A Role for *buttonhead* in the Early Head and Trunk Development in the Beetle *Tribolium castaneum*

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ABSTRACT : The head gap gene *buttonhead* (*btd*) is required for the patterning of head segments in the early *Drosophila* embryo. Mutant phenotypes of *btd* display a gap-like phenotype in which antennal, intercalary, mandibular and the anterior portion of the maxillary segments are eliminated. In agreement with the phenotypes, *btd* is expressed in a stripe covering the head segments at the blastoderm stage. During the early phase of the germband extension, *btd* is expressed in stripes with single segmental periodicity, which is required for the formation of the peripheral nervous system. In contrast to the key role of *btd* in *Drosophila* embryonic development, it has been suggested that *Tribolium* ortholog of *btd* (*Tc-btd*) is dispensable for embryonic head development. In order for better understanding of the requirement of *Tc-btd* in the early *Tribolium* embryo, we re-analyzed the expression patterns and functions of *Tc-btd* during embryonic segmentation. *Tc-btd* is expressed in segmental stripes at the stages of blastoderm and germband elongation. Up to 28.3% of embryos in which *Tc-btd* is knocked down displays the loss of antennal, mandibular and the pregnathal regions in the head, with abdominal segments being disrupted in the trunk. Our findings suggest that *Tc-btd* is required for the head and trunk development in the early *Tribolium* embryo.
Key words : *buttonhead*, Head gap gene, Segmentation, *Tribolium*

INTRODUCTION

During embryonic head development, all insects build segments that fuse to each other to form the head capsule bearing appendages with different roles in feeding and feeling (Snodgrass, 1935). The insect head consists of the anterior pregnathal and the posterior gnathal regions. While the anterior pregnathal region comprises the intercalary (IC), antennal (AN), ocular segments (OC), and other pre-antennal tissues, the posterior gnathal region is composed of the mandibular (MD), maxillary (MX), and labial (LB)

segments that constitute the mouthparts (Snodgrass, 1935; Rogers and Kaufman, 1997). The genetic mechanisms of head segmentation have been studied in most detail in *Drosophila melanogaster*. In *Drosophila*, the typical hierarchy of maternal, gap, pair-rule, and segment polarity gene interactions form the maxillary and labial gnathal segments (St Johnston & Nusslein-Volhard, 1992). For patterning the pregnathal and the mandibular segments, however, *Drosophila* utilizes the head gap genes, *orthodenticle* (*otd*), *empty spiracles* (*ems*) and *buttonhead* (*btd*), which are expressed in broad and overlapping do-

mains of the embryonic head region (Dalton et al., 1989; Cohen & Jurgens, 1990; Finkelstein & Perrimon, 1990; Walldorf & Gehring, 1992; Wimmer et al., 1993). Consistently, mutations in any head gap gene display phenotypes missing the pregnathal and the mandibular segments that correspond to the expression domains of each head gap gene. For example, *otd* mutants do not bear the antennal and ocular segments, and *ems* mutants do not have the antennal, intercalary, and the anterior portion of the mandibular segments (Dalton et al., 1989; Cohen & Jurgens, 1990; Walldorf & Gehring, 1992). In addition, loss of *btd* leads to the lack of antennal, intercalary, mandibular and the anterior portion of the maxillary segments (Wimmer et al., 1996).

It has been suggested that the genetic mechanisms of head segmentation in *Drosophila* might not be always applicable to other insect head segmentation. In *Drosophila*, activation of the head gap genes depends on Bicoid, which is the key maternal factor for patterning anterior regions along the anterior-to-posterior axis of *Drosophila* embryo (Finkelstein & Perrimon, 1990; Wimmer et al., 1995; Gao & Finkelstein, 1998). Interestingly, however, it has become clear that *bicoid* is absent from most other insects but probably arose recently in the lineage of dipterans during insect evolution (Stauber et al., 1999; Schroder, 2003; Chouard, 2008). Furthermore, *Drosophila* larva has the derived head morphology including the maggot's head being hidden within the thorax and the head structures being highly derived (Rogers & Kaufman, 1996). To understand the evolution of genetic mechanisms controlling head development, orthologs of *Drosophila* head gap genes have been analyzed in insects (Schinko et al., 2008). In the red flour beetle *Tribolium castaneum*, functional analyses of orthologs of *Drosophila* head gap genes revealed that the head segmentation mechanisms in *Tribolium* are distinct from those of *Drosophila*. Similar to *Drosophila*, *Tc-otd1* plays as a head gap gene for the early regionalization of all head segments formed during the blastoderm stage

and then for the later head patterning (Schinko et al., 2008). However, it has been suggested that *Tc-ems* and *Tc-btd* are not head gap genes; *Tc-ems* has a minor role for patterning the antennal segment and *Tc-btd* is not required for head development at all (Schinko et al., 2008). The dispensable role for *Tc-btd* in *Tribolium* head development is unexpected and remains arguable because *Tc-btd* is expressed in the future mandibular segment at the blastoderm stage, with its later expression in the antennal and intercalary segments (Schinko et al., 2008). In the milkweed bug *Oncopeltus fasciatus*, knock-down of *Of-otd* leads to the lack or reduction of the anterior region to the mandibular segment, with the mandibular and maxillary segments being malformed (Birkan et al., 2011). However, roles for *Of-ems* could not be analyzed because of the very low penetrance of RNAi effects (Birkan et al., 2011).

Here we reexamined the expression patterns and functions of *Tc-btd* during *Tribolium* segmentation for better understanding the role of *Tc-btd* in *Tribolium* development. *Tc-btd* is expressed in stripes with single segmental periodicity at the segmentation stages, with its later expression in the head segments and head lobes. Larval cuticles of *Tc-btd* RNAi (*Tc-btd*[RNAi]) embryos display the loss of pregnathal regions with the gnathal maxillary and labial segments remaining in the head and the defects in abdominal segmentation in the trunk. The defects are observed in the head and trunk regions of the growing germbands undergoing embryonic segmentation. Our observations suggest that *Tc-btd* is required for the head and trunk development in *Tribolium*.

MATERIALS AND METHODS

1. Isolation of *Tribolium btd* cDNA

Tc-btd sequence was identified in the *Tribolium* genome (GenBank accession number: NM_001114320; Tc number: TC011696). A cDNA fragment of *Tc-btd* spanning the Btd box and zinc finger domains was amplified by using

PCR (*Tc-btd*_F: 5'-GTGACTACTATGATGGCT-3' and *Tc-btd*_R: 5'-TTGCTCCCCCAAAGTAAT-3') then cloned into the pGEM®-T Easy Vector (Promega). The cDNA sequence is available in Genbank under the accession number of MK546416.

2. Sequence alignment and phylogenetic analysis

Phylogenetic analysis was carried out using the amino acid sequences of insect orthologs of Btd obtained from Genbank: (*Drosophila*-Btd, *Drosophila melanogaster* NP_511100; *Anopheles*-Btd, *Anopheles gambiae* XP_311200; *Drosophila*-Sp1, *Drosophila melanogaster* NP_727360; *Anopheles*- Sp1, *Anopheles gambiae* XP_311199; *Tribolium*-Sp8, *Tribolium castaneum* NP_001034509; Human-SP1, *Homo sapiens* NM_001251825). CLUSTAL Omega with default parameters was used to align the CDS of Tc-Btd with other Btd/Sp orthologs (Sievers et al., 2011). A maximum likelihood tree was constructed using the PhyML method with default parameters (Lefort et al., 2017).

3. Whole mount *in situ* hybridization and immunochemistry

Whole-mount in situ hybridization was performed as described with some modifications (Kim et al., 2013). The digoxigenin-labeled *Tc-btd* riboprobe was transcribed with 1 μg of the pGEM®-T Easy Vector containing the *Tc-btd* cDNA fragment using T7 RNA polymerase (Ambion). Wild-type (GA2) embryos were collected in 24 hours-post-crossing, which were temporally heterogeneous. The embryos were dechorionated in 50% liquid chlorine bleach and then fixed for 15 min in 4% formaldehyde in Phosphate-Buffered Saline (PBS). The fixed embryos were dehydrated in MeOH, rehydrated in PBT (PBS containing 0.1% Tween 20), then treated with xylene to increase permeability for the riboprobes. Embryos were hybridized with *Tc-btd* riboprobes in hybridization buffer (50% formamide / 5X Saline Sodium Citrate Buffer (SSC) / yeast RNA at 200 μg/mL / heterologous DNA at 100 μg/mL /

0.1% Tween 20). The hybridized riboprobes were detected by Anti-Digoxigenin-AP (Roche) in conjunction with BM purple (Roche). Immunochemistry was performed as described previously with a 1:5 dilution of mAb anti-En (4D9) from the Developmental Studies Hybridoma Bank (DSHB) at the University of Iowa (Patel et al., 1994). Embryos that were stained with *Tc-btd* riboprobe or anti-En (4D9) antibody were photographed on a BX50 compound microscope (Olympus, Melville, NY).

4. RNAi analysis

Parental RNAi for knocking down *Tc-btd* was performed as described (Bucher et al., 2002). A PCR fragment of *Tc-btd* that contains a T7 promoter on each end was generated with a T7-linked primer set (*Tc-btd*_T7F: 5'-TAATACGACTCACTATAGGGACCACCTGCACCA GGATGTAC-3' and *Tc-btd*_T7R: 5'- TAATACGACTCA CTATAGGGTTCACCCCGTCACTGCTTTT-3'). dsRNA for *Tc-btd* was synthesized with 1 μg of PCR DNA template using T7 RNA polymerase (Ambion). 500 ng/μL or 750 ng/μL of *Tc-btd* dsRNA were injected into wild-type (GA2) pupae. Injection buffer (0.1 mM Sodium Phosphate Buffer pH 6.8, 5 mM KCl) or 1 μg/μL of *Tc-fushi tarazu* (*FTZ*) dsRNA was injected as a negative control and produced no defects in the head and trunk, as reported previously (Choe et al., 2006). Embryos were collected every 48 hours for 2 weeks and then incubated at 30°C for 24 hours or for 4 days to analyze the RNAi phenotypes.

RESULTS

1. The *Tribolium btd* ortholog

The *Tribolium* ortholog of *btd* was identified in the *Tribolium* genome. This predicted 846 bp of *Tc-btd* that coded for a deduced protein of 282 amino acids. The deduced protein contained the C-X-C-P-X-C Btd box and C2H2 zinc finger domains (Wimmer et al., 1993). A fragment of *Tc-btd* containing the Btd box and zinc finger domains was

amplified from wild-type cDNA (Fig. 1A). Alignment of the deduced 104 amino acid sequences with orthologs of Btd/Sp-family members from other insects displayed the well-conserved Btd box and zinc finger domains, which were highlighted in yellow and green, respectively in Fig. 1A. Zinc finger domains of *Tribolium* Btd showed 83.95% identity with those of *Anopheles* and 69.62% identity with those of *Drosophila*. In addition, zinc finger domains of *Tribolium* Btd showed 91.36% identity with those of *Tribolium* Sp8, 92.59% identity with those of *Anopheles* Sp1,

and 92.59% identity with those of *Drosophila* Sp1. Phylogenetic tree constructed using maximum likelihood analysis from the complete deduced amino acid sequences of insect Btd/Sp-family members confirmed that the fragment of Btd box and zinc finger domains we isolated from *Tribolium* cDNA belonged to the insect *btd* gene (Fig. 1B).

2. Expression patterns of *Tc-btd* in *Tribolium* segmentation

Although the expression patterns of *Tc-btd* during seg-

Fig. 1. The *Tribolium* ortholog of *btd*. (A) Alignment of the Btd box (highlighted in yellow) and zinc finger domains (highlighted in green) of Tc-Btd with those of insect Btd/Sp family members. They are highly conserved within insect orthologs of Btd/Sp family members. (B) Phylogenetic analysis of the complete deduced amino acid sequences of the *Tribolium* ortholog of *btd*. This rooted phylogenetic tree was constructed using maximum likelihood (ML) based on multiple sequence alignments of the complete deduced amino acid sequences of the insect orthologs of *btd/sp* using *Homo sapiens* SP1 as an outgroup. Bootstrap values are shown at branches. In this ML tree, *Tribolium* Btd is grouped with the group of *Anopheles* Btd and *Drosophila* Btd with a 100% of bootstrap value, whereas *Tribolium* Sp8 is with the group of *Anopheles* Sp1 and *Drosophila* Sp1 with a 100% of bootstrap value. *Tribolium*, *Tribolium castaneum*; *Drosophila*, *Drosophila melanogaster*; *Anopheles*, *Anopheles gambiae*.

mentation were reported previously in *Tribolium* (Schinko et al., 2008), we re-analyzed its expression patterns with our own riboprobe that hybridized with 629 bp of *Tc-btd* mRNA. The first transcripts of *Tc-btd* were detected as a stripe at approximately 50% egg length from the posterior pole during the blastoderm stage (Fig. 2A). When the germ rudiment formed, the first stripe was located in the mandibular (MD) segment (Fig. 2B). As the germband grew, a series of *Tc-btd* stripes appeared sequentially in the maxillary (MX), labial (LB), thoracic (T), and abdominal (A) segments, with their expression being maintained throughout the germband extension stages (Fig. 2C-H). When the *Tc-btd* stripe arose in the third thoracic (T3) segment, it appeared in the antennal (AN) segment (arrows in Fig. 2E). As the germband grew, the antennal expression became stronger than its initial expression (arrows in Fig. 2F). In the fully extended germband, new expression domains of *Tc-btd* were observed in the pregnathal region (arrowheads in Fig. 2H). Compared to the early expression of *btd* in the future head regions of *Drosophila* embryos (Wimmer et al., 1993), *Tc-btd* expression in the head appeared in later stages of head development, as reported previously (Schinko et al., 2008). The late expression of *Tc-btd* in the pregnathal region might imply a role for *Tc-btd* in maintaining a segmental identity or promoting further differentiation of the head primordium, rather than a role in patterning the head segments per se. In addition, the segmental *Tc-btd* stripes in the thorax and abdomen of the growing germband suggest a role for *Tc-btd* in the trunk segmentation.

3. Defects in the head and abdomen of *Tc-btd*[RNAi] larva

We investigated the role of *Tc-btd* in the head and trunk segmentation using RNAi. First, we injected 500 ng/μL of *Tc-btd* dsRNA into wild-type (GA2) pupae. Among 127 cuticles of *Tc-btd*[RNAi] larvae, 10.2% (13/127) showed defects in the head and trunk while the remaining 89.8% (114/127) were normal. To increase RNAi efficiency, we
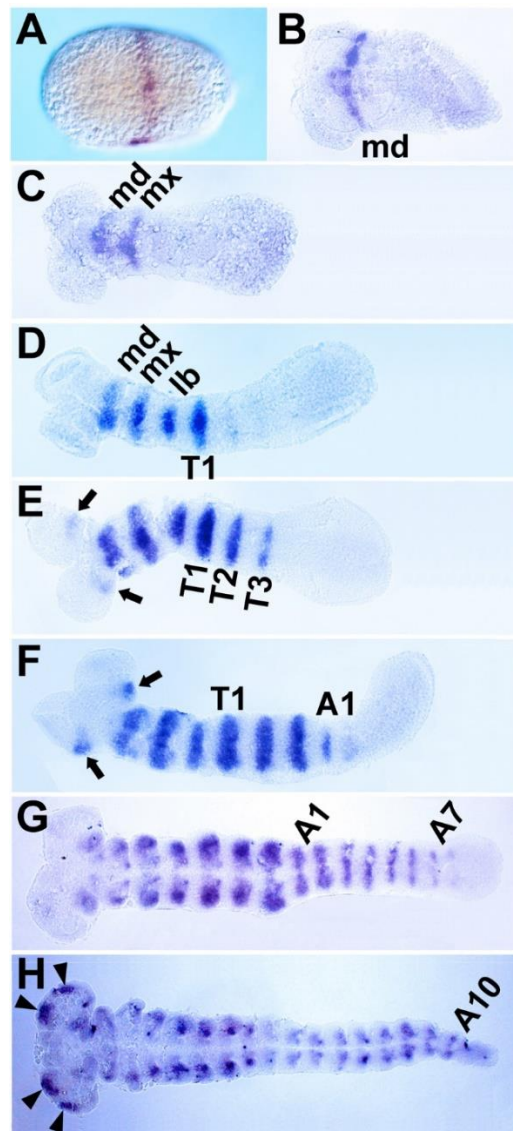

Fig. 2. *In situ* hybridization of *Tc-btd* in *Tribolium* embryonic segmentation. (A) A late stage of blastoderm embryo expressing *Tc-btd* at approximately 50% egg length from the posterior pole. (B) An early germ rudiment that expresses *Tc-btd* in the mandibular segment. (C-G) Elongating germbands that express stripes of *Tc-btd* sequentially in the pregnathal, thoracic, and abdominal segments. While *Tc-btd* is expressed in the trunk regions, it is not expressed in the posterior growth zone of each germband. *Tc-btd* expressions are seen in the antennal segments (arrows) in E and F. (H) A fully extended germband that completed segmentation. *Tc-btd* expression in the head lobe is marked with arrowheads. Anterior is to the left.

then injected 750 ng/µL of *Tc-btd* dsRNA into wild-type (GA2) pupae. In this case, 28.3% (49/173) showed similar defects in the head and trunk to those injected with 500 ng/µL of *Tc-btd* dsRNA. The remaining 71.7% (124/173) showed normal head and trunk segmentation. Although the penetrance of *Tc-btd*^RNAi was quite low (10.2% to 28.3%), *Tc-btd*^RNAi led to the reduced size of the head and the lack of several abdominal segments compared with wild types

(Fig. 3A, C). In most cases, the pregnathal region including the antenna (AN), and the mandible (MD) were lost and the maxillary (MX) was abnormally shaped in *Tc-btd*^RNAi larvae (Fig. 3B, D). However, the labium (LB) of *Tc-btd*^RNAi larvae appeared to be normal (Fig. 3B, D). Compared with wild types, in some cases, an uncharacterized head lobe formed on the dorsal of the thorax in addition to the reduced size of the head (Fig. 3E, F). As a nega-
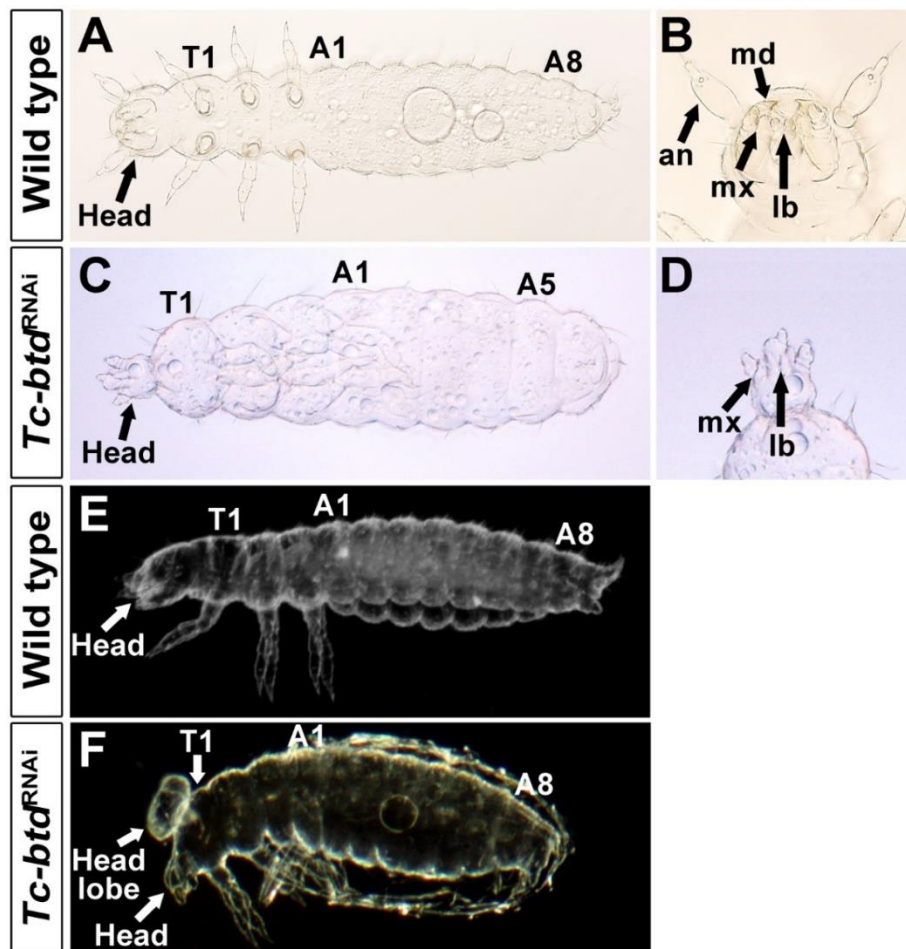
Fig. 3. Cuticle preparations in *Tc-btd* ^RNAi embryos. (A) Ventral view of wild-type larval cuticle with head, three thoracic segments bearing a pair of legs in each segment, and eight abdominal segments. (B) A magnified image of the wild-type head carrying the head appendages. (C) Ventral view of *Tc-btd* ^RNAi larva. This animal contains a small head, three thoracic, and five abdominal segments. (D) A zoom-in image of the small *Tc-btd* ^RNAi larval head. The maxilla and labium are present, but the pregnathal regions are missing. (E) Lateral view of wild-type larval cuticle with head, three thoracic segments, and eight abdominal segments. (F) Lateral view of *Tc-btd* ^RNAi larva. This animal has an uncharacterized head lobe on the dorsal of the thorax in addition to the small head. However, this animal develops normally three thoracic and eight abdominal segments. Note that the egg-shell is incompletely dissected from this late-stage embryo. (A, C, E, F) Anterior is to the left. (B, D) Anterior is to the top.

tive control, $1\times$ injection buffer or 1 μg/μL of *Tc-ftz* dsRNA, which was previously reported to have no effects on the segmentation of larval cuticles, was injected into wild-type (GA2) pupae (Choe et al., 2006). All cuticles of 1×injection buffer injected (n=109) or *Tc-ftz* dsRNA injected (n=146) larvae displayed the normal head and trunk. Although it was reported previously that *Tc-btd* was dispensable for the development of head in *Tribolium* (Schinko et al., 2008), our results indicated the role of *Tc-btd* in the trunk segmentation as well as head development.

4. Germband defects in *Tc-btd*^RNAi embryos

In order to verify the defects observed in the *Tc-btd*^RNAi larval cuticles, we examined embryonic segmentation process at the stage of germband extension. To do so, we analyzed expressions of Engrailed (Tc-En) in *Tc-btd*^RNAi embryos. In wild-type growing germbands, a series of Tc-En stripes arises sequentially in the gnathal, thoracic, and abdominal segments as well as in the antennal segment (Fig. 4A). In the growing germbands of *Tc-btd*^RNAi embryos, we observed irregular Tc-En stripes in the gnathal region, which was bifurcated (arrow in Fig. 4C). Interestingly, the fully extended germbands of *Tc-btd*^RNAi embryos that the segmentation was completed, were short and wide in addition to the irregular Tc-En stripes, compared with wild types (Fig. 4B, D). In particular, the head lobe including the pregnathal segments seemed to form abnormally or be absent in *Tc-btd*^RNAi germbands (arrows in Fig. 4D). In the same *Tc-btd*^RNAi embryo, we observed a trace of tissues (arrowhead in Fig. 4D'), which was anterior to the defective head region (arrow in Fig. 4D') before dissecting the germband from the yolk. Such a trace of tissues was never observed in wild-type embryos. The defects in the head and trunk of *Tc-btd*^RNAi germbands suggest that the abnormal head and abdomen of *Tc-btd*^RNAi larval cuticles might be caused by some defects occurred in the early segmenting germbands.

DISCUSSION

We have re-analyzed expression patterns and functions of *Tc-btd* in *Tribolium* segmentation. We confirm the segmental expression of *Tc-btd* with single segmental periodicity during embryonic segmentation, as reported previ-
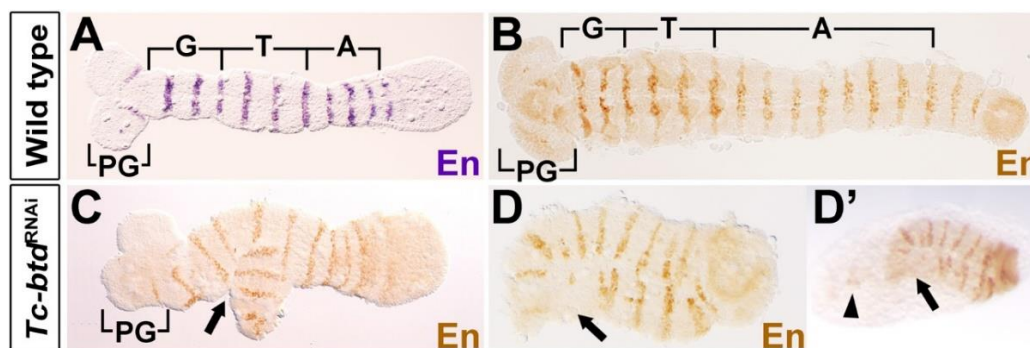

Fig. 4. Germbands of *Tc-btd* ^RNAi embryos. (A–D) *Tribolium* germbands stained with Anti-En antibody. (A) In the growing wild-type germband, Tc-En is expressed as stripes in the gnathal (G), thoracic (T) and abdominal (A) regions. Tc-En is also seen in the antennal segment of the pregnathal (PG) region. (B) Tc-En is still expressed as stripes in the trunk of the fully grown wild-type germband. (C) This elongating *Tc-btd* ^RNAi germband has bifurcated gnathal regions (arrow), with the striped Tc-En expression. (D) In this *Tc-btd* ^RNAi embryo, the shape of germband (arrow) is abnormal, with the anterior regions including the head lobe being unidentified. However, it still expresses Tc-En as stripes. (D') The *Tc-btd* ^RNAi embryo that remains intact with yolks before dissecting the germband (arrow) out. A trace of tissue (arrowhead) is seen in the anterior region of the embryo. Anterior is to the left.

ously (Schinko et al., 2008). However, we find that *Tc-btd* is required for the development of the head and trunk in the later larval cuticles as well as in the early embryonic germbands, which was not revealed in the previous report (Schinko et al., 2008). This finding raises immediately two issues on the role of *Tc-btd* in *Tribolium* development.

The first issue is why the requirement of *Tc-btd* in the head development was not revealed in the previous study that utilized the parental and embryonic RNAi and confirmed significant knock-down of *Tc-btd* (Schinko et al., 2008). A reason for that could be the different genetic background of strains that has a major effect on the penetrance and severity of defects for the loss of a gene. Indeed, it has been reported that phenotypes generated with RNAi knock-down depend on the genotype of the used strain in *Tribolium* (Kitzmann et al., 2013). To knock down *Tc-btd*, we utilized wild-type GA2 strain in the current study, whereas the wild-type stain used for knocking down *Tc-btd* was not properly documented in the previous report (Schinko et al., 2008). Thus, the different genetic background of the wild-type strains used in the previous and current studies might be a reasonable explanation for the different conclusion on the *Tc-btd* function in head development. However, we could not completely rule out a possibility that potential off-target effects by knock-down of *Tc-btd* may cause the defects in the head and trunk.

The second issue is on the effect of *Tc-btd* in the head and trunk development; is it necessary to pattern or shape the germbands? In order to shape the germband, first the germ rudiment forms with the condensation of blastomeres, and then extends its length fully through the combination of proliferation of cells at the posterior tip of the germ rudiment and the convergent extension of those cells (Brown et al., 1994; Cepeda et al., 2017). The head is patterned at the stage of blastoderm even before the germ rudiment forms, and the trunk is patterned at the stage of germband extension (Brown et al., 1994). The Tc-En stripes in the bifurcated head regions of the early growing

Tc-btd[RNAi] germband suggest that somehow head patterning is achieved but the morphogenesis of the germband is disrupted. The short and wide germband with the Tc-En stripes in the fully extended *Tc-btd*[RNAi] germband shows that germband growth is disrupted, whereas somehow the pattering of the trunk segmentation occurs. In addition, compared with other *Tribolium* segmentation genes that are expressed in the broad domains of the early blastoderm and in the posterior regions of the growing germbands (Choe et al., 2006; Choe & Brown, 2007; Sarrazin et al., 2012; El-Sherif et al., 2012), *Tc-btd* seems to be expressed too late for patterning the head and trunk segmentation in the blastoderm and growing germbands, respectively. Based on the phenotypes of *Tc-btd*[RNAi] germbands and the expression patterns of *Tc-btd*, we propose that *Tc-btd* could be involved in shaping rather than in patterning the germbands at the stages of blastoderm and germband extension. In this context, the tissues of the abnormally shaped *Tc-btd*[RNAi] germbands would not be patterned and survived properly in the later stages of head and trunk development, so would lead to the loss of the pregnathal and some abdominal segments in the *Tc-btd*[RNAi] larvae. Comparative functional analysis of *btd* orthologs in other insects would reveal to what extent *btd* plays a role in patterning or morphogenesis for the development of early germbands.

ACKNOWLEDGMENTS

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