Apparent role of Tribolium orthodenticl e in anteroposterior blastoderm patterning largely reflects novel functions in dorsoventral axis formation and cell survival

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SUMMARY
In the short-germ beetle Tribolium castaneum, the head gap gene orthodenticl e (Tc-otd) has been proposed to functionally substitute for bicoid, the anterior morphogen unique to higher dipterans. In this study we reanalyzed the function of Tc-otd. We obtained a similar range of cuticle phenotypes as in previously described RNAi experiments; however, we noticed unexpected effects on blastodermal cell fates. First, we found that Tc-otd is essential for dorsoventral patterning. RNAi depletion results in lateralized embryos, a fate map change that by itself can explain the observed loss of the anterior head, which is a ventral anlage in Tribolium. We find that this effect is due to diminished expression of short gastrulation (sog), a gene essential for establishment of the Decapentaplegic (Dpp) gradient in this species. Second, we found that gnathal segment primordia in Tc-otd RNAi embryos are shifted anteriorly but otherwise appear patterned normally. This anteroposterior (AP) fate map shift might largely be due to diminished zen-1 expression and is not responsible for the severe segmentation defects observed in some Tc-otd RNAi embryos. As neither Tc-sog nor Tc-zen-1 probably requires Otd gradient-mediated positional information, we posit that the blastoderm function of Tc-Otd depends on its initial homogeneous maternal expression and that this maternal factor does not provide significant positional information for Tribolium blastoderm embryos.

KEY WORDS: Tribolium, Short-germ insect, Anteroposterior patterning, Dorsoventral patterning, Orthodenticl e, Bicoid

INTRODUCTION
Morphogenesis in Drosophila is a result of progressive subdivision of the embryo along the dorsoventral (DV) and anteroposterior (AP) axes. Development of each of these axes is initiated by maternally supplied factors and development proceeds largely independently along the two axes (Lall and Patel, 2001). DV polarity in Drosophila depends on the nuclear concentration gradient of the Dorsal transcription factor, which subdivides the embryo into a series of domains with different fates: amnioserosa, dorsal ectoderm, neurogenic ectoderm and mesoderm. The boundaries of these domains are established by the activities of zygotic genes transcribed at different thresholds of the Dorsal protein (Moussian and Roth, 2005; Statopoulos and Levine, 2002). One zygotic target of Dorsal is the gene decapentaplegic (dpp), encoding a bone morphogenetic protein (BMP) homolog. Dpp specifies at high levels the extra-embryonic amnioserosa, whereas moderate BMP levels mediate dorsal ectoderm (Moussian and Roth, 2005; Statopoulos and Levine, 2002). At more-ventral positions, BMP activity is inhibited by Short gastrulation (Sog) and Brinker (Brk), which allow neuroectoderm specification (Ashe and Levine, 1999; Biels et al., 1996; Marques et al., 1997). Whereas Brinker is dispensable for Tribolium embryogenesis, Sog is essential to direct Dpp activity towards the dorsal side of the embryo, and the depletion of sog leads to the complete loss of neurogenic tissue (van der Zee at al., 2006).

Drosophila AP axis formation depends on spatial gradients of the maternal transcription factors Bicoid (Bcd) and Hunchback (Hb) (St Johnston and Nusslein-Volhard, 1992). Bcd activates multiple target genes, including gap and pair-rule genes (Blankenship and Wieschaus, 2001; Driever and Nusslein-Volhard, 1988; Gao and Finkelstein, 1998; Hulskamp et al., 1990; Rivar-Pomar et al., 1995; Small et al., 1992). In the absence of Bcd, the head, thorax and a subset of abdominal segments fail to develop and anterior terminal structures are transformed to a posterior fate (Driever and Nusslein-Volhard, 1988). However, although Bcd is essential to anterior patterning in Drosophila, no bcd ortholog has been isolated in any organism apart from the higher dipterans (Brown et al., 2001; Stauber et al., 1999; Stauber et al., 2002; Richards et al., 2008). It has been alleged that bcd evolved through duplication of an ancestral Hox-3/zen homolog and that afterwards one copy, zen, retained its function in specifying extra-embryonic tissues (Stauber et al., 1999), whereas the other copy (bcd) acquired new features, resembling those of orthodenticl e (otd, ac – FlyBase) (Finkelstein and Perrimon, 1990; Tahayato et al., 2003; Wimmer et al., 2000). In insects, Otd acts as a zygotic head gap gene (Cohen and Jurgens, 1990; Finkelstein and Perrimon, 1990; Finkelstein et al., 1990; Schinko et al., 2008; Schroder, 2003). Owing to this conserved function in head development and the Bcd-like DNA binding specificity, Otd has been proposed as an ancestral anterior patterning gene in insects (Lynch et al., 2006; Schroder, 2003; Wimmer et al., 2000).

In the short-germ beetle Tribolium castaneum (Klingler, 2004; Richards et al., 2008), two otd-related genes (Tc-otd-1 and Tc-otd-2) were identified, of which only Tc-otd-1 is expressed during early embryonic stages (Schroder, 2003). Although Tc-otd-1 mRNA is initially distributed uniformly, Tc-Otd-1 later withdraws from the posterior pole resulting in a transient anterior-to-posterior protein...
gradient (Schroder, 2003). As development proceeds, Tc-Otd-1 also retracts from the anterior pole, forming a domain that covers the anterior head, resembling \textit{otd} expression in \textit{Drosophila} (Cohen and Jurgens, 1990; Finkelstein and Perrimon, 1990; Finkelstein et al., 1990; Schinko et al., 2008; Schroder, 2003). In \textit{Drosophila}, depletion of \textit{otd} results in the deletion of the ocular and the antennal segments (Cohen and Jurgens, 1990; Finkelstein and Perrimon, 1990; Finkelstein et al., 1990). A comparable phenotype has been described for \textit{Tribolium} after knockdown of zygotic Tc-\textit{otd-1} by late blastodermal dsRNA injection (Schinko et al., 2008). Thus, it is feasible to assume that the zygotic function of \textit{otd} in patterning pregnathal segments is conserved among these insects (Schinko et al., 2008).

Knocking down Tc-\textit{otd-1} by parental RNAi results in larvae that are depleted of anterior segments (Schinko et al., 2008; Schroder, 2003). This segmentation phenotype is enhanced by the combined knockdown of Tc-\textit{otd-1} and Tc-\textit{hb}, which led to the proposition that Otd and Hb might substitute for Bcd as anterior morphogens in non-dipteran insects (Schroder, 2003). This idea is supported by functional studies in the long-germ wasp \textit{Nasonia}, where \textit{hb} and \textit{otd} are crucial to early patterning (Lynch et al., 2006; Pulzt et al., 2005). However, recent work in \textit{Tribolium} suggests that \textit{hunchback} functions in abdominal metamORIZATION only, whereas in the head and thorax it regulates segment identity (Marques-Souza et al., 2008), raising the question whether Otd can indeed provide concentration-dependent positional information, i.e. act as a Bcd-like morphogen, during \textit{Tribolium} blastoderm patterning.

In this study, we investigated the role of orthodenticle during early \textit{Tribolium} development. Surprisingly, we found that Tc-\textit{otd-1} RNAi results in cell lethality already during the differentiated blastoderm stage. To elucidate whether this phenotype is due to disruption of early AP patterning, we monitored gap and pair-rule gene expression patterns throughout blastoderm stages. We found that patterning of gnathal and thoracic segment primordia is not severely affected, whereas the anlagen of the pregnathal head and the serosa are reduced in size. Unexpectedly, we found Tc-\textit{otd-1} RNAi also to have a major impact on DV patterning. We identified Tc-\textit{zen-1} and Tc-\textit{sog} as major target genes of Tc-\textit{otd-1}. Both genes, however, are unlikely to receive concentration-dependent positional input from Tc-\textit{otd-1}. Instead, Tc-\textit{otd-1} appears to provide a more general function in AP and DV axis formation during \textit{Tribolium} short-germ development.

**MATERIALS AND METHODS**

**Histology**

Fixation, single in situ hybridization and immunofluorescence staining were performed using standard protocols (Patel et al., 1989; Tautz and Pfeifle, 1989). For double staining, fluorescein- and digoxigenin-labeled probes were detected using alkaline phosphatase and beta-galactosidase, the latter after signal enhancement via biotin deposition (Ppic et al., 2001). The rabbit polyclonal pMAD antibody (gift of Dr Morata, Centro de Biologia Molecular, Madrid, Spain) was used at a concentration of 1:3000. An alkaline-phosphatase-conjugated goat anti-rabbit antibody (1:2000, Dianova) was used for detection. Embryos were subsequently counterstained with Hoechst 33342. TUNEL assays were performed as previously described (Ppic and Damen, 2005). A detailed protocol is available from the authors.

**RNAi**

Parental RNAi was performed as described (Bucher and Klingler, 2004; Bucher et al., 2002). For Tc-\textit{otd-1} dsRNA synthesis, the previously described full-length clone (Schroder, 2003) and two non-overlapping PCR fragments were used as a template. RNAi for all three fragments yielded identical Tc-\textit{otd-1} RNAi phenotypes, excluding the possibility that an unrelated gene was affected. Tc-\textit{sog} and Tc-\textit{zen-1} dsRNAs were produced from previously described clones (Falciani et al., 1996; van der Zee et al., 2005). Double-stranded RNAs were injected into female pupae at a concentration of 1 \( \mu \text{g/\mu l} \) (Tc-\textit{otd-1} and Tc-\textit{zen-1}). To produce a phenotypic series of Tc-\textit{sog} RNAi phenotypes, dsRNA at concentration of 0.2 \( \mu \text{g/\mu l} \) was injected. Females were mated with wild-type males and reared under standard conditions.

**RESULTS**

Tc-\textit{otd-1} RNAi causes a fate map shift at the expense of the pregnathal region

Previous studies have indicated that the loss of Tc-\textit{otd-1} function causes the deletion of anterior segments including pregnathal, gnathal and, in the strongest cases, even thoracic segments (Schinko et al., 2008; Schroder, 2003). However, these authors did not elucidate how Tc-\textit{otd-1} affects the earliest stages of AP patterning. To understand how Tc-\textit{otd-1} regulates the blastoderm fate map, we investigated the expression patterns of the \textit{Tribolium} gap genes \textit{hunchback} (Tc-\textit{hb}), giant (Tc-\textit{gt}), knirps (Tc-\textit{kni}), mille-pattes (Tc-\textit{mlpt}) and Krüppel (Tc-\textit{Kr}) (Bucher and Klingler, 2004; Cerny et al., 2005; Cerny et al., 2008; Marques-Souza et al., 2008; Savard et al., 2006; Sommer and Tautz, 1993; Wolff et al., 1995) in Tc-\textit{otd-1} RNAi embryos (Fig. 1).

In wild type, Tc-\textit{hb} initially is expressed homogeneously. With the formation of the serosa, Tc-\textit{hb} fades from the anterior head, which results in the formation of a serosal and a gnathal domain (Fig. 1A) (Woff et al., 1995). Upon depletion of Tc-\textit{otd-1}, the gnathal Tc-\textit{hb} domain was shifted towards the anterior and the distance between this domain and the \textit{hb} serosa domain was strongly reduced (Fig. 1AB).

Tc-\textit{gt} forms at the differentiated blastoderm stage an anterior domain comprising pregnathal and gnathal segments, but excluding the serosa (Fig. 1C) (Bucher and Klingler, 2004). In Tc-\textit{otd-1} RNAi, this domain was shifted towards the anterior and its size was reduced (Fig. 1D). It appears that the less strongly expressing portion of the domain corresponding to the anterior head is reduced in size, whereas the strongly expressed gnathal part is less affected.

The expression of \textit{Tribolium} knirps (Tc-\textit{kni}) (Cerny et al., 2008) arises at the undifferentiated blastoderm stage in a rather broad central domain, which refines to a wedge-shaped domain covering the mandibular and pregnathal region (Fig. 1E). As for Tc-\textit{gt} and Tc-\textit{hb}, the depletion of Tc-\textit{otd-1} resulted in a shift of Tc-\textit{kni} expression towards the anterior and a concomitant reduction in size (Fig. 1F). These changes in Tc-\textit{hb}, Tc-\textit{gt} and Tc-\textit{kni} expression suggest that Tc-\textit{otd-1} RNAi embryos lack pregnathal anlagen already during early embryonic stages, whereas the gnathal region is expanded rather than reduced (see Fig. S2 in the supplementary material for Tc-empty-spiracles and Tc-buttonhead expression in Tc-\textit{otd-1} RNAi). This coordinated shift of expression domains might extend to \textit{Tribolium} Krüppel (Cerny et al., 2005; Sommer and Tautz, 1993), which appeared to expand toward the anterior in Tc-\textit{otd-1}-depleted embryos (Fig. 1IL). Unlike anterior gap gene domains in \textit{Drosophila} bicoid mutant embryos, none of these domains were lost in Tc-\textit{otd-1} RNAi embryos. The anterior Tc-\textit{mlpt} domain (Savard et al., 2006), however, was abolished by Tc-\textit{otd-1} RNAi (Fig. 1H), indicating
that, unlike other gap genes, Tc-mlpt requires Tc-otd-1 for its activation. However, given that the anterior Tc-mlpt domain has no obvious function in segmentation (Savard et al., 2006), it does not appear that the loss of Tc-mlpt activity contributes to the Tc-otd-1 RNAi phenotype.

Gnathal and thoracic anlagen are represented in Tc-otd-1 RNAi blastoderms

To further elucidate the function of Tc-otd-1 in patterning gnathal and thoracic segments, we visualized the emergence of segment primordia by analyzing the Tc-even-skipped expression pattern (Fig. 2). In wild-type embryos, Tc-eve is initially expressed in a double-segmental pattern, each stripe later resolving into secondary segmental stripes (Patel et al., 1994; Schoppmeier and Schroder, 2005). The first two primary Tc-eve stripes (Tc-eve stripe 1 and stripe 2; Fig. 2A), mark the maxillary and the first thoracic (T1) segment primordia, whereas the third primary stripe (Tc-eve stripe 3) covers the region where the growth zone will develop (Fig. 2C). In Tc-otd-1 RNAi embryos, these primary Tc-eve stripes were formed, but the position of the first and second primary stripes was shifted towards the anterior (Fig. 2B). In addition, stripe 2 arose much broader than in wild type and only later refined to nearly normal width (Fig. 2D). Thus, like in wild type, all three primary Tc-eve domains develop during blastodermal stages, and their position and width reflects an expanded fate map congruous to the shifted positions of gap gene domains as described above.

We then asked whether other primary pair-rule gene patterns are similarly shifted. To this end, we double-stained wild-type and Tc-otd-1-depleted embryos for Tc-eve and Tc-runt and Tc-eve and Tc-odd, respectively (Fig. 3). In wild type, the primary Tc-eve stripe 1 partially overlaps with the first primary runt stripe (Fig. 3A), whereas Tc-odd stripe 1 was expressed complementary to Tc-eve stripes 1 and 2 (Fig. 3B) (Choe et al., 2006). Also in Tc-otd-1 knockdown embryos, the expression domains of Tc-odd and Tc-run...
were shifted towards the anterior. Importantly, the stripes of all three pair-rule genes appeared to be shifted in a coordinated manner and phasing among pair-rule patterns was preserved (Fig. 3C,D). These results clearly demonstrate that knockdown of Tc-otd-1 does not affect the blastoderm fate map in a manner that could explain the loss of gnathum and thorax in RNAi larvae.

We further tested whether the combined knockdown of Tc-otd-1 and Tc-hb has a more severe impact on the early fate map (Schroder, 2003). In Tc-hb RNAi blastoderms, pair-rule gene expression was delayed to some degree, as the third, and occasionally the second, primary stripes did not form until the early germ rudiment (Marques-Souza et al., 2008). Nevertheless, the gnathal segment anlagen are formed in Tc-hb RNAi embryos (Marques-Souza et al., 2008). Although we found Tc-hb RNAi not to shift the blastodermal fate map (Fig. 2G), in double-knockdown embryos a similar shift of the early Tc-eve domain was evident as in the Tc-otd-1 RNAi situation (Fig. 2H). Thus, the Tc-otd-1 RNAI-mediated AP fate map shift is not enhanced by simultaneous knock-down of Tc-hb.

**Tc-otd-1 is required for serosa formation**

Depletion of Tc-otd-1 affects the morphological integrity of the serosa during differentiated blastoderm stages (Schroder, 2003). To analyze early patterning of the serosa anlage, we stained Tc-otd-1 RNAi embryos for zerknüllt-1 (Tc-zen-1), which in wild type is already expressed in the presumptive serosa before these cells become morphologically distinct from embryonic tissue (Fig. 4A) (Falciani et al., 1996; van der Zee et al., 2005). Knockdown of Tc-otd-1 restricted early Tc-zen-1 expression to a small region near the anterior pole of the egg, indicating that the anlage of the serosa was severely reduced but not completely absent (Fig. 4A,B). Thus, Tc-Otd-1 appears to contribute to the early serosal size regulation.

Strikingly, although in wild type the serosa-embryo boundary in the differentiated blastoderm is oblique, the smaller Tc-zen-1 domain in Tc-otd-1 RNAi embryos was rotationally symmetric with respect to the egg axis (Fig. 4B). Rotational symmetry of the Tc-zen-1 domain is indicative of defective DV patterning (Nunes da Fonseca et al., 2008; van der Zee et al., 2006). Thus, the loss of Tc-zen-1 expression asymmetry and the symmetric serosa-embryo boundary in Tc-otd-1 RNAi embryos (e.g. Fig. 1A,B,K,L; Fig. 2E,F; Fig. 4A,B,E,F) suggested that Tc-otd-1 might not only be involved in early serosa size regulation, but also influence DV axis formation.

![Fig. 3. Pair-rule gene expression in Tc-otd-1 RNAi. (A-D) Stage-matched wild-type (A,B) and Tc-otd-1 RNAi embryos (C,D) double-stained for even-skipped and runt (A,C) or even-skipped and odd-skipped (B,D). (A,C) In Tc-otd-1 RNAi, the anterior Tc-eve stripe 1 and Tc-runt stripe 1 domains shift towards the anterior. Both are still expressed in the same register, indicating that their interactions are largely unchanged. (B,D) Apart from slight variations, Tc-odd and Tc-eve are still expressed in complementary anterior domains upon Tc-otd-1 RNAi.

![Fig. 4. Tc-otd-1 RNAi affects dorsoventral patterning. (A-N) Stage-matched wild-type (A,C,E,G,I,K,M) and Tc-otd-1 RNAi embryos (B,D,F,H,J,L,N). Embryos in C,D were counterstained by Hoechst 33342 to visualize the morphology (E,F). (A,B) The serosal Tc-zen-1 domain is symmetric and reduced in Tc-otd-1 RNAi (arrowheads). (C-F) As indicated by the Tc-iro staining, the serosa embryo boundary has lost its obliqueness in Tc-otd-1. Moreover, the dorsal amnion (arrow in C) is not specified in Tc-otd-1 RNAi. (G,H) Whereas in the wild-type Tc-doc is restricted to the dorsal serosa, it becomes ubiquitously expressed in Tc-otd-1 RNAi (arrowheads in H point to the posterior border of the serosa). (I,J) The ventral Tc-sog domain is severely reduced in Tc-otd-1 RNAi. (K,L) Embryos were stained with an antibody recognizing phosphorylated MAD (pMAD). In Tc-otd-1 RNAi, pMAD becomes ubiquitously expressed in the serosa. (M,N) In Tc-otd-1 RNAi, Tc-dpp becomes symmetrically expressed. All panels are lateral views except for I,J, which are ventral views.
Tc-otd-1 controls Dpp activity via the regulation of sog expression

It has been shown previously that the obliqueness of the serosa-germ rudiment border in Tribolium depends on BMP signaling. In the beetle blastoderm, Short gastrulation (Sog) is required to direct Dpp activity towards the dorsal side of the embryo (van der Zee et al., 2006). Interestingly, BMP signaling in Tribolium also is required for head formation as the head is a ventral anlage (van der Zee et al., 2006). Consequently, ventralization of the embryo through Tc-dpp RNAi results in an enlarged head region at the expense of the serosa. In such embryos, the serosa-germ rudiment border is rotationally symmetric and located at a more anterior position (van der Zee et al., 2006). Consequently, ventralization of the embryo required for head formation as the head is a ventral anlage (van der Zee et al., 2006). Thus, there are obvious similarities between Tc-otd-1 and Tc-sog RNAi phenotypes, as in both phenotypes the serosa-germ rudiment boundary is rotation-symmetric and the anterior head is deleted.

To elucidate the impact of Tc-Otd-1 on DV axis formation, we monitored the expression of dorsocross (Tc-doc), which is asymmetrically distributed along the DV axis (Nunes da Fonseca et al., 2008; Tomoyasu et al., 2005; van der Zee et al., 2006). In wild type, Tc-doc is expressed in the dorsal 30% of the serosa (Fig. 4G). Tc-otd-1 knockdown resulted in Tc-doc expression expanding into a broad DV-symmetric domain covering most of the residual serosa anlage (Fig. 4H). This resembles the situation in Tc-sog RNAi embryos (Nunes da Fonseca et al., 2008; van der Zee et al., 2006). Thus, in both Tc-otd-1 and Tc-sog RNAi embryos, anterior tissue becomes dorsalized, as if the deletion of Tc-otd-1 would prevent the diffusion-driven ventral-to-dorsal transport of Dpp by Sog. To investigate this further, we analyzed the expression of Tc-sog and Tc-dpp in Tc-otd-1 RNAi embryos and monitored the activity of the Dpp signaling cascade by antibody staining against phosphorylated MAD (Mothers against Dpp; Fig. 4).

In wild type, Tc-sog is initially expressed in a broad ventral domain, which subsequently becomes more narrow and retracts from the serosa anlage (van der Zee et al., 2006). Tc-dpp mRNA is initially expressed near the anterior pole of the blastoderm embryo and later becomes localized in a stripe along the serosa-embryo boundary (Fig. 3M) (van der Zee et al., 2006). It has been shown that this anterior pattern of Tc-dpp expression is converted into a DV activity gradient (as monitored by pMAD staining) by Sog-mediated ventral-to-dorsal transport of Dpp (Fig. 4K) (van der Zee et al., 2006). We found that the ventral expression of Tc-sog was almost completely lost upon Tc-otd-1 RNAi, as only a few cells near the serosa-embryo boundary still expressed Tc-sog mRNA (Fig. 4J). Consistent with Tc-sog downregulation, pMAD activity was no longer restricted to the dorsal side of the serosa in Tc-otd-1 RNAi embryos but became ubiquitously distributed in this tissue (Fig. 4L), which is in accordance with the changes in expression of the Dpp target gene Tc-doc (Fig. 4H). Dpp activity in embryonic tissue, however, was largely absent (Fig. 4L), again resembling the situation after Tc-sog RNAi (van der Zee et al., 2006). These results indicate that Tc-Otd-1 positively regulates Tc-sog expression, and thus is required for establishing DV polarity.

Consistent with reduced Sog activity, not only ventral tissue, but also dorsal structures, are lost upon Tc-otd-1 RNAi. In the wild type, the iroquois gene (Tc-iro) (Nunes da Fonseca et al., 2008; Tomoyasu et al., 2005; van der Zee et al., 2006) is expressed in a narrow stripe marking the anterior border of the germ rudiment, which will give rise to the anterior amnion (Fig. 4C,E). In addition, Tc-iro is expressed in the dorsal amnion (Nunes da Fonseca et al., 2008). In Tc-otd-1 RNAi embryos, the anterior stripe of Tc-iro expression was maintained, but became rotation-symmetric, indicating that the anterior amnion anlagen was present while the dorsal amnion was lost, as no dorsal or posterior Tc-iro expression could be detected (Fig. 4D,F). Thus, as in Tc-sog RNAi embryos (van der Zee et al., 2006), anterior tissue is dorsalized after Tc-otd-1 RNAi, whereas more-posterior tissues lack peak levels of Dpp activity and become lateralized.

In conclusion, Tc-otd-1 is essential for the appropriate distribution of Dpp activity. In Tc-otd-1 RNAi embryos, Sog levels are severely reduced and Dpp activity does not become restricted to the dorsal side. As a consequence, the serosa-germ rudiment border remains straight and anterior embryonic tissue becomes dorsalized, which causes the loss of the anterior head.

Can the combined knockdown of sog and zen-1 phenocopy the effect of Tc-otd-1 RNAi?

Our discovery that Tc-otd-1 is involved in DV patterning raises the question of to which degree this function by itself can explain the loss of head structures in Tc-otd-1 RNAi larvae. To investigate if partial inactivation of Tc-sog results in head phenotypes similar to Tc-otd-1 RNAi larvae, we injected double-stranded Tc-sog RNA at low concentrations (200 ng/μl) into female pupae and analyzed their offspring for larval cuticle phenotypes (see Fig. S3, Table S2 in the supplementary material). In the first week of egg collection, most Tc-sog RNAi larvae displayed strong cuticle aberrations, including the ‘double dorsal’ phenotype described by van der Zee et al. (van der Zee et al., 2006) (see Fig. S3B-D in the supplementary material). As the RNAi effect subsides, normal cuticle morphology is restored in a posterior-to-anterior direction, ultimately resulting in larvae exhibiting normal DV polarity but lacking segments of the anterior head, resembling the situation after Tc-otd-1 RNAi (see Fig. S3F in the supplementary material). Thus, in weak Tc-sog, as in Tc-otd-1 RNAi, phenotypes, the anterior head (see Fig. S3F in the supplementary material) is deleted, indicating that the head defects in Tc-otd-1 RNAi might well be due to dorsoventral misspecification of anterior ventral tissue (Fig. 7A,B).

Tc-otd-1 RNAi alters DV positional information through regulation of sog, even though Tc-Otd-1 itself is rotation-symmetrically expressed. Evidently, the spatially specific expression of Tc-sog must be regulated by another factor, i.e. Dorsal (Nunes da Fonseca et al., 2008). This raises the question of whether the observed shift of gap and pair-rule gene domains reflects positional information provided by Tribolium Otd or if this blastoderm fate map shift might also be an indirect effect. It has been shown previously that inactivation of Tc-sog results in an expansion of anterior germ rudiment structures (van der Zee et al., 2005) and that Tc-zen-1 expression is reduced in Tc-otd-1 RNAi embryos (Fig. 4B). We wondered whether the combined knockdown of the Tc-Otd-1 target genes Tc-sog and Tc-zen can phenocopy the Tc-otd-1 RNAi fate map shift along the AP axis. Indeed, we found that the double-knockdown of the Tc-zen-1 and Tc-sog results in a coordinated shift of knirps, mlpt and even-skipped expression domains towards the anterior, a shift not observed in embryos depleted for Tc-sog alone (Fig. 5; see also Fig. S2 in the supplementary material). Interestingly, whereas Tc-otd-1 RNAi also results in the loss of the gnathal Tc-mlpt domain (Fig. 1H), in Tc-
sog Tc-zen-1 double-RNAi this Tc-mlpt domain is still present (Fig. 5C,D), suggesting that, in addition to Tc-sog and Tc-zen-1, Tc-Otd-1 activates at least one additional gene.

The combined knockdown of Tc-zen-1 and Tc-sog results in a phenotype that comprises most aspects of the Tc-otd-1 RNAi phenotype. As regulation of Tc-zen-1 is unlikely to depend on the Tc-Otd-1 gradient, it seems possible that Tc-Otd-1 does not provide concentration-dependent positional information at the blastoderm stage. **Tc-otd-1 knockdown causes early embryonic lethality**

In addition to the phenotypes described above, strongly affected Tc-otd-1 RNAi embryos suffer from additional severe defects. Only about 30% of eggs collected in the first week after eclosion of injected animals were capable of secreting a larval cuticle, whereas 70% of the embryos die prior to completion of embryonic development (see Fig. S1, Table S1 in the supplementary material). This phenotype differs from that of other DV or AP patterning studies (Schinko et al., 2008; Schroder, 2003). Interestingly, these more-posterior defects do not seem to fall into a clear AP progression, i.e. anterior thoracic defects are not more frequent than posterior thoracic defects, and these are not more frequent than abdominal defects (see Table S1 in the supplementary material). Thus, these trunk phenotypes cannot easily be ordered into a phenotypic series reflecting the AP Otd gradient. These randomly positioned defects can be explained, however, by randomly located apoptotic patches emerging in embryos where the degree of Tc-otd-1 inactivation approaches the threshold required for cell survival. This model implies that, in addition to Tc-sog and several segmentation genes, Otd has additional as yet unidentified target genes, which we admit remain to be identified (candidates include any early expressed housekeeping gene).

**DISCUSSION**

In this paper, we demonstrate two previously overlooked functions of Tc-otd-1, which are effected outside of the AP patterning paradigm but dominate the apparent segmentation phenotype of this gene in Tribolium. In consequence, we argue that the Tc-otd-1 segmentation function itself is more limited than previously thought, and that it shares few similarities with the role that Bicoid plays during Drosophila segmentation.

**Fig. 5. Tc-zen-1 and Tc-sog double RNAi results in a Tc-otd-1-like fate map shift.** (A-F) Stage-matched Tc-sog RNAi-differentiated blastoderms (A,C,E) and Tc-Sog Tc-zen-1 double RNAi embryos (B,D,F) stained for Tc-kni (A,B), Tc-mlpt (C,D) or Tc-eve (E,F). Wild-type expression is described in Fig. 1 (Tc-kni, Tc-sog) or Fig. 2 (Tc-eve), respectively. (A) In Tc-sog RNAi, the anterior kni domain is reduced but not shifted (bars). (B) In the Tc-sog and Tc-zen-1 double knockdown, an anterior shift of the kni domain is obvious, thus resembling the Tc-otd-1 RNAi phenotype (see Fig. 1). (C,D) In Tc-sog RNAi, the anterior mlpt domain is still present. In Tc-sog Tc-zen-1 double RNAi, this domain is shifted in the anterior direction (bars). (E) In Tc-sog RNAi, two primary Tc-eve domains are present. (F) Embryos depleted of Tc-sog and Tc-zen-1 resemble the Tc-otd-1 knockdown situation. To ensure that Tc-sog RNAi indeed did not result in Tc-otd-1-like fate map shifts, only ‘strong Tc-sog phenotypes’, which were obtained in the first week of egg collection, were analyzed. (see Fig. S3, Table S2 in the supplementary material).

**Fig. 6. Tc-otd-1 RNAi causes early cell lethality.** (A-F) Differentiated blastoderm wild-type (A) and Tc-otd-1 RNAi (C) embryos stained for TUNEL to visualize apoptosis. Embryos were counterstained by Hoechst 33342 (B,D). Stage-matched wild-type (E) and Tc-otd-1 RNAi (F) germ rudiment embryos stained for Hoechst 33342 to visualize the morphology. (A,B) In the wild type, no signs of apoptosis are obvious. (C,D) In Tc-otd-1 RNAi, most extra-embryonic and embryonic cells undergo apoptosis. (E,F) During subsequent stages of development, the morphology of Tc-otd-1 embryos is severely disrupted. Arrowheads in B and D point to the posterior boundary of the serosa. E, dorsal view; F, no obvious dorsoventral asymmetry.
The role of Tc-otd-1 in determining the AP fate map is less extensive than previously thought

There is no bicoid ortholog in Tribolium (Brown et al., 2001; Richards et al., 2008) and it has been proposed that Tc-otd-1 might play a similar role in directing patterning along the AP axis as Bcd does in Drosophila (Schröder, 2003). This conjecture was supported by a phenotypic series of Tc-otd-1 RNAi larvae – anterior deletions extending into gnathum and thorax – similar to that of bicoid mutants. Moreover, simultaneous knockdown of Tc-otd-1 and Tc-hb appeared to synergistically enhance the phenotype, reminiscent of synergistic activation of target genes by Bcd and Hb in Drosophila (Simpson-Brose et al., 1994). Given that in Drosophila the transcription factors Otd and Bcd have similar target specificities (Finkelstein and Perrimon, 1990; Tahayato et al., 2003; Wimmer et al., 2000), this proposition appeared to support a simple evolutionary model according to which, in higher dipterans, Bcd took over anterior regulatory functions previously fulfilled by Tc-otd-1 (see text for details).

Early homogeneous expression might account for all blastoderm target specificities (Finkelstein and Perrimon, 1990; Tahayato et al., 1995; Tautz, 1988). These domains are shifted anteriorly in a coordinated fashion (Fig. 1; see Fig. S2 in the supplementary material), which in principle is consistent with a long-range morphogen function of Tc-Otd-1. However, the observed fate map shift in Tc-otd-1 knockdown embryos is small and can only explain the pregnathal defects observed in the terminal phenotype, not the segmental deletions in gnathal and thoracic regions. This surprisingly small effect of Tc-otd-1 inactivation on AP patterning is confirmed at the level of pair-rule genes, where we find that the first three stripes of the primary pair-rule genes Tc-runt, Tc- eve and Tc-odd are formed normally, albeit shifted anteriorly (Figs 2, 3).

The pregnathal region – the interval between the first pair-rule gene stripe and the serosa-embryo boundary – is reduced in size in Tc-otd-1 RNAi blastoderms, consistent with the loss of pregnathal structures observed in the terminal phenotype. These pregnathal defects (see Fig. S2 in the supplementary material for Tc-ems expression) might be explained by the mild AP fate map shift we observed, but also the dorsoventral changes we find in Tc-otd-1 RNAi embryos must result in loss of the pregnathal head (see below).

It is a strange coincidence, however, that these pregnotal defects can also be explained by the dorsoventral changes we find in these RNAi embryos (see below). Moreover, it has been shown that inactivation of Tc-otd-1 at post-blastodermal stages also results in the loss of labrum and antennae (Schinko et al., 2008). Therefore, it is remarkable that all three obvious patterning effects of Tc-otd-1 can explain loss of the most-anterior head structures, not the segmental deletions in gnathal and thoracic regions in the terminal phenotype.

It had been previously proposed that Tc-Otd-1 and Tc-Hb cooperatively function as anterior morphogens in Tribolium (Schröder, 2003). However, the fate map shift in Tc-otd-1 RNAi blastoderms is not enhanced by the simultaneous knockdown of Tc-hb (Fig. 2G,H). Thus, there is no obvious synergistic effect of Tc-otd-1 and Tc-hb on blastodermal patterning. Marques-Souza and colleagues (Marques-Souza et al., 2008) already showed (and our unpublished results confirm this) that knockdown of Tc-hb results not in the loss of gnathal and thoracic segments but in their transformation to abdominal fates. Abdominal segments are deleted, however, in strong Tc-hb RNAi embryos. Thus, the severe double-RNAi defects (Schröder, 2003) should be interpreted as a superposition of anterior deletions (Tc-otd-1) and posterior deletions (Tc-hb) rather than synergistic morphogen action.

Given that the gnathal and thoracic region are enlarged rather than reduced in Tc-otd-1 RNAi blastoderm embryos, their absence in many RNAi larvae appears to be due to secondary loss at post-blastodermal stages. Several mechanisms could account for this secondary loss. According to one scenario, certain Tc-otd-1 target genes might be shifted or misexpressed to a larger degree than others, resulting in abnormal overlaps of expression activities, which might be incompatible with continued development of those primordia. However, we found that a number of segmentation gene domains shift in a coordinated fashion (Figs 1-3; see also Figs S2 and S5 in the supplementary material). Nevertheless, we cannot exclude that some other gene(s) might fail to obey that pattern. Our finding that the anterior domain of mlpt is affected differently than those of Tc-kni and Tc-gt (Fig. 1) could be seen as support for such a mechanism, even though this domain does not appear to be involved in segmentation (Savard et al., 2006). However, the pleiotropic nature of the Tc-otd-1 phenotype suggests a different explanation for the secondary loss of gnathal and thoracic primordia in these embryos (see below).
**Tc-otd-1 is essential for sog activation and dorsoventral patterning**

*Tribolium* wild-type embryos exhibit, at the differentiated blastoderm stage, a distinct dorsoventral polarity in that the serosa expands dorsally, displacing the embryonic tissue towards the ventral and posterior (Falciani et al., 1996; van der Zee et al., 2005). By contrast, *Tc-otd-1* RNAi embryos retain rotation symmetry at this stage and during subsequent gastrulation movements (Fig. 4). This phenotype is reminiscent of that caused by knockdown of dorsoventral genes (Nunes da Fonseca et al., 2008; van der Zee et al., 2006). Indeed, we find that mRNA expression of the pivotal ventral factor *Tc-sog* is severely reduced in these embryos. Similar to *Tc-sog* RNAi embryos, *Tc-otd-1* blastoderms are dorsetalized at an anterior-ventral level and lateralized at a posterior-dorsal level, as demonstrated by circumferential expression of a dorsal serosa marker (*doc*), loss of dorsal amnion (*pnr*) and a reduced dorsal activity of the zygotic dorsal morphogen *Tc-Dpp* (pMAD; Fig. 4).

The anterior head is a ventral primordium in *Tribolium* (van der Zee et al., 2006) and dorsetalized embryos lack anterior head structures. This is also the case in *Tc-sog* RNAi larvae (see Fig. S3 in the supplementary material) (van der Zee et al., 2006), and the severe reduction of *Tc-sog* expression principle could fully account for the loss of anterior head primordia in *Tc-otd-1* RNAi embryos (Fig. 7). As *Tc-sog* expression also requires activation by Dorsal (Nunes da Fonseca et al., 2008), our results indicate that DV axis patterning (and anterior head formation) in *Tribolium* depends on the combinatorial input of signals from DV (Tc-Dorsal) and AP (Tc-otd-1) patterning systems. Also in *Drosophila*, the formation of AP and DV axes is not entirely independent, as certain aspects of AP gene expression respond to DV positional information (Carroll et al., 1987; Gao and Finkelstein, 1998; Liaw and Lengyel, 1993; Rothe et al., 1994; Zeitlinger et al., 2007). However, segmentation gene expression in *Drosophila* is only modulated somewhat by the DV system, whereas in *Tribolium* the input of the ‘AP factor’ Otd-1 is absolutely essential for DV patterning.

An important difference between the fate map changes in *Tc-sog* and *Tc-otd-1* RNAi embryos, however, concerns the size of the serosa. Consistent with the oblique boundary of the serosa anlagen in wild type, in dorsetalized embryos the serosa expands at the expense of the (ventrally located) anterior head. In *Tc-otd-1* RNAi embryos, however, the serosa primordium is dramatically reduced in size, albeit rotation-symmetric as in dorsetalized blastoderms (e.g. Fig. 3). *Tc-zen-1* is required for serosa formation in *Tribolium* (van der Zee et al., 2005) and the expression of this gene is reduced in *Tc-otd-1* but not in *Tc-sog* RNAi. *Tc-zen-1*, therefore, is a probable third direct target activated by Tc-Otd-1, in addition to *Tc-sog* and *Tc-mpl* (Figs 1, 7).

**Early homogeneous expression might account for all blastoderm functions of Tc-otd-1**

The spatial localization of the *Tc-sog* domain is clearly provided through its activation by Tc-Dorsal (Nunes de Fonseca et al., 2008). Given that Tc-Otd-1 appears only to ‘boost’ a promoter activity whose spatial specificity is provided by another input, and given the early onset of *Tc-sog* expression, it is probably the initial homogeneous expression of Tc-Otd-1 that is used by the *sog* promoter to amplify the activating signal provided by the DV system.

Similarly, we would argue that *Tc-zen-1* also receives spatially specific regulatory information from another source, i.e. the terminal system, as in Tc-tor or Tc-tsl RNAi embryos the *Tc-zen-1* domain is much reduced in size (M.S., unpublished). Indeed, upon *Tc-otd-1* and Tc-tsl double RNAi, *Tc-zen-1* expression is lost (see Fig. S4 in the supplementary material). Although *Tc-otd-1* clearly influences the size of the *Tc-zen-1* domain (Fig. 4), it is unlikely that the *Tc-zen-1* expression boundary is read from the Tc-Otd-1 gradient as, at this anterior position in the egg, Tc-Otd-1 expression levels are rather uniform throughout blastoderm development (Schroder, 2003). Instead, the terminal system appears to provide a short-range graded signal near the anterior pole, which is enhanced by Tc-Otd-1 (Schoppmeier and Schroder, 2005).

It might well be that the similarity between *Tc-sog* and *Tc-zen-1* in receiving a general boost rather than spatial information from Tc-Otd-1 also extends to the way they mediate the fate map changes resulting from Tc-Otd-1 inactivation. In the case of *Tc-sog*, it is clear that all DV defects of *Tc-otd-1* RNAi can be accounted for by its effect on *Tc-sog* expression. Our *Tc-sog* *Tc-zen-1* double-RNAi experiment shows that much of the AP fate map effect of *Tc-otd-1* inactivation might be mediated through Tc-Zen-1. In double-knockdown embryos, also fates outside the serosa region, e.g. the anterior Tc-kni domain and the first two *eve* stripes, are shifted anteriorly (Fig. 5). It is conceivable, therefore, that all or most of the AP fate map shift caused by Tc-otd-1 knockdown can be explained by its activating effect on Tc-zen-1 alone.

A general requirement, presumably provided by early uniform Tc-Otd-1 expression, is also revealed by the cell lethality phenotype of *Tc-otd-1* RNAi embryos (Fig. 6). In *Drosophila*, segmentation mutants show a dramatic increase in apoptotic death (Adachi-Yamada and O’Connor, 2002; DiNardo et al., 1994; Klingensmith et al., 1989; Magrassi and Lawrence, 1988; Namba et al., 1997; White and Lehmann, 1986). However, although apoptosis in such *Drosophila* mutants is locally restricted, the situation in strong *Tc-otd-1* RNAi embryos is different, as all extraembryonic and embryonic cells undergo apoptosis (Fig. 6). Less severe apoptosis phenotypes, however, could explain the previously described stronger cuticle phenotypes (Schinko et al., 2008; Schroder, 2003), which we also observed (see Fig. S1, Table S1 in the supplementary material). In such embryos, apoptosis might be restricted to patches covering few segment primordia, which enables the residual (probably less affected) embryonic anlagen to escape lethality and to eventually secrete cuticle (see Fig. S1 and Table S1 in the supplementary material). Although we do not know the exact causes of early cell lethality in *Tc-otd-1* RNAi embryos, this apoptosis phenotype might well represent a third function of Tc-Otd-1, i.e. a function independent of its role in blastoderm patterning, which probably involves additional target genes, e.g. zygotically expressed early housekeeping genes that rely on homogeneously expressed Tc-Otd-1 for gene activation.

In conclusion, our data provide little reason to believe that the gradient of Tc-Otd-1 is more than a transitional stage between homogeneous blastoderm function and the subsequent head gap gene function (Schinko et al., 2008). In this respect, the Otd gradient in *Tribolium* might be more comparable to the *Drosophila* Caudal gradient, which only provides general activation rather than positional information (Cho et al., 2005; Hader et al., 1998; Macdonald and Struhl, 1986; Mlodzik et al., 1990; Niessing et al., 2002; Niessing et al., 1999; Rivera-Pomar et al., 1996).

**Anterior patterning in short-germ insects**

AP pattern formation in the early *Drosophila* embryo is initiated by three maternally provided systems (St Johnston and Nusslein-Volhard, 1992). In the short-germ beetle *Tribolium*, the terminal system (i.e. Torso receptor tyrosine kinase signaling) plays a
prominent role in early axis formation (Schoppmeier and Schroeder, 2005). At the posterior, Torsosignaling is required for growth zone formation and, at the anterior, it is involved in serosa formation via activation of Tc-zen-1 (Fig. 7). Patterning of gnathium and the thorax depends on a posterior-to-anterior Tc-Cad gradient (Copf et al., 2003). This gradient is formed through translational repression, mediated by combined activities of zygotically expressed Tc-Mex-3 and Tc-Zen-2 (Schoppmeier et al., 2009). However, the Tc-Cad gradient does not appear to provide concentration-dependent positional information (Schoppmeier et al., 2009). In Tc-otd-1 RNAi, Tc-Mex-3 expression resembles the effects of Tc-otd-1 RNAi on the Tribolium gap genes (see Fig. S5 in the supplementary material), indicating that Tc-Mex-3 also does not require Tc-Otd-1 for its activation.

As the posterior system (nanos and pumilio) also plays only a minor role in the Tribolium blastoderm (C. Schmitt and M.S., in preparation), we believe that additional maternal positional information systems must be present in this species. However, our data indicate that this function is not provided by the Tc-otd-Tc-hb system. Anterior patterning in long-germ insects involves localized determinants [e.g. bcd in Drosophila (Driever and Nusslein-Volhard, 1988) and otd, cad and gt in Nasonia (Brent et al., 2007; Lynch et al., 2006; Olsnicky et al., 2006)]. In Tribolium, only two localized mRNAs are known so far that have no patterning activity (Bucher et al., 2005). However, given that the localization machinery evidently is functioning during Tribolium oogenesis, we expect that additional maternally localized gradient systems remain to be identified in this species.

Finally, it should be noted that there is a stunning similarity between the fate map changes caused by the loss of Drosophila bcd and those caused by zen-1 RNAi in Tribolium as, in both, anterior regions of the early embryo are deleted and replaced by the expansion of more-posterior regions (van der Zee et al., 2005). Even though the effect of Tc-Zen-1 as a long-reaching AP regulator is though the effect of Tc-Zen-1 as a long-reaching AP regulator is expected to be identified in this species.

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