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brachyenteron is necessary for morphogenesis of the posterior gut but not for anteroposterior axial elongation from the posterior growth zone in the intermediate-germband cricket *Gryllus bimaculatus*

Yohei Shinmyo*, Taro Mito, Tomohiro Uda, Taro Nakamura, Katsuyuki Miyawaki†, Hideyo Ohuchi and Sumihare Noji‡

In the long-germband insect *Drosophila*, all body segments and posterior terminal structures, including the posterior gut and anal pads, are specified at the blastoderm stage. In short- and intermediate-germband insects, however, posterior segments are sequentially produced from the posterior growth zone, a process resembling somitogenesis in vertebrates, and invagination of the posterior gut starts after anteroposterior (AP) axial elongation from the growth zone. The mechanisms underlying posterior segmentation and terminal patterning in these insects are poorly understood. In order to elucidate these mechanisms, we have investigated the roles of the *Brachyury/brachyenteron* (*Bra/byn*) homolog in the intermediate-germband cricket *Gryllus bimaculatus*. Loss-of-function analysis by RNA interference (RNAi) revealed that *Gryllus byn* (*Gb'byn*) is not required for AP axial elongation or normal segment formation, but is required for specification of the posterior gut. We also analyzed *Gryllus caudal* (*Gb'cad*) RNAi embryos using in situ hybridization with a *Gb'byn* probe, and found that *Gb'cad* is required for internalization of the posterior gut primordium, in addition to AP axial elongation. These results suggest that the functions of *byn* and *cad* in posterior terminal patterning are highly conserved in *Gryllus* and *Drosophila* despite their divergent posterior patterning. Moreover, because it is thought that the progressive growth of the AP axis from the growth zone, controlled by a genetic program involving *Cdx/cad* and *Bra/byn*, might be ancestral to bilaterians, our data suggest that the function of *Bra/byn* in this process might have been lost in insects.

KEY WORDS: *Gryllus bimaculatus*, Intermediate-germband insect, *brachyenteron*, *caudal*, Posterior patterning, RNA interference

INTRODUCTION

Molecular mechanisms of anteroposterior (AP) patterning and segmentation are best understood in *Drosophila melanogaster*, and this knowledge provides a basis for investigating the evolution of these processes in the phylum Arthropoda. In the long-germband insect *Drosophila*, all body segments and posterior terminal structures, including the posterior gut and anal pads, are specified almost simultaneously at the blastoderm stage. This is in contrast to short- and intermediate-germband embryogenesis, which is thought to be a primitive feature of arthropods (reviewed by Davis and Patel, 2002). In short- and intermediate-germband insects, anterior segments are specified almost simultaneously at the blastoderm stage, whereas posterior segments are sequentially produced from the posterior growth zone, and invagination of the posterior gut starts after AP axial elongation from the growth zone. Thus, because this major difference in the developmental process of posterior patterning is observed among insects, changes in the mechanisms underlying this process are assumed to be key events in the evolutionary transition from short- and intermediate- to long-germband embryogenesis. However, we still do not know how the transition occurred at the molecular level, because the posterior patterning mechanisms in short- and intermediate-germband insects remain poorly understood.

Recent functional studies using RNA interference (RNAi) in short- and intermediate-germband insects have demonstrated that several transcription factors, including Caudal (Cad), Even-skipped (Eve) and Hunchback (Hb), and cellular signaling pathways, including Wingless (Wg)/Armadillo (Arm) and Torso signaling, are involved in elongation and/or segmentation from the posterior growth zone (Copf et al., 2004; Liu and Kaufman, 2004; Liu and Kaufman, 2005; Mito et al., 2005; Miyawaki et al., 2004; Schoppmeier and Schröder, 2005; Shinmyo et al., 2005). This has led to two tentative conclusions regarding the evolution of genetic mechanisms directing posterior patterning. First, because a number of homologs of these factors are involved in posterior terminal patterning in *Drosophila*, the terminal system found in *Drosophila* may be involved in AP axial specification from the growth zone. Second, because homologs of some of these factors are also involved in AP axial elongation from the primitive streak and tail bud in vertebrates, there may be common mechanisms for AP axial formation between arthropods and vertebrates. These hypotheses prompted us to investigate the role of the *Brachyury/brachyenteron* (*Bra/byn*) gene, which is involved in morphogenesis of the posterior gut in *Drosophila* and AP axial elongation in vertebrates, in short- and intermediate-germband insects.

Department of Biological Science and Technology, Faculty of Engineering, The University of Tokushima, 2-1 Minami-Josanjima-cho, Tokushima City 770-8506, Japan.

*Present address: Division of Developmental Neurobiology, Graduate School of Medical Sciences, Kumamoto University, Kumamoto 860-8556, Japan
†Present address: Division of Genetic Information, Institute for Genome Research, The University of Tokushima, Tokushima 770-8503, Japan
‡Author for correspondence (e-mail: noji@bio.tokushima-u.ac.jp)

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DEVELOPMENT

Bra/byn is the best-characterized T-box gene and has been isolated from many organisms. In vertebrates, *Bra* is expressed transiently around the blastopore during gastrulation, in the involuting mesoderm and endoderm, and subsequently its expression becomes restricted to the notochord and tailbud (Herrmann, 1991; Kispert and Herrmann, 1994; Kispert et al., 1995; Schulte-Merker et al., 1992; Smith et al., 1991; Wilkinson et al., 1990). Mouse (Wilson and Beddington, 1997; Wilson et al., 1995) and zebrafish (Melby et al., 1996; Schulte-Merker et al., 1994) mutants demonstrate that *Bra* is necessary for gastrulation, axial specification and caudal morphogenesis. Within arthropods, *byn* expression and function have been most extensively investigated in *D. melanogaster*. *Drosophila byn* (*Dm*'*byn*) is expressed in the posterior terminal region from 0 to ~20% egg length at the blastoderm stage, where the primordia of the posterior gut and anal pads are located, and continues to be expressed in the hindgut and anal pads throughout embryogenesis (Kispert et al., 1994). In *Dm*'*byn* mutant embryos, programmed cell death occurs in primordia of the hindgut and anal pads, resulting in a severe reduction of their structures (Kispert et al., 1994; Singer et al., 1996). Additionally, *Dm*'*byn* is known to be involved in the formation of the midgut constrictions, elongation of the Malpighian tubules and specification of the visceral mesoderm in *Drosophila* embryos (Kusch and Reuter, 1999; Singer et al., 1996). Conserved expression patterns of Byn were reported in the short-germband insects *Locusta migratoria* and *Tribolium castaneum* using the anti-TN1-123 antibody that binds specifically to the Byn protein in *Drosophila* embryos (Kispert et al., 1994). However, the precise expression patterns and functions of *byn* have not been investigated in short- and intermediate-germband insects.

We have examined *byn* in the intermediate-germband cricket *Gryllus bimaculatus*. *Gryllus byn* (*Gb*'*byn*) is expressed in the posterior terminal cells of the embryo during AP axial elongation, and continues to be expressed in the hindgut during late embryogenesis. Reduction of the *Gb*'*byn* expression level by RNAi resulted in defects in the posterior gut, but not in the posterior body segments. These results indicate that *Gb*'*byn* is not required for AP axial elongation or normal segment formation, but is required for morphogenesis of the posterior gut. We also examined the function of *Gryllus caudal* (*Gb*'*cad*) in posterior patterning by RNAi, and found that *Gb*'*cad* is required for internalization of the posterior gut primordium, in addition to AP axial elongation. We compare *cad* and *byn* function in *Gryllus* with their function in other bilaterians, and discuss the evolution of *Cdx/cad* and *Bra/byn* function in other animals.

MATERIALS AND METHODS

Animals

The two-spotted cricket *Gryllus bimaculatus* was reared at 28-30°C and 70% humidity under a 10-hour light, 14-hour dark photoperiod, as previously described (Niwa et al., 2000). Fertilized eggs were collected with wet kitchen towel and incubated at 28°C in a plastic dish.

Cloning of the *G. bimaculatus* homolog of *byn*

Total RNA was extracted from *G. bimaculatus* at various embryonic stages using Isogen (Nippon-Gene). mRNA was isolated using an Oligotex^{TM}-dT30 Super mRNA Purification Kit (TaKaRa). cDNA was synthesized using the Superscript First Strand Synthesis Kit (Invitrogen). To isolate a *Gb*'*byn* cDNA fragment by PCR, we used four primers (see Fig. 1 for primer position). The sequences of these guessmers were:
byn-5′A, 5′-ACNAAYGARATGATHGTNAC-3′;
byn-5′B, 5′-GAYCCNRMNGCNATGTAYAC-3′;
byn-3′A, 5′-AANGGRTTRTAYTTDATYTT-3′; and
byn-3′B, 5′-TCRTTYTGGTANGCNGTNAC-3′.

From the short fragment sequence obtained from the degenerate PCRs, we designed gene-specific primers and performed 5′ and 3′ rapid amplification of cDNA ends (RACE) using the SMART RACE cDNA Amplification Kit (Clontech). The following primers were used:
primary 3′RACE PCR, 5′-AACGCC GTCTACGTGCACCCCGAG-3′;
nested 3′RACE PCR, 5′-CAACGGACAGATAATGCTTAACTC-3′;
primary 5′RACE PCR, 5′-GAGTTAAGCATTATCTGTCCGTTG-3′; and
nested 5′RACE PCR, 5′-CCACTCGCCGTTCACGTACTTCCA-3′.

The *Gb*'*byn* cDNA sequence has been deposited in the DNA Data Bank of Japan (DDBJ) under Accession Number AB246318.

Embryo fixation, in situ hybridization and RNAi

Embryo fixation and in situ hybridization with a digoxigenin (DIG)-labeled antisense RNA probe were performed as previously described (Niwa et al., 2000; Zhang et al., 2005). The dsRNA used in parental and embryonic RNAi was in vitro transcribed from PCR fragments amplified using primers containing T7 phage promoter sequences. Sense and antisense RNA were synthesized using the MEGAscript Kit (Ambion). The RNA was denatured in boiling water and then annealed at room temperature overnight. The resulting dsRNA was ethanol precipitated and then resuspended in water at a final concentration of 20 μM for *Gb*'*byn* (309 bp), *Gb*'*cad* (426 bp) and *DsRed2* (660 bp). In all RNAi experiments, *DsRed2* dsRNA was used as a negative control (Miyawaki et al., 2004). Injections for embryonic and parental RNAi were performed as previously described (Zhang et al., 2002; Mito et al., 2005). For parental RNAi, the injected females were mated with untreated males, and eggs were collected 5-10 days after injection.

RESULTS

Cloning of the *Gryllus brachyenteron* homolog

In order to clone *Gb*'*byn*, we designed degenerate primers to the conserved T domain of *byn* homologs from other organisms. We then performed RT-PCR using these primers and isolated a short *Gb*'*byn* clone. This fragment allowed us to design specific primers for 5′ and 3′ RACE and, thereby, to isolate fragments of the gene. The *Gb*'*byn* gene is predicted to encode 357 amino acids, with a highly conserved T domain. The alignment of T domains of *Gb*'Byn and Bra/Byn proteins of other animals is shown in Fig. 1.

Expression pattern of *Gb'byn* during early embryogenesis

Embryogenesis in *Gryllus* has been described previously (Miyawaki et al., 2004; Niwa et al., 1997; Zhang et al., 2005). Briefly, in terms of segmentation, the germ anlage is formed in the ventral side of the posterior quarter of the egg by stage 3.0. Anterior segmentation occurs almost simultaneously by stage 4.0, at least to the level of the segment polarity genes, because *Gryllus wingless* (*Gb*'*wg*) is expressed in five vertical stripes corresponding to the mandibular through second thoracic segments. Then, the remaining posterior segments are sequentially produced through germband elongation from the posterior growth zone. The specification of the posterior segments can be tracked by the appearance of *Gb*'*wg* stripes, which appear one by one in the third thoracic segment at stage 4.3, and then in abdominal segment 1 at stage 4.4. At stage 7.5, the posterior-most stripe appears in abdominal segment 10.

We observed the expression pattern of *Gb*'*byn* during early embryogenesis by whole-mount in situ hybridization (Fig. 2). We were unable to detect any *Gb*'*byn* expression prior to stage 3.0 (data not shown). *Gb*'*byn* transcripts were first detected as two spots in the posterior terminal region of the embryo at stage 3.8 (Fig. 2A). *Gb*'*byn* expression appeared more strongly at stage 4.3 (Fig. 2B). During germband elongation, *Gb*'*byn* continued to be expressed in the terminal region of the embryo within the ectoderm, where the

DEVELOPMENT

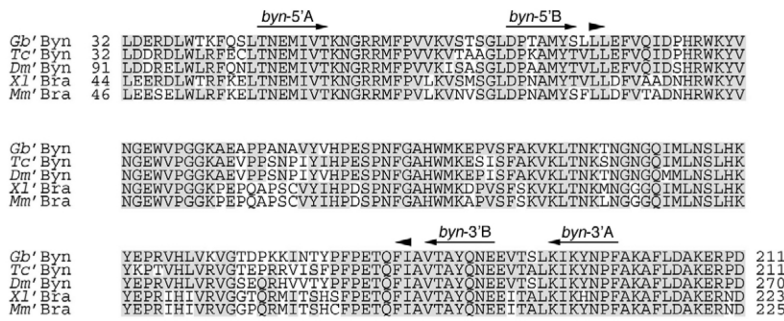

Fig. 1. Alignment of the conserved T-domain of Bra/Byn homologs in *G. bimaculatus* and other species. *Gb*, *Gryllus bimaculatus*; *Tc*, *Tribolium castaneum*; *Dm*, *Drosophila melanogaster*; *Xl*, *Xenopus laevis*; *Mm*, *Mus musculus*. Conserved amino acid residues are highlighted in gray. Arrows indicate the positions of degenerate primers. Arrowheads indicate the 5′ and 3′ ends of the fragment used for synthesis of dsRNA.

hindgut primordium is presumably located (Fig. 2C-E). The *Gb*'*byn*-expressing cells of the terminal region started to sink inward just after the completion of germband elongation and segmentation (Fig. 2F,G).

Expression patterns of *Gb'byn* and *Gb'cad* during late embryogenesis

Next, we observed the expression pattern of *Gb*'*byn* during late embryogenesis, and compared this with the expression of *Gb*'*cad* (Fig. 3). At stage 8, when the proctodeum has extended and is positioned parallel to the body axis, *Gb*'*byn* was expressed in the developing hindgut (Fig. 3A,B). This *Gb*'*byn* expression was maintained during late embryogenesis (Fig. 3C,D). There was no expression of *Gb*'*byn* in the mesoderm surrounding the hindgut, posterior midgut and Malpighian tubules.

We have previously reported the expression pattern of *Gb*'*cad* during early embryogenesis (Shinmyo et al., 2005). Here, we focused on its expression during late embryogenesis. At stage 9, *Gb*'*cad* was expressed in a region adjacent to the hindgut (Fig. 3E). At stage 11, the expression domain was subdivided into two, corresponding to the Malpighian tubules and developing posterior midgut (Fig. 3F). In addition, *Gb*'*cad* was expressed throughout late embryogenesis in the cerci and in the region surrounding the orifice of the hindgut (Fig. 3E,F). Double staining indicated that the spatial patterns of *Gb*'*cad* and *Gb*'*byn* expression were almost complementary to one another in the posterior gut, i.e. in the posterior midgut, Malpighian tubules and hindgut (Fig. 3G,H).

Gb'byn RNAi nymphs exhibited severe defects in the posterior gut

To examine the function of *Gb*'*byn* during *Gryllus* embryogenesis, we used RNAi to deplete *Gb*'*byn* transcripts and produce knockdown phenocopies. Two RNAi methods have been established in *Gryllus*: embryonic RNAi (eRNAi) (Miyawaki et al., 2004), which involves microinjection of dsRNA into the early eggs; and parental RNAi (pRNAi) (Mito et al., 2005), which involves injection of dsRNA into adult virgin females to yield knockdown phenocopies. We confirmed that no qualitative phenocopy differences were produced when using eRNAi or pRNAi, and mainly used pRNAi for our analyses because it does not produce any injection artefacts. As with the wild type, eggs from the *Gb*'*byn* RNAi-injected females developed and hatched nymphs 12-13 days after egg laying. No obvious difference was observed in the cuticle patterns of wild-type and *Gb*'*byn* RNAi nymphs (Fig. 4A,B). However, most *Gb*'*byn* RNAi nymphs (95%, n=118 out of 124) exhibited inhibited growth in the first instar and died before reaching the second instar. To investigate the effects of *Gb*'*byn* depletion on gut formation, we compared the morphology of the alimentary canal in first-instar *Gb*'*byn* RNAi nymphs (Fig. 4D,E) with that of the wild type (Fig. 4C). The alimentary canal of the wild-type nymph consists of the foregut, including crop and proventriculus, the midgut, including gastric caecum and Malpighian tubules, and the hindgut, including the small and large intestines and rectum sac (Fig. 4C). The majority of *Gb*'*byn* RNAi nymphs exhibited severe morphological defects in the posterior gut (95%, n=38 out of 40; Fig. 4D,E), whereas the crop and proventriculus in the foregut, and the

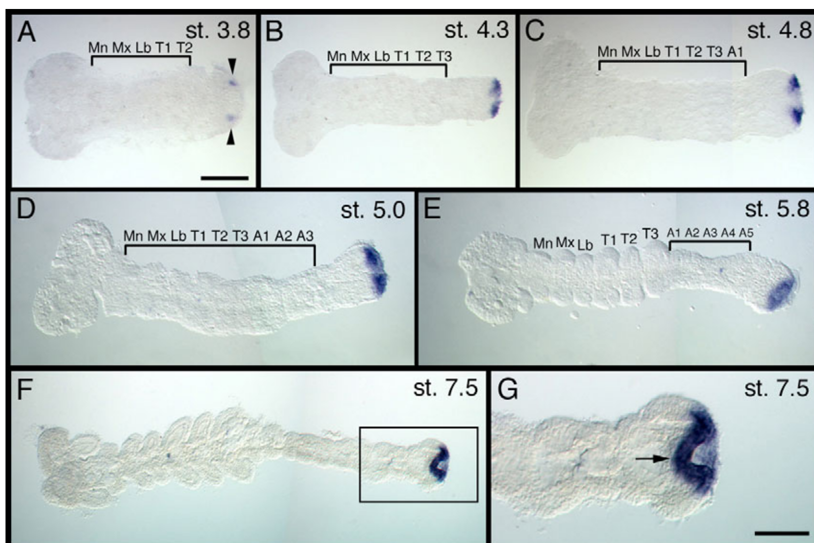

Fig. 2. *Gb'byn* expression patterns during early embryogenesis of *G. bimaculatus*. (**A-G**) Expression patterns of *Gb'byn* at stages 3.8 (A), 4.3 (B), 4.8 (C), 5.0 (D), 5.8 (E) and 7.5 (F,G). The boxed area in F is enlarged in G. In order to illustrate the segmentation process of the *Gryllus* embryo, specified segmental regions are labeled based on the appearance of *Gb'wg* stripes: Mn, mandible; Mx, maxilla; Lb, labium; T1-T3, thoracic segments 1-3; A1-A5, abdominal segments 1-5. *Gb'byn* expression is first detected as two spots (arrowheads) in the posterior terminal region (A). *Gb'byn* continues to be expressed in the terminal region until germband elongation is completed (B-E). The proctodeum that expresses *Gb'byn* starts to invaginate (arrow) after germband elongation and segmentation are completed (G). Anterior is to the left in all embryos. Scale bars: in A, 200 μm for A-F; in G, 100 μm.

DEVELOPMENT

anterior region of the midgut, including the gastric caecum, seemed to be formed normally. All affected nymphs shared severe defects in the hindgut and posterior region of the midgut, whereas the severity of the disruption of the Malpighian tubules varied. In most of the affected *Gb'byn* RNAi nymphs (74%, *n*=28 out of 38; Fig. 4D), the tubules were much shorter than those of the wild type (Fig. 4C). In the most strongly affected nymphs, the tubules were virtually absent (26%, *n*=10 out of 38; Fig. 4E). Thus, *Gb'byn* RNAi nymphs that lack the posterior gut seem unable to absorb food, or to resorb water and ions, resulting in starvation in the first instar. These results indicate that *Gb'byn* is required for the formation of the posterior gut, but not for posterior elongation and segmentation.

Gb'byn is necessary for differentiation of the posterior midgut and hindgut, and for elongation of the Malpighian tubules

To further investigate the effects of *Gb'byn* depletion on posterior terminal patterning, we examined the expression patterns of *Gb'wg*, *Gryllus hedgehog* (*Gb'hh*) and *Gb'cad* during *Gb'byn* RNAi embryogenesis. First, we confirmed that *Gb'byn* expression in the terminal region was reduced in the *Gb'byn* RNAi embryos at stage 5.2 (Fig. 5A,B). In late stages, *Gb'byn* expression in the hindgut (Fig. 5C) provides a useful marker for characterizing defects. In *Gb'byn* RNAi embryos, the *Gb'byn* expression domain was greatly reduced in the hindgut (92%, *n*=23 out of 25; Fig. 5D). Although this indicated a severe reduction of the hindgut, there was still a hindgut remnant that expressed *Gb'byn* in all *Gb'byn* RNAi embryos. This suggested that the hindgut primordium invaginated normally in *Gb'byn* RNAi embryos, but that subsequent development of the hindgut did not occur normally, thereby implying a requirement for *Gb'byn* in hindgut development post-invagination. However, we cannot rule out the possibility that *Gb'byn* is required for the hindgut invagination itself, because almost all *Gb'byn* RNAi embryos might show hypomorphic phenocopies, as judged by the fact that weak *Gb'byn* expression was detected in almost all *Gb'byn* RNAi embryos (Fig. 5B). In this case, hindgut development after invagination would be more sensitive to *Gb'byn* reduction than development before invagination.

Although *Gb'hh* expression is observed in the terminal region during germband elongation (Miyawaki et al., 2004), overlapping with *Gb'byn* expression, *Gb'hh* expression patterns were unaffected in the *Gb'byn* RNAi embryos (data not shown). During invagination of the proctodeum in wild-type embryos, *Gb'hh* is expressed in the developing hindgut (Inoue et al., 2002). At stage 11-12, the expression domain became subdivided into three regions: strong expression in the small intestine and rectum sac, and weak expression in the large intestine (Fig. 5E) (Inoue et al., 2002). In the *Gb'byn* RNAi embryos, abnormal expression of *Gb'hh* was observed in the hindgut remnant, probably as a combined pattern of the small intestine and rectum sac expression domains, with reductions in both (100%, *n*=25; Fig. 5F). This indicates a dramatic defect in the large intestine. In addition, *Gb'hh* expression in the Malpighian tubules was also disrupted in the *Gb'byn* RNAi embryos (Fig. 5F).

Gb'wg is expressed in the posterior growth zone during germband elongation (Miyawaki et al., 2004). This expression pattern was unaffected in the *Gb'byn* RNAi embryos (data not shown). In the wild-type embryos at stage 11-12, *Gb'wg* expression was detected in two regions, the anterior region of the small intestine and the posterior rectum of the hindgut (Fig. 5G) (Inoue et al., 2002). In the *Gb'byn* RNAi embryos, *Gb'wg* expression was detected in both anterior and posterior regions of the severely reduced hindgut, with

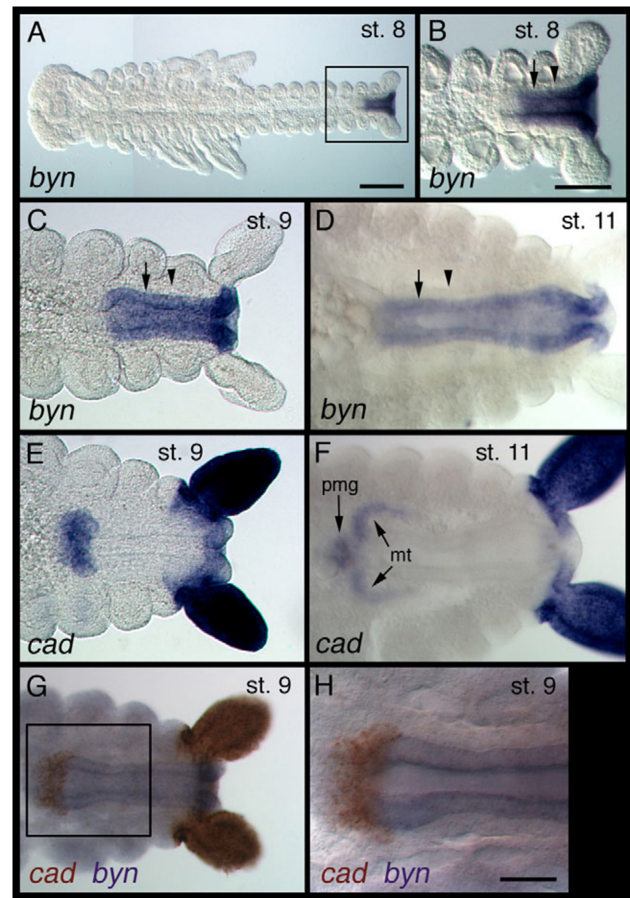

Fig. 3. Expression patterns of *Gb'byn* and *Gb'cad* during late embryogenesis of *G. bimaculatus*. (**A-D**) Expression of *Gb'byn* at stages 8 (A,B), 9 (C) and 11(D). The boxed area in A is enlarged in B. *Gb'byn* expression is detected in the ectodermal epithelium of the hindgut (arrows), whereas no *Gb'byn* expression is detectable in the mesoderm surrounding the hindgut (arrowheads). (**E**,**F**) Expression patterns of *Gb'cad* at stages 9 (E) and 11 (F). *Gb'cad* is expressed in the region surrounding the orifice of the hindgut, Malpighian tubules (mt), and developing posterior midgut (pmg) of stage 11 embryos. (**G,H**) Embryo double stained for *Gb'cad* (brown) and *Gb'byn* (blue). The boxed area in G is enlarged in H. Very little, if any, overlap can be seen in the expression patterns of the two genes. Anterior is to the left in all embryos. Scale bars: in A, 200 μm; in B,100 μm for B-G; in H, 50 μm.

reduced expression domains (92%, *n*=23 out of 25; Fig. 5H). This result indicates a dramatic defect in the large intestine of *Gb'byn* RNAi embryos, as well as relatively mild defects in the small intestine and rectum, consistent with the pattern of *Gb'hh* expression in the *Gb'byn* RNAi embryos.

Gb'cad is expressed in the posterior growth zone during germband elongation (Shinmyo et al., 2005). This expression pattern was unaffected in *Gb'byn* RNAi embryos (data not shown). In wild-type embryos at stage 9, *Gb'cad* was expressed in the region surrounding the orifice of the hindgut and in the region adjacent to the hindgut (Fig. 3E, Fig. 5I). *Gb'cad* expression in both domains was greatly reduced in the *Gb'byn* RNAi embryos (100%, *n*=10; Fig. 5J). Additional domains of *Gb'cad* expression in the cerci were not affected in the *Gb'byn* RNAi embryos (Fig. 5, compare I with J). At stages 11-12, *Gb'cad* expression was detected in the region surrounding the orifice of the hindgut, the Malpighian tubules and the

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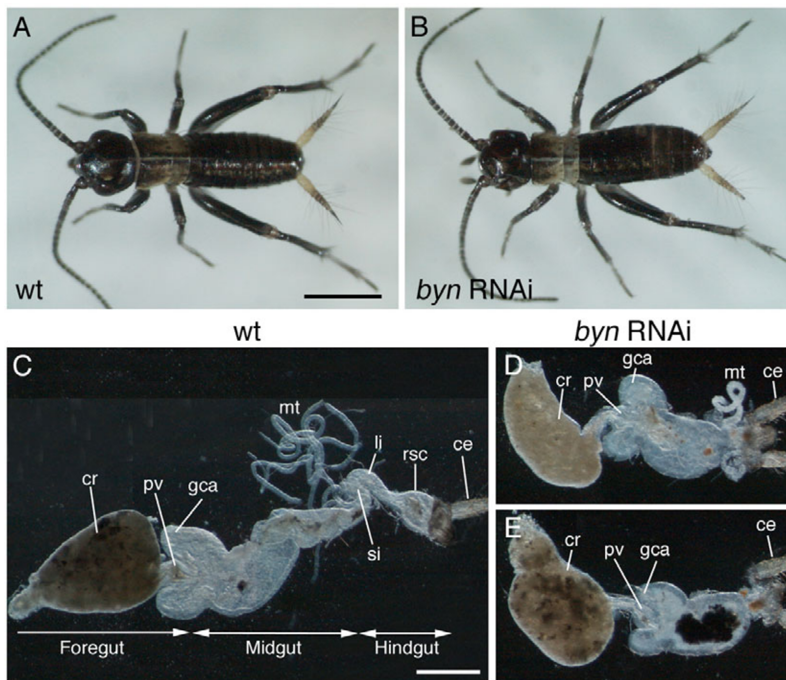

Fig. 4. Effect of *Gb'byn* RNAi on *G. bimaculatus* nymphs. (**A**,**B**) Wild-type (A) and *Gb'byn* RNAi (B) nymphs in the first instar. (**C**) The alimentary canal of the wild-type nymph in the first instar, consisting of the fore-, mid- and hindgut. Cr, crop; pv, proventriculus; gca, gastric caecum; mt, Malpighian tubules; si, small intestine; li, large intestine; rsc, rectum sac; ce, cercus. (**D**,**E**) Representative alimentary canals of *Gb'byn* RNAi nymphs in the first instar. In most *Gb'byn* RNAi nymphs, the hindgut and the posterior region of the midgut were severely reduced, and the Malpighian tubules were decreased in size (D). In the more strongly affected nymphs, the Malpighian tubules were severely reduced (E). Scale bars: in A,1 mm for A,B; in C, 500 μm for C-E.

posterior midgut in wild-type embryos (Fig. 3F, Fig. 5K). *Gb*'*cad* expression in the region surrounding the orifice of the hindgut and posterior midgut was greatly reduced in the *Gb*'*byn* RNAi embryos (88%, *n*=22 out of 25; Fig. 5L), indicating a reduction in these structures. We also found that *Gb*'*cad* was weakly expressed in the very small remnant of the Malpighian tubules seen in all *Gb*'*byn* RNAi embryos (100%, *n*=25; Fig. 5L), indicating that the primordium of the Malpighian tubules was formed in *Gb*'*byn* RNAi embryos. This suggests that the disruption of the Malpighian tubules observed in the *Gb*'*byn* RNAi nymphs (Fig. 4D,E) resulted from an inhibition of tubule elongation. This interpretation is supported by the fact that the shortened Malpighian tubules were formed in most *Gb*'*byn* RNAi nymphs (Fig. 4D). Thus, in *Gb*'*byn* RNAi embryos, the expression patterns of the marker genes for the posterior gut suggest that *Gb*'*byn* is necessary for differentiation of the posterior midgut and hindgut, and for elongation of the Malpighian tubules.

Gb'cad is necessary for internalization of the hindgut primordium

In *Drosophila*, *cad* is essential for invagination and maintenance of the hindgut primordium (Wu and Lengyel, 1998). Although it has been shown that *cad* is required for the formation of all trunk segments in short- and intermediate-germband insects (Copf et al., 2004; Shinmyo et al., 2005), the role of *cad* in posterior terminal patterning in these insects has not been investigated. To determine *Gb*'*cad* function in *Gryllus*, we generated embryos depleted of *Gb*'*cad* by pRNAi, and examined the expression patterns of *Gb*'*byn* during *Gb*'*cad* RNAi embryogenesis. First, we confirmed that most *Gb*'*cad* RNAi embryos obtained by pRNAi exhibited severe defects in the trunk segments (Fig. 6A-C), as described previously in *Gb*'*cad* eRNAi experiments (Shinmyo et al., 2005). In wild-type embryos at stage 4, *Gb*'*byn* was expressed in the posterior terminal region (Fig. 6D), and this remained unaffected in *Gb*'*cad* RNAi embryos (100%, *n*=10; Fig. 6E). This suggests that *Gb*'*cad* is not involved in establishing the hindgut primordium. In wild-type embryos, *Gb*'*byn*-expressing cells in the terminal region sink inwards at stage 7.5 and are completely internalized by stage 9 (Fig. 3C, Fig. 6F). In the *Gb*'*cad* RNAi embryos, the *Gb*'*byn*-expressing cells failed to invaginate at stage 9, remaining on the outside of the embryo (Fig. 6G,H).

We also examined the expression pattern of *Gb*'*hh*, which is also used as a marker gene for the hindgut (Fig. 6I). In the *Gb*'*cad* RNAi embryos, *Gb*'*hh* expression was observed in the external hindgut remnant (Fig. 6J,K). These observations indicate that the invagination of the hindgut primordium did not occur in the *Gb*'*cad* RNAi embryos, suggesting that *Gb*'*cad* is not necessary to establish the hindgut primordium, but is required for internalization of the primordium. However, we cannot rule out the possibility that the hindgut primordium, in which *Gb*'*byn* is normally expressed, is not correctly specified in the *Gb*'*cad* RNAi embryos. In this case, *Gb*'*cad* and *Gb*'*byn* would be activated independently in the hindgut primordium, and both genes would be necessary for the establishment of the hindgut primordium. Further expression analyses of hindgut markers in *Gb*'*cad* RNAi embryos will be required to determine *Gb*'*cad* function in specification of the hindgut primordium.

DISCUSSION

We have isolated the *Gb*'*byn* gene from the intermediate-germband cricket *Gryllus bimaculatus* and investigated its developmental function using RNAi. We found that *Gb*'*byn* is not required for AP axial elongation or normal segment formation, but is required for the specification of the posterior gut. We also found that *Gb*'*cad* is required for internalization of the hindgut primordium, in addition to AP axial elongation. Here, we discuss the functions of *Gb*'*cad* and *Gb*'*byn* in *Gryllus* embryogenesis, and compare them with their functions in other bilaterians.

Specification of the hindgut primordium appears to occur independently of posterior segment specification in *Gryllus*

In *Drosophila*, all segments and posterior terminal structures are specified by the blastoderm stage. By contrast, in *Gryllus*, posterior segments are specified in an anterior to posterior direction through

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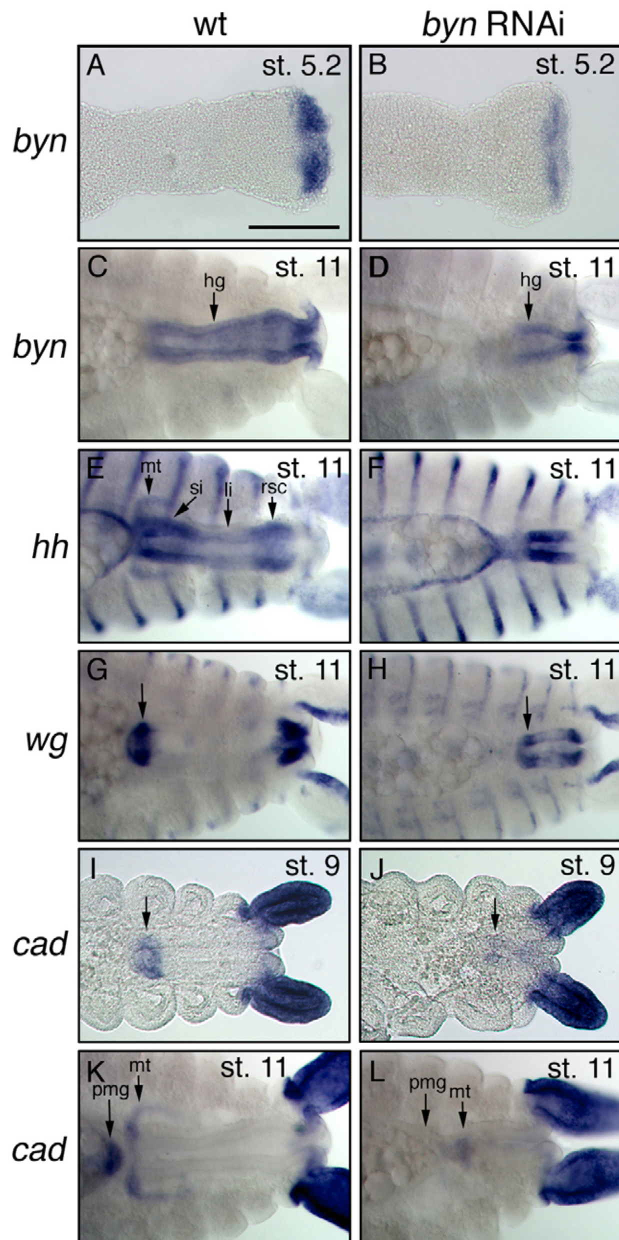

Fig. 5. Expression patterns of marker genes for the posterior gut in *Gb'byn* RNAi embryos. (**A-D**) Expression pattern of *Gb'byn* in wild-type (A,C) and *Gb'byn* RNAi (B,D) embryos, at stages 5.2 (A,B) and 11 (C,D). *Gb'byn* expression in the hindgut (hg) is greatly reduced in *Gb'byn* RNAi embryos, but some *Gb'byn*-expressing tissues do remain (D). (**E**,**F**) Expression patterns of *Gb'hh* in wild-type (E) and *Gb'byn* RNAi (F) embryos at stage 11. In wild-type embryos, *Gb'hh* is strongly expressed in the small intestine (si) and rectum sac (rsc) of the hindgut, and weakly expressed in the large intestine (li) and Malpighian tubules (mt). In *Gb'byn* RNAi embryos, *Gb'hh* expression was observed abnormally in the hindgut remnant, probably as a combined pattern from the small intestine and rectum sac. (**G**,**H**) Expression of *Gb'wg* in wild-type (G) and *Gb'byn* RNAi (H) embryos at stage 11. In wild-type embryos, *Gb'wg* is expressed in the anterior region of the small intestine (arrow) and in the posterior rectum. *Gb'wg* expression in these domains was reduced in the *Gb'byn* RNAi embryos. (**I-L**) Expression of *Gb'cad* in wild-type (I,K) and *Gb'byn* RNAi (J,L) embryos at stages 9 (I,J) and 11 (K,L). In wild-type embryos at stage 9, *Gb'cad* is expressed in the region surrounding the orifice of the hindgut and in a region adjacent to the hindgut; this expression was much reduced in *Gb'byn* RNAi embryos (compare I and J, arrow). In wild-type embryos at stage 11, *Gb'cad* expression was observed in the Malpighian tubules and posterior midgut (pmg; K). In *Gb'byn* RNAi embryos, *Gb'cad* expression was observed in a very small remnant of the Malpighian tubules, and *Gb'cad* expression in the posterior midgut was greatly reduced (L). In all embryos, anterior is to the left. Scale bar in A: 200 μm for A-L.

elongation of the posterior growth zone, and invagination of the posterior gut starts after the specification of the posterior segments. It is not clear how and when the posterior segments and terminal structures are specified, or how developmental timing of their structures is controlled during *Gryllus* embryogenesis. We found that *Gb'byn* is expressed as two spots in the posterior terminal cells of embryos, where the hindgut primordium is presumably located, before AP axial elongation (Fig. 2A). This suggests that the specification of the hindgut primordium occurs before AP axial elongation, and independently of posterior segment specification. This interpretation is supported by our previous data indicating that the terminal structures, such as the hindgut and cerci, are formed in *Gb'hb* or *Gb'krüppel* RNAi embryos, in which posterior segments generated from the growth zone are severely defective (Mito et al., 2005; Mito et al., 2006). Further support comes from studies showing that in *Gryllus* embryos subjected to lethal doses of radiation, the most posterior segment carrying the cerci is always present, even if many other segments are missing (Sander, 1976).

The interpretation may also be supported by the observation that *Gb'byn* expression appears normal in early *Gb'cad* RNAi embryos, which presumably lack all trunk segments at late stages (Fig. 6E).

It is important to note that *tailless*, which acts upstream of *byn* in *Drosophila* terminal patterning, is already expressed at the blastoderm stage at the posterior pole of *Tribolium* embryos. This suggests that there is a group of cells within the posterior growth zone that is determined at the blastoderm stage to produce the terminal structures in *Tribolium* (Schröder et al., 2000). This conceivably might also apply to *Gryllus* embryogenesis.

Roles of *Cdx/cad* and *Bra/byn* in posterior gut patterning

We found that the posterior gut, consisting of the posterior midgut, Malpighian tubules and hindgut, was severely reduced in *Gb'byn* RNAi nymphs (Fig. 4, Fig. 7A). Furthermore, detailed analysis of the expression patterns of tissue-specific markers revealed that *Gb'byn* is necessary for differentiation of the midgut and hindgut, and for elongation of the Malpighian tubules (Fig. 5, Fig. 7A). In *Drosophila byn* mutants, the posterior gut is severely reduced as a consequence of massive apoptosis in the gut primordia (Kispert et al., 1994; Singer et al., 1996). It remains unclear whether apoptosis contributes to the reduced posterior gut in *Gb'byn* RNAi embryos because of a technical problem associated with the TUNEL staining. However, the similarities in phenotype suggest that *byn* function during embryogenesis is highly conserved between long- and intermediate-germband insects. *Bra* is not reported to be involved in gut formation in vertebrates, but it is expressed in the posterior gut endoderm of hemichordates (Peterson et al., 1999) and echinoderms (Gross and McClay, 2001; Shoguchi et al., 1999). Although the posterior gut endoderm of these animals is substantially different from the hindgut ectoderm of insects, these similarities suggest that the involvement of *Bra/byn* in specification of the posterior gut

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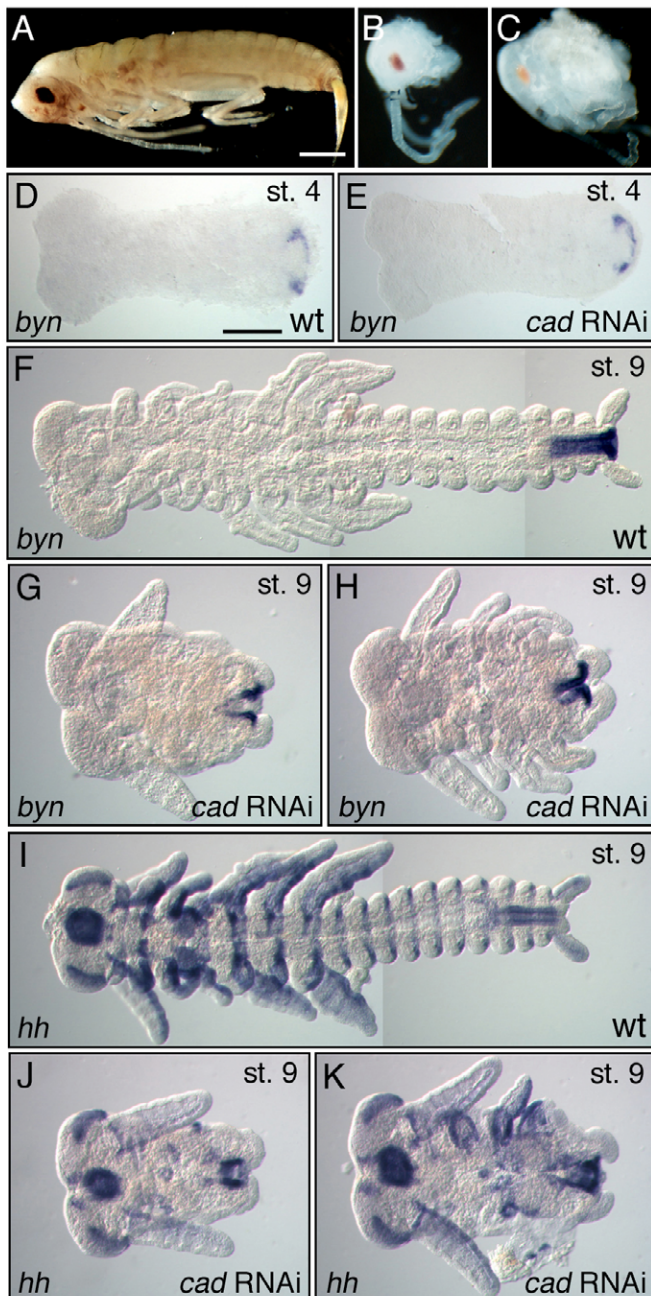

Fig. 6. Effect of *Gb'cad* RNAi on posterior terminal patterning. (**A-C**) Wild-type (A) and *Gb'cad* RNAi (B,C) embryos at 12 days after egg laying. In the most severe cases (64%, *n*=178 out of 280), *Gb'cad* RNAi embryos completely lack gnathum, thorax and abdomen, whereas the anterior head is formed normally (B). In other cases (31%, *n*=87 out of 280), the normal anterior head and part of the trunk segments are formed (C). (**D-H**) Expression of *Gb'byn* in wild-type (D,F) and *Gb'cad* RNAi (E,G,H) embryos at stages 4 (D,E) and 9 (F-H). At stage 4, *Gb'byn* expression in the terminal regions was normal in all *Gb'cad* RNAi embryos (D,E). In wild-type embryos at stage 9, *Gb'byn* was expressed in the internalized hindgut (F). In the *Gb'cad* RNAi embryos at this stage, the *Gb'byn*-expressing cells failed to invaginate, remaining on the outside of the embryo (G,H). (**I-K**) Expression of *Gb'hh* in wild-type (I) and *Gb'cad* RNAi (J,K) embryos at stage 9. In wild-type embryos, *Gb'hh* expression was observed in the internalized hindgut, whereas, in the *Gb'cad* RNAi embryos, *Gb'hh* expression was observed in the external remnant.

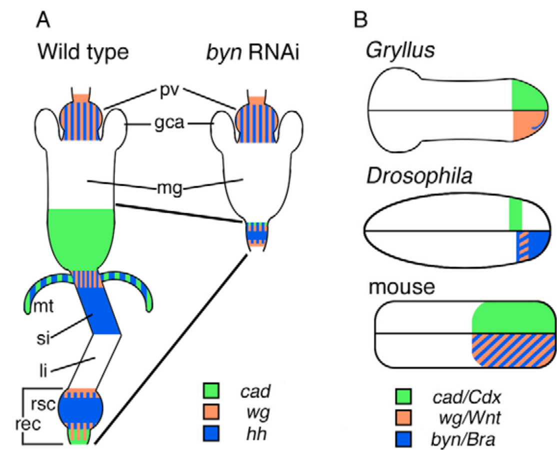

Fig. 7. Schematics of the profile of *Gb'byn* expression and function. (**A**) A comparison of extrapolated expression patterns of *Gb'hh*, *Gb'wg* and *Gb'cad* in the gut of wild-type (left) and *Gb'byn* RNAi (right) nymphs. In *Gb'byn* RNAi nymphs, *Gb'hh* expression is observed in the severely reduced hindgut as a probable combined pattern from the small intestine and rectum sac, and in the small remnant of the Malpighian tubules. *Gb'wg* expression seems to be observed in the small intestine and posterior rectum of the severely reduced hindgut of *Gb'byn* RNAi nymphs. *Gb'cad* expression in the *Gb'byn* RNAi nymph seems to be observed in the small remnant of the Malpighian tubules, and is almost completely eliminated in the region surrounding the orifice of the rectum and posterior midgut. The hatched regions indicate overlapping expression patterns. pv, proventriculus; gca, gastric caecum; mg, midgut; mt, Malpighian tubules; si, small intestine; li, large intestine; rsc, rectum sac; rec, rectum. (**B**) Comparison of the expression of *Gb'byn*, *Gb'wg* and *Gb'cad* in early embryos of *Gryllus* with those of *Drosophila* and mouse. In early *Gryllus* embryos, *Gb'wg* and *Gb'cad* expression is detected in the posterior growth zone (Miyawaki et al., 2004; Shinmyo et al., 2005), whereas *Gb'byn* expression is restricted in the posterior terminal region (see Fig. 2). In *Drosophila*, all three genes are expressed in the posterior terminal region at the cellular blastoderm stage (Hoch and Pankratz, 1996; Kispert et al., 1994; Singer et al., 1996; Wu and Lengyel, 1998). In mouse, all three genes are expressed in the primitive streak of early embryos (Kispert and Herrmann, 1994; Liu et al., 1999; Meyer and Gruss, 1993).

might be ancestral to bilaterians. A similar presumption might also extend to the role of *Cdx/cad* in gut development. In *Gryllus*, *Gb'cad* is expressed in the Malpighian tubules and posterior midgut endoderm during late embryogenesis (Fig. 2). Our RNAi analysis shows that *Gb'cad* is necessary for internalization of the posterior gut primordium (Fig. 6). In *Drosophila*, *Dm'cad* is known to be expressed in the Malpighian tubules and posterior midgut endoderm of older embryos (Macdonald and Struhl, 1986; Mlodzik et al., 1985), and to be essential for internalization and maintenance of the posterior gut primordium (Wu and Lengyel, 1998). Thus, the expression pattern and function of *cad* in posterior gut development are highly conserved between *Gryllus* and *Drosophila*. In vertebrates, *Cdx* genes are expressed in the gut endoderm during late embryogenesis (reviewed by Freund et al., 1998), and *Cdx2* mutant mice develop intestinal tumors (Chawengsaksophak et al., 1997). In *Caenorhabditis elegans*, the *cad* homolog *pal-1* is expressed zygotically in mesoderm cells of the posterior gut (Edgar et al., 2001). On the basis of these data, we hypothesize that the involvement of *Cdx/cad* and *Bra/byn* in the specification of the posterior gut might be an ancestral feature of bilaterians.

DEVELOPMENT

It should be noted that morphogenesis of the Malpighian tubules and posterior midgut is blocked in *Gb*'*byn* RNAi nymphs, and that *Gb*'*cad* expression in these tissues is reduced in *Gb*'*byn* RNAi embryos, even though *Gb*'*byn* expression is not detected in these tissues in *Gryllus* embryos. There are two possible explanations for this phenomenon. First, it might be that very low levels of *Gb*'*byn* expression, which are below detectable levels, are sufficient for the development of these tissues. In this case, *Gb*'*byn* would be required for the formation of the Malpighian tubules and posterior midgut through the direct or indirect regulation of *Gb*'*cad* expression in these structures. Second, morphogenesis of the Malpighian tubules and posterior midgut depends upon signaling from the contiguous hindgut, where *Gb*'*byn* is expressed. In this case, the reduction in *Gb*'*cad* expression in the Malpighian tubules and posterior midgut of the *Gb*'*byn* RNAi embryos would result from the inhibition of hindgut development. A similar phenomenon is also observed in *Drosophila byn* mutants (Singer et al., 1996), suggesting conservation in the mechanisms of terminal patterning.

Evolution of *Cdx*/*cad* and *Bra*/*byn* function in AP axial elongation

The progressive growth of AP axial structures from a posterior region is observed in such diverse animals as chordates, short- and intermediate-germband arthropods, annelids and molluscs. In short- and intermediate-germband arthropods, posterior segments are sequentially produced from the posterior growth zone, where *cad* is expressed (Copf et al., 2003; Dearden and Akam, 2001; Schulz et al., 1998; Shinmyo et al., 2005) (Fig. 7B) and required for AP axial elongation from the growth zone (Copf et al., 2004; Shinmyo et al., 2005). *cad* expression in the growth zone is likely to be regulated by Wg/Arm signaling in *Gryllus* embryos (Shinmyo et al., 2005) (Fig. 7B). Segmentation in short- and intermediate-germband arthropods resembles somitogenesis in vertebrates, in which somites are generated progressively from a posteriorly located presomitic zone (reviewed by Peel et al., 2005). In addition, the *Cdx* genes, which are regulated by Wnt signaling, are expressed in the nascent mesoderm of the primitive streak (Ikeya and Takada, 2001; Marom et al., 1997; Meyer and Gruss, 1993) (Fig. 7B), and are involved in axial elongation and somitogenesis (Epstein et al., 1997; Subramanian et al., 1995; van den Akker et al., 2002). These similarities suggest that the molecular mechanisms underlying these processes are conserved between short- and intermediate-germband arthropods and vertebrates. Recently, it has been shown that *even-skipped* (*eve*) is expressed in the posterior growth zone and is required for AP axial elongation in the intermediate-germband insect *Oncopeltus fasciatus* (Liu and Kaufman, 2005). This fact may also suggest conserved mechanisms for these processes because, in vertebrates, *Evx1* (the *eve* homolog) is known to be expressed in the primitive streak and tail bud, although its function has not been investigated (Dush and Martin, 1992). These data suggest that AP axial formation from the posterior growth zone is ancestral to bilaterians. A similar hypothesis has been proposed, based on a comparison of *Bra* expression patterns in molluscs and vertebrates. In vertebrates, *Bra* is also expressed in the nascent mesoderm of the primitive streak and tail bud (Kispert and Herrmann, 1994; Knezevic et al., 1997; Wilkinson et al., 1990) (Fig. 7B), and is necessary for AP axial formation (Wilson and Beddington, 1997). Because *Bra* expression in the posterior pole of the AP axis, up to the end of mollusc larval development, is similar to that in vertebrates, Lartillot et al. (Lartillot et al., 2002) have proposed that *Bra* might have a conserved role in the regulation of AP patterning among bilaterians, through maintenance of the posterior growth zone. This hypothesis implies that the role of *Bra/byn* in AP axial elongation might be ancestral to bilaterians. Importantly, we found that *Gb'byn* is expressed exclusively in the posterior terminal region (Fig. 7B), and is not involved in AP axial elongation from the growth zone. Therefore, if the hypothesis is correct, our results suggest that the function of *Bra*/*byn* in AP axial elongation might have been lost in insects. More data from a wider range of protostomes will be required to confirm this.

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