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Developmental Biology 364 (2012) 224–235

Contents lists available at SciVerse ScienceDirect

Developmental Biology

journal homepage: www.elsevier.com/developmentalbiology

Evolution of Developmental Control Mechanisms

A dual role for *nanos* and *pumilio* in anterior and posterior blastodermal patterning of the short-germ beetle *Tribolium castaneum*

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ARTICLE INFO

Article history:
Received for publication 30 September 2010
Revised 9 January 2012
Accepted 20 January 2012
Available online 4 February 2012

Keywords:
Tribolium
Short germ segmentation
nanos
hunchback
Gap genes

ABSTRACT

Abdominal patterning in *Drosophila* requires the function of Nanos (*nos*) and Pumilio (*pum*) to repress posterior translation of *hunchback* mRNA. Here we provide the first functional analysis of *nanos* and *pumilio* genes during blastodermal patterning of a short-germ insect. We found that *nos* and *pum* in the red flour beetle *Tribolium castaneum* crucially contribute to posterior segmentation by preventing *hunchback* translation. While this function seems to be conserved among insects, we provide evidence that Nos and Pum may also act on *giant* expression, another gap gene. After depletion of *nos* and *pum* by parental RNAi, Hunchback and *giant* remain ectopically at the posterior blastoderm and the posterior *Krüppel* (*Kr*) domain is not being activated. *giant* may be a direct target of Nanos and Pumilio in *Tribolium* and presumably prevents early *Kr* expression. In the absence of *Kr*, the majority of secondary gap gene domains fail to be activated, and abdominal segmentation is terminated prematurely. Surprisingly, we found Nos and Pum also to be involved in early head patterning, as the loss of Nos and Pum results in deletions and transformations of gnathal and pre-gnathal anlagen. Since the targets of Nos and Pum in head development remain to be identified, we propose that anterior patterning in *Tribolium* may involve additional maternal factors.

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Introduction

Anterior–posterior pattern formation in the early *Drosophila* embryo is initiated by distinct maternally provided systems (St Johnston and Nüsslein-Volhard, 1992). While anterior patterning is mediated by the Bicoid morphogen (Driever and Nüsslein-Volhard, 1988), posterior patterning largely depends on Nanos (Nos) and Pumilio (Pum) proteins (Barker et al., 1992; Macdonald, 1992; Nüsslein-Volhard et al., 1987; Wang and Lehmann, 1991). Both are part of a repression complex regulating the translation of maternal Hunchback (Hb), which is crucial to promote abdominal patterning (Barker et al., 1992; Hülskamp et al., 1989; Irish et al., 1989; Macdonald, 1992; Murata and Wharton, 1995; Struhl, 1989; Struhl et al., 1992; Tautz, 1988; Wreden et al., 1997). During oogenesis, *nos* mRNA is localized to the posterior pole of the embryo, and its translational activation during embryogenesis leads to a gradient of Nos protein emanating from the site of localization (Gavis and Lehmann, 1994; Wang and Lehmann, 1991; Wang et al., 1994). Pum, which is distributed ubiquitously, binds to the so-called Nanos response element (NRE) in the 3′ UTR of the *hb* mRNA and mediates – together with Nanos and additional proteins recruited by the complex – the deadenylation of *hb* mRNA (Sonoda and Wharton, 1999, 2001; Wharton and Struhl, 1991; Wreden et al., 1997). Translational repression of Hb in the posterior confines this protein to the anterior half of the embryo, allowing correct posterior expression of gap- and pair rule genes (Eldon and Pirrotta, 1991; Gaul et al., 1987; Kraut and Levine, 1991a,b; Struhl, 1989). Consequently, in *nanos* or *pumilio* mutants, abdominal segments cannot be formed due to ectopic Hb expression in the posterior of the blastoderm (Barker et al., 1992; Hülskamp et al., 1989; Irish et al., 1989; Macdonald, 1992; Nüsslein-Volhard et al., 1987).

While maternal systems were investigated in great detail in *Drosophila*, comparative studies in other insects revealed a high degree of divergence (Rosenberg et al., 2009). The determination of anterior–posterior polarity by Bcd, for instance, is a derived developmental mechanism limited to cyclorrhaphan flies (Stauber et al., 1999).

The red flour beetle *Tribolium* develops as a short germ embryo, where – in contrast to the long germ mode of *Drosophila* – the majority of segments get patterned in a secondary growth process from a so-called growth zone (Richards et al., 2008). While growth zone formation in *Tribolium* depends on the localized activity of the Torso-pathway (Schoppmeier and Schröder, 2005), it is still under debate, to which degree early short-germ embryogenesis involves maternal gradient systems (Bucher et al., 2005; Schoppmeier and Schröder, 2005; Schröder, 2003).

At the anterior, Otd and Hb were postulated to substitute for Bcd as anterior morphogens in non-dipteran insects (Lynch et al., 2006; Schroder, 2003). However, recent work in *Tribolium* suggests that neither Hb nor Otd provide concentration dependent positional information

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doi:10.1016/j.ydbio.2012.01.024

(Kotkamp et al., 2010; Marques-Souza et al., 2008). Instead Hb functions in abdominal segmentation and Hox-Gene regulation (Marques-Souza et al., 2008), while Otd was found to fulfil a more general function for anterior–posterior and dorso-ventral axis formation, which likely depends on early ubiquitous Otd distribution (Kotkamp et al., 2010). Still, *Tribolium* Otd and Hb are both initially expressed ubiquitously. Subsequently, both proteins retract from the posterior pole, indicating translational regulation (Schröder, 2003; Wolff et al., 1995). Schröder (2003) proposed Nanos and Pumilio to act on HB and OTD translational regulation, which is supported by the existence of NREs in the 3′ UTRs of *Tribolium hb* and *otd* (Schroder, 2003; Wolff et al., 1995).

To gain additional insights into the posterior system of *Tribolium*, we analysed the functions of *Tc-nanos* and *Tc-pumilio* genes in blastodermal patterning. We found Nos and Pum to be required for abdominal segmentation and for translational repression of Hb. While this repressive function is conserved in insects, we provide evidence that *giant* expression is under control of the posterior group genes too.

Methods

Cloning of genes

Single *Tribolium* Nanos and Pumilio orthologs were identified by Basic Local Alignment Search Tool (BLAST) analysis of the *Tribolium* genome (Richards et al., 2008). Candidate genes were amplified from cDNA, cloned into pBluescript KS vector and sequenced to confirm their identity. For *Tribolium nanos*, subsequent RACE and amplification of additional fragments from cDNA revealed 5′ and 3′ UTR sequences.

Expression analysis

Fixation, single in situ and immunofluorescence stainings, were performed using standard protocols (Patel et al., 1989; Tautz and Pfeifle, 1989). For double staining, fluorescein- and digoxigenin-labelled probes were detected using alkaline phosphatase and beta-galactosidase, the latter after signal enhancement via biotin deposition (Prpic et al., 2001). The rabbit polyclonal Otd antibody (gift of Reinhard Schröder, University Rostock) was used at a concentration of 1:200. Hb protein expression was detected using the anti-*Nv*-Hb antibody (Pultz et al., 2005) at a dilution of 1:500. An alkaline phosphatase conjugated goat anti-rabbit antibody (1:2000, Dianova) was used for detection. Embryos were subsequently counterstained with Hoechst 33342.

Expression of *nanos* mRNA was analysed by PCR using cDNA of different developmental stages as template. Total RNA was extracted from unfertilized eggs, stage-matched embryos, whole larvae, male and female pupae, and adult beetles using Trizol (Invitrogen). RNA was treated with TurboDNase (Ambion) and phenol–chloroform extracted. 1.5 μg RNA was used for cDNA synthesis with the Transcriptor High Fidelity cDNA Synthesis Kit (Roche) and oligo(dT) primers. 0.5 μl of cDNA was used for PCR with Primers specific for *nanos* (5′-ACG TGC TCA AAT GTC TTT C-3′, 5′-GTG GTT GAT AAA TTT GGT CG-3′). As a control, the expression of the ribosomal protein rp49 mRNA was monitored (5′-ATG GCA AAC TCA AAC GCA AC-3′, 5′-TAG CAT GTG CTT CGT TTT GG-3′) (Konopova and Jindra, 2007). To exclude contamination with genomic DNA or other *nanos* DNA fragments, control reactions without reverse transcriptase were performed. Moreover, we used forward primers spanning an exon border for both genes, preventing amplification of genomic fragments.

Parental RNAi

RNA interference experiments were essentially carried out as previously described (Bucher et al., 2002). The following dsRNA concentrations were used: *pumilio* (3 μg/μl), *nanos* (4 μg/μl), *nanos* and *pumilio* double RNAi (4 μg/μl each). As a control we injected *dsRed* dsRNA at a concentration of 8 μg/μl. The template for *dsRed* control RNAi was amplified from a minos-transposon vector (Lorenzen et al., 2007) using specific primers carrying additional promoter sequences for the T7-RNA-polymerase (5′-TAA TAC GAC TCA CTA TAG GAG TTC ATG CGC TTC AAG GTG-3′ and 5′-TAA TAC GAC TCA CTA TAG GTG GTG TAG TCC TCG TTG TGG-3′).

First instar larvae were cleared in lactic acid/10% ethanol overnight at 60 °C. After washing once with lactic acid, cuticles were transferred to a drop of lactic acid on a slide. Cuticle autofluorescence images were captured on a Zeiss Axiophot and maximum projection images were generated from image stacks.

Results

Tribolium nanos and pumilio genes

A single *Tribolium nanos* ortholog (*Tc-nos*, 1081 bp, EEZ99428) was identified in the *Tribolium* genome by tBLASTn analysis (Fig. S1). Since the 5′ UTR and the correct start codon of *nanos* were not included in an initial automatic annotation of the gene, we performed 5′ RACE, which revealed the start codon and 246 bp of the *nos* 5′ UTR sequences. The putative 3′ UTR (382 bp) was identified in-silico and verified by RT PCR (Betlebase: TC030446). For subsequent RNAi studies, an 810 bp fragment spanning the CDS and the 3′ UTR was used.

Nanos proteins encode two evolutionarily conserved CCHC zinc finger motifs (Curtis et al., 1995, 1997). The zinc-binding residues and their relative position within the motif are conserved among all analysed metazoan species, while within the motif a low level of sequence conservation is prevailing. *Tribolium* and *Drosophila*, for instance, share 46% of the positions, which is comparable to the similarity among other insect Nos proteins (Fig. S1).

We used *Drosophila pumilio* as query sequence in a tBLASTn search of the *Tribolium* genome and identified a single *pumilio* gene (*Tc-pum*, 3309 bp, TC005073). Pumilio proteins encode eight highly conserved PUF repeats, forming the Pumilio homology domain (Pum-HD), which is necessary and sufficient for sequence specific RNA binding and can mediate protein–protein interactions (Sonoda and Wharton, 1999; Wang et al., 2002; Wickens et al., 2002; Zamore et al., 1997). Within the PUF repeats, *Tribolium* Pumilio shows 87% identity to *Drosophila* Pumilio (Fig. S1). For subsequent expression and RNAi studies, an 850 bp fragment spanning most of the PUF domain was used.

As in *Drosophila* (Macdonald, 1992), *Tribolium pumilio* is expressed during oogenesis (not shown) and remains transcribed ubiquitously throughout embryonic development (Fig. S2). Thus, in both *Tribolium* and *Drosophila*, the distribution of *pum* exhibits no axial asymmetry (Barker et al., 1992; Macdonald, 1992).

Unexpectedly, it was not possible to observe *nos* expression by in-situ hybridisation in embryonic or in ovarian tissue. To detect *nos* expression, we used probes corresponding to the full-length open reading frame (ORF), as well as probes corresponding to different ORF and UTR fragments of various lengths. In addition, we applied numerous probe labelling, detection, and amplification methods (not shown). In none of these approaches, we observed any specific signal as compared to control sense *nos* probes (not shown). To monitor Nos protein expression, we raised antibodies against two independent Tc-Nos peptides, which however, neither recognized Nanos protein in situ nor in western blotted protein extracts (not shown).

Still, we were able to detect *nos* expression by RT-PCR (Fig. S2). As compared to *rp49* mRNA (ribosomal protein 49) expression (Konopova and Jindra, 2007), *nos* mRNA gets maternally provided to the egg and is expressed throughout embryonic and post-embryonic *Tribolium* development (see Fig. S2 for details). While we could confirm that *Tc-nos* is indeed transcribed, it remains obscure why we could not observe

expression by in-situ hybridisation. We assume that *nos* expression levels may be below the detection thresholds of the in-situ hybridisation protocol. Considering the sequence homology and that *nos* RNAi leads to strong and reproducible RNAi phenotypes (see below), we are convinced that we indeed identified the functional *Tribolium nanos* ortholog (see also discussion).

nanos and pumilio parental RNAi results in abdominal and head defects

Parental RNA interference (pRNAi) allowed us to analyse embryos lacking both, maternal and zygotic *Tribolium nanos* (*nos*) and *pumilio* (*pum*) function (Fig. 1).

Depletion of either *nos* or *pum* resulted in severe abdominal truncations (Figs. 1C and E). Affected larvae developed only one to two regular abdominal segments, followed by a region of irregular cuticle and remnants of terminal structures, i.e. urogomphi, and pygopodia. Unexpectedly, we also observed anterior phenotypes. Larvae depleted of *nos* or *pum* show deletions and transformations of gnathal and pregnathal segments (Figs. 1B, D, and F) (see below).

While *nos* and *pum* pRNAi resulted in identical segmentation phenotypes, we observed an additional effect of *pum* RNAi on appendage development, which was not obvious in *nos* RNAi (Figs. 1C and E). Podomers and gnathal appendages were considerably shortened in proximo-distal direction and malformed. This finding can be correlated with an increase of *pum* expression in the developing limb buds (Fig. S2). Although we did not analyse this phenotype in detail, these results indicate that there are some Pum functions that may – analogous to *Drosophila* (Gamberi et al., 2002) – not require Nos activity.

Given that Nos and Pum proteins interact with each other to fulfil their function for segmentation, we expected the double knockdown phenotype to resemble the single RNAi phenotype. Indeed, we

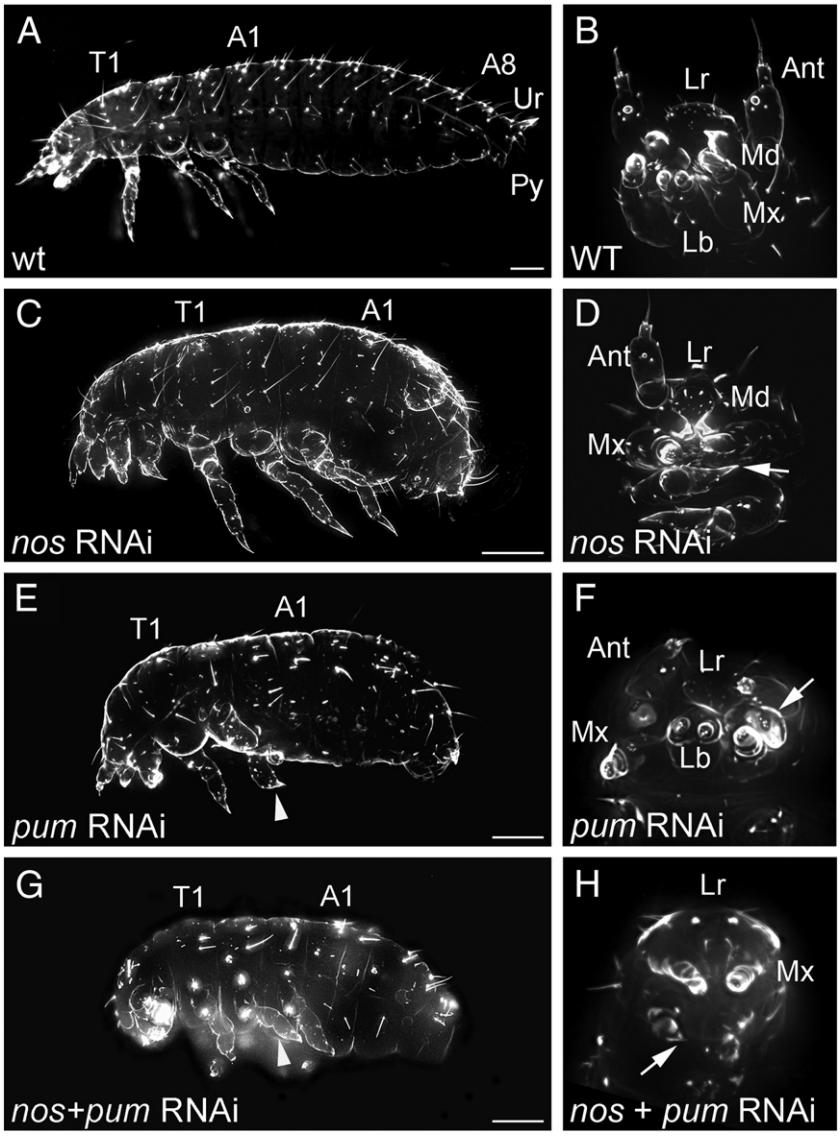


Fig. 1. Larval *nanos* and *pumilio* RNAi phenotypes. Cuticles of wildtype (A,B), *nanos* RNAi (C,D), *pumilio* (E,F) and *nanos* and *pumilio* double RNAi larvae (G,F). (C,E,G) Lateral views of *nos* (B), *pum* (E), and *nos*/*pum* double RNAi (G) larvae, which display severe posterior truncations. While the first abdominal segment (A1) is formed regularly, subsequent segments show a various degree of aberration and eventually abdominal segment formation stops. (B,D,F,H) Ventral views of larval heads. *Nos* (D), *pum* (F), and *nos*/*pum* double RNAi (H) larvae display transformations and deletions of pre-gnathal and gnathal segments, such as transformations of labial towards thoracic identity (e.g. D, H, arrows) or mandibular towards unclear gnathal identity (F, arrow). Frequently, head segments are lost (D,F,H), ultimately resulting in larvae that only process the Labrum (Lr), Maxillae (Mx) and a partially transformed Labium (arrow, H). Arrowheads (E,G) point to the additional leg phenotype that is only obvious in *pum* or *pum*/*nos* double RNAi (see text for details). Scale bars: 100 μm. Lr: Labrum; Ant: Antennae; Md: Mandible; Mx: Maxilla; Lb: Labium; T: thoracic segment; A: abdominal segment; Ur: Urogomphi, Py: Pygopodia.

observed the same segmentation defects in all experiments (Figs. 1G and H) (Table S1). As the penetrance of strong phenotypes was higher in double RNAi-experiments, we decided to use the double-RNAi background for further analysis.

nanos and pumilio parental RNAi results in the premature breakdown of abdominal segmentation

To determine whether segmentation defects after *nos*/*pum* RNAi reflect early patterning or a subsequent segment maintenance functions, we analysed the expression of the segmentation genes *even-skipped* (*eve*) (Patel et al., 1994) and *wingless* (*wg*) (Nagy and Carroll, 1994) during germband elongation (Fig. 2). In the wild-type, *wg* is expressed in segmental stripes and in a more complex pattern in the head (Nagy and Carroll, 1994). Thoracic *wg* stripes appear normal in *nos*/*pum* RNAi germband embryos (Figs. 2A and B). Anterior abdominal *wg* stripes, however, appear disorganized, while more posterior *wg* domains are clearly aberrant (Fig. 2B). Eventually, the segmentation process ceases prematurely (Fig. 2B).

During germ band elongation, *Tribolium eve* domains arise in the anterior region of the posterior growth zone and subsequently split into segmental stripes. While, the formation of thoracic *eve* domains is not affected by *nos* and *pum* RNAi (not shown), segmentation defects become evident at subsequent stages (Figs. 2D and E). Although *eve* stripe 5 does split into segmental stripes 5a and 5b, these segmental stripes are irregular in shape and less intense (Fig. 2D). Eventually, these domains fade away. The primary *eve* stripe 6 forms very similar to wild type, but never divides into segmental stripes 6a and 6b (Fig. 2E).

Our results show that *nos* and *pum* are required for proper patterning of the posterior half of the *Tribolium* embryo, similar to the situation observed for *Drosophila.* In *Drosophila nanos* or *pumilio* mutants, abdominal segments cannot be formed due to ectopic Hb expression (Barker et al., 1992; Gabbrielli, 1957; Hülskamp et al., 1989; Irish et al., 1989; Macdonald, 1992; Nüsslein-Volhard et al., 1987; Struhl, 1989), raising the question, whether Nos and Pum act as Hb translational repressors in *Tribolium* as well.

Hunchback de-repression in absence of nanos and pumilio

Previously, potential *nanos*/*pumilio* target sites (nanos-response elements, NREs) were not only identified in the 3′ UTR of *Tribolium hunchback* but also of *orthodenticle* (Schroder, 2003; Wolff et al., 1995). Therefore, we analysed the effect of *nos* and *pum* RNAi on Hb and Otd protein expression (Fig. 3).

Tribolium Otd is expressed in a highly dynamic pattern (Schinko et al., 2008; Schröder, 2003). During undifferentiated blastoderm stage, Otd protein is distributed ubiquitously and successively becomes restricted to a head domain in the differentiated blastoderm. As this transition from ubiquitous expression to the later head domain involves posterior and subsequently also anterior cessation of Otd expression, it has been proposed that Otd forms a Nos/Pum dependent transient gradient, spanning the posterior half of the embryo (Schröder, 2003). However, we did not observe any obvious impact of Nos and Pum on early Otd expression (Figs. 3G and H). In both single and double RNAi, the distribution of Otd protein – including the transient gradient – is unchanged, suggesting that early Otd expression does not depend on translational repression by Nos and Pum.

Interestingly, however, we observed changes of Otd distribution later during *Tribolium* embryogenesis (Fig. S5). In germband stages, Otd is expressed in the head, as well as in three rows of mid-line cells. After *nos*/*pum* RNAi this expression expands into neighbouring cells (Fig. S5B). Although it remains to be elucidated, if this is a direct effect, these results may explain the presence of a NRE in the *otd* mRNA.

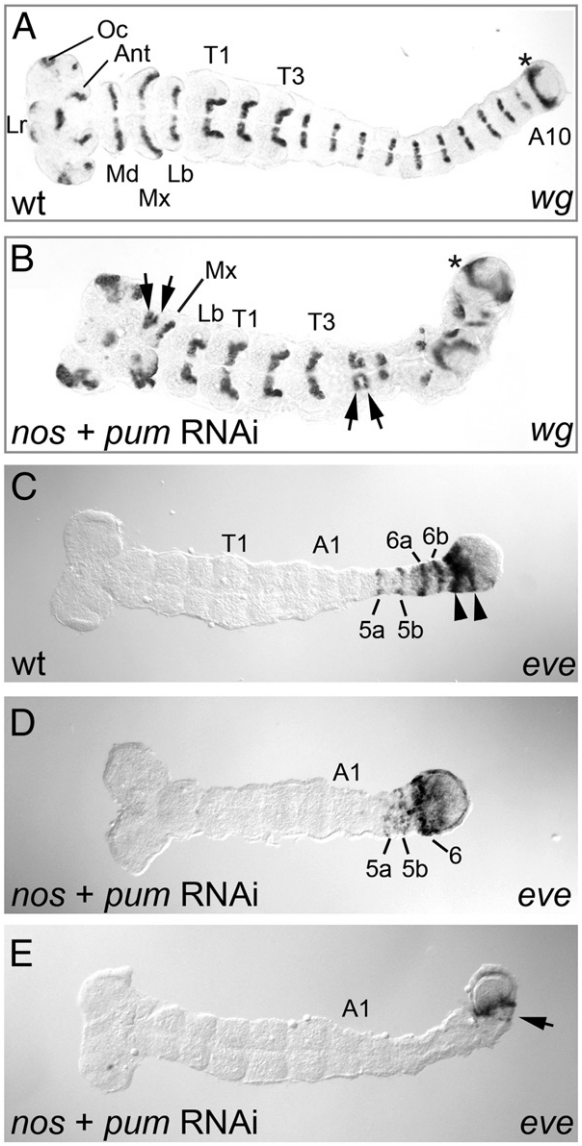


Fig. 2. *nanos* and *pumilio* double RNAi affects abdominal segmentation. Flat mounted wild-type (A,C) and *nos*/*pum* double RNAi embryos (B,D,E) stained for *wingless* (A,B) or *even-skipped* (C–E) by in-situ hybridisation. (A) In wildtype elongated germband embryos, *wg* is expressed in segmental stripes and in the head. In addition, a domain in the posterior part of the growth zone is obvious (asterisk) (B) Upon *nos*/*pum* double RNAi, anterior abdominal *wg* stripes become irregular (arrows) and eventually no additional *wg* domains are formed. The posterior domain is still present (asterisk). Also the *wg* pattern in the head is severely disturbed. Antennal *wg* domains are absent, ocular and mandibular *wg* domains are malformed, and the distance between mandibular and maxillary *wg* stripe is severely reduced (arrows). (C) In wildtype elongated germband embryos, *eve* is expressed in two primary domains in the growth zone (arrowheads). These double-segmental domains eventually give rise to secondary segmental domains. (D) *nos*/*pum* double RNAi embryo slightly younger than the embryo in (C). Secondary *eve* domains are visible (*eve* stripes 5a and 5b), but misshaped. In the growth zone, only a single primary *eve* domain is obvious (*eve* stripe 6). (E) At later stages of development, no secondary *eve* expression domains are visible. In the growth zone, only a single primary *eve* domain can be recognized (arrow). Lr: Labrum; Ant: Antennae; Oc: Occular domain; Md: Mandible; Mx: Maxilla; Lb: Labium; T: thoracic segment; A: abdominal segment. Anterior to the left, ventral views.

To analyse Hb expression, we used a cross-reacting antibody against the *Nasiona vitripennis* protein (Pultz et al., 2005), (Figs. 3A–D; see also Fig. S3 for details). In the early blastoderm, *Tribolium* Hb is expressed ubiquitously. Subsequently, Hb is cleared from the posterior pole (Figs. 3C and D), while *hb* mRNA is still present in this area, suggesting a translational control mechanism (Wolff et al., 1995). In embryos depleted of *nos* and *pum*, Hb protein persists at the posterior

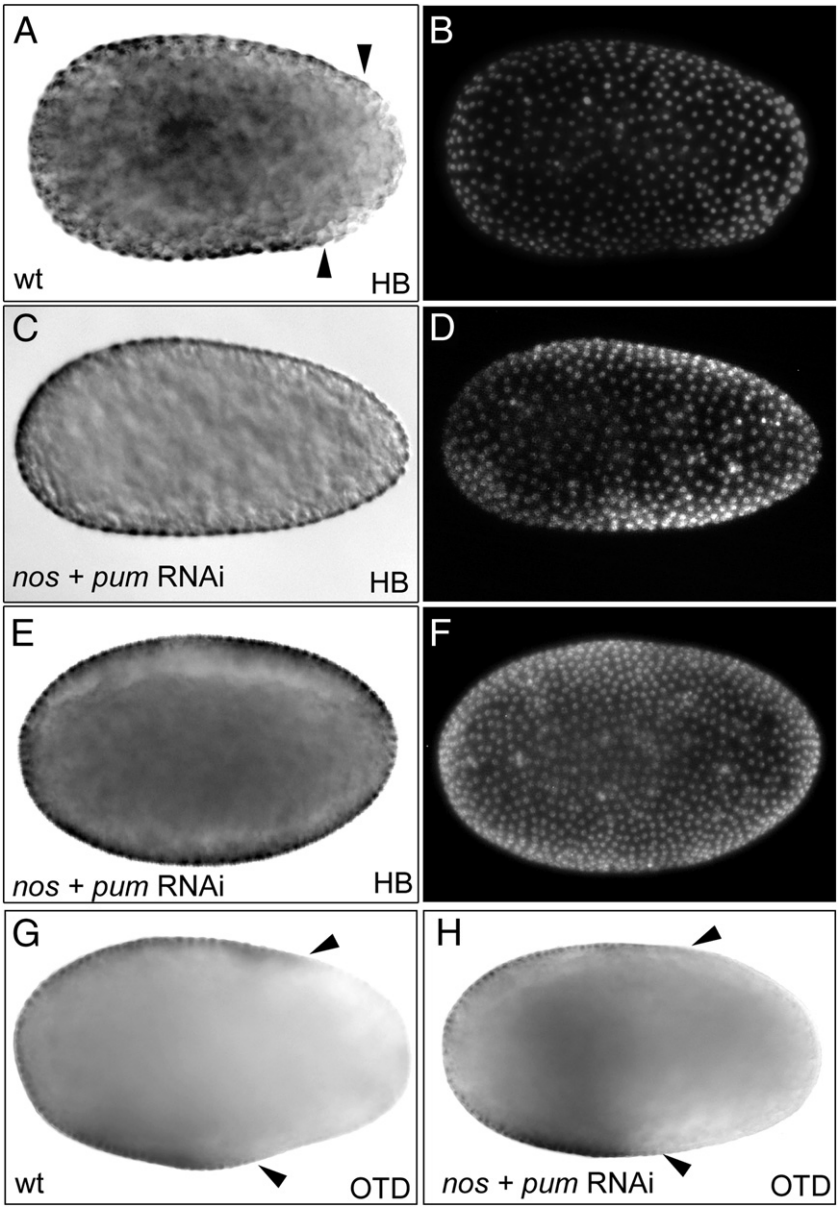


Fig. 3. Expression of Hb and Otd. (A–H) Stage matched undifferentiated blastoderm wildtype (A,B,G) and *nos*/*pum* double RNAi embryos (C–F,H), stained for Hb (A–F) and Otd (G,H) protein. (E,F) Late undifferentiated blastoderm *nos*/*pum* double RNAi embryo stained for HB. Wildtype (G) and *nos*/*pum* depleted (H) embryos stained for Otd. Embryos in (A,C,E) were subsequently stained for the nuclear marker Hoechst 33342 to visualize morphology (B,D,F). (A,B) In early wildtype embryo, HB retracts from the posterior pole (arrowheads). (C,D) Upon *nos*/*pum* double RNAi, HB protein remains ectopically at the posterior pole. (E,F) Also during subsequent stages, HB cessation was not observed. (G) During early blastoderm stages, Otd forms a transient gradient, (H) which appears to be unaffected by knock-down of *nos* and *pum*. Arrowheads mark the posterior border of Otd expression. Anterior to the left, lateral views.

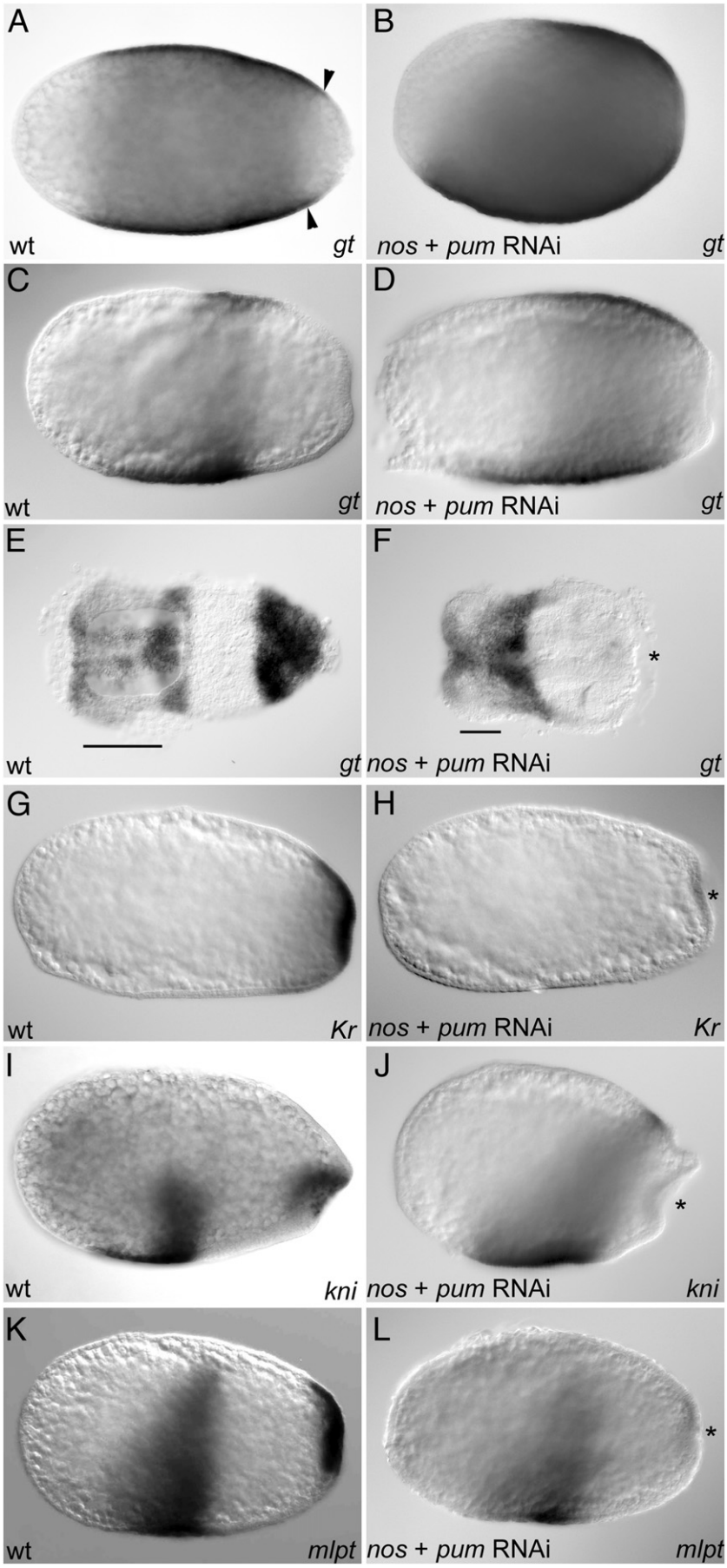
pole (Figs. 3C–F), indicating a conserved role of Nos and Pum as repressors of Hb translation.

nanos and pumilio are required to activate posterior blastodermal gap gene domains

In *Drosophila*, Hb derepression in *nos* and *pum* mutant embryos affects abdominal segmentation, which is mediated by the alteration of blastodermal *giant*, *knirps*, and *Krüppel* domains (Eldon and Pirrotta, 1991; Gaul et al., 1987; Kraut and Levine, 1991a,b). To uncover effects of *Tribolium nos* and *pum* on early patterning, we examined the expression of *Tribolium* gap genes *giant* (*gt*), *Krüppel* (*Kr*), *knirps* (*kni*), and *milles-pattes* (*mlpt*) (Bucher and Klingler, 2004; Cerny et al., 2005, 2008; Savard et al., 2006; Sommer and Tautz, 1993) (Fig. 4).

In wild type embryos, *gt* is initially expressed homogeneously. Later, expression retracts from both poles (Fig. 4A), eventually forming an anterior domain comprising pre-gnathal and gnathal segments but excluding the serosa (Bucher and Klingler, 2004) (Fig. 4C). While *gt* is

Fig. 4. Expression of gap-genes in *nos* and *pum* RNAi.(A–L) Wildtype (A,C,E,G,I,K) and *nos*/*pum* double RNAi embryos (B,D,F,H,J,L), stained for *gt* (A–F), *Kr* (G,H), *kni* (I,J), and *mlpt* (K, L). (A–D) As compared to wildtype (A, arrowheads show posterior border of expression), *gt* remains ectopically at the poster pole in *nos*/*pum* RNAi (B). Eventually, *gt* mRNA retracts from the posterior pole and the head domain is established (C,D). (E,F) Flat mounted germ-rudiment stage embryos. The secondary *gt* domain (E) is not activated in *nos*/*pum* RNAi (F). Bars in (E,F) resemble the distance between the anterior rim of the head anlagen and the maxillary *gt* expression domain. (G-H) The posterior *Kr* domain (G) is not established in absence of *nos* and *pum* (H, asterisk). (I–L) While *kni* (I,J) and *mplt* (K,L) head expression domains are basically present, secondary (posterior) domains are not activated in *nos* and *pum* RNAi embryos (asterisks in J,L). Anterior to the left, (A–D, G–L) lateral views and (E,F) ventral views.


A
wt
gt
B
nos + pum RNAi
gt
C
wt
gt
D
nos + pum RNAi
gt
E
wt
gt
F
*
nos + pum RNAi
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G
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still cleared from the anterior after *nos* and *pum* RNAi (Fig. 4B), *gt* expression remains at the posterior pole throughout early blastodermal stages. Eventually, also posterior *gt* expression ceases (Fig. 4D) and at early germ rudiment stage the anterior *gt* domain forms (Fig. 4F). A second *gt* domain arises de novo at the posterior pole of wild type embryos at the late differentiated blastoderm stage (Bucher and Klingler, 2004) (Fig. 4E). Upon *nos* and *pum* depletion, however, this secondary domain is not established (Fig. 4F).

The gap domain of *Kr* arises at the posterior pole of blastoderm stage embryos (Fig. 4G). Given that *Tribolium* is a short germ insect, the position of this domain is largely conserved with respect to the *Drosophila* fate map (Cerny et al., 2005; Sommer and Tautz, 1993). This prominent *Kr* expression domain is lost upon *nos* and *pum* knock-down (Fig. 4H).

Tribolium kni expression arises at the undifferentiated blastoderm stage in a broad central domain, which refines to a wedge shaped domain covering the mandibular and pre-gnathal region (Fig. 4I) (Cerny et al., 2008). In addition, a posterior domain of *kni* emerges at late differentiated blastoderm stage (Fig. 4I). While in *nos* and *pum* knock-down embryo the anterior *kni* domain is established, the posterior *kni* domain is not activated (Fig. 4J).

In wildtype differentiated blastoderm stage, *mlpt* is expressed in a wedge shaped domain, covering the head region and a posterior domain (Fig. 4K) (Savard et al., 2006). Again, the anterior domain is present, the posterior expression domain, however, is – analogous to *gt*, *kni*, and *Kr* – lost in *nos* and *pum* RNAi embryos (Fig. 4L).

Previously, it has been shown that the depletion of *Tribolium gt*, *Kr*, *kni*, or *mlpt* results in the loss of abdominal segments (Bucher and Klingler, 2004; Cerny et al., 2005, 2008; Savard et al., 2006). Hence, the loss of posterior *gt*, *Kr*, *kni*, and *mlpt* expression domains may explain the breakdown of abdominal segmentation in *nos* and *pum* RNAi. Unexpectedly, however, *nos* and *pum* knock-down not only causes ectopic posterior HB distribution, but also results in persisting *gt* expression at the posterior pole, indicating some – direct or indirect – regulatory input of Nos and Pum on *gt* expression.

Tribolium giant mRNA has a putative Nanos-response element

Translational regulation of *Drosophila hunchback* is mediated by the binding of Pumilio to Nanos Response Elements (NREs) within the 3′ UTR, and the subsequent recruitment of Nanos and Brain Tumor to form a quarternary complex (Murata and Wharton, 1995; Sonoda and Wharton, 2001). Fig. 5 shows a putative NRE in the 3′ UTR of *Tribolium giant*, which is largely identical to the consensus motifs of Box A and Box B of the *Drosophila* NREs (Curtis et al., 1997; Gerber et al., 2006; Murata and Wharton, 1995; Sonoda and Wharton, 1999; Zamore et al., 1997). Together with ectopic *gt* expression in *nos* and *pum* RNAi, the presence of candidate NREs suggests that Nos and Pum may act on *gt* translational regulation in *Tribolium*.

Head patterning defects occur early in embryogenesis

Unexpectedly, we also observed anterior phenotypes after *nos* and *pum* RNAi. Larvae depleted of *nos* or *pum* exhibited deletions and transformations of head segments (Figs. 1D, F, and H). Even though these phenotypes showed some variability, we found antennal and mandibular segments to be deleted, while in weaker phenotypes the labium was frequently transformed towards thoracic identity. As revealed by analysis of the *wg* pattern in *nos*/*pum* double RNAi germband stages, loss of gnathal and pre-gnathal anlagen already occurs early in embryogenesis (Fig. 2B) and thus, is not due to subsequent segment maintenance defects. At that stage, *wg* domains corresponding to the antennal and the mandibular segment anlagen were either lost entirely or severely disturbed.

To further elucidate the function of *nos* and *pum* in head patterning, we visualized the emergence of pregnathal and gnathal anlagen by expression of *Tribolium six3*, *buttonhead* (*btd*), and *even-skipped* (Fig. 6) (Posnien and Bucher, 2009; Schinko et al., 2008). In wild type differentiated blastoderm embryos, *six3* is expressed in a triangle-shaped domain at the anterior rim of the germ rudiment (Fig. 6A). This domain covers anterior non-segmental tissue and the anlagen of the labrum. Upon *nos* and *pum* RNAi, *six3* expression is severely down regulated (Fig. 6B).

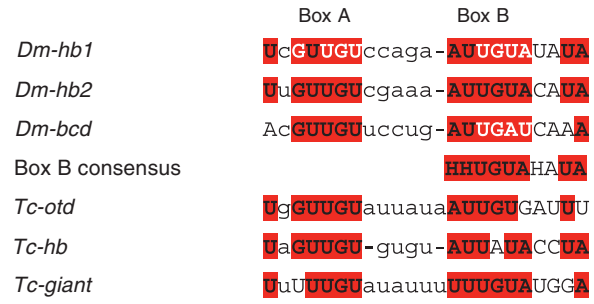


Fig. 5. A candidate Nanos response element in the *Tribolium giant* mRNA.Alignment of candidate NREs from the 3′ UTR of *Tribolium giant* with the *Tribolium hb* and *otd* NREs (Schroder, 2003) and NREs of *Drosophila hb* and *bicoid*. The sequences of *Dm-hb* (first and second NRE, red) and *Dm*-bcd mRNA are shown with nucleotides that affect *Drosophila* Pumilio binding when mutated (white) (Curtis et al., 1997; Gerber et al., 2006; Murata and Wharton, 1995; Sonoda and Wharton, 1999; Zamore et al., 1997). The RNA consensus motif of Box B was identified in a genome-wide survey of mRNAs associated with Pum (Gerber et al., 2006).

Tribolium btd expression first can be detected in late blastoderm. In the differentiated blastoderm stage, it forms a narrow stripe that later comes to lie in the mandibular segment (Fig. 6C). Upon *nos* and *pum* RNAi, *btd* expression is lost (Fig. 6D), suggesting that patterning of gnathal anlagen is affected already early in embryogenesis.

At the differentiated blastoderm, the pair-rule gene *eve* is expressed in three double segmental domains, which correspond to gnathal anlagen and the first thoracic segment primordia (Figs. 6E and G) (Patel et al., 1994). In *nos* and *pum* knock-down embryos, we found the first primary *eve* stripe not to split into two segmental domains (Figs. 6F and H), reflecting the loss of the mandibular segment in RNAi larvae. Posterior *eve* domains (i.e. *eve* stripe 2 and *eve* stripe 3) were unaffected (Fig. 6F). Furthermore, the distance between the most anterior *eve* stripe and the serosa–germ-rudiment boundary is decreased in *nos* and *pum* RNAi embryos, again revealing patterning defects of anterior head regions.

Although the head is a ventral anlage in *Tribolium* (Kotkamp et al., 2010; van der Zee et al., 2006), we did not detect any impact of *nos* and *pum* on dorso-ventral patterning. In wildtype embryos, the border between the extraembryonic serosa and the germ rudiment exhibits a distinct dorsoventral polarity. This polarity is lost and the border gets perpendicular to the embryonic ap-axis in ventralized or dorsalized embryos (van der Zee et al., 2006). In *nos* and *pum* RNAi differentiated blastoderm embryos, we did not detect any changes of the expression of the serosa marker *zerknüllt-1* (*zen-1*) and the serosa–germ rudiment border remains oblique (Figs. 6C and D). Hence, *nos*/*pum* RNAi head phenotypes are not due to impaired dorso-ventral patterning.

To elucidate the origin of the transformation phenotype, we monitored the expression of *Tribolium Antennapedia* in *nos* depleted embryos (Figs. 6I and J). In the wildtype, *Antp* is restricted to a strong thoracic domain and in addition, is weakly expressed in a broad abdominal domain (Fig. 6I). In *nos* RNAi we observed an anterior expansion of *Antp* into the labial segment primordia (Fig. 6J), which may account for the transformation of the labium to thoracic identity. Thus, the homeotic phenotype might be explained by imperfect Hox-gene regulation.

Our results demonstrate that *nos* and *pum* are also required for early patterning of pregnathal and gnathal segment anlagen. Upon *nos* and *pum* RNAi, head primordia are malformed or partially lost. In addition, we observed transformations of head segments towards thoracic identity. Since we could not observe any obvious impact of *nos*/*pum* RNAi on anterior HB and Otd distribution (Fig. 3), the direct target genes of *nos* and *pum* in head patterning remain to be identified.

Discussion

In this paper we provide the first functional analysis of posterior group genes in a short germ insect. We show that *Tribolium nanos* and *pumilio* RNAi results in the breakdown of abdominal segmentation and additional anterior defects. These segmentation defects are most likely due to the derepression of HB and *gt* in the posterior of the *Tribolium* blastoderm. We argue that the activation of the *Kr* domain is crucial in this respect. *Kr* expression depends on the activity of HB in the early blastoderm and requires the posterior retraction of *gt*. In the absence of *nos* and *pum*, *gt* and Hb remain ectopically expressed in the posterior blastoderm and the *Kr*-dependent activation of posterior blastodermal gap-gene domains fails, which results in the premature termination of abdominal segmentation.

Tribolium nanos is expressed at low abundance

Surprisingly, we were not able to recognize *nanos* expression by in-situ hybridisation. While we can basically exclude technical reasons, our finding can be explained in two directions. Either *nos* is expressed below the detection level of our in-situ hybridisation protocols, or we did not identify the functional *Tribolium nos* ortholog. To rule out the latter we evaluated the genomic locus of *Tribolium nanos*. The *Tribolium* genome does not show a region with shared synteny to the *Drosophila nos* locus. *Drosophila nos* is located on the 2nd chromosome in an intron of CG11779 and overlaps with the 3′ UTR of CG42358. The *Tribolium nos* is located on chromosome 2 as well and is located in an intron of TC000252, which shows no homology to CG11779. We also could not identify any nos-like genes in the proximity of the *Tribolium* orthologs of CG11779 (TC008130 on chromosome 4) or CG42358 (LOC657361 on chromosome 9).

Even though we cannot completely exclude another *nos* homolog in the thus far unsequenced regions of the genome, we consider this as unlikely. *nos* RNAi lead to strong and reproducible phenotypes and in addition, we could verify *nos* expression by RT-PCR, RACE-PCR, and sequencing of ovarian, maternal and post-embryonic *Tribolium* transcriptomes (MS, unpublished). Therefore, we posit that *nos* is expressed below the detection level of the in-situ hybridisation protocols and that we indeed identified the functional *Tribolium nanos* ortholog.

Nanos and Pumilio may act as translational repressors of hunchback and giant

The posterior *nos* and *pum* RNAi phenotypes suggest a posterior and locally restricted function of Nos, while the effects on head patterning indicate that Nos activity extends towards the anterior. Thus, even though we could not directly observe posterior *nos* mRNA localization, our functional data suggests that blastodermal patterning in *Tribolium* could involve a posterior to anterior Nos gradient. This is in accordance with expression data from other species, including long and short germ insects (Chang et al., 2006; Curtis et al., 1995; Dearden, 2006; Goltsev et al., 2004; Juhn et al., 2008; Lall et al., 2003; Lemke and Schmidt-Ott, 2009; Lynch and Desplan, 2010; Nakao et al., 2008).

While posterior *nos* expression is largely conserved among insect species, the functional relevance for early embryonic patterning is unproved in most cases. As yet, evidence for translational regulation of Hb by Nos and Pum was only provided for long-germ insects (Lemke and Schmidt-Ott, 2009; Lynch and Desplan, 2010), while only spatial correlations of *nos* expression with either Hb protein or mRNA were shown for short-germ insects without any functional data (Lall et al., 2003). We now provide evidence that a HB/Nos system is also conserved in the short-germ beetle *Tribolium*. In *Tribolium nos* and *pum* RNAi, Hb is derepressed at the posterior pole (Fig. 3), indicating that translational repression of Hb by Nos and Pum is indeed an ancestral feature of early insect development.

In addition to Hb, we found *gt* expression to depend on Nos/Pum. In early wild type embryos, maternal *gt* transcripts are distributed homogeneously throughout the syncytial blastoderm. Later, expression withdraws from both poles and intensifies along the posterior edge of this domain (Bucher and Klingler, 2004). In embryos depleted of *nos* and *pum*, *gt* mRNA remains at the posterior pole, while anterior retraction is unaffected (Fig. 4). This effect is most likely not due to ectopic Hb expression, as the formation of the initial *gt* domain is basically unchanged in *hb* RNAi embryos (Fig. S4A, B). Also in *Drosophila*, anterior *gt* expression does not depend on Hb (Eldon and Pirrotta, 1991).

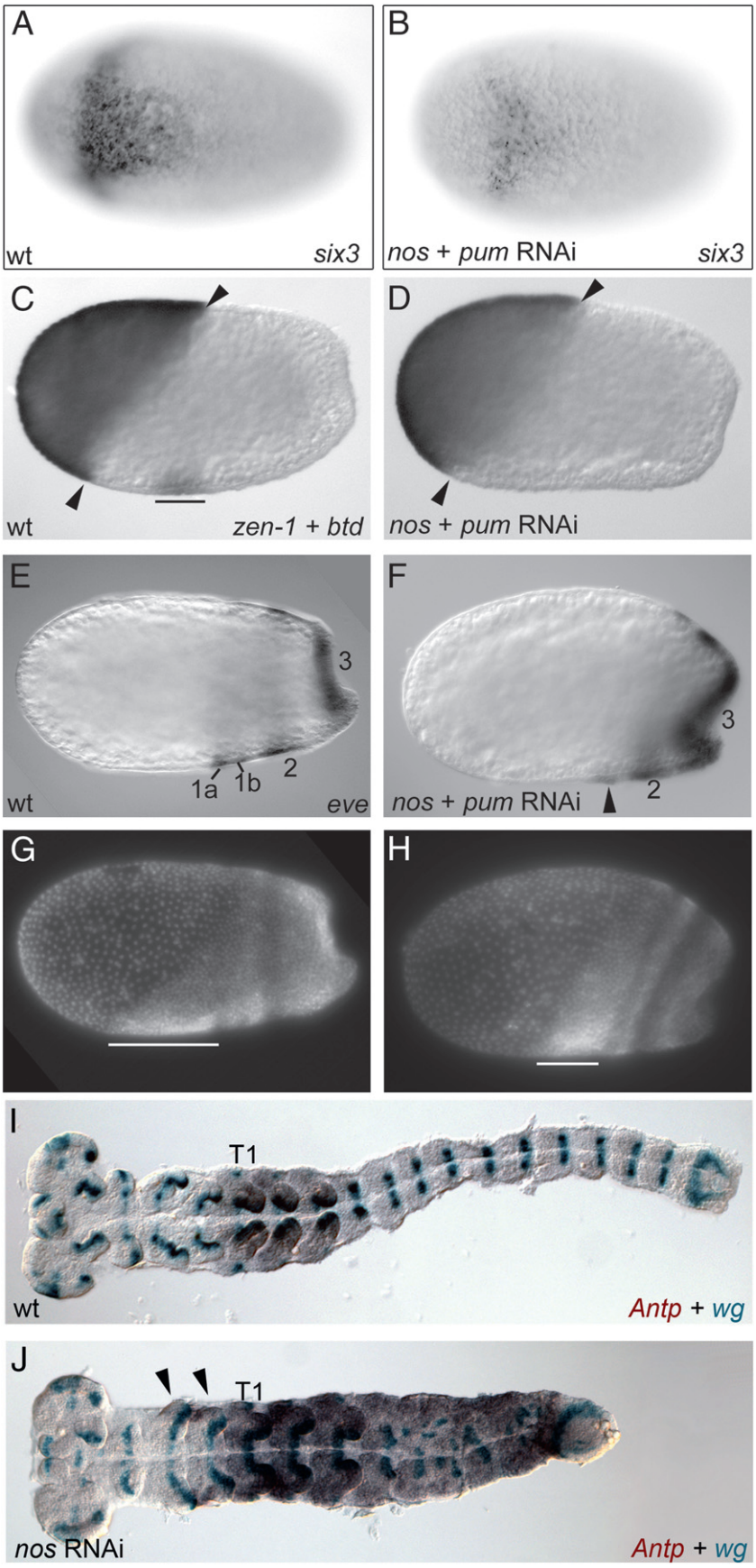
Hence, posterior *gt* cessation in *Tribolium* either may depend on transcriptional repression by a thus far unidentified posterior factor or alternatively, maternal *gt* mRNA translation and/or stability could be repressed at the posterior pole by Nos and Pum. The latter is supported by the presence of a candidate NRE in the 3′ UTR of *Tribolium gt* mRNA (Fig. 5). While the translational regulation of *gt* mRNA as well as a direct interaction with Nos and Pum certainly needs to be confirmed, our results nevertheless demonstrate that Nos and Pum are important for shaping maternal *giant* and Hunchback expression, which in turn allows the proper activation of zygotic gap-gene domains (Fig. 7) (see below).

Nos and Pum establish blastodermal gap gene domains

The activation of *Krüppel* expression by Hunchback, is a well conserved feature among insects and also in *Tribolium*, *Kr* expression is lost in *hb* RNAi (Figs. Fig. S4E–H) (Marques-Souza et al., 2008). Unexpectedly, *nos* and *pum* RNAi – although leading to ectopic Hb expression – did not result in an expansion of *Kr* expression, but causes the loss of the primary *Kr* domain (Fig. 4H), indicating additional regulatory input on *Kr*. Previously, Gt has been suggested to act as a repressor of *Kr* expression (Cerny et al., 2005). We analysed *Kr* expression in *gt* knockdown embryos and indeed found *Kr* expanding anteriorly, from the posterior border of *eve* stripe 2a, where the anterior border of the *Kr* domain is located in wild type embryos, to the posterior border of *eve* stripe 1b (Fig. S4I and J). This expansion is responsible for the gnathal transformations towards thoracic fate in these embryos (Cerny et al., 2005). Given that *gt* expression remains ectopic in embryos depleted of *nos* and *pum*, we posit that prolonged posterior *gt* expression prevents the formation of the primary *Kr* domain in the *Tribolium* blastoderm.

Based on our results, we propose that the formation of the blastodermal *Kr* domain in *Tribolium* depends on positive regulatory input from HB and on repression by Gt (Fig. 7). Given that posterior clearance of *Tribolium gt* depends (direct or indirect) on Nos and Pum, rather than on HB, this situation is different from *Drosophila* where HB activates *Kr*, but represses the posterior *Dm-gt* domain (Hulskamp et al., 1990; Kraut and Levine, 1991b).

The loss of the *Kr* domain seems to be a central aspect of the *nos* and *pum* RNAi phenotype. In embryos depleted for *nos* and *pum*, secondary gap-gene domains of *gt* and *kni* are not established, which resembles the situation in *Kr* ^{jaws} mutant embryos (Figs. Fig. S4K–N). This indicates that posterior (secondary) *gt* and *kni* domains are activated by *Kr*. Along that line the loss of the posterior *gt* domain in *hb*


A
wt
six3
B
nos + pum RNAi
six3
C
wt
zen-1 + btd
D
nos + pum RNAi
E
3
1a
1b
2
wt
eve
F
3
nos + pum RNAi
2
G
H
I
T1
wt
Antp + wg
J
T1
nos RNAi
Antp + wg

knockdown embryos (Figs. Fig. S4A–D) is likely due to the hb-dependent loss of *Krüppel* activity (Cerny et al., 2005).

Our results indicate that ectopic *gt* expression at the posterior pole of early blastoderm embryos lacking *nos* and *pum* activity inhibits *Kr* expression, which in turn leads to the loss of posterior *gt* and *kni* domains (Fig. 7). Again, this situation is different from *Drosophila*, where Kr represses posterior *gt* expression.

Misregulation of posterior gap-genes may account for segmentation defects

In *Dm-nos* and *Dm-pum* mutants, alteration of posterior gap gene domains results in the loss of abdominal segments (Eldon and Pirrotta, 1991; Gaul et al., 1987; Kraut and Levine, 1991a,b). Likewise, abdominal segmentation is disturbed in *nos* and *pum* knockdown in *Tribolium*. The morphology of the larval cuticle (Fig. 1) and the embryonic expression pattern of *eve* and *wg* (Fig. 2) illustrate that anterior abdominal segments do not form regular and that segmentation is terminated prematurely. As described before, such embryos lack posterior *gt*, *Kr*, *kni*, and *mlpt* expression domains (Fig. 4).

It is evident from their phenotypes that gap gene orthologs are also involved in abdominal segmentation of short germ insects (Ben-David and Chipman, 2010; Bucher and Klingler, 2004; Cerny et al., 2005; Liu and Kaufman, 2004; Mito et al., 2005, 2006; Savard et al., 2006). In *Tribolium Kr* ^{jaws} mutant embryos, segmentation breaks down after the formation of five *eve* stripes (Cerny et al., 2005), reflecting the situation in *nos* and *pum* knock-down (Fig. 2D). Comparable phenotypes were also observed in *mlpt* or *gt* RNAi (Bucher and Klingler, 2004; Savard et al., 2006). The inactivation of *gt* leads to a disturbed pattern of *eve* and the segment polarity gene *engrailed* (Bucher and Klingler, 2004) very similar to the *eve* and *wg* pattern we observed in *nos* and *pum* RNAi. Germ band growth is disrupted whenever pair-rule patterning is affected, and the defects are reflected by irregular expression of segment polarity genes (Choe et al., 2006). However it is not clear, how exactly *Tribolium* gap genes influence segmentation in the growth zone. While stripe specific regulation of pair rule genes or triggering of a pair-rule based segmentation clock seem to be possible explanations, additional functions for cell fate specification and survival in the growth zone cannot be excluded.

While, the exact origin of segmentation defects in gap-gene and pair-rule RNAi remains to be elucidated, we posit that the truncation phenotype in *nos* and *pum* RNAi embryos is due to the loss of posterior expression domains of the gap genes *Kr*, *kni*, *gt* and *mlpt*, which in turn affects pair-rule gene and subsequently, segment polarity gene expression.

Blastodermal patterning in Tribolium

In *Drosophila*, maternal gradients control the expression of gap genes, which act as the first zygotic transcription factors in patterning the anterior–posterior axis. In *Tribolium*, posterior blastodermal patterning depends on a posterior-to-anterior Cad gradient (Copf et al., 2004), which is formed by combined activities of zygotically expressed *Mex-3* and *Zen-2* (Schoppmeier et al., 2009). While the Cad gradient does not appear to provide concentration-dependent positional information (Schoppmeier et al., 2009), the question remains, how posterior gap-gene domains are established. We now show that Nos and Pum are involved in posterior blastodermal patterning as well and are required to set up the early Hb and *gt* domains, in turn regulating posterior gap-gene expression (Fig. 7). While Nos is thus likely part of an ancestral posterior patterning centre, anterior patterning reveals a higher degree of divergence.

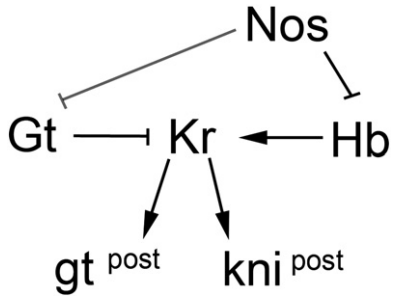


Fig. 7. Summary of posterior gap–gene interaction. Nos (together with Pum) is required for posterior retraction of Hb and Gt. While Hb activates the posterior Kr domain, Gt likely acts (either direct or indirect) as a repressor of Kr. Kr in turn is necessary for the activation of the secondary (posterior) domains of *gt* and *kni* (see text for details).

An important role for localized maternal determinants has long been postulated for several insect taxa including crickets, beetles, and flies (Rosenberg et al., 2009). However, the existence of an anterior morphogenetic centre has thus far only been proven for the long-germ insects *Drosophila* (i.e. *bcd*) and *Nasonia* (i.e. *otd* and *hb*) (Brent et al., 2007; Lynch et al., 2006; St Johnston and Nüsslein-Volhard, 1992). Our data now suggests that an anterior maternal positional information system may exist in *Tribolium*.

We found *nos* and *pum* genes to be involved in head patterning. The depletion of *nos* and *pum* results in deletions and transformations of pre-gnathal and gnathal segments. Interestingly, Pumilio was already found to modulate the perdurance of *bicoid* mRNA and protein in anterior regions also in *Drosophila*, leading to head defects in *pum* mutants (Gamberi et al., 2002). Hence, it is likely that in *Tribolium* Nos and Pum act on an anterior factor as well, which in turn ensures proper expression of head patterning genes. Since we did not observe any obvious impact on anterior HB and/or Otd distribution, we suggest that anterior patterning in *Tribolium* may involve an additional – as yet unknown – anterior patterning factor. Such a hypothetical factor X could either be unlocalized or may form a gradient, which is shaped by Nos and Pum dependend translational repression. This factor may in turn ensure proper expression of head patterning genes. Given the comparatively weak and variable *nos* and *pum* RNAi head phenotypes, a proposed gradient would rather be modulated than formed by Nos and Pum. This would be in analogy to *Drosophila*, where *pum* modulates the perdurance of *bicoid* mRNA and protein in the anterior (Gamberi et al., 2002). Although we cannot exclude that subtle alterations of Hb or Otd levels may contribute to the *nos*/*pum* head phenotype, we consider the presence of an unidentified factor to be more likely, as there is no known involvement of *hb* in *Tribolium* head development (Marques-Souza et al., 2008) and *otd* seems to act through the dorso-ventral system (Kotkamp et al., 2010).

Additional evidence for an unidentified anterior patterning gene comes from *Tribolium Mex-3*, which substitutes for *Drosophila* Bcd in translational repression of Caudal (Schoppmeier et al., 2009). *Mex-3*

Fig. 6. Head patterning defects in *nos* and *pum* RNAi. (A–H) Stage matched wildtype (A,C,E,G) and *nos*/*pum* double RNAi (B,D,F,H) blastoderm embryos, stained for *six3, zen-1*/*btd,* and *eve*. *zen-1* and *btd* mRNA probes (C,D) were labelled identically and signals were developed simultaneously using the same substrate. Embryos in (E,F) were counterstained for Hoechst 33342 (G,H) to visualize morphology. (I,J) Wildtype (I) and *nos* RNAi (J) elongated germ stage embryos double stained for *wg* and *Antp*. (A,B) In *nos* and *pum* double RNAi, the *six3* domain is strongly reduced in intensity and size. (C,D) While serosal *zen-1* expression is unchanged in *nos*/*pum* RNAi (arrowheads label the posterior margin of the extra embryonic serosa), the *btd* domain is lost, reflecting the loss of the mandibular anlagen. The bar in (C) corresponds to the *btd* domain in wildtype. (E,F) In differentiated wildtype blastoderm embryos, *eve* is expressed in three primary, double segmental domains (*eve* stripes 1–3), which split and give rise to secondary segmental domains (*eve 1a* and *eve 1b*). (E–H) Upon *nos*/*pum* double RNAi, the first primary *eve* domain, which corresponds to the mandibular and maxillary segment anlagen, is severely reduced and does not longer split into segmental domains (arrowhead). Bars in (G,H) correspond to the size of the pre-gnathal anlagen. (I) Wildtype embryo double stained for the segmental marker *wg* (green) and for *Antp* (brown). *Antp* is restricted to a strong thoracic domain and in addition, is expressed in broad, but rather weak abdominal domain. (J) In *nos* RNAi *Antp* expands into the labial and maxillary segment primordia (arrows). All panels anterior to the left, (C–H) lateral views and (A,B,I,J) ventral views. T: thoracic segment.

mRNA, however, is not localized maternally but expressed zygotically in a dynamic pattern. Since transcriptional regulation of *Mex-3* is independent of known anterior patterning genes (Schoppmeier et al., 2009) there might be indeed some other, thus far unidentified factors involved in anterior patterning during early *Tribolium* development.

Supplementary materials related to this article can be found online at doi:10.1016/j.ydbio.2012.01.024.

Acknowledgments

We are grateful to Martin Klingler for continuous support. We wish to thank Reinhard Schröder for providing the Otd and Jeremy Lynch for the Hb antibody. The lab of M.S. is funded through grants by the German Research Foundation (DFG).

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