 2004; Chipman, 2010). These debates include ongoing discussion of the definition of a ‘segment’ and which phyla display ‘body segmentation’ (e.g. Schmidt-Rhaesa and Rothe, 2006) as opposed to simply possessing any serially repeated morphological unit (Budd, 2001; Scholtz, 2002), such as phytomers in plants (Howell, 1998). Traditionally, members of the arthropods, annelids (marine worms) and chordates are considered to have segmented body plans (Brusca and Brusca, 2003). Because these segmented body plans are highly complex, they were once considered to be derived from a common segmented ancestor (Grobben, 1908). However, it has since become clear that these phyla are not monophyletic but are more closely related to several unsegmented phyla than they are to each other (Aguinaldo et al., 1997). This raises the question of whether their segmented body plans arose independently or are the result of common ancestry (homology). Data from paleontology, morphology and molecular developmental analyses have been used to argue for both hypotheses (e.g. Couso, 2009; Chipman, 2010). Even within the protostomes, arthropods and annelids belong to distinct clades (Eernisse et al., 1992), each containing several unsegmented phyla

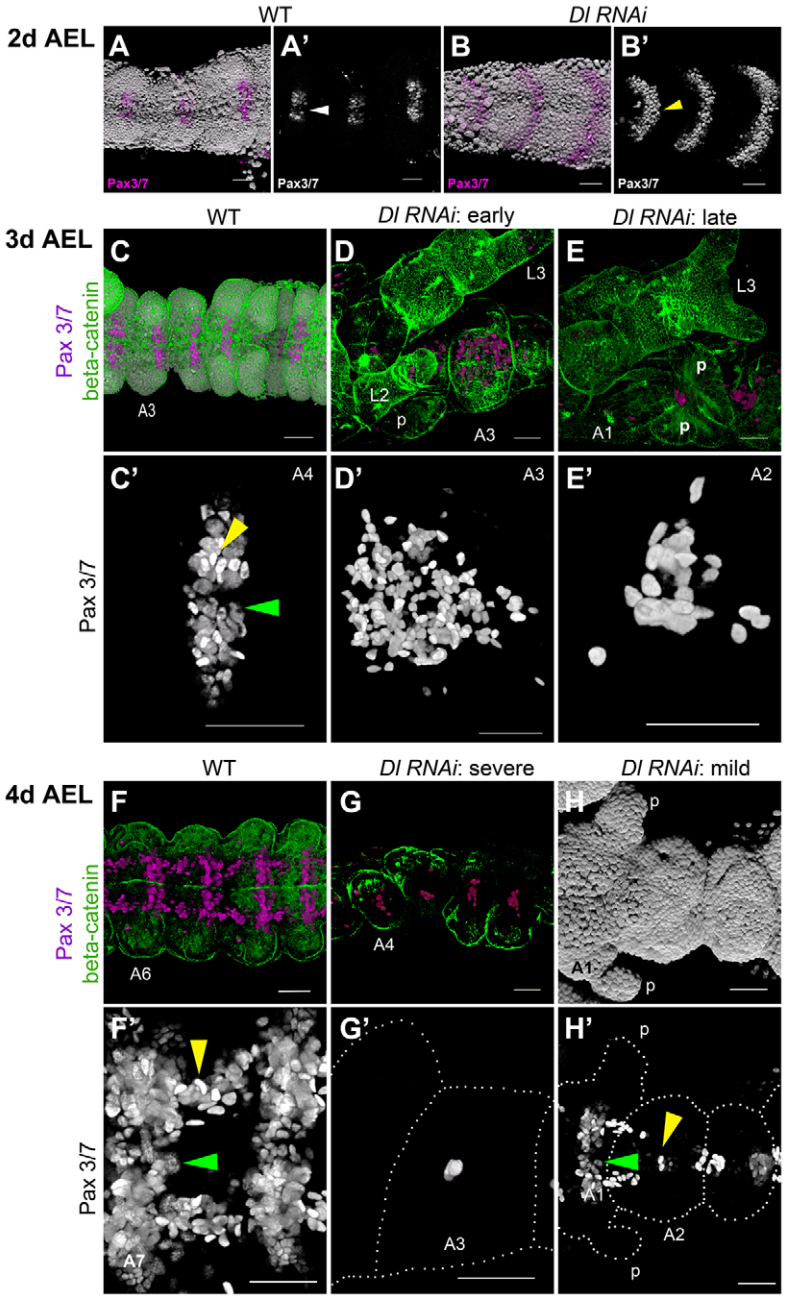

Fig. 8. *Dl*^{RNAi} embryos show early neurogenic phenotypes followed by neuronal and ectodermal loss. Pax3/7-expressing neurons (magenta) in the thorax and abdomen of wild-type (A,C,F) and *Dl*^{RNAi} (B,D,E,G,H) *G. bimaculatus* embryos throughout abdominal segment patterning (2-4d AEL). Green, anti-β-catenin. (**A-B'**) More neurons are specified in 2d AEL *Dl*^{RNAi} embryos (B,B') than in controls (A,A'). Loss of neuroectoderm in 2d AEL *Dl*^{RNAi} embryos (B) causes fusion of lateral neuronal bands (arrowhead in B'). (**D**) Neurogenic phenotype persists until early 3d AEL. (**C**,**E-H**) By late 3d and 4d AEL, *Dl*^{RNAi} embryos (E,G,H) have fewer neurons than controls (C,F). In D and E, loss of central thoracic tissue causes fusion of legs (L3). (**C'-G'**) Higher magnification of neurons in a single segment of embryos shown in (C-G); segment number at top right. Arrowheads indicate lateral neurons with strong Pax3/7 expression (yellow) and ventral neurons with weaker Pax3/7 expression (green). Dotted outline in G' indicates segment borders. (**H**) Three-dimensional reconstruction of a *Dl*^{RNAi} embryo 4d AEL with a phenotype that is variable across abdominal segments. (**H'**) Pax3/7 expression of the same embryo shown in H. A1 (first abdominal segment) shows a milder phenotype than A2, where all central Pax3/7-expressing neurons (green arrowhead) are absent and only a few lateral Pax3/7-expressing neurons (yellow arrowhead) remain. L2 and L3, second and third thoracic legs; p, pleuropodium. The anteriormost abdominal segment is labelled in each panel. Anterior is to the left. Scale bars: 50 μm.

(Brusca and Brusca, 2003). However, anatomical and molecular developmental data from annelids and arthropods (Scholtz, 2002; Prud'homme et al., 2003; Dray et al., 2010; Janssen et al., 2010) suggest that a protostome ancestor might have been segmented. Approaching the even deeper question of whether a bilaterian ancestor had a segmented body plan is a challenge that should be explored at several phenotypic levels, including cellular behaviour, development and anatomy, and gene regulatory networks. Because Notch/Delta signalling is crucial for vertebrate segmentation, several studies have focused on this pathway as a major indicator of the possibility of a segmented bilaterian ancestor. In this context we investigated the precise role of this pathway in early segment patterning in a basal insect.

We have shown that *Gb-Dl* is expressed in an iterated pattern in the abdomen, but find no evidence for cyclic expression of *Dl* during cricket segmentation, in contrast to what has been observed in vertebrates with a Notch-coordinated segmentation clock (Jiang et al., 2000; Maruhashi et al., 2005). If *Gb-Dl* participates in a segmentation mechanism that patterns future segments before they become defined morphologically, its expression should precede the expression of the late segmental marker *Gb-wg* (Oppenheimer et al., 1999). However, *Gb-Dl* is expressed in abdominal segments only after expression of *Gb-eve* has been established, and simultaneously with the appearance of *Gb-wg*. Although maternal depletion of *Gb-Dl* results in loss of growth zone maintenance and in posterior elongation defects (Mito et al., 2011), our analyses of zygotic *Gb-Dl* knockdown show that Notch signalling is not specifically required for abdominal segment patterning. However, in a small percentage (<2%) of embryos, developmental delays can create an initial impression that segmentation is disrupted. Moreover, final segment morphologies are clearly abnormal at later stages, which can add to the impression of disrupted segmentation.

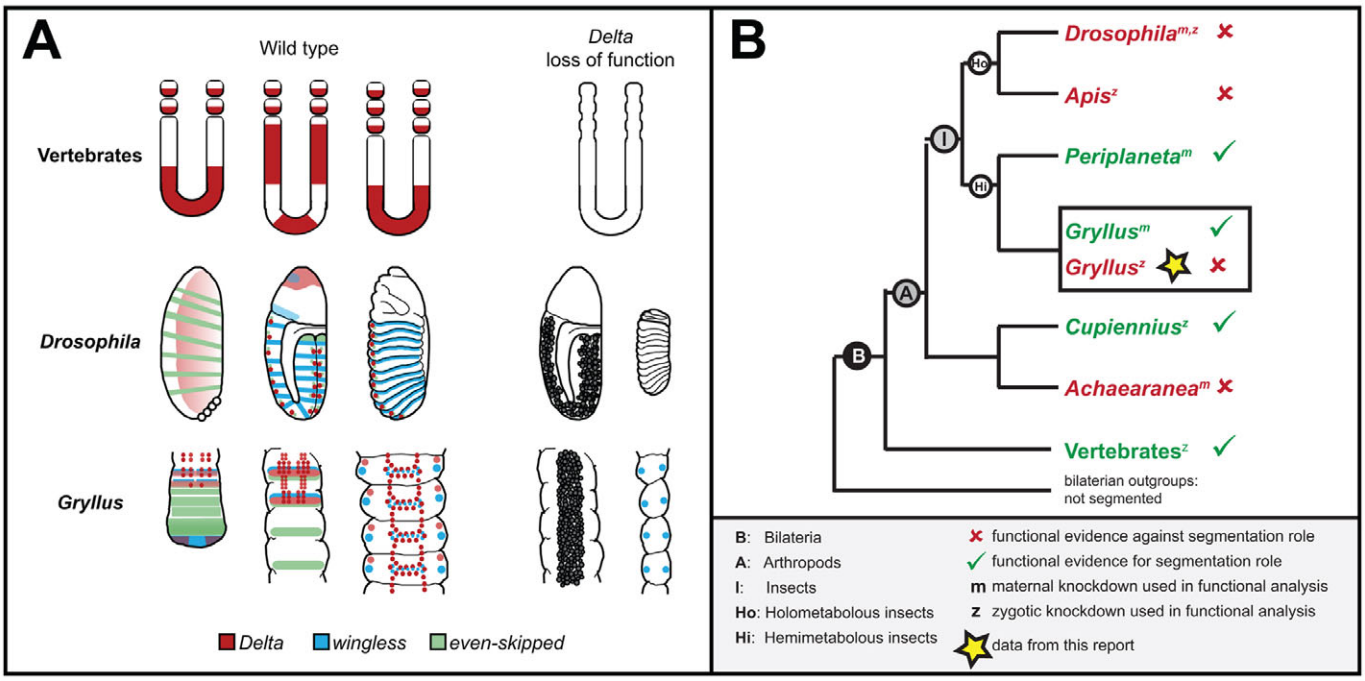

Fig. 9. Comparison of *Delta* loss-of-function phenotypes with respect to segment formation in animals. (**A**) Vertebrate somites form from anterior to posterior in response to oscillating gene expression, the maintenance of which involves the Notch pathway, including *Dl*. Loss of *Dl* function disrupts somitogenesis (Dequéant and Pourquie, 2008). In *D. melanogaster* embryos, *Dl* (red) is expressed early in neurogenic territories and later in the nervous system (Vässin et al., 1987; Kooh et al., 1993). Loss of *Dl* function causes neuroblast overproliferation (grey) and loss of ectoderm, but segment patterning and formation proceed normally (Lehmann et al., 1983). In *G. bimaculatus*, *Dl* is expressed early in neurogenic territories, later in the nervous system, and at lower levels in the ectoderm of abdominal segments following expression of the segmental markers *eve* (green) and *wg* (purple). Loss of *Gb-Dl* function causes neuroblast overproliferation and loss of ventral ectoderm, but segment patterning and formation proceed normally. (**B**) Phylogenetic context for the role of Notch/Delta signalling in arthropod and vertebrate segmentation based on functional studies (Lehmann et al., 1983; Stollewerk et al., 2003; Schoppmeier and Damen, 2005; Dequéant and Pourquie, 2008; Pueyo et al., 2008; Wilson et al., 2010; Mito et al., 2011). Data from this study (star) do not support an ancestral role of Notch singalling in segment generation across arthropods.

We have elucidated the causes of morphological abnormalities in Dl^{RNAi} embryos, and show that mesoderm specification is not affected by *Gb-Dl* knockdown. However, the mesectoderm is lost in early Dl^{RNAi} embryos, and early neurogenic phenotypes are followed by caspase-mediated apoptosis of the ectopic neural tissue. *Delta* loss of function also enhances caspase-dependent apoptosis in flies and mice (Murata-Ohsawa et al., 2004; Müller et al., 2005). Loss of ventral tissue in Dl^{RNAi} embryos is also apparent in previous studies on the function of Notch signalling in spider segmentation (Stollewerk et al., 2003; Schoppmeier and Damen, 2005). This suggests that conserved roles for Notch/Delta signals in apoptosis and neurogenesis can lead to secondary defects in segment morphologies. In summary, our data show that Notch-based signalling does not oscillate via *Delta* and is not required for early segment patterning or morphological formation of segment boundaries in the cricket *G. bimaculatus*.

Notch/Delta signalling in short-germ insect segmentation

Crickets are basally branching insects with respect to beetles and flies (Ishiwata et al., 2010). Our observations therefore do not support the hypothesis that Notch-based segmentation is ancestral for insects (Fig. 9). The apparent differential requirement for zygotic Notch signalling in two different basally branching insects, the cricket *G. bimaculatus* (this study) and the cockroach *P. americana* (Pueyo et al., 2008), means that establishing the relative phylogenetic position of these two insects is crucial to determining whether Notch-based segmentation is ancestral in insects. Many phylogenetic analyses of insects yield a sister group relationship for the clades Blattodea (containing cockroaches) and Orthoptera (containing crickets) (Kristensen, 1981; Wheeler et al., 2001; Ishiwata et al., 2010). However, some intraordinal analyses support a derived position for cockroaches relative to termites and mantids, the other members of its parent clade Dictyoptera (Maekawa et al., 1999; Kjer et al., 2006; Cranston and Gullan, 2009). This might mean that the involvement of Notch signalling in cockroach segmentation, rather than representing an ancestral insect state, could instead be a distinct cockroach-specific mechanism that is not shared by other short-germ insects.

Implications for the evolution of segmentation

To incorporate these insect data into a robust hypothesis for the ancestral mechanism of arthropod segmentation, functional data from the other major arthropod clades must be considered. Myriapods are the sister group to insects and crustaceans (Pancrustacea) (Regier et al., 2010), and as such are of interest in considering putative ancestral pancrustacean states. Unfortunately, functional data are not available for myriapods. However, although reported dynamic expression patterns of Notch pathway genes (Kadner and Stollewerk, 2004; Chipman and Akam, 2008) could be snapshots of oscillating expression, descriptions of early

embryogenesis suggest that extensive cellular movements are required for shaping the germ band, including the posterior growth zone, during segmentation (Heymons, 1901; Chipman et al., 2004). This makes it likely that cell movements contribute to the observed expression patterns. Cell rearrangements during axis elongation have not yet been examined in any short-germ arthropod, but contribute substantially to elongation during segmentation in vertebrates (Zhang et al., 2008). Future work will be necessary to determine the extent and role of these movements during sequential arthropod segmentation.

Chelicerates are likely to be the most basally branching arthropod clade (Regier et al., 2010). In this group, although zygotic loss of Notch signalling appears to disrupt segmentation in *C. salei* (Stollewerk et al., 2003; Schoppmeier and Damen, 2005), data from a different spider suggest that embryos defective in maternal Notch signalling could fail to form segments because of defective mesoderm-ectoderm cell fate decisions rather than a defect in segment patterning per se (Oda et al., 2007). These data are reminiscent of some of the differences that exist between the results of maternal (Mito et al., 2011) and zygotic (this study) knockdown in *G. bimaculatus*. However, this also demonstrates the difficulty of consolidating data from maternal and zygotic knockdowns performed in different species to infer evolutionary scenarios. Future studies that combine both types of knockdown experiments could be helpful in addressing the question of ancestral arthropod functions for Notch/Delta in segmentation.

This study highlights the importance of detailed phenotypic analysis for inferring deep homologies and establishing criteria for evolutionary developmental hypotheses. In short-germ arthropods, segmentation takes place simultaneously with several other developmental events, including mesoderm morphogenesis, neuroectoderm differentiation and neurogenesis. Consequently, disruption of several processes besides those required for segment patterning per se can yield what appear to be segmentation disruption phenotypes. Moreover, we found that examining RNAi phenotypes at several different developmental time points and contrasting the results of maternal and zygotic knockdown studies were crucial to understanding the roles of *Delta* in cricket embryogenesis. Following the progression of the *Dl* RNAi phenotype over time allowed us to detect tissue-specific effects on neural development, as well as generic developmental delays, which are a common feature of loss-of-function mutations in Notch pathway members (Ballard et al., 2010; Julian et al., 2010; Reis et al., 2011). These observations helped us to distinguish such neural and developmental delay phenotypes from specific effects on segmentation. Given the high degree of pleiotropy of Notch signalling in metazoan development, it is crucial to examine the complex spatiotemporal integration of multiple developmental processes when analysing and interpreting Notch pathway disruption phenotypes. Although whether Notch signalling was essential for an ancestral arthropod segmentation programme remains unknown, our work indicates that the molecular developmental basis for the hypothesis of a common origin of segmentation across bilaterians might be weaker than is often suggested.

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Competing interests statement
The authors declare no competing financial interests.

Author contributions
All authors designed the research; F.K., B.E.-C. and C.G.E. performed experiments, collected and analysed data; C.G.E. wrote the paper with input from F.K. and M.A.; C.G.E. and M.A. obtained funding for the research.

Supplementary material
Supplementary material available online at
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