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Developmental Biology 298 (2006) 87–94
DEVELOPMENTAL BIOLOGY
www.elsevier.com/locate/ydbio

Knockdown of *spalt* function by RNAi causes de-repression of Hox genes and homeotic transformations in the crustacean *Artemia franciscana*

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Received for publication 22 May 2006; revised 21 July 2006; accepted 22 July 2006
Available online 28 July 2006

Abstract

Hox genes play a central role in the specification of distinct segmental identities in the body of arthropods. The specificity of Hox genes depends on their restricted expression domains, their interaction with specific cofactors and selectivity for particular target genes. *spalt* genes are associated with the function of Hox genes in diverse species, but the nature of this association varies: in some cases, *spalt* collaborates with Hox genes to specify segmental identities, in others, it regulates Hox gene expression or acts as their target. Here we study the role of *spalt* in the branchiopod crustacean *Artemia franciscana*. We find that *Artemia spalt* is expressed in the pre-segmental 'growth zone' and in stripes in each of the trunk (thoracic, genital and post-genital) segments that emerge from this zone. Using RNA interference (RNAi), we show that knocking down the expression of *spalt* has pleiotropic effects, which include thoracic to genital (T→G), genital to thoracic (G→T) and post-genital to thoracic (PG→T) homeotic transformations. These transformations are associated with a stochastic de-repression of Hox genes in the corresponding segments of RNAi-treated animals (AbdB for T→G and Ubx/AbdA for G→T and PG→T transformations). We discuss a possible role of *spalt* in the maintenance of Hox gene repression in *Artemia* and in other animals.

Keywords: Hox gene regulation; Genital structures; Appendages; Homeosis

Introduction

spalt (*sal*) was originally identified as a gene required for the development of posterior head and posterior abdominal segments in *Drosophila* embryos (Jurgens, 1988). Subsequent studies revealed that *spalt* and its sister gene, *spalt-related* (*salr*), encode transcription factors with several conserved zinc-finger domains and play a number of important roles in development, including the specification of segmental identities (together with Hox genes) in the embryo, tracheal patterning, the establishment of a central domain in the wing blade, the selection of photoreceptor cell fate in the eye and cell fate decisions in the nervous system (de Celis et al., 1996; Dong et al., 2002; Franch-Marro and Casanova, 2002; Kuhnlein et al., 1994; Mollereau et al., 2001; Rusten et al., 2001). Homologues of *spalt* have been cloned in several animals, including *C. elegans* (*sem-4* gene), fish, *Xenopus*, mice and humans (*SALL1–4* paralogues), where they are known to have diverse developmental roles (Grant et al., 2000; Harvey and Logan, 2006; Koster et al., 1997; Nishinakamura et al., 2001; Onai et al., 2004; Parrish et al., 2004; Toker et al., 2003). In humans, mutations in the *SALL1* and *SALL4* genes are associated with the Townes–Brocks syndrome and Okihiro syndrome, respectively, which include developmental anomalies in the ear, limbs, anus, kidneys and heart (Al-Baradie et al., 2002; Kohlhase, 2000; Kohlhase et al., 1998, 2002, 2005).

The function of *spalt* genes has been closely associated with the function of Hox genes in several developmental contexts: in *Drosophila* embryos, *spalt* acts in combination with Hox genes to specify segmental identities of gnathal and posterior abdominal segments (Jurgens, 1988; Kuhnlein et al., 1994); in *Drosophila* wing/haltere development, *spalt* is a direct target of repression by the Hox gene *Ubx* (Galant et al., 2002); in *C. elegans*, the *spalt* homologue *sem-4* directly regulates the expression of the Hox gene *egl-5* in the context of touch receptor specification (Toker et al., 2003) and the Hox gene *lin-39* in the

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0012-1606/$ - see front matter © 2006 Elsevier Inc. All rights reserved.
doi:10.1016/j.ydbio.2006.07.024

context of vulval development (Grant et al., 2000). In vertebrates, some anomalies associated with mutations in *SALL1* (Townes–Brocks syndrome) are similar to the phenotypes of some Hox genes, leading to speculation that their functions may also be linked (Toker et al., 2003). Thus, *spalt* genes are associated with the function of Hox genes in several contexts, but the nature of this relationship is not constant: *spalt* genes appear to be acting as cofactors, regulators or targets of different Hox paralogues in each of these cases.

So far, no clear parallels have been found in the way *spalt* genes interact with Hox genes in *Drosophila*, *C. elegans* or mice, suggesting that these roles may have evolved independently in these animals. Studies of *spalt* functions in additional species may shed light on the origin of these diverse functions. Here we study the function of a *spalt* orthologue – equally related to *Drosophila spalt* and *spalt-related* (Copf et al., 2003) – in the branchiopod crustacean *Artemia franciscana*. We have raised antibodies to study the expression of this gene in *Artemia* and used RNA interference (RNAi) to knock down gene expression and study its effects during the generation of trunk segments.

Materials and methods

Antibody against Artemia Spalt and immunohistochemical stainings

A 240 bp fragment of *A. franciscana spalt* (*AfSal*), corresponding to an exon encoding zinc-finger 2, was amplified from genomic DNA using the primers 5′-cgggatccTGCGTCATTTGTCATAGAG-3′ and 5′-cggaattcAAGTCTTA-TATGTTGTTG-3′, based on a previously published sequence (accession AJ567454; Copf et al., 2003). The fragment was cloned in the pGEMT-Easy vector (Promega), verified by sequencing and subcloned as a *Bam*HI–*Hin*dIII fragment into the pET-23a expression vector (Novagen) to generate a His-tagged protein fusion. The protein was expressed in BL21(pLys) cells and purified on an Ni-NTA column (QIAGEN), as described in the manufacturer's manual. Antibodies were raised against this bacterially expressed protein by repeated immunizations in three mice and a rabbit (carried out by Davids Biotechnologie) over a period of 6–8 months. The rabbit anti-AfSal serum was affinity purified on an Affigel-10 column (Biorad) carrying the bacterially expressed and purified *Artemia* Spalt protein (Harlow and Lane, 1988). The sera were used for whole mount immunohistochemical stainings on *Artemia* larvae at 1:50–1:500 dilutions, as described previously (Copf et al., 2003). The best immunostainings were obtained with the affinity purified rabbit anti-AfSal serum; similar patterns were also observed with two of the mouse sera. Double immunostainings with Engrailed or Nubbin/Pdm were carried out using the 4F11 and 2D4 monoclonal antibodies, respectively (Averof and Cohen, 1997; Damen et al., 2002; Patel et al., 1989b).

Artemia spalt RNAi

The 240 bp fragment of *AfSal* was subcloned as a *Bam*HI–*Eco*RI fragment from the pGEMT-Easy clone into pBluescript II KS (Stratagene). Double stranded RNA was produced from this clone using T3 and T7 RNA polymerase and was injected into stage L1–L3 larvae, as described previously (Copf et al., 2004). The larvae were then cultured for approximately 2 weeks before collection and fixing.

Analysis of RNAi phenotypes by SEM and immunohistochemical stainings

RNAi-treated animals were fixed for 30 min at room temperature with 4% formaldehyde in seawater and washed extensively in 100% methanol. Their phenotypes were initially examined and categorized under a Leica MZ6 stereoscope. Selected animals were then examined by scanning electron microscopy (SEM), as described previously (Copf et al., 2003). Specimens were often examined by SEM and then dissected, re-coated and re-examined to reveal structures hidden by the mature thoracic limbs. The expression of Distal-less, AbdB, Nubbin/Pdm and Ubx/AbdA was examined in a small number of individuals by whole mount immunochemical stainings, as described previously (Copf et al., 2003), using a mouse polyclonal anti-Dll (Panganiban et al., 1995), a mouse polyclonal anti-AbdB (Copf et al., 2003), the mouse monoclonal 2D4 (Averof and Cohen, 1997; Damen et al., 2002) and the mouse monoclonal FP6.87 (Kelsh et al., 1994) antibodies, respectively.

Results

Expression of Artemia spalt during the formation of trunk segments

We previously cloned a fragment of *spalt* from *Artemia* and examined its expression by in situ hybridization in newly hatched larvae (Copf et al., 2003). At this stage, the animal consists of the head region, the pre-segmental 'growth zone' from which all the trunk segments will subsequently arise and a telson at the posterior end. During this stage, *spalt* transcripts are specifically localized to the growth zone (Fig. 1A). Problems associated with the in situ hybridization technique in older stages did not allow us to examine the expression of *spalt* during subsequent larval stages, when the trunk segments and their appendages are generated from this growth zone. To overcome this problem, we raised antibodies to detect the distribution of *Artemia* Spalt protein by immunohistochemical stainings.

During larval development, Spalt is detected in the pre-segmental zone that lies between the newly formed segments and the telson, the area that is broadly defined as the 'growth zone' (Figs. 1B, C). Furthermore, Spalt is detected in a series of stripes that correspond to individual trunk segments as they emerge from the growth zone (Figs. 1B, C). These stripes appear in a similar fashion in all the thoracic, genital and post-genital segments. They become broader and less sharp as the segments mature and eventually fade away. Double stainings with antibodies for Spalt and Engrailed (which is expressed in the posterior compartment of each segment; Manzanares et al., 1993; Patel et al., 1989a) show that the Spalt stripe is straddling the anteroposterior (AP) compartment boundary (between Engrailed- and non-Engrailed expressing cells) in each segment (Fig. 1D). In the maturing thoracic segments, Spalt expression becomes more prominent in the distal parts of the growing limb buds (Fig. 1E) and then becomes localized to a small number of cells within the phyllopodous thoracic appendages (Fig. 1F). Spalt expression is also detected in cells within the central nervous system (seen in Fig. 1D), in a pair of large cells associated with the posterior part of the gut and in the ommatidia of the compound eyes (not shown).

spalt RNAi phenotypes

In a previous study, we developed an effective methodology for knocking down gene expression by RNAi in *Artemia*; this was achieved by injecting double-stranded RNA (dsRNA) into the hemocoel of early larvae and detecting systemic RNAi phenotypes in the same individuals during later larval stages

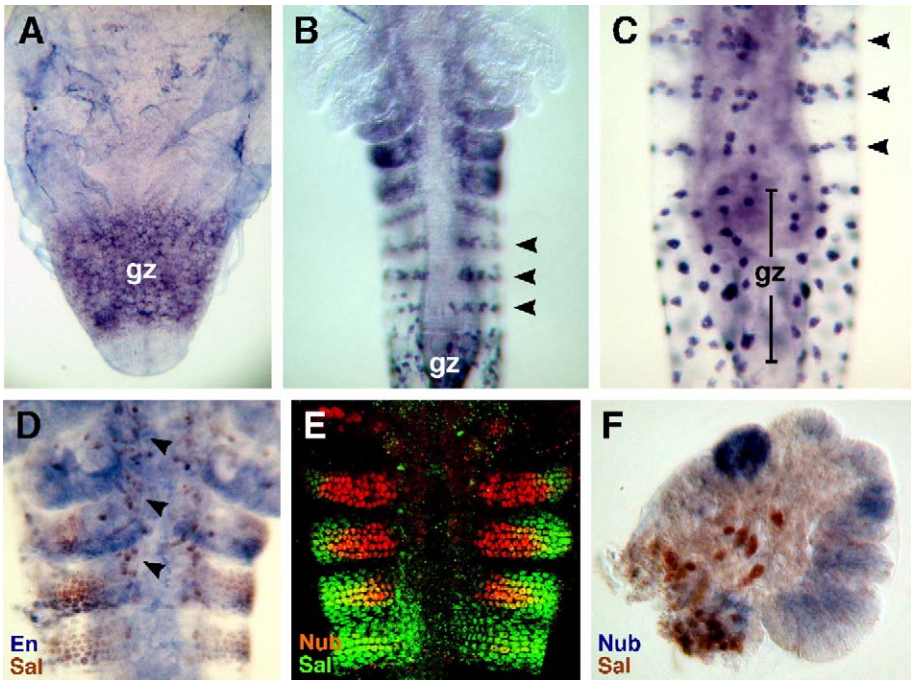

Fig. 1. Expression of *Artemia spalt* during the formation of trunk segments. (A) In situ hybridization for *spalt* just prior to hatching. *spalt* is expressed in a broad domain that corresponds to the pre-segmental growth zone (gz). (B) Immunostaining with an antibody raised against the *Artemia* Spalt protein. Staining is detected in the growth zone (gz) and in newly emerging segmental stripes (arrowheads marking stripes on T7–T9). Similar staining is observed during the generation of all the thoracic, genital and post-genital segments. The staining becomes more diffuse and eventually fades away in the more anterior (mature) segments. (C) Higher magnification of the growth zone (gz) and newly emerging segmental stripes, showing specific nuclear localization of the Spalt protein. (D) Double immunostaining for Spalt (brown) and Engrailed (Blue), showing that the expression of Spalt is straddling the AP compartment boundary (seen in the youngest/posterior-most segments). Staining is also observed within the central nervous system (arrowheads). (E) Confocal image of double fluorescent immunostaining for Spalt (green) and Nubbin/Pdm (red) in successive thoracic segments. Spalt expression becomes most prominent in the distal part of appendage primordia; more mature segments are at the top. (F) Double immunostaining for Spalt (brown) and Nubbin/Pdm (blue) in a dissected immature thoracic appendage. From this stage onwards, Spalt is only expressed in a small number of cells in the developing thoracic appendages, while Nubbin/Pdm expression becomes restricted to the distal epipodite (Averof and Cohen, 1997). Anterior is up in all panels.

(Copf et al., 2004). Here we use this technique to study the effects of reducing *spalt* function in *Artemia*. As in our previous study, we find that a significant proportion of injected individuals have no discernible phenotype, presumably due to incomplete inactivation of the gene. The remaining individuals have variable but severe defects in their appendages, including the replacement of thoracic appendages by genital structures and the appearance of thoracic appendage structures in the genital and/or post-genital segments. The latter phenotypes, where segmental structures are transformed into structures that normally arise on different segments, represent homeotic transformations in two opposite directions: thoracic to genital (T→G) and genital or post-genital to thoracic (G/PG→T). In addition to these phenotypes, some segmental anomalies including segment malformations or fusions are also observed (see Fig. 4C).

In a representative experiment, we observed that 49% (97/199) of individuals that that survive to late larval stages have no obvious phenotype, while the remaining individuals (102/199) have variable but severe defects in at least some of their appendages. The majority of these animals have thoracic appendages that are severely malformed (lacking the multi-lobed phyllopodous structure and setae of normal thoracic appendages; Figs. 2D, E), rudimentary or entirely missing (Figs. 2C, E). About 8% (15/199) have genital structures replacing some of the last thoracic appendages (Fig. 3), and about 4% (7/199) have thoracic appendage structures appearing in at least some of their genital or post-genital segments (Fig. 4). These frequencies represent the phenotypic effects of *spalt* RNAi as scored on a stereoscope. The frequency of severe phenotypes may be underestimated since strongly affected individuals are less likely to survive to the late larval stages when the phenotypes were scored.

These phenotypes were never observed previously in animals injected with a control dsRNA or with *caudal* dsRNA (see Copf et al., 2004), suggesting that these results are specific to *spalt* dsRNA injection. Conversely, although some segmental anomalies are observed, we find that *spalt* RNAi has no obvious effects on axis elongation or on the number of segments that are generated from the growth zone, unlike the phenotypes observed with *caudal* RNAi (Copf et al., 2004).

Thoracic to genital (T→G) transformations are associated with de-repression of AbdB expression

In approximately 8% of the individuals scored for *spalt* RNAi phenotypes, individual thoracic appendages appear to be transformed to resemble external genital structures (genitalia)

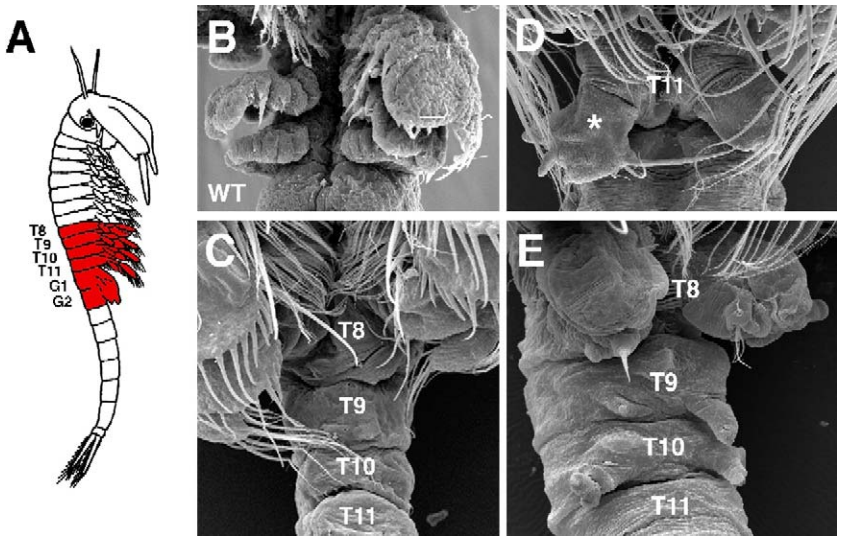

Fig. 2. *Artemia spalt* RNAi phenotypes: malformed, rudimentary and missing appendages. (A) Illustration highlighting the region of the body where malformed, rudimentary or missing appendages occur, including posterior thoracic (T8–T11) and genital (G1 and G2) segments. (B) SEM of successive thoracic appendages in a normal individual, during mid-larval stages, showing the characteristic morphology of immature, growing phyllopodous appendages. (C–E) SEM of RNAi-treated individuals during late larval stages: (C) individual with missing appendages in segments T9–T11; (D) individual with malformed appendage (marked by asterisk) in T11, showing clear abnormalities compared to normal phyllopodous appendages or to immature appendages (compare to panel B); (E) individual with rudimentary and malformed (in T9 and T10) or missing (in T11) thoracic appendages. Anterior is up in all panels.

that normally develop only in the genital segments. The strength and extent of these transformations are variable: some appendages appear completely transformed, while others show intermediate characters that suggest a partly thoracic and partly genital identity. These transformations are consistently observed in the most posterior thoracic segments (T10 or T11) and they are most prominent in males.

We used scanning electron microscopy (SEM) to examine these transformations in more detail. In males, the transformed appendages appear as outgrowths of similar shape and size to the normal male genitalia. In many cases, this resemblance extends to details such as the presence of a genital pore (in the correct position), the presence of an area of smoother cuticle on the median side of the appendage and the absence of setae (Figs. 3B, C). It is interesting to note that, although the genitalia normally arise from a partial fusion of the two genital segments (G1 and G2), these T→G transformations produce genitalia that arise from single thoracic segments (see Fig. 3C).

Immunostainings with antibodies for Distal-less, which has distinctive expression patterns in thoracic and genital appendages, confirm the overall resemblance of these outgrowths to genital appendages (Figs. 3F, G). In these stainings, it is also possible to visualize an internal structure that resembles the evertible penis of normal genitalia (arrowheads in Fig. 3G). In females, some of the phenotypes can be interpreted as partial T→G homeotic transformations, but these transformations are generally less complete than in males (Fig. 3D).

Based on previously described expression patterns of Hox genes in *Artemia*, the genital structures are thought to be specified by strong and persistent expression of *AbdB* (Averof and Akam, 1995; Copf et al., 2003). *AbdB* expression also extends to posterior thoracic segments during late larval stages, but this expression is significantly weaker than in the genital segments (Copf et al., 2003). To investigate whether the T→G transformations are associated with changes in the expression of Hox genes, or independent of them, we used immunohistochemical stainings to examine the expression of AbdB in affected individuals. These stainings showed that AbdB is ectopically expressed in the transformed regions (Fig. 3H). The levels of ectopic expression are comparable to those of normal AbdB expression in the genital segments and significantly stronger than the late AbdB expression seen in posterior thoracic segments of wild type animals (Copf et al., 2003).

Genital to thoracic (G→T) and post-genital to thoracic (PG→T) transformations are associated with de-repression of Ubx/AbdA expression

Approximately 4% of the individuals examined for *spalt* RNAi phenotypes have ectopic thoracic appendage structures in their genital and/or post-genital segments. The number and morphology of these ectopic structures vary. In many cases, these have characteristics of well-developed thoracic appendages, such as a phyllopodous appearance and characteristic setae arranged along the margin of the appendage (Figs. 4B–D); in other cases, they appear as small outgrowths or mere rows of setae (arrowheads in Fig. 4E). Distal-less stainings confirm that these are genuine appendages with a characteristic thoracic identity (Fig. 4F).

To examine whether these homeotic transformations are restricted to appendages or whether they also affect other tissues, we carried out immunostainings for Nubbin/Pdm, which is expressed in the developing neuromeres of the central nervous system. Normally, these neuromeres develop in the

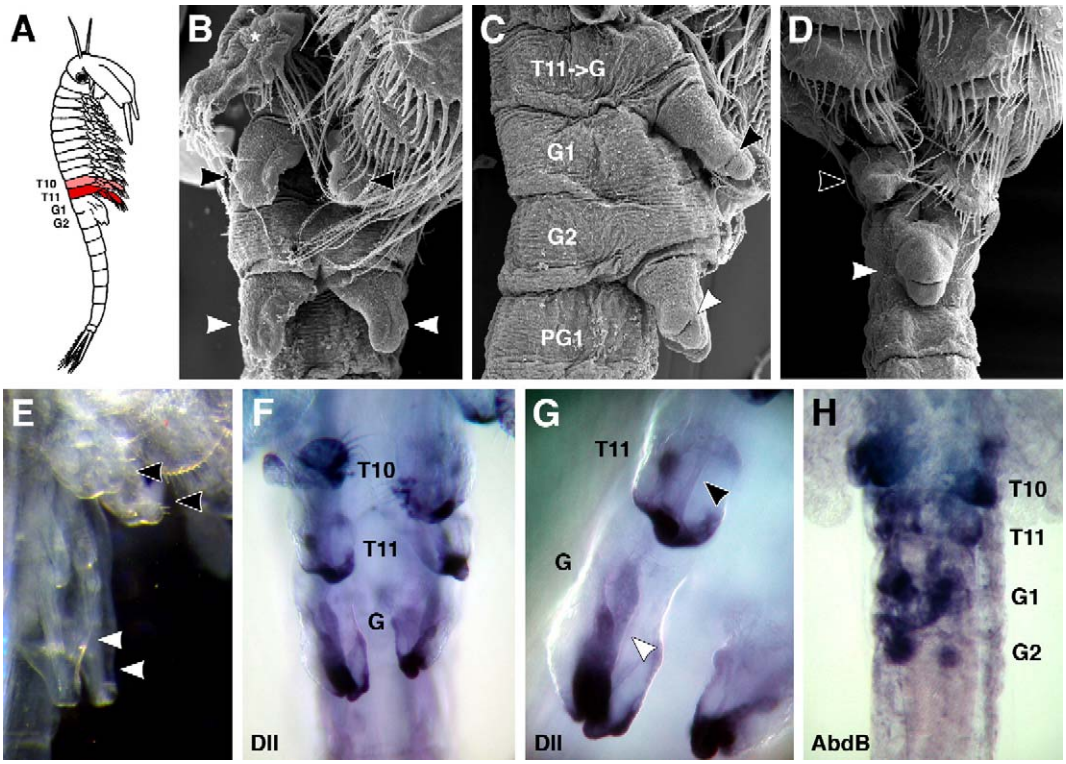

Fig. 3. *Artemia spalt* RNAi phenotypes: thoracic to genital (T→G) transformations. (A) Illustration indicating that T→G transformations are observed primarily in the thoracic segment T11, with partial transformations also observed in T10. (B, C) SEMs of a male individual with perfect transformations of both T11 appendages to genitalia (indicated by black arrowheads). These structures appear to be identical to the normal genitalia (indicated by white arrowheads), with striking similarities in their overall shape and size, the absence of setae, the presence of smoother cuticle on their medial side (seen in panel B) and the presence of a genital pore (arrowheads in panel C). Note that normal genitalia arise by partial fusion of G1 and G2, whereas the ectopic genitalia arise only from T11. The developing T10 thoracic appendage (asterisk) is reduced in size but not homeotically transformed. (D) SEM of a female individual with partially transformed appendages on the right side of her body. The normal female genitalia (white arrowhead) arise in G1 and G2 by the medial fusion of the left and right genital primordia. The T11 appendage on the right side of the body (black arrowhead) is partially transformed but has failed to fuse with the left appendage of T11, which is untransformed. The right appendage on T10 is also mildly affected. (E) View of affected male individual on the light microscope, showing the normal male genitalia (white arrowheads) and transformed T11 appendages (black arrowheads). (F, G) Immunostaining for Distal-less in a male with complete T→G transformations of T11 appendages and partial transformations in T10 (particularly on the left side of the body). The pattern of Distal-less staining in T11 is identical to the pattern in genitalia. (G) Higher magnification, showing the outline of the evertible penis in normal genitalia (white arrowhead) and in the transformed T11 appendage (black arrowhead). (H) Immunostaining for AbdB, showing ectopic expression in an individual with partial T→G transformations. Staining is observed in the genital segments (where AbdB is normally expressed; Copf et al., 2003) and in posterior thoracic segments, at similar intensities. The expression appears to be patchy and asymmetric. Anterior is up in all panels.

thoracic and genital segments but are absent from the post-genital segments, which become innervated by the neuromeres of the genital segments (Criel, 1991). After *spalt* RNAi, ectopic Nubbin/Pdm staining was observed in the post-genital segments (3/42 stained larvae), in groups of cells that resemble neuromeres (Fig. 4G). This indicates that *spalt* RNAi can also produce PG→T homeotic transformations in the central nervous system.

Previous studies on Hox genes suggest that the thoracic segmental identity is likely to be specified by *Ubx* and *AbdA* in *Artemia* (Averof and Akam, 1995; Y. Shiga and S. Hayashi, personal communication). To examine whether the ectopic thoracic appendages obtained by *spalt* RNAi are associated with ectopic expression of Ubx and/or AbdA, we carried out immunostainings with a monoclonal antibody that detects both these proteins. Ectopic Ubx/AbdA expression was detected in ectopic appendages and in ectopic neuromeres of the central nervous system (Figs. 4H, I).

The majority of individuals with ectopic thoracic appendages also have severely malformed or missing thoracic and genital appendages (see Figs. 4D, F), and some show defects in segmentation (Fig. 4C), suggesting that these might represent the most severe *spalt* RNAi phenotypes.

Discussion

Expression of spalt is not segmentally restricted in Artemia

In *Drosophila*, *spalt* is expressed in two sub-terminal regions of the embryo, where it cooperates with the Hox genes to specify the identity of gnathal and posterior abdominal segments (Jurgens, 1988; Kuhnlein et al., 1994). Later, during larval stages, *spalt* is expressed in the wing primordium, but not in the haltere primordium where it is a direct target of Ubx repression; its expression in the wing contributes to patterning of characteristic wing features such as the veins (de Celis et al.,

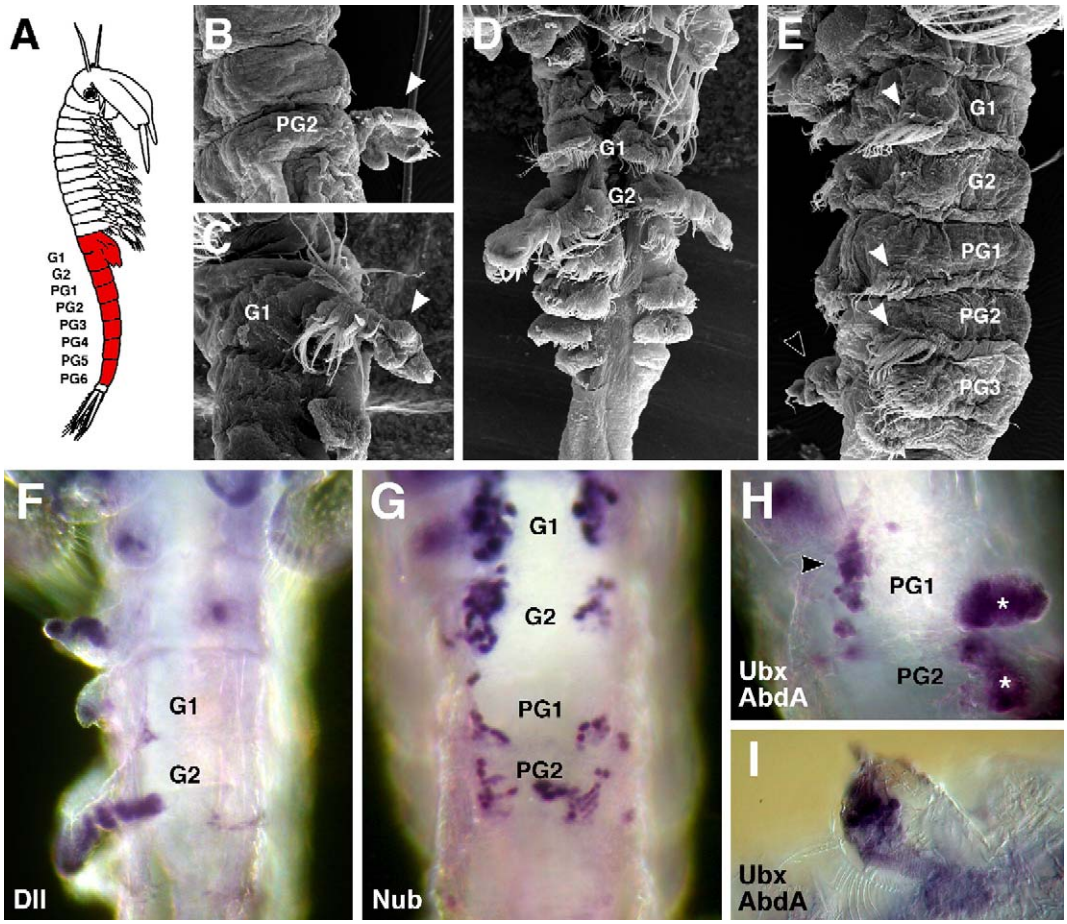

Fig. 4. *Artemia spalt* RNAi phenotypes: genital and post-genital to thoracic (G/PG→T) transformations. (A) Illustration highlighting the region where ectopic thoracic appendages are observed, including the genital (G1 and G2) and post-genital (PG1–6) segments. (B) SEM of an individual with an isolated ectopic appendage in the second post-genital segment. The appendage (arrowhead) appears only in one side of the animal; it bears characteristic features of the thoracic phyllopodous appendages, including a flattened multi-lobed appearance with setae emerging along the margin of the appendage (compare to Fig. 2B). (C) Individual with a unilateral ectopic appendage in the genital region. Segmentation appears to be disrupted in the genital region. (D) Individual with a series of ectopic immature phyllopodous appendages emerging from genital and post-genital segments. The development of normal phyllopodous appendages in the posterior thoracic segments is also disrupted. (E) Ventro-lateral view of an individual with ectopic rows of setae (white arrowheads) and a small outgrowth (black arrowhead) emerging from genital and post-genital segments; these structures are likely to represent partial G/PG→T transformations. (F) Immunostaining for Distal-less in an individual with ectopic appendages on one side of the genital segments. The staining in these appendages (particularly in G2) is characteristic of Distal-less expression in the phyllopodous thoracic appendages (Panganiban et al., 1995). The development of posterior thoracic appendages is also disrupted. (G) Immunostaining for Nubbin/Pdm, following *spalt* RNAi, showing expression in the neuromeres of the genital segments (G1 and G2) and in ectopic neuromeres in two post-genital segments (PG1 and PG2). (H) Immunostaining showing ectopic Ubx and/or AbdA expression associated with what appear to be ectopic neuronal cells (arrowhead) and ougrowths (asterisks) in the post-genital region. Ubx and AbdA are normally not expressed in this region (Averof and Akam, 1995; Copf et al., 2003). (I) Ectopic Ubx/AbdA expression in an ectopic appendage forming on the first post-genital segment. Anterior is up in all panels except panel I.

1996; Galant et al., 2002). These two roles exemplify how the segmentally restricted expression of *spalt* can contribute to the development of segment-specific features, either in parallel to Hox genes or as their downstream target.

Contrary to *Drosophila*, in *Artemia spalt* is smilarly expressed during the generation of all the trunk segments. It is initially expressed in the growth zone, from which all these segments arise, and is then expressed in stripes of cells that run across each newly formed segment (Fig. 1). There is no evidence for segment-specific regulation of *spalt* during the early stages of segment and appendage formation. Later, *spalt* expression becomes modulated in the growing thoracic appendages, but this occurs only after these appendages have acquired their distinctive morphology. Thus, in *Artemia*, the expression of *spalt* does not appear to be under the control of Hox genes and is unlikely to convey any segment-specific information within the trunk.

Range of spalt RNAi phenotypes

About 50% of *spalt* RNAi-treated animals that survived to late larval stages had an overt phenotype — a level of penetrance that is not unusual for RNAi experiments (Copf et al., 2004; Kamath et al., 2003). We believe this is explained by the fact that RNAi reduces, but does not completely abolish, expression of the targeted gene (e.g. Caplen et al., 2001; Copf et al., 2004). Moreover, the frequency of severe phenotypes is likely to be underestimated in these experiments as severely affected animals are less likely to survive to the late larval stages when the phenotypes were scored.

The knockdown of *spalt* by RNAi produced a variety of phenotypes affecting the development and/or the identity of posterior thoracic appendages. The effect on appendages is consistent with the expression of *spalt* in a band straddling the AP compartment boundary in each segment, which is where the appendages arise. The most common phenotype was the development of malformed or rudimentary appendages (Fig. 2). The restriction of this phenotype to the appendages of posterior thoracic segments (T8–T11) is probably due to the timing of *spalt* dsRNA injections and the delayed onset of RNAi, as documented previously for *caudal* RNAi (Copf et al., 2004). Injections carried out in stage L1 larvae produced defects in somewhat more anterior appendages compared to injections at stages L2–3 (data not shown).

In contrast, no obvious differences were observed in the segmental range of T→G transformations, which were observed only in T10 or T11. The susceptibility of T10 and T11 to T→G transformations may be related to the fact that, in normal development, these segments express low levels of AbdB during late larval stages (Copf et al., 2003). Ectopic thoracic appendages (G/PG→T transformations) were recorded in most genital and post-genital segments, with no obvious segmental preferences.

Homeotic transformations are associated with a stochastic de-repression of Hox genes

Probably the most puzzling result of these *spalt* RNAi experiments is the recovery of homeotic transformations in two opposite directions: posteriorward T→G and anteriorward G→T and PG→T transformations. What could be the basis for these transformations in opposite directions? The timing of dsRNA injections is not likely to be responsible as no obvious differences were found in the direction of transformations when injections were carried out in L1 or L2–3 larvae. But a hint for the resolution of this puzzle may come from the phenotypic variability observed *within* individual animals. The fact that, often, different phenotypes are obtained on the left and right sides of the same individual (e.g. Figs. 3D and 4B, C, F, H) suggests that there is a strong stochastic element to these transformations.

We have shown that these homeotic transformations are associated with the ectopic expression of Hox genes. The particular Hox genes that are ectopically expressed correlate with the nature of the transformation: AbdB is associated with T→G transformations, while Ubx/AbdA are associated with G/PG→T transformations. Given the central role that Hox genes are known to play in the specification of segmental/regional identity in insects and vertebrates (McGinnis and Krumlauf, 1992), there is likely to be a direct causal relationship between the ectopic expression of these genes and the homeotic transformations that we observe in *Artemia*. Thus, the variability and stochasticity of these transformations may be reduced to stochasticity in Hox gene de-repression. Indeed, the ectopic expression of AbdB and Ubx/AbdA was usually asymmetric and patchy, closely mirroring the spatial distribution of the morphological transformations (Figs. 3H and 4H, I). Thus, de-repression of either of these Hox genes appears to be an infrequent event that is triggered stochastically in different patches of cells as a result of *spalt* RNAi.

Based on these results, we propose that *Artemia spalt* is required to maintain a robust repression on Hox genes in body regions where they should not be expressed. Unfortunately, we are not able to address whether this stochasticity is due to uneven silencing of *spalt* by RNAi in different groups of cells or due to the stochastic effects of lower *spalt* expression on its target Hox genes.

Maintenance of Hox gene repression may be a widely conserved role of spalt

Our experiments suggest that *Artemia spalt* acts on Hox genes to ensure that they are stably repressed in segmental domains where their expression would be inappropriate. *spalt* does not appear to convey any segment-specific information as it is similarly expressed in all the trunk segments and it affects the expression of at least two different Hox genes. These characteristics are typically associated with the function Polycomb group (PcG) genes in *Drosophila*, which act as general repressors to maintain the correct spatial domains of Hox gene expression once these domains have been established by segment-specific regulators (Ringrose and Paro, 2004).

Some authors have previously suggested that Spalt proteins may function as part of a general repression complex, similar to the PcG proteins, in diverse animals (Toker et al., 2003). In *Drosophila*, this is supported by the genetic interaction of *spalt* with at least two PcG genes (enhancement of *Polycomb* and *polyhomeotic* mutations in double heterozygotes; Landecker et al., 1994) and by the ectopic expression of *Ubx* in *spalt* mutants (Casanova, 1989; Castelli-Gair, 1998). In *C. elegans*, there is clear evidence that the *spalt* homologue *sem-4* directly represses expression of the Hox gene *egl-5* in the context of touch receptor specification (Toker et al., 2003). And, although the molecular basis of the SALL1 phenotypes is not known, it has been shown that vertebrate SALL1 proteins can interact with chromatin remodeling complexes and act as strong transcriptional repressors; they are able to recruit histone deacetylase complexes, they co-localize with heterochromatin and with HP1 and they physically interact with a protein that binds to telomeric heterochromatin (Kiefer et al., 2002; Netzer et al., 2001, 2006).

Taken together, these observations suggest that Spalt proteins are likely to have a conserved role in transcriptional repression through chromatin modification. This appears to be linked to the regulation of Hox gene expression in some animals but may also extend to the regulation of many other genes in various developmental contexts (including some Hox target genes). Our data from *Artemia* are consistent with this hypothesis and suggest that the role of *spalt* in maintaining Hox gene expression deserves further investigation in *Drosophila* and in other arthropod species.

Acknowledgments

We thank Habib Boulekbache, David Montero and UMR7138 from Université Pierre and Marie Curie for support with SEMs and Tassos Pavlopoulos, Maura Strigini, Michael Akam and Andrew Peel for comments on the manuscript. This work was supported by a research fellowship from the Irakleitos programme of the Greek Ministry of Education.

References

Al-Baradie, R., Yamada, K., St Hilaire, C., Chan, W.M., Andrews, C., McIntosh, N., Nakano, M., Martonyi, E.J., Raymond, W.R., Okumura, S., et al., 2002. Duane radial ray syndrome (Okihiro syndrome) maps to 20q13 and results from mutations in SALL4, a new member of the SAL family. Am. J. Hum. Genet. 71, 1195–1199.

Averof, M., Akam, M., 1995. Hox genes and the diversification of insect and crustacean body plans. Nature 376, 420–423.

Averof, M., Cohen, S.M., 1997. Evolutionary origin of insect wings from ancestral gills. Nature 385, 627–630.

Caplen, N.J., Parrish, S., Imani, F., Fire, A., Morgan, R.A., 2001. Specific inhibition of gene expression by small double-stranded RNAs in invertebrate and vertebrate systems. Proc. Natl. Acad. Sci. U. S. A. 98, 9742–9747.

Casanova, J., 1989. Mutations in the spalt gene of *Drosophila* cause ectopic expression of Ultrabithorax and Sex combs reduced. Roux's Arch. Dev. Biol. 198, 137–140.

Castelli-Gair, J., 1998. Implications of the spatial and temporal regulation of Hox genes on development and evolution. Int. J. Dev. Biol. 42, 437–444.

Copf, T., Rabet, N., Celniker, S.E., Averof, M., 2003. Posterior patterning genes and the identification of a unique body region in the brine shrimp *Artemia franciscana*. Development 130, 5915–5927.

Copf, T., Schroder, R., Averof, M., 2004. Ancestral role of caudal genes in axis elongation and segmentation. Proc. Natl. Acad. Sci. U. S. A. 101, 17711–17715.

Criel, G.R.J., 1991. Morphology of *Artemia*. In: Browne, R.A., Sorgeloos, P., Trotman, C.N.A. (Eds.), Artemia Biology. CRC Press, Boca Raton, Florida, pp. 119–153.

Damen, W.G., Saridaki, T., Averof, M., 2002. Diverse adaptations of an ancestral gill: a common evolutionary origin for wings, breathing organs, and spinnerets. Curr. Biol. 12, 1711–1716.

de Celis, J.F., Barrio, R., Kafatos, F.C., 1996. A gene complex acting downstream of dpp in *Drosophila* wing morphogenesis. Nature 381, 421–424.

Dong, P.D., Dicks, J.S., Panganiban, G., 2002. Distal-less and homothorax regulate multiple targets to pattern the Drosophila antenna. Development 129, 1967–1974.

Franch-Marro, X., Casanova, J., 2002. spalt-induced specification of distinct dorsal and ventral domains is required for *Drosophila* tracheal patterning. Dev. Biol. 250, 374–382.

Galant, R., Walsh, C.M., Carroll, S.B., 2002. Hox repression of a target gene: extradenticle-independent, additive action through multiple monomer binding sites. Development 129, 3115–3126.

Grant, K., Hanna-Rose, W., Han, M., 2000. sem-4 promotes vulval cell-fate determination in *Caenorhabditis elegans* through regulation of lin-39 Hox. Dev. Biol. 224, 496–506.

Harlow, E., Lane, D., 1988. Antibodies; A Laboratory Manual. Cold Spring Harbor Laboratory Press, Cold Spring Harbor.

Harvey, S.A., Logan, M.P., 2006. sall4 acts downstream of tbx5 and is required for pectoral fin outgrowth. Development 133, 1165–1173.

Jurgens, G., 1988. Head and tail development of the *Drosophila* embryo involves spalt, a novel homeotic gene. EMBO J. 7, 189–196.

Kamath, R.S., Fraser, A.G., Dong, Y., Poulin, G., Durbin, R., Gotta, M., Kanapin, A., Le Bot, N., Moreno, S., Sohrmann, M., et al., 2003. Systematic functional analysis of the *Caenorhabditis elegans* genome using RNAi. Nature 421, 231–237.

Kelsh, R., Weinzierl, R.O., White, R.A., Akam, M., 1994. Homeotic gene expression in the locust Schistocerca: an antibody that detects conserved epitopes in Ultrabithorax and abdominal-A proteins. Dev. Genet. 15, 19–31.

Kiefer, S.M., McDill, B.W., Yang, J., Rauchman, M., 2002. Murine Sall1 represses transcription by recruiting a histone deacetylase complex. J. Biol. Chem. 277, 14869–14876.

Kohlhase, J., 2000. SALL1 mutations in Townes–Brocks syndrome and related disorders. Hum. Mutat. 16, 460–466.

Kohlhase, J., Wischermann, A., Reichenbach, H., Froster, U., Engel, W., 1998. Mutations in the SALL1 putative transcription factor gene cause Townes–Brocks syndrome. Nat. Genet. 18, 81–83.

Kohlhase, J., Heinrich, M., Schubert, L., Liebers, M., Kispert, A., Laccone, F., Turnpenny, P., Winter, R.M., Reardon, W., 2002. Okihiro syndrome is caused by SALL4 mutations. Hum. Mol. Genet. 11, 2979–2987.

Kohlhase, J., Chitayat, D., Kotzot, D., Ceylaner, S., Froster, U.G., Fuchs, S., Montgomery, T., Rosler, B., 2005. SALL4 mutations in Okihiro syndrome (Duane-radial ray syndrome), acro–renal–ocular syndrome, and related disorders. Hum. Mutat. 26, 176–183.

Koster, R., Stick, R., Loosli, F., Wittbrodt, J., 1997. Medaka spalt acts as a target gene of hedgehog signaling. Development 124, 3147–3156.

Kuhnlein, R.P., Frommer, G., Friedrich, M., Gonzalez-Gaitan, M., Weber, A., Wagner-Bernholz, J.F., Gehring, W.J., Jackle, H., Schuh, R., 1994. *spalt* encodes an evolutionarily conserved zinc finger protein of novel structure which provides homeotic gene function in the head and tail region of the *Drosophila* embryo. EMBO J. 13, 168–179.

Landecker, H.L., Sinclair, D.A., Brock, H.W., 1994. Screen for enhancers of Polycomb and Polycomblike in *Drosophila melanogaster*. Dev. Genet. 15, 425–434.

Manzanares, M., Marco, R., Garesse, R., 1993. Genomic organization and developmental pattern of expression of the engrailed gene from the brine shrimp Artemia. Development 118, 1209–1219.

McGinnis, W., Krumlauf, R., 1992. Homeobox genes and axial patterning. Cell 68, 283–302.

Mollereau, B., Dominguez, M., Webel, R., Colley, N.J., Keung, B., de Celis, J.F., Desplan, C., 2001. Two-step process for photoreceptor formation in *Drosophila*. Nature 412, 911–913.

Netzer, C., Rieger, L., Brero, A., Zhang, C.D., Hinzke, M., Kohlhase, J., Bohlander, S.K., 2001. SALL1, the gene mutated in Townes–Brocks syndrome, encodes a transcriptional repressor which interacts with TRF1/PIN2 and localizes to pericentromeric heterochromatin. Hum. Mol. Genet. 10, 3017–3024.

Netzer, C., Bohlander, S.K., Hinzke, M., Chen, Y., Kohlhase, J., 2006. Defining the heterochromatin localization and repression domains of SALL1. Biochim. Biophys. Acta 1762, 386–391.

Nishinakamura, R., Matsumoto, Y., Nakao, K., Nakamura, K., Sato, A., Copeland, N.G., Gilbert, D.J., Jenkins, N.A., Scully, S., Lacey, D.L., et al., 2001. Murine homolog of SALL1 is essential for ureteric bud invasion in kidney development. Development 128, 3105–3115.

Onai, T., Sasai, N., Matsui, M., Sasai, Y., 2004. *Xenopus* XsalF: anterior neuroectodermal specification by attenuating cellular responsiveness to Wnt signaling. Dev. Cell 7, 95–106.

Panganiban, G., Sebring, A., Nagy, L., Carroll, S., 1995. The development of crustacean limbs and the evolution of arthropods. Science 270, 1363–1366.

Parrish, M., Ott, T., Lance-Jones, C., Schuetz, G., Schwaeger-Nickolenko, A., Monaghan, A.P., 2004. Loss of the Sall3 gene leads to palate deficiency, abnormalities in cranial nerves, and perinatal lethality. Mol. Cell. Biol. 24, 7102–7112.

Patel, N.H., Kornberg, T.B., Goodman, C.S., 1989a. Expression of engrailed during segmentation in grasshopper and crayfish. Development 107, 201–212.

Patel, N.H., Martin-Blanco, E., Coleman, K.G., Poole, S.J., Ellis, M.C., Kornberg, T.B., Goodman, C.S., 1989b. Expression of engrailed proteins in arthropods, annelids, and chordates. Cell 58, 955–968.

Ringrose, L., Paro, R., 2004. Epigenetic regulation of cellular memory by the Polycomb and Trithorax group proteins. Annu. Rev. Genet. 38, 413–443.

Rusten, T.E., Cantera, R., Urban, J., Technau, G., Kafatos, F.C., Barrio, R., 2001. Spalt modifies EGFR-mediated induction of chordotonal precursors in the embryonic PNS of *Drosophila* promoting the development of oenocytes. Development 128, 711–722.

Toker, A.S., Teng, Y., Ferreira, H.B., Emmons, S.W., Chalfie, M., 2003. The *Caenorhabditis elegans* spalt-like gene sem-4 restricts touch cell fate by repressing the selector Hox gene egl-5 and the effector gene mec-3. Development 130, 3831–3840.