Case studies on the genes *zerknüllt*, *decapentaplegic* and *short gastrulation* in the beetle *Tribolium* illustrate concepts in evolutionary developmental biology

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Als Gregor Samsa eines Morgens aus unruhigen Träumen aufwachte,
fand er sich in seinem Bett zu einem ungeheueren Ungeziefer verwandelt.
Er lag auf seinem panzeratig harten Rücken und sah,
wenn er den Kopf ein wenig hob,
seinen gewölbten, braunen,
von bogenförmigen Versteifungen geteilten Bauch,
auf dessen Höhe sich die Bettdecke,
zum gänzlichen Niedergleiten bereit,
kaum noch halten konnte.
Seine vielen,
im Vergleich zu seinem sonstigen Umfang kläglich dünnen Beine
flimmerten ihm hilflos vor den Augen.

Franz Kafka: *Die Verwandlung*

Cover illustration: B. van der Zee (1917-1997)

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CHAPTER 1

General introduction

A history of Evo-Devo

Evolutionary developmental biology (Evo-Devo) aims to unveil how developmental processes and mechanisms become modified during evolution and how from these changes the past and present biodiversity arose (Baguna and Garcia-Fernandez, 2003). Evo-Devo is often called a "new science" or a "revolution" (Carroll, 2005a); however, as soon as evolution entered scientific thinking, development was intimately associated with it. In the eighteenth century, scientists left the static view of Aristotle that all organisms are unchangeable and can be ordered from simple to complex in a fixed "Chain of Being". Organisms were rather supposed to "march up" this Ladder of Being during evolution, as Lamarck later formulated it. This progressive view forced the static embryological theory of that time – preformationalism – to change. Preformationalism teaches that development merely makes visible the organism, which is already present in all its details in the egg (or sperm). And in this miniature organism in the egg, even smaller, similar miniatures of its own progeny are already present, and so on. There was no space for evolutionary change. The preformationalist Bonnet (1720-1793) was the first to construct a synthesis, paraphrased by Gould, (Gould, 1977):

"Why, after all, should the succession of encapsulated generations in the ovaries of a primal Eve all bear the same form. We might open the Russian doll in this primal ovary and find only ten dolls of identical form; inside the tenth we might discover a vastly superior creature, and after ten similar boxes another being of still more perfect design."

In the nineteenth century, the idea of unchanging species and preformationalism were left for good. A new theory joining development and evolution became popular – recapitulation – with Haeckel as its later and most fanatic proponent. He formulated the biogenetic law: ontogeny is a recapitulation of phylogeny. That means: during its own embryological development, a higher organism quickly passes through the adult stages of its paleontological ancestors. Haeckel thought that new, higher organisms arise by the addition of development at the end of the current development, a process called terminal addition. Of

course, in order to prevent that development would finally become endless in the highest organisms, the ancestral development was shortened, or "condensed" (Gould, 1977). Although there is some superficial truth in Haeckels observations, the statement that an early stage in human development literally is, for example, an adult fish, is too dogmatic.

Von Baer (1792-1876) put the resemblances of embryonic stages of mammals with, for example, adult fish in a better perspective. He observed that development proceeds from the most general to the most specialized. If one goes back in embryological development of a vertebrate, one will find the general form of vertebrates. A mammalian embryo never passes through a real fish state, but fish are simply less different from their early embryos. Von Baer notes (Von Baer, 1828):

"It is only because the least developed animals are but little removed from the embryonic condition, that they retain a certain similarity with the embryos of higher animal forms."

For Darwin (1809-1882) this was inspiring. He realized that similarity of embryos reveals a common ancestry. Paraphrased by Gould (1977):

"The gill slits of the human fetus represent no ancestral adult fish: we see no repetition of adult states, no recapitulation. Yet adult fish, as primitive ancestors, have departed least from this embryological condition of all vertebrates."

Hence, embryology can give important clues about phylogeny and can help to determine true homologies. I will call this the first principle connecting development and evolution. Despite this important finding, developmental biology lost its importance for evolution in the beginning of the twentieth century. Haeckels student Roux encouraged embryologists to leave the seashore and go into the laboratory, thus announcing the start of experimental embryology in 1894. In the same year, Bateson claimed that the embryological method has failed when it came to the mechanisms of evolution (Gilbert, 2003). Famous experimental embryologists like Spemann entered the scene. Furthermore, the formulation of the Modern Synthesis (around 1940) providing a cause for evolution by integrating genetics and evolutionary biology, made evolutionary biology go its own way.

However, there have always been scientists trying to bridge development and evolution. Garstang (1922), for example, claimed: "Ontogeny does not recapitulate phylogeny; it creates it.", or De Beer (1954), who stressed the importance of heterochrony (both in: Gilbert, 2003). After the formulation of the Modern Synthesis, there were even scientists who tried to integrate genetics, evolutionary and developmental biology. Most notably Goldschmidt, Waddington

and Schmalhausen. Goldschmidt speculated that mutations in developmentally important genes could cause big changes in the body plan ('hopeful monsters'), enabling macroevolution. Waddington plead for the study of the process that turns the genotype into a phenotype (= development), and developed important modern concepts, like developmental constraint, canalization and genetic assimilation, the latter originally proposed by Baldwin. Canalization and genetic assimilation were independently discovered by Schmalhausen (Gilbert, 2003).

Concepts in modern Evo-Devo

Canalization is the property of developmental pathways to produce standard phenotypes despite mild genetic or environmental perturbations. *Genetic assimilation* is the phenomenon that an environmentally induced reaction can easily come under genetic control, if this is advantageous. Both can be illustrated by sex determination in reptiles. In some reptiles, sex determination is temperature dependent. Canalization achieves that an individual does not have an intermediate sex, when developed at an intermediate temperature, but that an individual is either male or female. Genetic assimilation effectuated that sex determination came under genetic control in some reptiles (Kirschner and Gerhart, 2005).

In 1977, Stephen Jay Gould wrote the important book 'Ontogeny and Phylogeny', rediscovering the classical evolutionary embryologists. The book is mainly about heterochrony. Heterochrony assumes that developmental processes are more or less dissociated and can be independently *accelerated* or *retarded*. The results can be *paedomorphosis* (the new adults look more like the embryo of the ancestor) or *recapitulation* (the new embryos look like adults of the ancestor). Paedomorphosis can be caused by *progenesis* (acceleration of maturation with regard to somatic development) or by *neoteny* (retardation of shape/somatic development with regard to maturation). Recapitulation can be caused by *hypermorphosis* (retardation of maturation with respect to somatic development) or by *acceleration* (acceleration of shape/somatic development with respect to developmental stage). Some people call this book about developmental mechanisms of evolutionary change the conception (Gilbert, 2003), or even the birth of Evo-Devo (Hall, 2003).

However, the biggest impetus for modern Evo-Devo was provided by the integration of genetics and molecular biology into embryology. The discovery of Hox genes, and the screen for developmental genes in *Drosophila* by Christiane Nüsslein-Volhard and Eric Wieschaus made clearer how genes direct development. Although Ernst Mayr warned that "the search for homologous genes is quite futile except in very close relatives", it was soon discovered that many

developmental genes (for example Hox genes) are evolutionary conserved among phyla (McGinnis et al., 1984). The comparison of developmental genes between species became the new discipline of Evo-Devo (Carroll, 2005a).

The main idea is that mutations in developmental genes cause a change in development ("developmental reprogramming"; Arthur, 2004) and lead to evolutionary novelty. By comparing genes among organisms with known phylogenetic relationships, those changes can be traced and evolutionary scenarios can be inferred. I will call this the second principle connecting development and evolution. An example is the discovery of a mutation in the abdominal Hox protein Ultrabithorax (Ubx) in higher insects. This mutated Ubx protein represses Distal-less expression. Thus, abdominal legs are repressed in higher insects whereas other arthropods, which do not have this mutation, bear abdominal legs (Galant and Carroll, 2002; Hittinger et al., 2005; Ronshaugen et al., 2002). The evolutionary scenario would be that higher insects acquired this mutation and lost the abdominal legs.

However, the Ubx mutation did probably not happen in a 'hopeful monster' way. Ubx is partially redundant with the Hox gene Abdominal-A which also represses abdominal legs (Hittinger et al., 2005; Tour et al., 2005). And, although ectopic Ubx can repress thoracic legs, removal of the mutation does not simply lead to abdominal legs (Hittinger et al., 2005; Tour et al., 2005). Evolutionary novelty is hardly ever reducable to a mutation in a protein. Changes in proteins are usually deleterious. *Gene duplication* (Ohno, 1970) can circumvent this problem. After duplication, one copy of the gene could continue to carry out the ancestral function, while the other copy can acquire mutations and adopt a new function.

Another way to achieve morphological novelty without affecting protein functionality, is changing cis-regulatory sequences (Carroll, 2005a; Carroll, 2005b). By the creation of new binding sites for transcription factors in cis-regulator*y* sequences of genes, those genes or gene duplicates can be recruited to perform their task - or a new task - in novel tissues or at novel moments in development. This process can be called *co-option* (Raff, 1996). A beautiful example is the co-option of NADPH:quinone oxidoreductase as the lens protein ζ-crystallin in guinea pigs (Lee et al., 1994). Co-option can occur at the morphological level as well when existing structures become recruited for a new function, for example the reptilian jaw articulation elements, which became the bones of the middle ear in mammals. Thus, nature works with "what is already there", rather than inventing completely new things. Evolution is tinkering, as Jacob formulated it (Jacob, 1977).

This tinkering requires that the "tinkering units" can be dissociated and are not highly connected. Therefore, life must have a *modular* organization. The separable modules can be used again and again in different combinations by nature (Raff, 1996). *Modularity* and *dissociation* stay at the basis of many Evo-Devo concepts. Heterochrony, for example, can only occur when developmental processes can be independently regulated. Regulatory sequences of genes also have a modular organization; the ζ-crystallin of guinea pigs, for example, has a lens specific and an enzyme specific promoter. An important way to achieve modularity in the spatial dimension is segmentation. This divides the animal body into independent modules, of which for example appendages can be independently modified, without affecting other appendages. This is also called *compartmentation* (Kirschner and Gerhart, 2005).

The word compartmentation can also be used in describing gene networks. Genes can be considered nodes and their regulatory connections can be considered links in a network (Barabasi, 2002). It appears that gene networks have a modular organization too, with high connectivity within, but low connectivity among the subnetworks, or compartments (Niehrs, 2004). The high connectivity within a compartment is caused by only a few genes with lots of links:"hubs" (Somogyi et al., 2004). The way nodes are connected gives the network certain properties (Barabasi, 2002). By changes in cis-regulatory sequences, the connections in a network and thus the properties of the network change. By recruiting a hub, the whole subnetwork is co-opted. Network growth by gene duplication will enhance the formation of hubs, because a duplicating gene is probably connected to a hub. Since duplicated genes will initially retain their ancestral connections, the hub has received an extra link by the duplication event (Barabasi, 2002).

If modules are not well dissociated, evolution becomes constrained. If a gene is for example involved in two processes, favorable mutations for one process might be disadvantageous for the other process. Thus, *pleiotropy* can keep genes at a suboptimal level. Gene duplication can sometimes circumvent this problem, since gene duplication can be followed by *subfunctionalization*: both copies specialize in a specific function of the ancestral gene (Force et al., 1999; Lynch et al., 2001; Piatigorsky and Wistow, 1991). However, pleiotropy and highly connected modules form *developmental constraints*.

A beautiful example of a developmental constraint is the conserved number of cervical vertebrae in mammals, caused by the pleiotropic effects of the Hox genes determining this number (Galis, 1999). Especially during the phylotypic stage, modules seem to be highly connected and evolution highly

constrained (Galis and Metz, 2001). Beacuse embryology constrains certain changes and facilitates the reuse of already present modules (Kirschner and Gerhart, 2005), embryos 'steer' evolution (Arthur, 2004). Of course, random mutations are always required, but the chance for some type of changes to happen is bigger than for other types. I will call this the third principle connecting development and evolution. Although this is only recently more appreciated (Arthur, 2004; Kirschner and Gerhart, 2005; Schlosser and Wagner, 2004) it has already intelligently described by Rupert Riedl in 1975 (Riedl, 1975), whom I herewith boldly declare the Father of Evo-Devo.

Summarizing, there are three important principles that connect embryology to evolution. First, as the classical comparative embryologists already discovered, embryology supplies important clues about phylogenic relationships and about true homologies. Second, by comparing developmental genes among organisms with known phylogenetic relationships, changes that caused developmental reprogramming can be traced and evolutionary scenarios can be inferred. Third, embryos can steer evolution by constraining certain types of changes and by facilitating other types.

Aim of this thesis

In this thesis, the function of developmental genes in the red flour beetle *Tribolium castaneum* is compared to the function of their homologues in other animals, like the fruitfly *Drosophila melanogaster*. The phylogeny of insects is well known (Whiting, 2004). It appears that *Drosophila* belongs to a derived group of Diptera. The development of *Drosophila* displays a lot of derived characters: the anterior segments are specified by the maternal gene bicoid, pattern formation largely takes place in the syncytial blastoderm, all segments are present in the blastoderm and no growth zone is present, the legs develop from imaginal discs and the larval head is folded inward. *Tribolium* represents a more ancestral state with regard to these characters and its genes probably retained their ancestral function. By comparing those gene functions to the function of their homologues in *Drosophila*, I attempt to reconstruct what evolutionary changes occurred in the lineage leading to *Drosophila*.

The thesis consists of two case studies. In the first one (chapter 2), the function of the two *zerknüllt* genes in *Tribolium* is investigated. One of the biggest morphological differences between *Tribolium* and *Drosophila* embryos is the architecture of their blastoderm and their extraembryonic membranes. Nearly the whole *Drosophila* blastoderm will give rise to the embryo proper and only a small

part will give rise to a very reduced extraembryonic membrane. In contrast, a big part of the *Tribolium* blastoderm is occupied by two extraembryonic membranes which later play a role in important morphological processes like dorsal closure. In this respect, most insects develop like *Tribolium* (Roth, 2004). *Drosophila zerknüllt* has been shown to specify the extraembryonic membrane. One of the questions I hope to answer by studying *zerknüllt* function in *Tribolium* is how *Drosophila* could reduce its extraembryonic membranes so much without affecting important morphogenetic processes like dorsal closure.

In the second case study (chapter 3), the functions of *decapentapegic* (*dpp*) and *short gastrulation (sog)* are examined. The vertebrate homologues of these genes are called BMP and chordin respectively. BMPs belong to a well known family of ligands, the TGFβ family. BMPs play a role in a lot of processes, but also in dorsoventral patterning in vertebrates and in *Drosophila*. Sog and chordin are BMP inhibitors. In vertebrates, BMP has to be inhibited anteriorly to form the head. This seems not to be the case in *Drosophila*. Since the only functional study of Dpp and Sog in arthropods has been carried out in *Drosophila*, it is still unclear whether BMPs play a role in dorsoventral patterning in all arthropods, and if Sog really does not have a role in head formation in other arthropods. By investigating the function of Sog and Dpp in *Tribolium*, I investigate another arthropod and hope to resolve those uncertainties.

In chapter 4, the conclusions and results of the case studies are discussed in a bigger perspective: I explore to what extent the introduced concepts of Evo-Devo apply to them. It turns out that the comparative studies of developmental genes in *Tribolium* and their evolutionary implications illustrate many concepts in evolutionary developmental biology.

CHAPTER 2

Distinct functions of the *Tribolium zerknüllt* genes in serosa specification and dorsal closure

2.1 SUMMARY

Background: In the long germ insect *Drosophila* a single extraembryonic membrane, the amnioserosa, covers the embryo at the dorsal side. In ancestral short germ insects an inner membrane, the amnion, covers the embryo ventrally and an outer membrane, the serosa, completely surrounds the embryo. An early differentiation step partitions the uniform blastoderm into the anterior-dorsal serosa and the posterior-ventral germ rudiment giving rise to amnion and embryo proper. In *Drosophila* amnioserosa formation depends on the dorsoventral patterning gene *zerknüllt* (*zen*), a derived Hox3 gene.

Results: The short germ beetle *Tribolium castaneum* possesses two *zen* homologues, *Tc-zen1* and *Tc-zen2*. *Tc-zen1* acts early and specifies the serosa. The loss of the serosa after *Tc-zen1* RNAi is compensated by an expansion of the entire germ rudiment towards anterior. Instead of the serosa, the amnion covers the embryo at the dorsal side and later size regulation normalizes the early fate shifts, revealing a high degree of plasticity of short germ development. *Tc-zen2* acts later and is required for the amnion and serosa fusion, necessary for dorsal closure. After *Tc-zen2* RNAi, the amnion and serosa stay apart and the embryo closes ventrally, assuming a completely everted (inside-out) topology.

Conclusions: In *Tribolium*, the duplication of the *zen* genes was accompanied by subfunctionalization. One of the paralogues, *Tc-zen1*, acts as an early anterior-posterior patterning gene by specifying the serosa. In absence of the serosa, *Tribolium* embryogenesis acquires features of long germ development with a single extraembryonic membrane. I discuss implications for the evolution of insect development including the origin of the *zen*-derived anterior determinant *bicoid.*

2.2 INTRODUCTION

One of the evolutionary innovations that probably contributed to the unparalleled success of the insects is the formation of two protecting extraembryonic membranes: the amnion and the serosa (Zeh et al., 1989). These membranes are absent from all other arthropods (Machida and Ando, 1998; Roth, 2004). The prominent distinction between cells of the presumptive serosa and cells of the germ rudiment, giving rise to the amnion and the embryo proper, is the first differentiation step that occurs within the uniform blastoderm. This stage is called the "differentiated blastoderm" (Roth, 2004). Remarkably, this stage is absent in a small group of insects, the higher dipterans, to which *Drosophila* belongs (Schmidt-Ott, 2000; Schwalm, 1988). In *Drosophila,* the germ rudiment occupies the entire blastoderm. A single extraembryonic membrane, the amnioserosa, develops from a dorsal stripe of the blastoderm and covers the yolk at the dorsal side of the egg during further development.

The red flour beetle *Tribolium castaneum* may be a representative of all other insects, given that it still possesses an amnion and a serosa and the differentiated blastoderm stage. At this stage, the prospective serosa is visible as a dorsally tilted anterior cap of flattening cells. During gastrulation, the serosa begins to cover the embryo proper at the posterior pole, forming the posterior amniotic fold, together with the amnion. The same occurs to a lesser extent at the anterior pole, forming the anterior amniotic fold. When the crests of the amniotic folds meet, a round serosal window is formed. Finally, the serosal window closes, forming a continuous outer membrane, the serosa and an inner membrane, the amnion (Handel et al., 2000) (see also Fig. 9A-D for schematic representation).

In *Drosophila*, amnioserosa formation requires the gene *zerknüllt* (*zen*) which encodes a homeobox transcription factor (Rushlow and Levine, 1990) and is a target gene of maternal and zygotic DV morphogen gradients. During early blastoderm stages, the maternal NF-κB/Dorsal protein gradient represses *zen* in the ventral half of the embryo and thus confines its transcription to a broad dorsal domain (Rushlow et al., 1987b). During gastrulation, a zygotic BMP gradient with peak levels along the dorsal midline activates *zen* in a 5 cell-wide dorsal expression domain, comprising the cells that give rise to the amnioserosa (Raftery and Sutherland, 2003). In *zen* mutants, the amnioserosa is lost and is replaced by dorsal ectoderm. *zen* maps to the Antennapedia complex (ANT-C) between *deformed* and *proboscipedia* (Wakimoto et al., 1984). This region harbours two closely linked transcription units, *zen* and *z*2, with identical expression patterns (Rushlow et al., 1987a). Since *zen* alone could rescue a deletion covering both *zen*

and *z*2, it was suggested that *z*2 is dispensible (Rushlow et al., 1987a). This was later confirmed by producing a deletion specific for *z2* (Pultz et al., 1988).

zen homologues have also been found in *Tribolium castaneum* and the grasshopper *Schistocerca gregaria*. *Schistocerca-zen* is expressed in the serosa and late amnion, while *Tribolium-zen* is expressed in the serosa (Dearden et al., 2000; Falciani et al., 1996). Later sequencing of the *Tribolium* ANT-C revealed two *zen* homologues: *Tc-zen1*, which corresponds to the homologue cloned by Falciani et al. (Falciani et al., 1996), and *Tc-zen2* (Brown et al., 2002). Notably, the duplication of *zen* in *Drosophila* and *Tribolium* has happened independently, as inferred from phylogenetic analysis (Brown et al., 2002) and the absence of *z*2 in *Drosophila pseudoobscura* (Randazzo et al., 1993). So far, expression of *Tc-zen2* has not been investigated. The expression of *Schistocerca-zen* and *Tribolium-zen1* suggests a conserved function for *zen* in the patterning or differentiation of extraembryonic tissues in all insects. However, until now no functional study has been conducted except in *Drosophila*.

In this chapter, I show that after duplication, the *zen* genes of *Tribolium* acquired partially different expression patterns and diverged completely with regard to their function. RNAi with *Tc-zen2* results in incorrect dorsal closure, generating completely everted (inside-out) larvae. RNAi with *Tc-zen1* leads to the complete loss of the anteriormost cell fate, the serosa, and a compensating expansion of the more posterior germ rudiment. The fact that *Tc-zen1* is involved in early specification of an anterior structure might have been a favourable condition for the evolution of the *zen*-derived anterior determinant *bicoid* which specifies head and thorax in higher dipterans (St Johnston and Nusslein-Volhard, 1992). Despite absence of the serosa after *Tc-zen1* RNAi, size regulation generates normal larvae which exhibit perfect dorsal closure. This developmental plasticity sheds light on the evolution of long germ insects with a single extraembryonic membrane (the amnioserosa).

2.3 MATERIALS AND METHODS

Stock maintenance

Beetles were kept on wheat flour (Diamant extra, type 405) supplemented with dried bakers yeast in plastic boxes at 30°C. For egg collection, beetles were placed for one to three days on instant flour (Diamant instant, type 405). Beetles were sieved out with a 710 µm maze sieve and eggs collected with a 300 µm maze sieve (Retsch). All food was kept at least one night at -20°C and all sieves were kept at 60°C in order to prevent infections.

Embryo fixation

Embryos were dechorionized with a hypochlorite solution and fixed for 30 minutes in 4 ml PEMS (0.1 M PIPES, 2mM MgSO4, 1mM EGTA, pH 6.9), 5 ml heptane and 1ml 20% formaldehyde. Subsequently, the water phase was removed and embryos were devitellinized by a methanol shock and stored in methanol at -20°C. Older embryos, difficult to devitellinize by a methanol shock, were devitellinized with forceps and needle.

Cloning of *Tc-zen2*

RNA was islolated by a trizol (Invitrogen) extraction and cDNA was made using the Cloned AMV First Strand Synthesis Kit (Invitrogen). *Tc-zen2* was amplified from this cDNA with the forward primer CCATTCTCGGGGC TTTTCATAG and the reverse primer ACAATTCTTCCCTTGGTAATACTG at an annealing temperature of 60°C and cloned into TOPO vector (Invitrogen).

Synthesis of dsRNA and in situ probes

dsRNA was synthesized with the MEGAscript RNAi kit of Ambion according to the manufacturers protocol. DIG-labeled in situ probes were made with the MAXIscript T7/T3 Kit (Ambion), but with DIG RNA Labeling mix (Roche). Preferentially linearized plasmids were used as template instead of PCR products. *lacZ* dsRNA: A fragment including the *lacZ* gene and a T7 site was amplified from the TOPO II vector with the forward primer AGCGCCC AATACGCAAACCG and the reverse primer CACACCCGCCGCGCTTAATG at an annealing temperature of 65°C and cloned into the TOPO 2.1 vector (Invitrogen). Fragments with two opposing T7 sites were excised with PvuII, purified (Purification Kit, Amersham) and used as template for dsRNA synthesis.

Parental RNAi

Female pupae were collected shortly before hatching and the dorsal side of the terminal segment was fixed on a microscope slide with Fixogum (Marabu). Approximately 0.2 µl of a 0.5-1.0µg/µl dsRNA solution was ventrally injected between the third and fourth abdominal segment (see Fig. 2 for total amounts). After 5 days, wild type male beetles were added and offspring was collected and analyzed. (Bucher et al., 2002)

In situ hybridizations and immunostainings

In situ hybridizations were performed as described (Tautz and Pfeifle, 1989), but without proteinase K treatment. In situ stained embryos were counterstained with DAPI. Immunostainings were essentially carried out as described in (Roth et al., 1989). The Engrailed antibody 4D9 was used 1:5 and the anti-phospho-histon3 antibody was used 1:1000 in PBST.

Cuticle preparation

Eggs were transferred to a 96 well plate. After 5 days, hatching rates were counted and a few drops of a 9:1 lactic acid:ethanol mixture were added to every well. After one night at 60°C, cuticles were studied under a darkfield binocular or mounted for light microscopy.

TUNEL assays

Assays were essentially performed as described in (Prpic, 2004b). However, 0.1 % Tween-20 was added to the TdT and DNAse I buffer.

Preparation of cross sections for confocal microscopy

Embryos in their vitelline membrane were cut with a razor blade into 5 or 6 slices in PBST, treated with RNAse and washed in PBST. After refixing 10 minutes in 4% formaldehyde in PBST, sections were washed with PBST, blocked for one hour in 1% bovine serum albumin and 3% normal goat serum in PBST and subsequently incubated with undiluted anti-phospho-tyrosine antibody overnight at 4°C. After washing with PBST and 30 minutes blocking, sections were incubated for two hours with Alexa 555 anti-mouse antibody (Molecular Probes, 1:400) and YOYO-1 (Molecular Probes, 1:25.000) in PBST at room temperature. After washing with PBST, sections were embedded in vectashield (Vector Laboratories) and examined under a confocal microscope.

2.4 RESULTS

Expression of the *zerknüllt* genes in *Tribolium*

I first asked whether the duplication of the *zen* genes in the lineage leading to *Tribolium* led to differences in the expression pattern of the two paralogues. *Tc-zen2* is expressed similar to *Tc-zen1* (Falciani et al., 1996) in the presumptive serosa at the anterior pole of blastoderm embryos (Fig. 1A). The flat cells of the presumptive serosa are easily recognised by the wider spaced nuclei (Fig. 1B). During and after gastrulation, when the serosa covers the embryo, *Tc-zen2* continues to be expressed in the serosa, like *Tc-zen1* (Fig. 1C). As for *Tc-zen1*, no expression was detected in the embryo proper or in the early amnion. Whereas *Tc-zen1* expression remains restricted to the serosa, *Tc-zen2* shows a new expression domain in the late amnion (Fig. 1D). *Tc-zen2* transcripts can be detected in the flattened amniotic cells which cover the posterior head and the anterior thorax region (Fig. 1D). This expression is initiated shortly before the amnion and serosa begin to fuse (see below).

Functional analysis of the *zerknüllt* genes by parental RNAi

To investigate the function of the *Tribolium zen* genes, I performed knock-down experiments using the parental RNAi technique As a negative control, female pupae were injected with *lacZ* dsRNA. Aside from 13% unfertilized or undeveloped eggs, all developing young embryos from these mothers were indistinguishable from wild type (Fig. 2, penultimate bar). After injection of 0.1 μg *Tc-zen1* or *Tc-zen2* dsRNA, similar frequencies of unfertilised or undeveloped embryos were observed (Fig. 2). The developing embryos however, displayed distinct, specific phenotypes which are described in the next sections. Control in situ hybridizations confirmed the absence of *Tc-zen1* or *Tc-zen2* transcripts after the respective dsRNA injection and the absence of both transcripts after the combined injections. Furthermore, I monitored the interdependence of the paralogues. *Tc-zen2* does not control *Tc-zen1* expression, since normal *Tc-zen1* expression was detected after *Tc-zen2* RNAi. In contrast, the early expression of *Tc-zen2* is dependent on *Tc-zen1*, since no early *Tc-zen2* transcripts were found after *Tc-zen1* RNAi. However, late amniotic expression of *Tc-zen2* could be detected after *Tc-zen1* RNAi, demonstrating that this late expression is independent of *Tc-zen1* and that a non-specific knock down of *Tc-zen2* transcripts by *Tc-zen1* dsRNA injections can be excluded.

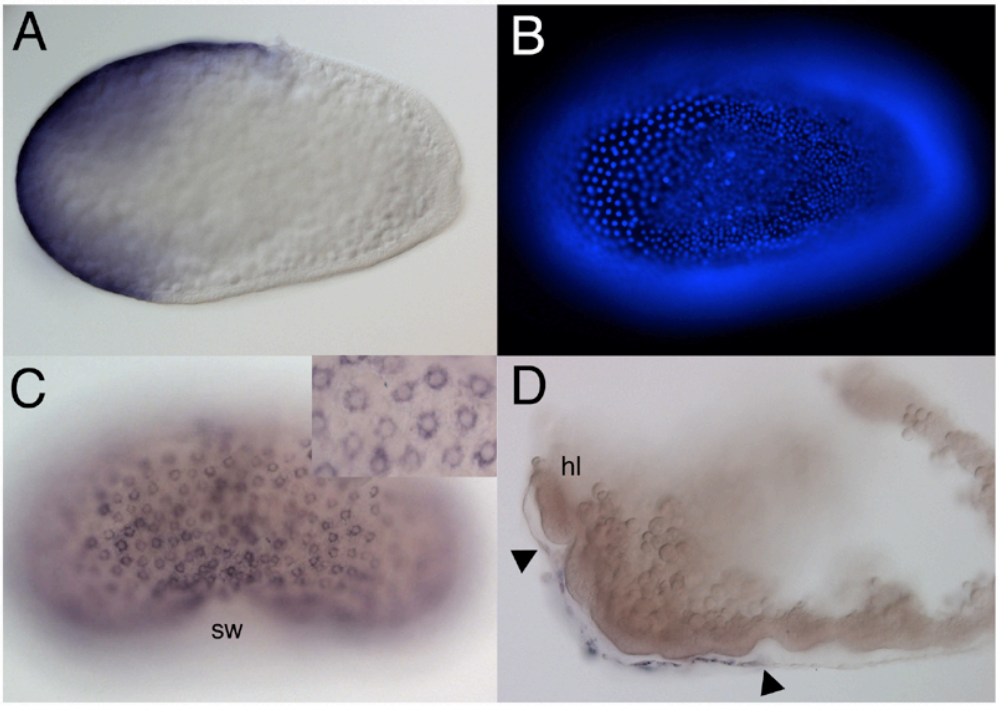


Fig. 1. Expression of *Tc-zen2*

All embryos show in situ hybridisations for *Tc-zen2*. (A) Optical midsection of a differentiated blastoderm embryo. *Tc-zen2* is expressed in a tilted anterior cap. (B) Fluorescence image of the surface of the embryo shown in A. DAPI staining visualizes the nuclei. *Tc-zen2* is expressed in the wider spaced nuclei of the presumptive serosa. (C) Serosal window stage. *Tc-zen2* is expressed in the serosa, which completely covers the embryo. Inset: serosal surface at larger magnification. *Tc-zen2* transcripts are found in the cytoplasm surrounding the nuclei of the stretched-out serosal cells. (D) Extended germband stage. *Tc-zen2* is expressed in the amnion, covering the thoracic and posterior head region. The serosa has been removed. Arrowheads indicate the limits of the expression. sw= serosal window, hl = head lobe.

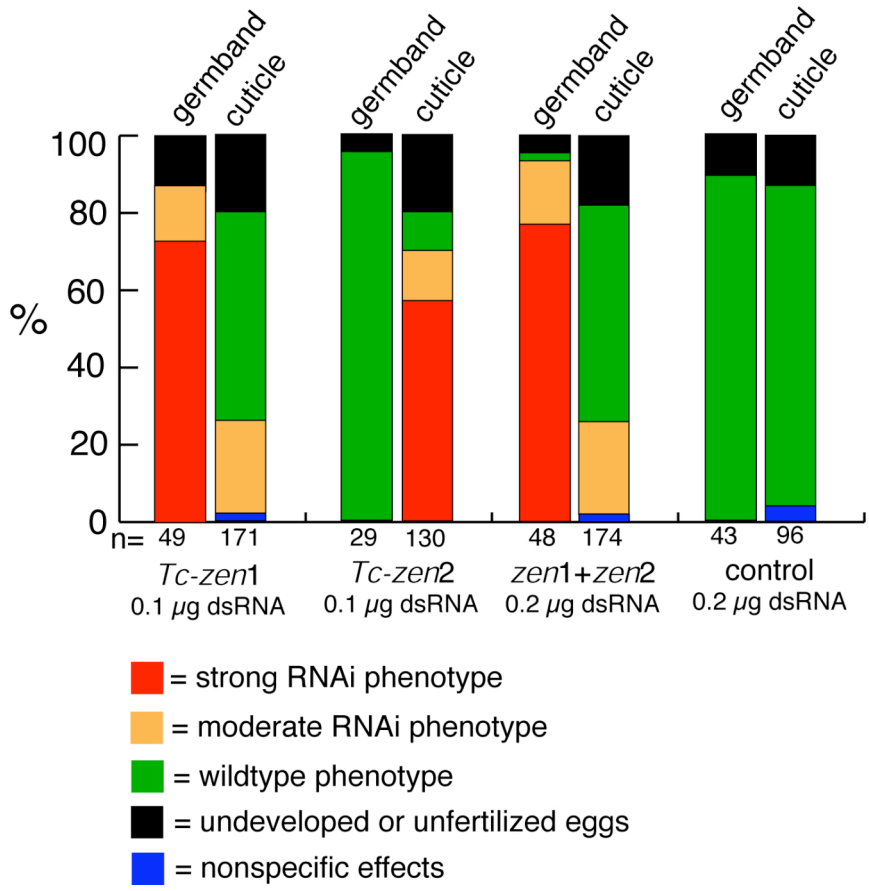


Fig. 2. Phenotypic frequencies for parental RNAi with the two *Tc-zen* homologues.

Frequencies of phenotypes after *Tc-zen1* RNAi, *Tc-zen2* RNAi, combined RNAi and a control injection with *LacZ* dsRNA. Every first bar represents the germband stages analyzed (12-18h). Every second bar represents the cuticles analyzed (>96h). Strong RNAi phenotypes are shown in red, moderate RNAi phenotypes in orange, wildtype phenotypes in green, unfertilized or undeveloped eggs in black and non-specific effects in blue. *Tc-zen1* RNAi has an early effect, the abnormal phenotype is later restored (compare first and second bar). *Tc-zen2* RNAi has a late effect (compare third and fourth bar). The combined RNAi is similar to *Tc-zen1* RNAi. Short description of the categories: Germ band extension phenotypes after *Tc-zen1* RNAi: Strong phenotype = big head, no serosa. Moderate phenotype = normal appearing head, no serosa. Cuticle phenotypes after *Tc-zen1* RNAi: Moderate phenotype = unhatched, strongly curved larvae. Cuticle phenotypes after *Tc-zen2* RNAi: Strong phenotype = completely everted. Moderate phenotype = partially everted. Combined injection: see *Tc-zen1* RNAi. Note that after *Tc-zen1* RNAi, all differentiated blastoderm embryos (6-9h) lack the serosa and have an expanded germ rudiment (see text). The first bar, however shows a slightly later stage (12-18h), in which 71% displays dramatic consequences of this phenotype (red), but 14% are already partially recovered (orange).

Loss of *Tc-zen1* causes a transformation of presumptive serosa into germ rudiment

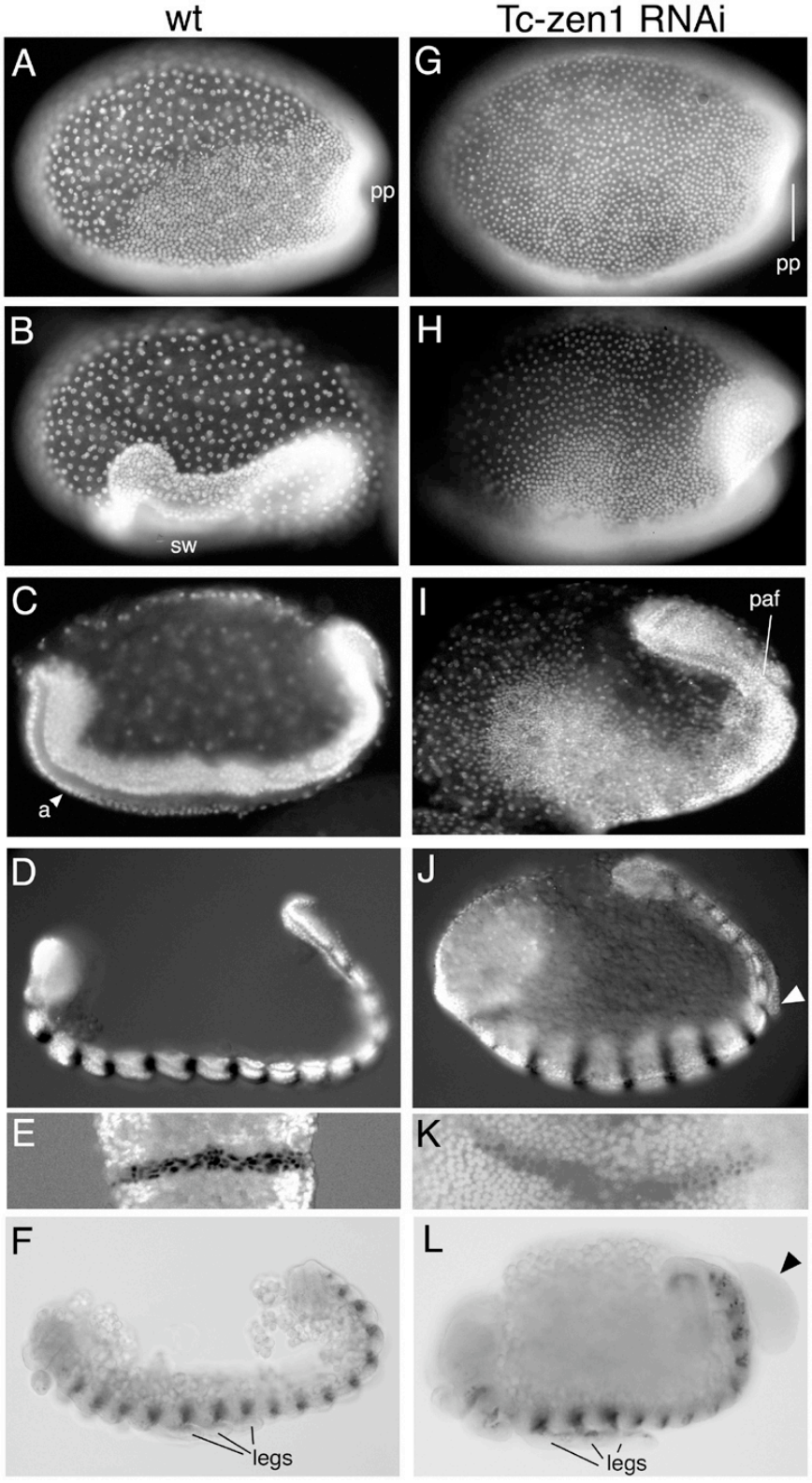
In wild type embryos at the differentiated blastoderm stage, a clear distinction arises between the wider spaced serosal cells and the more densely spaced cells of the germ rudiment (Fig. 1B). This difference is even more pronounced at the onset of gastrulation, marked by the formation of the primitive pit at the posterior pole (Fig. 3A). At this stage the serosal nuclei become larger than those of the germ rudiment since they undergo polyploidisation. After *Tc-zen1* RNAi, the embryos never partition the blastoderm into regions with distinct nuclear spacing (Fig. 2 and Fig. 3G). The nuclear density throughout the embryo resembles that of the germ rudiment. Thus, high nuclear densities are also found in the anterior third of the embryo, where the serosa would form normally. At the anterior tip a slight decrease in nuclear density is observed (Fig. 3G, 4J). However, even here the nuclei are more densely packed than in the presumptive serosa of a wild type embryo at the corresponding stage. Furthermore, in this region the nuclear size does not increase as in serosal nuclei and there is no sharp boundary separating this region from the remaining cells.

The high nuclear density throughout the embryo can be explained by assuming that *Tc-zen1* RNAi causes a loss of the serosal cell fate. Almost all cells of the blastoderm appear to adopt the fate of germ rudiment. The slight decrease in nuclear density at the anterior pole could be due to an incomplete knock-down of *Tc-zen1* after dsRNA injection. However, the later development of *Tc-zen1* RNAi embryos described below suggests another explanation. To test the assumption that the germ rudiment expands at the expense of the serosa after *Tc-zen1* RNAi, I analysed the expression of five marker genes.

Tc-twist served as a ventral marker. In wild type at the uniform blastoderm stage, *Tc-twist* is weakly expressed in a ventral stripe along the entire AP axis. Shortly before primitive pit formation however, *Tc-twist* is excluded from the emerging serosa and is strongly upregulated in the germ rudmiment (Fig. 4A,B; (Handel et al., 2005; Sommer and Tautz, 1994). In *Tc-zen1* RNAi embryos, I observed this strong upregulation along the entire AP axis (Fig. 4I,J). The expanded stripe of strong *twi* expression includes the slightly wider spaced cells at the anterior tip, demonstrating that they do not behave like serosal cells. The change in *twi* expression suggests that the whole ventral side of the embryo adopted the cell fate of the germ rudiment.

Fig. 3. The development of embryos after parental *Tc-zen1* RNAi

(A-F) Wild type embryos, lateral views, unless otherwise indicated. (G-L) Embryos of the corresponding age after *Tc-zen1* RNAi. (A-E, G-K) DAPI stainings. (D-F, J-L) engrailed antibody stainings. (A) Onset of gastrulation (primitive pit stage). The nuclei of the serosa are larger and wider spaced than those of the germ rudiment. (B) Serosal window stage. The amnion and the serosa grow over the head and abdomen of the embryo proper. (C) Extending germ band stage. After closure of the serosal window, the amnion and the serosa cover the embryo as continuous membranes. The amnion (a) is well visible. The serosa has been removed. (D) Extended germ band stage. Amnion and serosa have been removed. (E) Ventral view of the third engrailed stripe of an extending germ band embryo. (F) Retracting germ band stage. (G) After *Tc-zen1* RNAi the distinction between the widely spaced nuclei of the serosa and the densely spaced nuclei of the germ rudiment is absent. All nuclei are densely spaced. (H) During gastrulation, cells condense slowly to the ventral side. No serosal window is visible. (I) More cells than in wildtype contribute to the head, which develops slower. The posterior amniotic fold forms. (J) The cells of the big head express Engrailed. The En stripes are laterally expanded and further apart in the anteroposterior dimension. Development of the head is delayed with regard to development of the abdomen. (K) Ventral view of the third engrailed stripe of an extending germband embryo. The stripe consists of 1.5 times more cells, which are less densely spaced. (L) At the retracted germband stage, *Tc-zen1* RNAi embryos look rather normal. Arrowheads point at the double extraembryonic membrane, covering the abdomen of embryo. a = amnion, paf = posterior amniotic fold, pp = primitive pit, sw = serosal window.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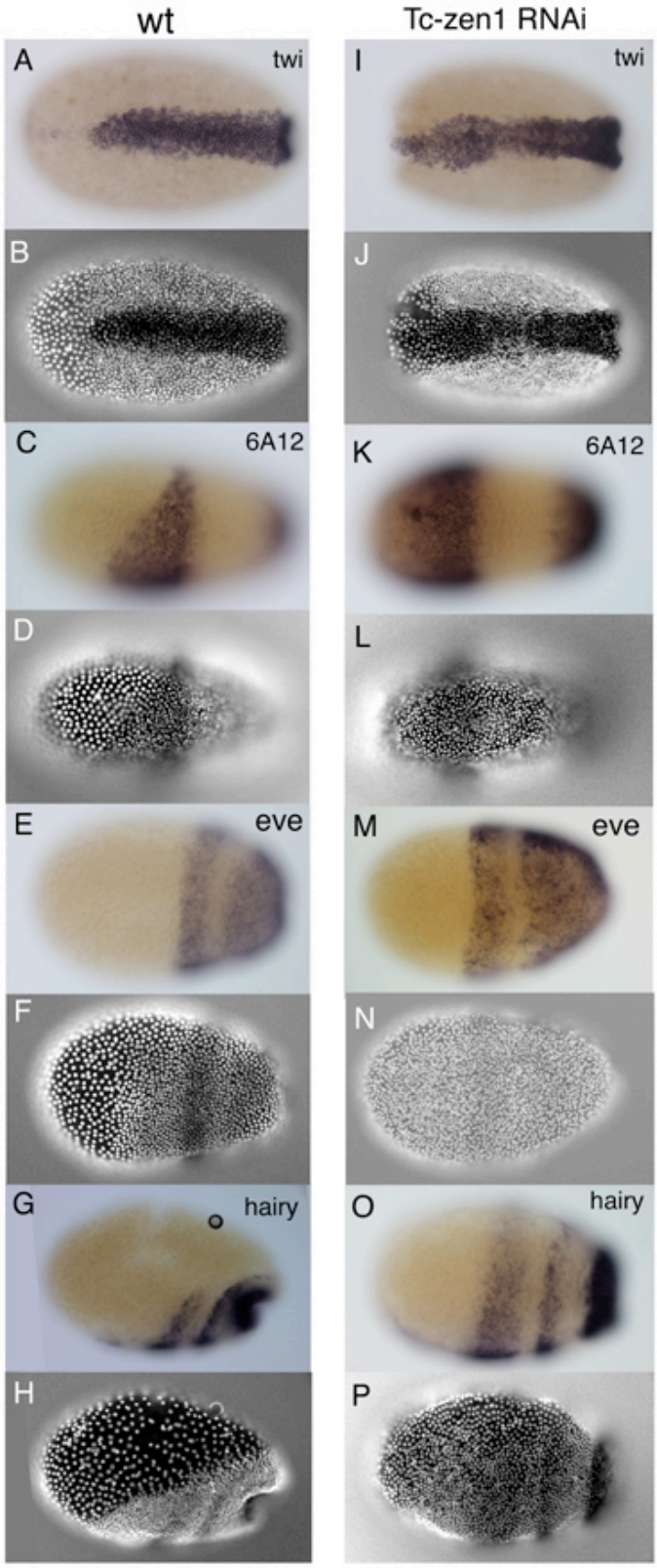
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Fig. 4. Expansion of the germ rudiment after *Tc-zen1* RNAi.

(A-G) Wild type differentiated blastoderm stages. (A-F) and early gastrulation (G,H). Lateral views unless indicated otherwise. (I-P) Corresponding stages after *Tc-zen1* RNAi. (B, D, F, H) and (J, L, N, P) show DAPI counterstainings of the embyos shown above. (A, B) Ventral view. *Tc-twist* is expressed in the germ rudiment, but not in the flattened cells of the serosa characterized by wider spaced nuclei. (C,D) Tc006A12 expression in the anterior germ rudiment, just posterior of the serosa. Onset of expression in the primitive pit allows accurate staging of the embryos. (D, E) *Tc-evenskipped* at the onset of the differentiated blastoderm stage. (G, H) *Tc-hairy* expression at the onset of gastrulation. (I, J) Ventral view. *Tc-twist* upregulation has expanded to the anterior tip. Some slightly wider spaced nuclei are present at the anterior tip. However, they express *twi* and are therefore not of serosal origin. (K,L) The anterior domain of Tc006A12 expression shows a dramatic expansion towards anterior. The more posterior domain also seems to be expanded. (M, N) *Tc-eve* expression at the primitive pit stage. Both the posterior cap and the stripe are expanded towards anterior and are enlarged. No serosal nuclei are present. (O, P) All three *Tc-hairy* stripes expanded towards dorsal and anterior, and shifted anteriorly. The effect is diminished for the more posterior stripes.

To investigate how the AP coordinates of the germ rudiment change after *Tc-zen1* RNAi, the expression of the novel gene Tc006A12 (Fig. 4C,D; GENBANK accession number CB335374 (Savard, 2004)) was analyzed. In wild type embryos at the primitive pit stage, Tc006A12 is expressed in a wedge-shaped domain, immediately posterior the serosa (Fig. 4C,D). The onset of expression of an additional posterior Tc006A12 domain (Fig. 4C,D) allows accurate staging of *Tc-zen1* RNAi embryos in comparison to wildtype. In *Tc-zen1* RNAi embryos, the anterior Tc006A12 domain expands and is shifted towards the anterior pole, covering the area, which harbours the serosa in wild type (Fig. 4K,L). Since the expansion is more pronounced at the dorsal in comparison to the ventral side, the domain becomes more symmetrical along the DV axis. These data suggest that the serosa is replaced by enlarged anterior regions of the germ rudiment.

To investigate if also more posterior fates of the germ rudiment expand, the pair-rule genes *Tc-eve* (Brown et al., 1997) and *Tc-hairy* (Sommer and Tautz, 1993) were used as markers. In wildtype, *Tc-eve* is expressed in a broad posterior cap and in one anterior stripe at the very onset of primitive pit formation (Fig. 4E, F). At this stage, both domains are expanded and shifted anteriorly after *Tc-zen1* RNAi (Fig. 4M,N). The same was observed for the expression of *Tc-hairy*. Thus, all cell fates of the germ rudiment appear to be expanded towards anterior, including the more posterior ones. Furthermore, the *Tc-eve* stainings allowed me to assess when *Tc-zen1* starts to act. At the early uniform blastoderm stage, *Tc-eve* is expressed in a posterior cap, with its anterior border in the middle of the blastoderm. At this stage, no dramatic differences between wildtype and *Tc-zen1* RNAi embryos could be detected, suggesting that the fateshifts towards anterior do not take place until shortly before the differentiated blastoderm stage.

At the onset of gastrulation, *Tc-eve* and *Tc-hairy* are expressed in three stripes, restricted to the condensing germ rudiment (Fig. 4G,H for *Tc-hairy*). *Tc-zen1* RNAi causes an expansion and a shift of all stripes towards anterior. This effect is most pronounced for the anterior stripe and decreases towards posterior (Fig. 4O,P for *Tc-hairy*). Furthermore, *Tc-hairy* is expressed along the entire DV axis in *Tc-zen1* RNAi embryos. The same was observed for *Tc-eve*. However, at this stage the expansion of the serosa has lead to a considerable ventral condensation of the germ rudiment in wild-type embryos. The absence of this process is probably the main reason why the *Tc-hairy* stripes show a dramatic dorsal expansion after *Tc-zen1* RNAi (see discussion).

In summary, these data demonstrate that the earliest overt cell differentiation step of the *Tribolium* embryo, the differentiation into serosal and germ rudiment cells, depends on *Tc-zen1*. The absence of serosal cells after knock-

down of *Tc-zen1* is compensated by an anterior expansion of the entire germ rudiment, including the more posterior fates. In absence of the serosa, both the posterior and ventral condensation of the germ rudiment are blocked. Concomitantly, the expression domains of early embryonic patterning genes cover a larger area of the embryo surface. This leads to a situation resembling long-germ development.

After loss of *Tc-zen1* the amnion covers the yolk at the dorsal side of the embryo

In gastrulating wild type embryos, the germ rudiment condenses to the ventral side and the amnion and serosa start to cover the abdomen by formation of the posterior amniotic fold (Fig. 3B, see also Fig. 9 for schematic representation). After formation of the fifth *engrailed* (*en*) stripe, the amnion and the serosa have completely covered the embryo proper (Fig. 3C shows only the amnion). During later stages of *Tc-zen1* RNAi embryos, cells also condense towards the ventral side and an increasing number of wider spaced nuclei becomes visible at the anterior and dorsal side (Fig. 3H, I). However, this process is delayed in comparison to wild type, and no sharp boundary forms between the area of the wider spaced nuclei and the developing germ band. In addition, the abdomen starts to be covered by a double layer of extraembryonic tissue (Fig. 3I), which resembles the posterior amniotic fold of wild type embryos. However, this process is much delayed and a serosal window never forms. The embryo will never be completely covered by extraembryonic cell layers, even when 17 *en* stripes are present (Fig. 3J).

The appearance of widely spaced nuclei and a double layer of extraembryonic tissue seem to indicate the presence of serosal cells. Three lines of evidence, however, strongly suggest that all extraembryonic tissue in these embryos is of amniotic origin. First, cell divisions could still be detected by anti-phosphohiston3 (αPH3) staining among the wider spaced nuclei (Fig. 5A, B). Serosal cells never show αPH3 staining since they stop dividing prior to gastrulation and later become polyploid. In contrast, amniotic cells maintain mitotic activity even after they become flattened and widely spaced during wild type development (Handel et al., 2005). Second, the serosal marker Tc004A04 ((Savard, 2004); GENBANK accession number CB335138) is not expressed in these flattened cells (Fig. 5C, D). Third and most importantly, these cells express the amniotic marker gene *Tc-pannier (pnr)* (Fig. 6 and (Berns, 2001)). In wild type at the primitive pit stage *Tc-pnr* expression is excluded form the serosa. *Tc-pnr*

starts to be expressed in a dorsal stripe of the germ rudiment which extends into the posterior amniotic fold (Fig. 6A). Thus, early *Tc-pnr* expression occurs presumably in the amniotic anlagen. In accordance with this assumption, during germ band extension *Tc-pnr* becomes highly expressed in the amniotic cells which have folded over the embryo at the ventral side (Fig. 6D). In *Tc-zen1* RNAi embryos all wider spaced, stretched-out cells that emerge anterior and dorsal to the condensing embryonic anlagen express *Tc-pnr* (Fig. 6B, C). The expression level increases during further development and the border towards the condensing embryonic anlagen sharpens (Fig. 6E, F). *Tc-pnr* is also expressed in the double layer of cells, partially covering the abdomen after *Tc-zen1* RNAi (Fig. 6E). Since no stretched-out cells exist after *Tc-zen1* RNAi, which lack *Tc-pnr* expression, I conclude that all serosal cells are lost and all stretched-out cells are amniotic. The flattening of these cells occurs with the same time course as that of wild type amniotic cells, i.e. much later and more continuous than the flattening of the serosal cells. This may explain the delayed condensation of the embryonic anlagen after *Tc-zen1* RNAi (Fig. 3). The slightly wider spaced nuclei at the anterior tip of early *Tc-zen1* RNAi embryos might be the first sign of amnion formation (Fig. 3G; Fig. 4J).

Size regulation after loss of *Tc-zen1*

The “big head” phenotype is the most striking consequence of a loss of *Tc-zen1* and is clearly visible throughout germ band elongation (Fig. 3I, J). The head and thorax region of the embryo is enlarged in comparison to wild type (Fig. 3C, D, I, J). The development of the head and thorax is also considerably delayed, as indicated by a retarded formation of the head lobes and segmental grooves in this area. The abdominal segments, however, look normal, probably because they emerge from a growth zone and are unaffected by fate shifts in the blastoderm.

To get a quantitative measure for these changes, I analysed the size of the Engrailed (En) stripes (Fig. 3E, K). The third En stripe consisted of 1.5 times as many cells in the lateral dimension compared to the wild type stripe (n=4). The total area occupied by this stripe is even larger than 1.5 times the area of a wild type stripe. Hence, not only does the anterior of the embryo consist of more cells, those cells are also less densely packed than in wild type. The latter may be the consequence of a slower condensation of head and thoracic cells.

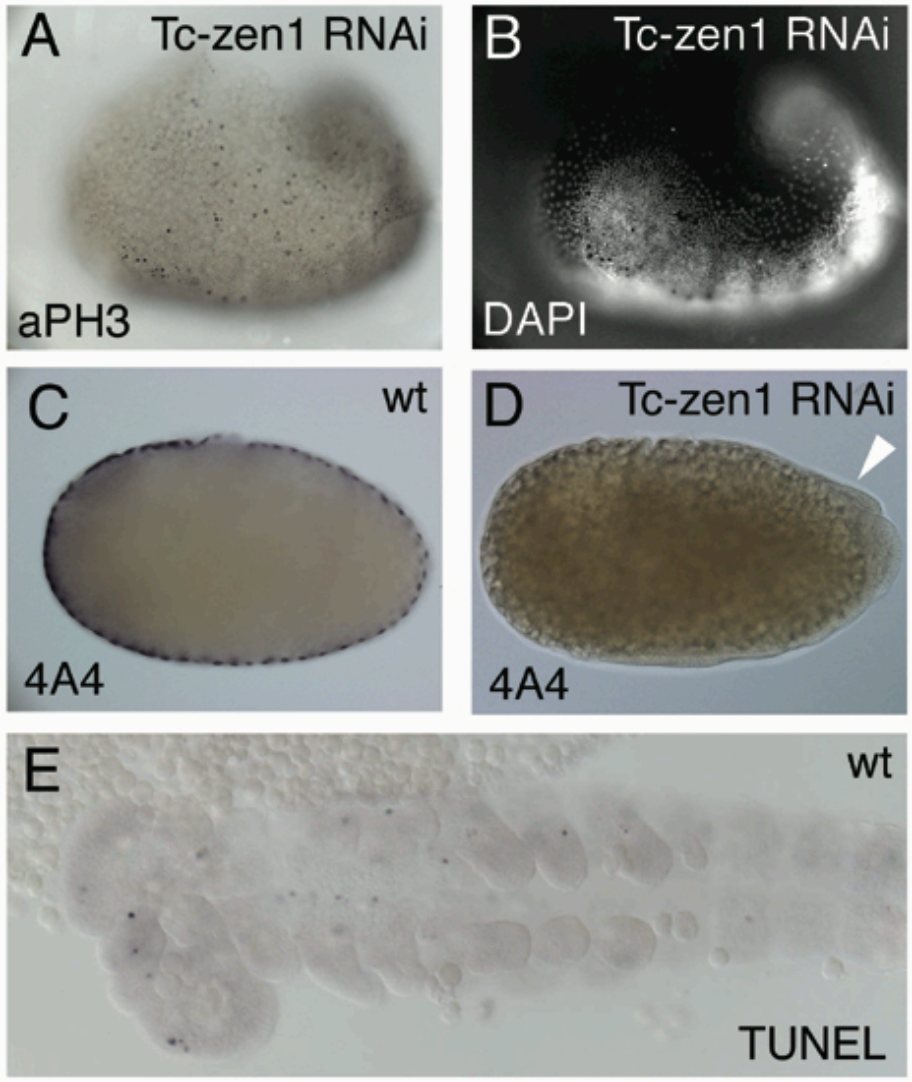


Fig. 5. αPH3, A4A staining and TUNEL assay

(A) Extending germband (big head) after *Tc-zen1* RNAi. Black dots are staining for antiphosphohiston3 and indicate cell divisions. Ectopic divisions are detected in the serosa-like wider spaced nuclei, not belonging to the germ rudiment, demonstrating that they are amniotic. (B) DAPI image at a slightly different focal plane of the same embryo as in (A). (C) Wildpype staining of the serosal marker Tc004A04. (D) After Tc-zen1 RNAi, no 4A4 staining could be detected, even in the double extraembryonic memebrane (white arrowhead). (E) TUNEL assay in wild type. Black dots reveal cell death. At the extending germband stage, irregular and sparse cell death was detected. After *Tc-zen1* RNAi, no difference could be detected within the germ rudiment (not shown).

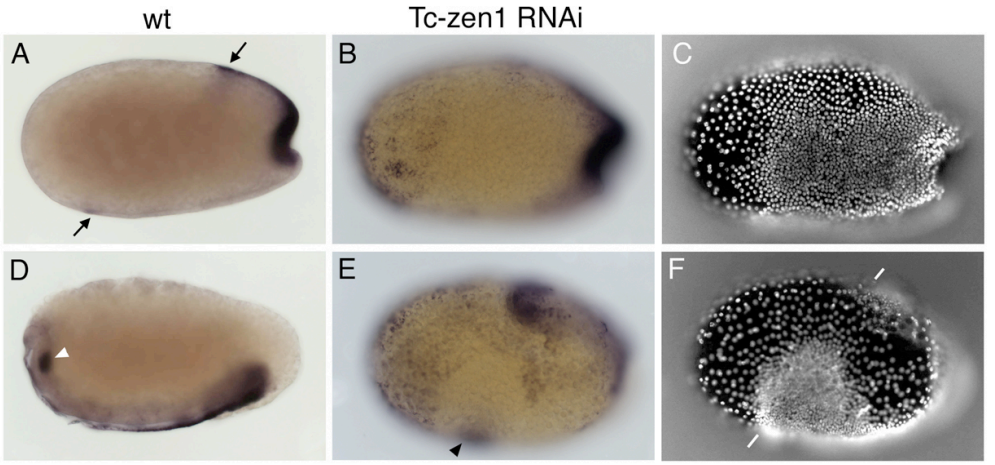


Fig. 6. The amnion covers the embryo at the dorsal side of *Tc-zen1* RNAi embryos.
(A,D) Wild type embryos. (B, E) Embryos after *Tc-zen1* RNAi. (C, F) DAPI counterstainings of embryos shown in (B, E). (A) Primitive pit stage. *Tc-pnr* is expressed dorsally, in the presumptive amnion and is absent in the flattened cells of the serosa. On the surface a faint rim of expression can be observed, just posterior to the serosa (arrows, out of focus in lateral regions). (B, C) *Tc-zen1* RNAi embryo at a later stage than (A). At this stage cells start to flatten at the anterior pole as seen form the wider spaced nuclei (C). These cells all express *Tc-pnr*. (D) Extending germ band stage. All cells of the amnion express *Tc-pnr*. The arrowhead points to a small group of cells within the head region, expressing *Tc-pnr*. (E, F) After *Tc-zen1 RNAi* all flattened cells express *Tc-pnr*. The arrowhead points at the small group of cells within the head region, expressing *Tc-pnr*. The white lines in (F) demarcate the anterior and posterior end of the embryo proper.

The enlargement of head and thorax is restored during further development, leading to strikingly normal embryos at the time of dorsal closure (Fig. 2, Fig. 3F, L). The double membrane covering the abdomen is still present (Fig. 3L), but disappears during dorsal closure. Finally, 78 % of the larvae secreted a largely normal cuticle: 54% of the larvae even hatched, while 24% did not hatch, but their cuticle was indistinguishable from wild type except for the head being bent towards the ventral side (Fig. 2, second bar). To investigate whether the enlargement of the head and thorax during early development led to an increased size of the larvae, I compared the length of wild type and *Tc-zen1* knock-down larvae. No significant difference was detected (*Tc-zen1* RNAi: 324 µm, N=6, s.d. = 18 µm; wild type: 335 µm, N=10, s.d. = 28 µm). To check if regulation of cell number was taking place by decreased cell divisions or by increased cell death, I performed αPH3 stainings and TUNEL essays, respectively. However, within the head and thorax region no apparent differences were detected between wild type and *Tc-zen1* knock-down embryos (Fig. 5E).

In summary, these observations show that not only can *Tribolium* embryos develop without a serosa, but that they also possess the regulative capacity to ameliorate a severe anterior expansion of the early fate map of the germ rudiment. The mechanisms responsible for this size regulation are however not obvious (see discussion).

Tc-zen2 is required for dorsal closure

In contrast to *Tc-zen1*, *Tc-zen2* RNAi causes no abnormalities before dorsal closure (Fig. 2). The embryos form a normal serosa, which expresses *Tc-zen1* (data not shown). Of the collected cuticles, however, 57% turned out to be completely everted (Fig. 2, third and fourth bar; Fig. 7). In these cuticles, the legs, bristles and urogomphi are enclosed by the body wall (Fig. 7C, D), whereas the tracheae and hindgut lay outside (data not shown). In order to understand this phenotype, wild type dorsal closure was investigated.

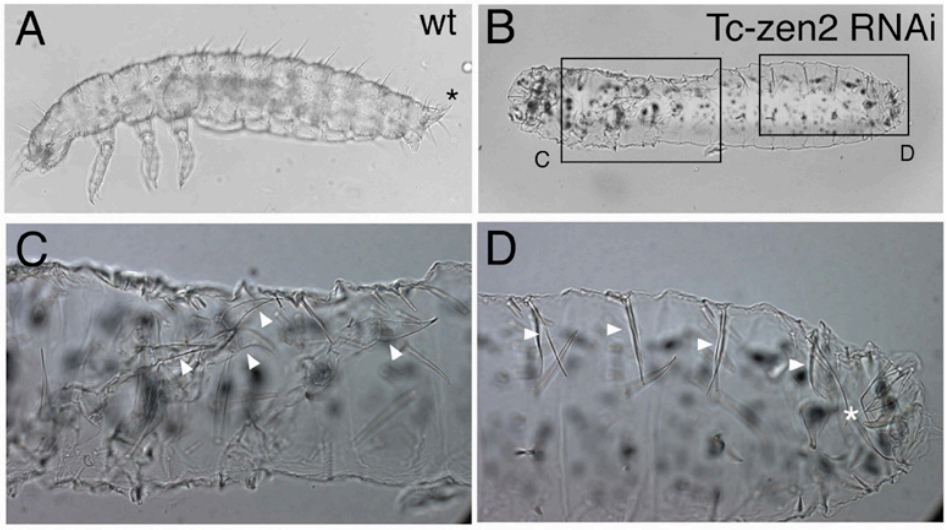
After closure of the serosal window, the amnion and serosa cover the embryo as separate cell layers. At the fully extended germband stage, however, the amnion fuses with the serosa beneath the thorax and posterior segments of the head (Fig. 8A; see also Fig. 9 for schematic representation). The sections suggest that this fusion takes place by intercalation of amniotic and serosal cells. The fusion proceeds in lateral-to-dorsal direction (Fig. 8B), pulling the lateral sides of the embryo towards dorsal.

Fig. 7. Everted larval cuticle after *Tc-zen2* RNAi.

(A) Wild type first instar larva. (B) Everted (inside out) larva after *Tc-zen2* RNAi. (C, D) Magnified regions from (B). (C) The legs are enclosed by the body wall. Arrowheads mark the tips of the legs. (D) Bristles, marked by arrowheads, and urogomphi, marked by an asterix, point to the inside and are enclosed by the body wall.

Fig. 8. Wild type dorsal closure and the origin of the everted *Tc-zen2* RNAi phenotype.

Confocal images of cross sections through the thoracic region of wild type (A-C) and *Tc-zen2* RNAi embryos (D, E). Cell membranes are stained with anti-phosphotyrosine (red) and cell nuclei with YOYO (green). A', B' and D' show magnifications of the area in the white squares in A, B and D, respectively. Schematic drawings are presented to the right. (A) Extended germ band stage, thoracic region. The amnion fused with the serosa at the ventral side. (B) Wild type retracted germ band stage. The fusion of amnion and serosa proceeded more towards the dorsal side. (C) Wild type dorsal closure. The fused amnion and serosa disappeared. The remaining serosa connects the two sides of the embryo above the yolk. The asterix marks the dorsal organ. (D) After *Tc-zen2* RNAi, the amnion and the serosa stay apart as two separate membranes, even at this retracted germ band stage. (E) After *Tc-zen2* RNAi, the embryos are completely everted. The nerve cord remains outside. The legs are enclosed by the bodywall. l = leg, n = nerve cord.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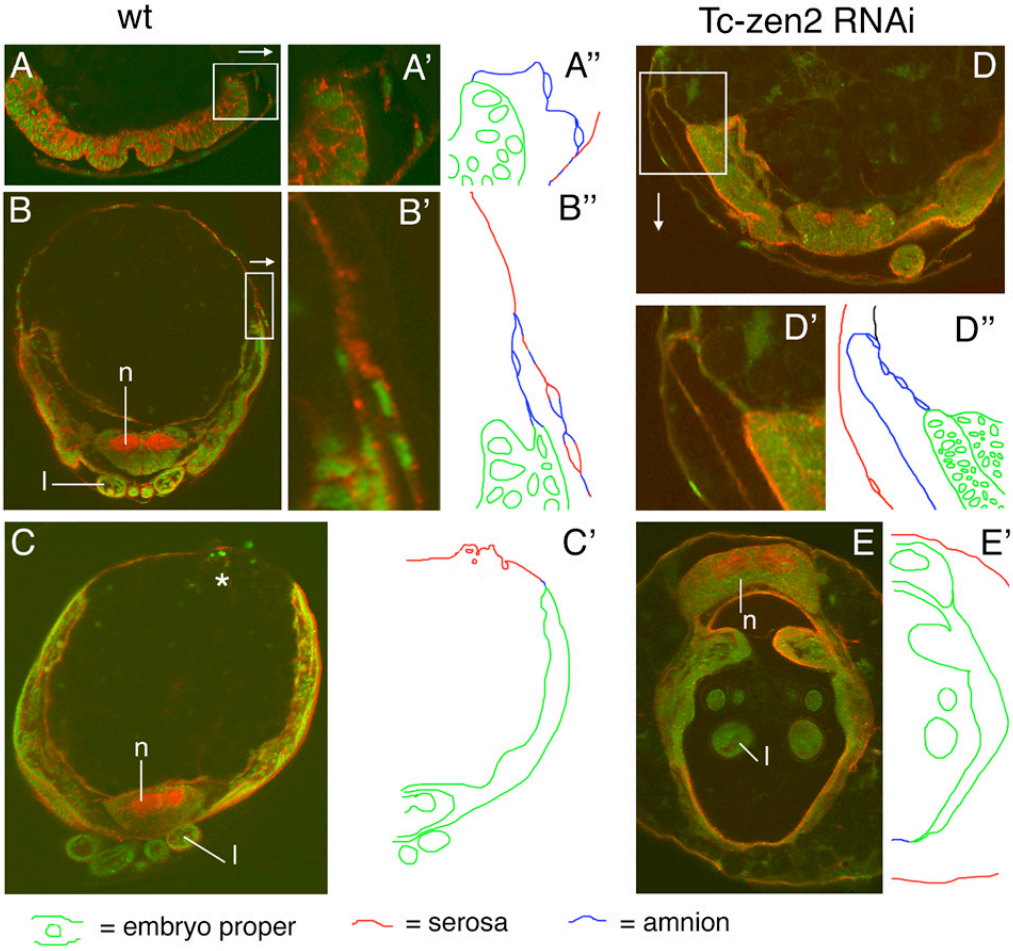
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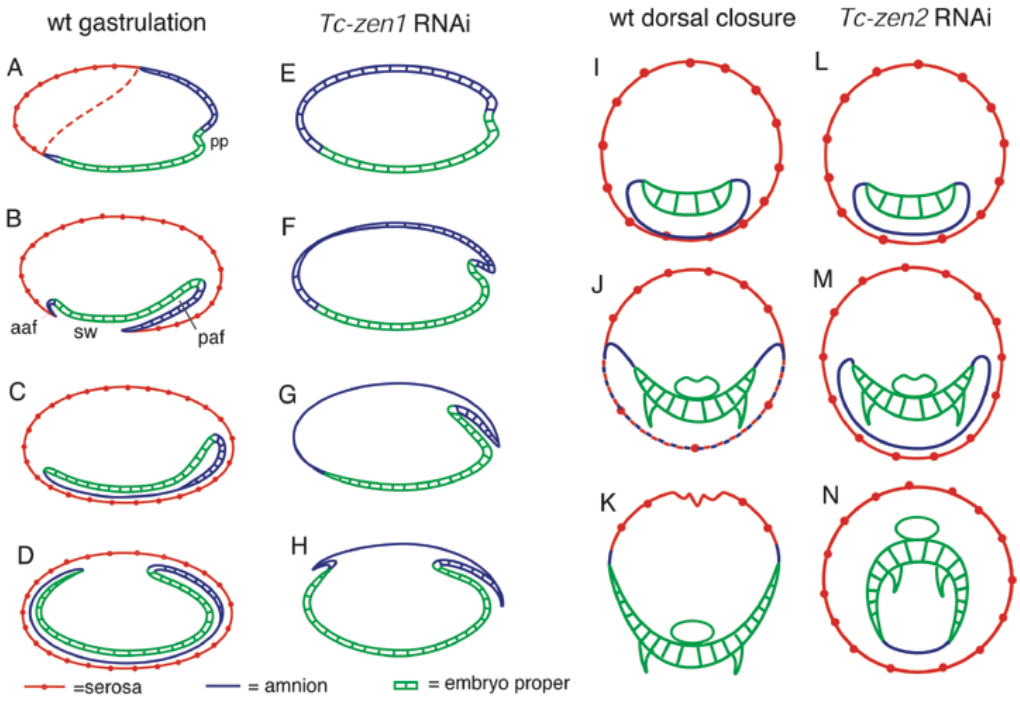

Fig. 9. Schematic drawings of the *Tc-zen* loss-of-function phenotypes.

(A-D) Wild type gastrulation and germ band extension. (E-H) The *Tc-zen1* RNAi phenotype. (I-K) Wild type dorsal closure. (L-N) The *Tc-zen2* RNAi phenotype. (A) The onset of gastrulation can be recognized by the formation of the primitive pit. A clear border forms between the streched-out cells of the serosa and columnar cells of the germ rudiment (dashed line).The dorsal cells of the germ rudiment will form the amnion. (B) Amnion and serosa grow over the posterior end of the embryo proper, forming the posterior amniotic fold. This occurs to a lesser extent at the anterior, forming the anterior amniotic fold. The crests of the folds form the serosal window. (C) After closure of the serosal window, the amnion and the serosa are visible as separate membranes. (D) Germ band extension. (E) After *Tc-zen1* RNAi, no serosa is present and the whole embryo consists of germ rudiment. The presumptive amnion occupies the whole anterior and dorsal side of the egg. The anlagen of the embryo proper are enlarged. (F) The amniotic cells start to flatten and the cells of the embryo proper condense. (G) The posterior amniotic fold has formed. The head and thorax of the embryos consist of more cells, which are less dense, generating the typical "big head" phenotype. (H) When the germband starts to retract, an anterior amniotic fold forms. (I) At the extended germ band stage, the amnion and the serosa start to fuse beneath the thorax. (J) The fusion proceeds towards dorsal, pulling the lateral sides of the embryo towards dorsal. (K) The fused amnion and serosa disappears. At the dorsal tip the remaining serosa starts to crumble, forming the dorsal organ and pulling the sides of the embryo over the yolk. This finally leads to dorsal closure. (L, M) After *Tc-zen2* RNAi the amnion and serosa do not fuse. (N) The amnion still connects the two lateral sides of the embryo ventrally, forcing the embryo to close ventrally, enclosing the legs and leaving the nerve cord and yolk outside. aaf= anterior amniotic fold, paf = posterior amniotic fold, pp= primitive pit, sw = serosal window.

At this stage, the embryo is surrounded ventrally only by the fused amnion and serosa (Fig. 8B). This fused amnion and serosa subsequently disappears (Fig. 8C). The remaining serosa, which connects the two lateral sides of the embryo and covers the yolk at the dorsal side, starts to crumble (Fig. 8C), forming the dorsal organ (Anderson, 1972). When the dorsal organ is absorbed into the yolk, the embryo closes dorsally.

After *Tc-zen2* RNAi however, the amnion does not fuse with the serosa. Amnion and serosa are visible as separate cell layers even at the stage of germ band retraction (Fig. 8D). Consequently, the dorsal sides of the embryo are forced to close ventrally, enclosing the legs and leaving the nerve cord outside (Fig. 8E). Fig. 9I-N shows a schematic comparison of wild type dorsal closure with the "ventral" closure of the embryo after *Tc-zen2* RNAi, generating an everted embryo.

Since *Tc-zen2* is expressed in the late amnion, where fusion of the amnion and the serosa starts, and given the absence of fusion after *Tc-zen2* RNAi, I conlcude that *Tc-zen2* is necessary for the proper intercalation of the amnion and the serosa.

Simultaneous loss of *Tc-zen1* and *Tc-zen2* rescues the *Tc-zen2* knock-down phenotype

Since the late amniotic expression of *Tc-zen2* is independent of *Tc-zen1*, I asked whether combined injections of *Tc-zen1* and *Tc-zen2* dsRNA would result in an additive phenotype. Surprisingly, combined injections lead to phenotypes indistinguishable from *Tc-zen1* dsRNA injections alone. The embryos do not display the *Tc-zen2* phenotype and exhibit normal dorsal closure (Fig. 2 fifth and sixth bar). This indicates that the only function of *Tc-zen2* is the fusion of the amnion with the serosa. Since there is no serosa after *Tc-zen1* RNAi, the fusion of the amnion and the serosa is not required. The amnion is oriented in a manner allowing dorsal closure to occur normally (compare Fig. 9H and K).

2.5 DISCUSSION

In this case study, I show that the two *zerknüllt* homologues in *Tribolium* have very disctinct, non redundant functions. *Tc-zen1* has an early function and specifies the serosal fate at the blastoderm stage. RNAi with *Tc-zen1* results in loss of serosal fate and expansion of the germ rudiment fate, including the amnion. *Tc-zen2* has a late function and is responsible for the fusion, crucial for dorsal closure, of amnion and serosa at the extended germ band stage. RNAi with *Tc-zen2* results in completely everted embryos. A schematic interpretation of the RNAi phenotypes is presented in Fig. 9.

Although the Tc-zen1 and the Tc-zen2 proteins have only 38% amino acid identity and thus might bind to different cis-regulatory elements, it is more likely that the distinct phenotypes are generated by diverged transcriptional regulation. I found that early serosal *Tc-zen2* expression is dependent on *Tc-zen1*, which explains why *Tc-zen2* does not rescue the serosa after *Tc-zen1* RNAi. In addition, *Tc-zen1* is not expressed in the late amnion, which explains why *Tc-zen1* cannot rescue dorsal closure. This makes the duplication of *zen* in *Tribolium* a textbook example of subfunctionalisation (Force et al., 1999; Lynch et al., 2001).

Expansion of the germ rudiment

In *Drosophila, zerknüllt* mutations lead to cell fate shifts along the DV axis, as the loss of the amnioserosa is compensated by an expansion of the dorsal ectoderm towards the dorsal midline. Accordingly, *zen* expression in *Drosophila* is largely controlled by the dorsoventral patterning systems, at both the maternal and zygotic level (Raftery and Sutherland, 2003; Rushlow et al., 1987b).

In *Tribolium*, loss of *Tc-zen1* causes an enlargement of the germ rudiment at the expense of the serosa. The early expansions, visualized by molecular markers, occur primarily along the AP axis (Fig. 4C-F, K-N), suggesting an AP patterning role for *Tc-zen1*. This is consistent with the earliest expression of *Tc-zen1* which starts symmetrically at the anterior pole without a dorsal tilt (Chen et al., 2000; Falciani et al., 1996). Furthermore, ventralized and dorsalized *Tribolium* embryos possess a dorsoventrally symmetrical serosa at the anterior pole (see chapter 4). This shows that the serosa is primarily established under control of the AP patterning system and only later tilts dorsally under influence of the DV system. The latter is probably a direct regulatory input of the DV pathway on *Tc-zen1*, since *Tc-zen1* expression slightly expands at the dorsal side before the

morphological distinction of the serosa and germ rudiment cells becomes apparent. Such a DV input might be the starting point for evolutionary changes, which culminate in the situation found in *Drosophila* where *zen* is a DV patterning gene. In *Tribolium*, however, *zen1* is primarily an AP patterning gene.

Tc-zen1 might act like a gap gene in *Drosophila*. This could even account for the enlargement of the more posterior germ rudiment fates after *Tc-zen1* RNAi, since deletion of gap genes in *Drosophila* lead to long range fateshifts (Rivera-Pomar and Jackle, 1996). However, a substantial part of the posterior enlargement is probably a secondary consequence of loss of *Tc-zen1*. Since the germ rudiment does not condense in absence of the serosa, the (shifted) spatial coordinates of the uniform blastoderm stage are maintained until the beginning of gastrulation. The entire blastoderm behaves now like the germ rudiment in wild type and the cell number increases throughout the embryo surface. This results in true fate map shifts along both body axes, as revealed by our analysis of the Engrailed stripes in *Tc-zen1* RNAi embryos. Thus, one of the main functions of the serosa prior to gastrulation might be the coordinated scaling down of an early expanded fate map. Absence of *Tc-zen1* maintains the expanded fatemap, generating a situation similar to long germ development, in which genes like *twist* are expressed along the entire AP axis.

The shrinkage of an early expanded fate map during transition from uniform to differentiated blastoderm is probably an ancestral feature of short germ development, as it has been observed by fate map studies on dragon flies (odonata) which represent one of the most primitive hemimetabolous insect orders (Seidel, 1935).

Size regulation

The dramatic early fateshifts after *Tc-zen1* RNAi result in embryos with an enlarged head and thorax. This enlargement is caused by (1) a lower density and (2) an increased number of cells contributing to head and thorax compared to wild type. How is it possible that normally sized larvae arise from these embryos?

The lower density of cells in the head region is caused by the absence of flattening serosal cells. In wildytpe the serosa enables the cells of the germ rudiment to condense ventrally at the onset of gastrulation. Instead of serosal cells, amniotic cells are found at anterior and dorsal positions of *Tc-zen1* RNAi embryos. Although the amniotic cells flatten much later and more gradual than the serosal cells, the amniotic cells eventually compensate for the loss of the serosa after *Tc-zen1* RNAi and enable the head and thorax to condense slowly and normalize the cell density.

It is less clear how the increase in cell number after *Tc-zen1* RNAi is corrected. In *Drosophila* embryos with multiple copies of *bicoid*, the head and thorax regions are enlarged at the expense the abdominal region (Frohnhofer and Nusslein-Volhard, 1986; St Johnston and Nusslein-Volhard, 1992). Nevertheless, larvae emerge with an almost normal cuticle pattern (Busturia and Lawrence, 1994). In this case it was shown that local changes in cell division and cell death rates could account for size regulation (Namba et al., 1997). However, neither anti-PH3 stainings for cell divisions nor TUNEL essays for cell death showed obvious differences between *Tc-zen1* knock-down and wild type embryos. Thus, either our methods to record changes in cell division or cell death rates are not sufficiently accurate or alternative mechanisms of size regulation exist.

One alternative mechanism could be a cell fate shift of embryo proper to amnion. Such a mechanism is conceivable for two reasons. First, a surplus of amniotic cells does not harm the embryo, because an enlarged amnion will just fold further over the abdomen and the head of the embryo proper (Fig. 9H). An excess of amnioserosa cells does not harm development in *Drosophila* as well. In *Drosophila* embryos with four copies of *decapentapledgic* (*dpp*), the amnioserosa consists of up to 400 cells instead of the usual 135, without compromising viability (Wharton et al., 1993). Second, the fate decision between the amnion and the embryo proper is probably more flexible than the fate decision between the serosa and the germ rudiment. In ligation experiments with the camel cricket *Tachycines* for example, amniotic cells can be induced to form missing parts of the germ band. (Krause, 1952; Sander, 1976b). Therefore, a progressive transformation of embryonic into amniotic tissue may contribute to the size regulation of the enlarged head and thorax in *Tc-zen1* RNAi embryos.

Dorsal closure

The classical description of dorsal closure in short germ insects assumes a reversal of the movements which have generated amnion and serosa in the first place. The serosa fuses with the amnion and a serosal window forms which widens and finally releases the embryo at the ventral side. However, this course of events is found only in ancestral hemimetabolous insect orders (Krause, 1952). In the majority of cases the serosa stays connected to the inner eggshell or the serosal cuticle, while dorsal closure appears to be largely driven by morphogenetic movements within the amnion or dorsal ectoderm of the embryo (Sander, 1976a; Sander, 1976b).

I have seen a similar phenomenon in *Tribolium* (Fig. 9I-K). The fusion of amnion and serosa ventrally does not lead to a renewed formation of a serosal window. The amnion appears to intercalate with the serosa, dragging the two halves of the embryonic ectoderm towards the dorsal side of the embryo. This function in dorsal closure is probably ancestral for *zen*, since *zen* is also expressed in the late amnion in *Schistocerca* (Dearden et al., 2000). More importantly RNAi with *zen* in the hemipteran *Oncopeltus fasciatus* results in everted embryos (Panfilio et al., 2006).

The function of *Tc-zen2* is restricted to mediating the fusion between amnion and serosa. After loss of the serosa due to *Tc-zen1* RNAi, *Tc-zen2* is not required anymore for dorsal closure. The remaining amnion then connects the two sides of the embryo dorsally and covers the yolk, enabling normal dorsal closure (compare Fig. 9H and K). The situation resembles normal development in *Apis* or *Drosophila*. In *Apis*, the amnion does not enclose the embryo by forming an amniotic cavity, but covers the yolk at the dorsal side (Fleig and Sander, 1988). In *Drosophila* a single extraembryonic cell layer, the amnioserosa, covers the yolk and embryo dorsally and is indispensable for dorsal closure.

Taken together, the loss of the serosa in the *Tribolium* embryo reveals an unexpected plasticity of the extraembryonic membrane system in a short germ insect. A situation is generated which shows similarity to normal development of more derived long germ embryos from other holometabolous insect orders (Hymenoptera and Diptera). The plasticity seen in *Tribolium* might represent the ancestral condition and might have facilitated the evolutionary changes which lead to the reduction of the extraembryonic membranes in hymenopterans and higher dipterans.

The function of the serosa

Normally sized larvae and perfect dorsal closure after the loss of the serosa leaves us with a baffling problem: why do *Tribolium* embryos need a serosa in the first place? Protection against desiccation or rupture (Zeh et al., 1989) is not essential, since the *Tc-zen1* RNAi embryos without serosa survived the dry laboratory conditions and relatively rough handling with sieves. Nevertheless, I can neither exclude an essential physical protection against conditions which are found only in the natural habitat, nor a small contribution to the survival rate due to physical protection which still would be highly significant under conditions of natural selection. However, an interesting alternative to physical protection has recently been suggested. In *Manduca*, extraembryonic tissues appear to harbour

innate immune functions that protect the embryo against bacterial infections (Gorman et al., 2004). In *Tribolium*, the NF-κB protein Dorsal is expressed at high levels in the serosa (Chen et al., 2000). Since the Toll-rel/NF-κB pathway is crucial for innate immunity (Anderson, 2000; Hoffmann et al., 1999), an immune function had been suggested for the serosa in *Tribolium* as well (Chen et al., 2000). Such a function would be apparent only if the embryos were challenged by pathogens.

Hox3 and bicoid

It has been assumed that *zen* is derived from an ancestral class 3 Hox gene (Brown et al., 2001). In more primitive arthropods the Hox3/*zen* homologue is expressed in a canonical Hox3-like fashion, as shown for the spider *Cupiennius salei* and the mite *Archegozetes longisetosus* (Damen and Tautz, 1998; Telford and Thomas, 1998). The primitive wingless insect *Thermobia*, provides an interesting intermediate case (Hughes et al., 2004). There, *zen* is expressed as a Hox3 gene in head segments, but also in the emerging amnion, where it possibly fulfils a morphogenetic role. In the lineage leading to the winged insects, Hox3 completely lost its function in specifying segment identity and expanded its role in the morphogenesis and specification of the extraembryonic tissues. Concomitantly, it became involved in specifying the serosa which arises from anterior egg regions in the majority of insects. The anterior expression required for this function of *zen* might be due to a new enhancer element. However, it might also be derived from an enhancer element of the Hox3 ancestor which still had binding sites for AP patterning genes. In the latter case, the AP patterning function of *Tc-zen1* would be a vestige of its homeotic origin.

Irrespective of the evolutionary path by which *zen* acquired a function in serosa formation, this event was probably crucial for the evolution of the anterior maternal determinant *bicoid* which is involved in head and thorax formation in higher dipterans (St Johnston and Nusslein-Volhard, 1992; Stauber et al., 1999; Stauber et al., 2002; Stauber et al., 2000). Indeed, it is striking that there is a formal similarity between the phenotypes caused by loss of *bicoid* in *Drosophila* and those caused by loss of *zen1* in *Tribolium*. In both cases anterior regions of the early embryo are deleted and replaced by the expansion of more posterior regions. In a long germ insect like *Drosophila* the anterior regions correspond to the anlagen of head and thorax, in a short germ insect like *Tribolium* they correspond to the anlagen of the serosa. However, the serosa is not only the anteriormost fate of the blastoderm in most insects, the decisions between serosa and germ rudiment

is also one of the earliest cell differentiation events. Thus, *zen* had to acquire an early anterior expression. This requirement has probably favoured evolutionary changes which allowed early transcription and anterior localization, including maternal expression. Both *Schistocerca zen* (Dearden et al., 2000) and *Tc-zen1* are already maternally expressed (S. Brown and L. Farzana personal communitcation). Taken together, it is conceivable that the involvement *zen* in serosa specification provided a favourable starting point for the evolution of *bicoid* in higher dipterans (Stauber et al., 1999; Stauber et al., 2002; Stauber et al., 2000).

2.6 ACKNOWLEDGMENTS

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CHAPTER 3

Sog/chordin is required for ventral-to-dorsal Dpp/BMP transport and head formation in a short germ insect

3.1 SUMMARY

BMP signaling plays a major role in dorsoventral patterning in vertebrates and in *Drosophila*. Inconsistently, in the beetle *Tribolium*, early BMP/*dpp* exhibits differential expression along the anteroposterior axis. However, the BMP/Dpp inhibitor Sog/chordin is produced in a ventral domain and establishes a dorsoventral Dpp activity gradient by transporting Dpp towards the dorsal side. *Tc-dpp* knock down abolishes dorsal cell fates. *Tc-sog* depleted embryos do not establish normal dorsoventral polarity in the ectoderm, and lack the complete neurogenic ectoderm. In contrast, the presence of other BMP antagonists still allows neurogenesis in vertebrate or *Drosophila sog*/*chordin* mutants. Suprisingly, similar to vertebrates, knock down of BMP antagonism (*Tc-sog* RNAi) in *Tribolium* reduces the head, while knock down of BMP signaling (*Tc-dpp* RNAi) enlarges the head. This could be a specific consequence of the *Tribolium* blastoderm fate map, but could also reveal an ancestral involvement of BMP signaling in head formation in Bilateria.

3.2 INTRODUCTION

Bone Morphogenetic Proteins (BMPs) pattern the mesoderm and ectoderm of the early vertebrate embryo along the dorsoventral axis (De Robertis and Kuroda, 2004; Hammerschmidt and Mullins, 2002). A ventral centre in the embryo expresses BMPs and induces the ectoderm to become epidermal (nonneural). A dorsal organizer expresses BMP antagonists like *chordin, noggin* and *follistatin* which prevent BMP signaling and allow the formation of neurectoderm.

BMPs play a similar role in the ectoderm of *Drosophila*. Two BMP homologues are known to be involved in embryonic dorsoventral (DV) patterning: *decapentaplegic* (*dpp*) and *screw* (*scw*) (Parker et al., 2004). *dpp* is expressed at the dorsal side and high BMP signaling levels are found along the dorsal midline where an extraembryonic tissue, the amnioserosa, is specified (Ferguson and Anderson, 1992; Wharton et al., 1993). Moderate levels induce the non-neurogenic ectoderm. At more ventral positions, BMP activity is antagonized by several inhibitory mechanisms allowing the specification of neurogenic ectoderm. These mechanisms include inhibition of Dpp signaling by the secreted chordin-like BMP-inhibitor *short gastrulation* (*sog*) (Biehs et al., 1996; Francois et al., 1994). Thus, in both *Drosophila* and vertebrates, BMP signalling acts anti-neurogenic and similar molecular mechanisms establish a BMP activity gradient. However, the orientation of the gradient in these animals is exactly inverted with regard to their DV axes.

These observations have lead to the suggestions that the BMPs together with their antagonists represent a conserved pattering system which was present in the common ancestor of arthropods and vertebrates. Indeed, *Drosophila sog* and *dpp* can respectively rescue and inhibit notochord development in *Xenopus* (Holley et al., 1995), while the vertebrate BMPs and can induce dorsal ectoderm in *Drosophila* (Padgett et al., 1993). These functional replacements, together with the similar relative expression domains of BMPs and their antagonists, have been used to propose an inversion of the DV axis in the early evolution of vertebrates (Arendt and Nubler-Jung, 1994). Support for a conserved function of the pathway in DV patterning comes from studies on enteropneusts. Those hemichordates differentially express BMPs and chordin along their dorsoventral axis, although this axis is oriented like in protostomes (Gerhart et al., 2005).

Loss of *chordin/sog* in *Drosophila* or vertebrates does not lead to the complete loss of neurogenic ectoderm. Several other mechanisms are present to prevent BMP signaling at the neuronal side. First, transcriptional repression of BMPsis involved. In zebrafish, the *bozozok* gene represses BMP2 expression at the

dorsal side (Hammerschmidt and Mullins, 2002). In *Drosophila*, the maternal Dorsal gradient and its zygotic target gene brinker repress *dpp* transcription in the ventral 60% of the embryo. Second, redundant BMP antagonists play a role. In vertebrates, at least two other secreted BMP inhibitors are present: Noggin and Follistatin. Double mutants with these inhibitors are required to observe strong phenotypes (Bachiller et al., 2000; De Robertis and Kuroda, 2004). In *Drosophila*, another type of redundancy evolved. There, the ventrally expressed *brinker* represses the transcription of dpp target genes. Only *brk sog* double mutants result in the complete loss of neurogenic ectoderm (Jazwinska et al., 1999)

In *Drosophila,* Sog is not only an inhibitor of Dpp/Scw, but is also required to achieve high levels of BMP signaling. In the early *Drosophila* embryo, the BMPs have broad expression domains which do not reflect the regions in which BMP signaling actually occurs. *scw* is homogenously transcribed throughout the entire embryo, while *dpp* is present in the dorsal 40% of the embryonic circumference (Arora et al., 1994; Ray et al., 1991; St Johnston and Gelbart, 1987). Nevertheless, the highest levels of BMP proteins and BMP signaling activity are found in a narrow dorsal stripe (5% of the embryonic circumference), which will give rise to the amnioserosa (Dorfman and Shilo, 2001; Shimmi and O'Connor, 2003; Sutherland et al., 2003). This dorsal stripe of high BMP signaling depends on Sog. Sog protein is expressed in ventral cells and by diffusion forms a gradient which decreases towards the dorsal side (Srinivasan et al., 2002). Since Sog binds Dpp homodimers and Dpp/Scw heterodimers, it transports these BMPs to the dorsal side. In a broad dorsal domain, Sog is cleaved by the metalloprotease Tolloid (Tld). This cleavage releases the BMPs which then can bind and activate their receptors. The combination of these reaction and diffusion processes leads to high BMP signaling levels in a narrow dorsal stripe far away from the ventral domain of *sog* transcription (Ashe and Levine, 1999; Eldar et al., 2002; Marques et al., 1997; Shimmi et al., 2005; Wang and Ferguson, 2005). Thus, because of the aforementioned redundant patterning by *brinker*, the most salient feature of Sog mutants is not the loss of neurogenic ectoderm, but the loss of the dorsalmost cell fate, the amnioserosa. A similar transport mechanism has been suggested for zebrafish (Hammerschmidt and Mullins, 2002), as chordin mutants also loose a fate far from the expression domain of *chordin*: the ventral tail fin.

Two aspects of BMP signaling, however, seem fundamentally different between *Drosophila* and vertebrates. First, vertebrate BMPs primarily pattern the mesoderm, whereas the *Drosophila* mesoderm only secondarily receives BMP signals from the patterned ectoderm. Second, vertebrate BMP signalling is

involved in head and forebrain development. Depletion of *chordin* and *noggin* results in a reduction of the head and forebrain (e.g. Bachiller et al., 2000; Anderson et al., 2002 for mouse), whereas BMP knock down enlarges the head and forebrain (Reversade et al., 2005 for Xenopus). This is not the case in *Drosophila*.

To get a better understanding of how the derived states represented by *Drosophila* and vertebrates relate to the common bilaterian ancestor, the analyis of potential evolutionary intermediates, like more primitive chordates or arthropods, would be useful. Dpp homologues from a variety of arthropods have been studied with respect to appendage formation (Hammerschmidt and Mullins, 2002; Jockusch et al., 2000; Niwa et al., 2000; Prpic, 2004a; Prpic, 2004b; Prpic et al., 2003; Yamamoto et al., 2004). However, only few data are available on their possible role in DV patterning. *dpp* of the spider *Achaearanea tepidariorum* is expressed in a group of so-called cumulus cells (Akiyama-Oda and Oda, 2003), which are necessary to induce dorsal development (Holm, 1952). Furthermore, the hemipteran *Oncopeltus fasciatus* expresses *dpp* at the dorsal edge of the site of germ band invagination. RNAi with *O. fasciatus dpp* leads to failures in germband invagination and all later embryonic processes (Angelini and Kaufman, 2005). Finally, grasshopper (*Schistocerca gregaria*) and the late *Tribolium dpp* expression have been proposed to be involved in DV patterning (Dearden and Akam, 2001; Sanchez-Salazar et al., 1996). However, the early anterior *Tribolium dpp* expression (Sanchez-Salazar et al., 1996) and its later refinement to the ventral-anterior rim along the germ rudiment (Chen et al., 2000) are difficult to reconcile with a role in DV patterning.

In this case study, RNAi expexperiments with *Tc-dpp* (Sanchez-Salazar et al., 1996) and *Tc-sog* (Stockhammer, 2003) are peformed. With antibody stainings against pMad, I demonstrate that *Tc-sog* is necessary for the dorsal localization of Tc-Dpp activity in the blastoderm. Similar to *Drosophila*, *Tc-dpp* knock down leads to the expansion of the neurogenic ectoderm, at the expense of the dorsal ectoderm and amnion. In contrast to *Drosophila* and vertebrates, *sog* RNAi results in a complete loss of the neurogenic ectoderm. Reminiscent of vertebrates, knock down of BMP antagonism (*Tc-sog* RNAi) leads to the absence of the head and brain, whereas knock down of BMP signaling (*Tc-dpp* RNAi) results in an enlarged head and brain.

3.3 MATERIALS AND METHODS

Stock keeping, embryo fixation, synthesis of dsRNA, in situ hybridizations, immunostainings and cuticle preparation were performed as described in chapter 2.

Cloning of *Tc-doc*

Tc-doc was predicted in silico in the available *Tribolium* genome (Baylor) and a 521 bp fragment was cloned with the specific primers ATCCGCCGACTA CTGCCTCTTCCT and CTAACTGTTTCCGCTTCGCACTCG. With RACE (Rapid Amplification of cDNA Ends) the 5' and 3' ends of the gene was obtained (BD SMART RACE cDNA Amplification Kit, BD Biosciences, according to the manufacturers protocol). All fragments were cloned in the TOPO II vector (Invitrogen). The *Tc-doc* sequence was submitted to GENEBANK, accession number DQ211693.

Parental RNAi

Since pupal *Tc-dpp* RNAi interfered with female maturation and resulted in sterility, all dsRNA injections were performed in adult beetles. Mature, female beetles were cooled on ice for two minutes and were ventrally fixed on a microscope slide with double sided tape. One elytrum was lifted and 0.1 µl of a 0.5-1.0 µg/µl dsRNA solution was dorsally injected. The females were immediately released from the tape and allowed to recover for one night before males were added. Eggs were collected 1-3 days thereafter for analysis.

Araldite sections

After in situ hybridization and immunostaining, embryos were dehydrated (successively 5 min in 50% EtOH, 70% EtOH, 100% EtOH, 100% dehydrated EtOH and 100% dehydrated acetone), transferred into a 1:1 acetone:araldite solution, and, after 2 hours, embedded in 100% araldite. After one night at 60°C, araldite blocks were sectioned (8µm sections) with a Leica RM2255 microtome.

3.4 RESULTS

The *Tc-sog*-like fragment cloned by Stockhammer (Stockhammer, 2003) codes for a protein that contains four Cysteine Rich (CR) domains displaying high percentages of aminoacid identity to the 4 CR domains of known Sog/chordin molecules (Fig. 10A). Alignment of the cDNA to the newly available *Tribolium* genome sequence reveals that the gene is composed of seven exons. The first intron is remarkably large (10kb), like in *Drosophila*. No other *sog*-like genes, neither homologues of the BMP antagonist *noggin* were found within the *Tribolium* genome sequence. The position of the protein in a distance tree (Fig. 10B) and the following expression data and functional analyses, strongly support the assumption that the identified *sog*-like gene is the true sog orthologue of *Tribolium* and I therefore will refer to to it as *Tc-sog* (*Tribolium castaneum sog*).

sog is expressed in a ventral domain of early *Tribolium* embryos, while *dpp* expression initially lacks dorsoventral asmmetry

In the following, I compare the expression of the *Tc-sog* in early *Tribolium* embryos to that of *Tc-dpp*, the likely target of inhibition by the Sog protein. Like in *Drosophila*, the early blastoderm of *Tribolium* embryos consists of a single layer of morphologically identical cells surrounding the yolk (Fig. 11H). At this stage, *Tc-dpp* is expressed in all cells, with higher levels in an anterior domain (Fig. 11G). The pattern lacks DV asymmetry. *Tc-sog*, however, is expressed in a broad ventral domain between 20% and 80% egg length (Fig. 11B). This expression domain overlaps with the area where nuclear Dorsal protein is present in *Tribolium* (Chen et al., 2000), suggesting that *Tc-sog* is a target of the maternal Dorsal gradient.

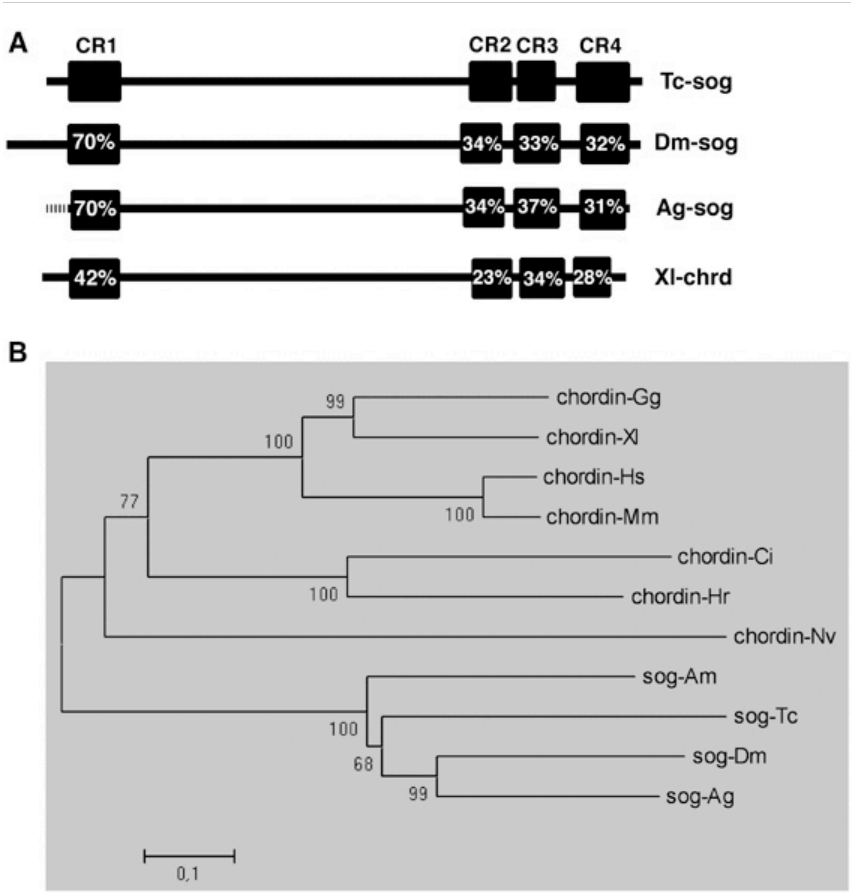


Fig. 10 Comparisons of Tc-Sog to known Sog/chordin sequences

(A) Overview of *Drosophila melanogaster* Sog (Dm-sog), *Anopheles gambiae* Sog (Ag-sog) and *Xenopus laevis* Chordin (Xl-chrd). Black boxes indicate the Cystein Rich (CR) domains and display the percentage of identity of identical amninoacids to the *Triblium castaneum* Sog sequence (Tc-Sog). (B) Neighbor-joining distance tree using Chordin (Sog) protein sequences from metazoan species. Sequences were loaded in MEGA 3.1 software. 1000 replicates were used for bootstrap analysis.Species name and accession genebank/ensembl numbers areshown in brackets. Chordin-Gg (*Gallus gallus*, NP_990311), Chordin-Xl (*Xenopus laevis*), Chordin-Hs (*Homo sapiens*, AAG35767), Chordin-Mm (*Mus musculus,* AAD19895), Chordin-Ci (*Ciona intestinalis*, BAE06347), Chordin-Hr (Urochordata, *Halocynthia roretzi*, AAK83138), Chordin-Nv (Cnidaria, *Nematostella vectensis* DQ358700.1), Sog-Am (*Apis mellifera*, XP_393520.1), Sog-Tc (*Tribolium castaneum*), Sog-Dm (*Drosophila melanogaster*, Q24025), Sog-Ag (*Anopheles gambiae* ENSF00000008622).

The next stage of development deviates from *Drosophila* and reveals an important feature of a type of embryogenesis that is found in insects more basal to *Drosophila*. The blastoderm is divided into the anterior-dorsal presumptive serosa characterized by widely spaced non-dividing nuclei, and the posterior ventral germ rudiment showing higher nuclear density (Fig. 11J). The presumptive serosa later forms the outer extraembryonic membrane, which surrounds the entire yolk mass and the embryo, while the germ rudiment gives rise to the embryo proper and an inner extraembryonic membrane. At this stage, *dpp* expression is confined to a stripe at the border between serosa and germ rudiment (Fig. 11I). Except for its obliqueness, this stripe rather reflects an AP pattern than a DV pattern. Slightly later, *dpp* transcripts appear at the dorsal side of the primitive pit (Fig. 12A).

The expression domain of *Tc-sog* becomes narrower, but remains rather broad in the anterior of the germ rudiment. A small gap in the *Tc-sog* domain is observed at the border of the serosa and germ rudiment, where *dpp* is expressed (Fig. 11C, D, arrow heads). Shortly thereafter, *Tc-sog* expression retracts from the presumptive serosa. The expression domain largely overlaps with the mesoderm. After gastrulation, the transcripts disappear from the invaginated mesoderm and are detected in the ventral ectoderm (Fig. 11E). At this stage, *Tc-dpp* is expressed in the dorsalmost ectoderm (Fig. 11K, arrows). Thus, *Tc-sog* and *Tc-dpp* obtain opposite expression domains in the ectoderm. *Tc-sog* expression later retracts from the ventral midline, but remains in the ectoderm from which the neuroblasts will delaminate (Wheeler et al., 2003). *Tc-dpp* is additionally expressed in the legs, but maintains expression along the dorsal margins.

In contrast to *Drosophila,* only head and thoracic segments have formed by the end of mesoderm invagination. The more posterior segments are formed from a double-layered posterior growth zone during germband extension. Similarly, the inner extraembryonic layer, the amnion, is only in part derived from the blastoderm and mainly extends from this growth zone. In the growthzone, *Tc-sog* is expressed in cells of the Inner Layer (IL) that lie on and in between the Outer Layer (OL) (Fig. 11F). The IL is continuous with the mesoderm and possibly contributes to it. In the growthzone, *Tc-dpp* is weakly expressed in the amnion, the ventral part of the OL ("a" in Fig. 11L). Additionally, strong *Tc-dpp* expression is found in two stripes flanking the IL (Fig. 11L, arrowheads).

In summary, the early ventral expression of *Tc-sog* suggests that *Tc-sog* is a target gene of Dorsal, like in *Drosophila*. In contrast, *Tc-sog* is initially also expressed in the presumptive mesoderm, while *Drosophila-sog* is immediately repressed by mesodermal *Dm-snail* (Markstein et al., 2002). Most importantly, *Tc-dpp* expression in the blastoderm reflects an AP pattern, whereas *Tc-sog* expression shows a strong dorsoventral asymmetry. Only after gastrulation *Tc-dpp* expression is restricted to dorsal areas.

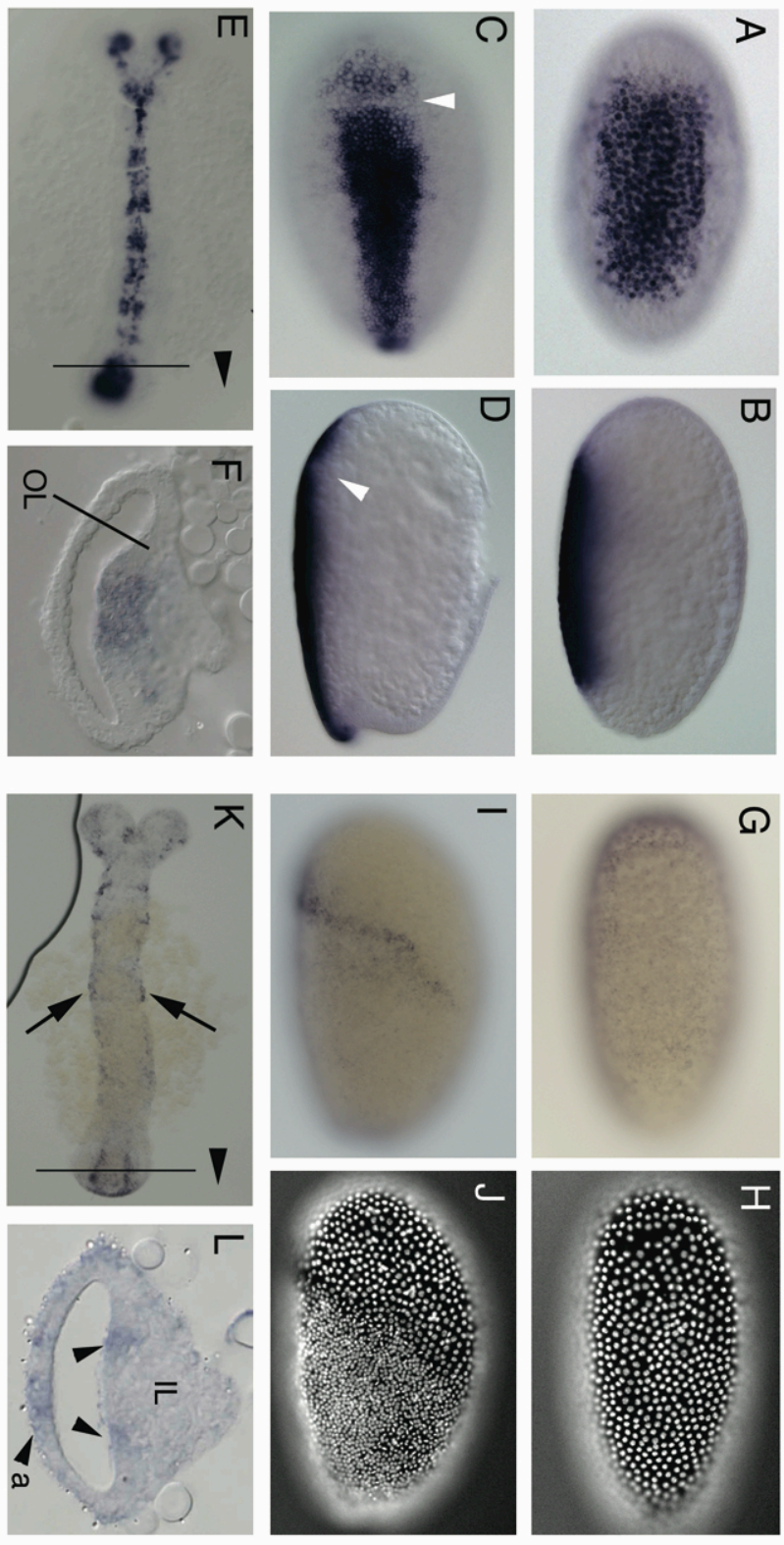
Tc-sog directs Dpp activity to the dorsal side

The two domains of *dpp* expression at the differentiated blastoderm stage (at the border of the germ rudiment and in the primitive pit) do not correspond to the dorsal side of the embryo (Fig. 12A). However, in *Tribolium,* the pattern of Dpp activity deviates from that of *dpp* expression even more profoundly than in *Drosophila*. I visualized Dpp activity with antibody stainings against pMAD, the phosphorylated SMAD which is only produced in cells with activated BMP receptors (Dorfman and Shilo, 2001; Persson et al., 1998; Tanimoto et al., 2000). pMAD accumulates along the whole dorsal side of the embryo at the differentiated blastoderm stage, but most prominently in the germ rudiment (Fig. 12D). Both in the serosa and the germ rudiment the pMAD concentration is graded with highest levels at the dorsal side and decreasing levels laterally, suggesting that a DV gradient of Dpp signaling activity exists at this stage in the *Tribolium* embryo.

In *Drosophila, pannier* (*pnr*) and *dorsocross* (*doc*) are target genes of the embryonic Dpp gradient and are required for dorsal ectoderm and amnioserosa specification, respectively. Therefore, I analysed the expression pattern of the homologs of both genes in *Tribolium* (*Tc-doc*, see materials and methods; *Tc-pnr*, see chapter 2). *Tc-doc* is expressed in the dorsal part of the serosa (Fig. 12G, J) indicating that the serosa consists of dorsoventrally disctinct cell populations, despite its uniform appearance. *Tc-pnr* is expressed at the dorsal side of the germ rudiment and in the primitive pit (Fig. 12M). Thus, *Tc-doc* and *Tc-pnr* expression correspond to high levels of Dpp signaling in the serosa and germ rudiment, respectively.

Fig. 11. Expression of *Tc-sog*, compared to expression of *Tc-dpp*.

(A-F) In situ hybridizations with *Tc-sog*. (G-L) In situ hybridizations with *Tc-dpp*.
(A) Uniform blastoderm stage, ventral view. *Tc-sog* is expressed in a broad ventral domain. (B) Lateral view of the embryo shown in A. (C) Differentiated blastoderm stage, ventral view. The *Tc-sog* expression domain becomes narrower, but remains rather broad at the anterior of the germ rudiment. A gap is observed at the border of germ rudiment and serosa (white arrowhead). (D) Lateral view of the embryo shown in C. (E) Extending germ band. *Tc-sog* is expressed in a ventral, ectodermal domain. The midline becomes free of transcripts. Except for the growthzone, *Tc-sog* is not expressed in the mesoderm.
(F) Section of the growthzone, at a position indicated with a black line in E. *Tc-sog* is expressed in cells of the Inner Layer in between the Outer Layer (OL). (G) Uniform blastoderm stage, lateral view. *Tc-dpp* is ubiquitously expressed, with stronger expression at the anterior pole. (H) DAPI counterstaining of the embryo shown in G. The nuclei have a uniform distribution. (I) Differentiated blastoderm, lateral view. *Tc-dpp* is expressed in in a stripe along the border of the serosa and the germ rudiment. (J) DAPI counterstaining of the embryo shown in I. The serosa can be recognized by big, widely spaced nuclei; the germ rudiment by smaller, dense nuclei. (K) Extending germ band. Except for the growth zone, *Tc-dpp* is expressed along the dorsal borders of the embryo (arrows). (L) Section of the growth zone at a position indicated by a line in K. *Tc-dpp* is weakly expressed in the amnion (a) and in some cells of the Outer Layer directly flanking the Inner Layer (IL).
Fotos A-D from: Stockhammer, 2003.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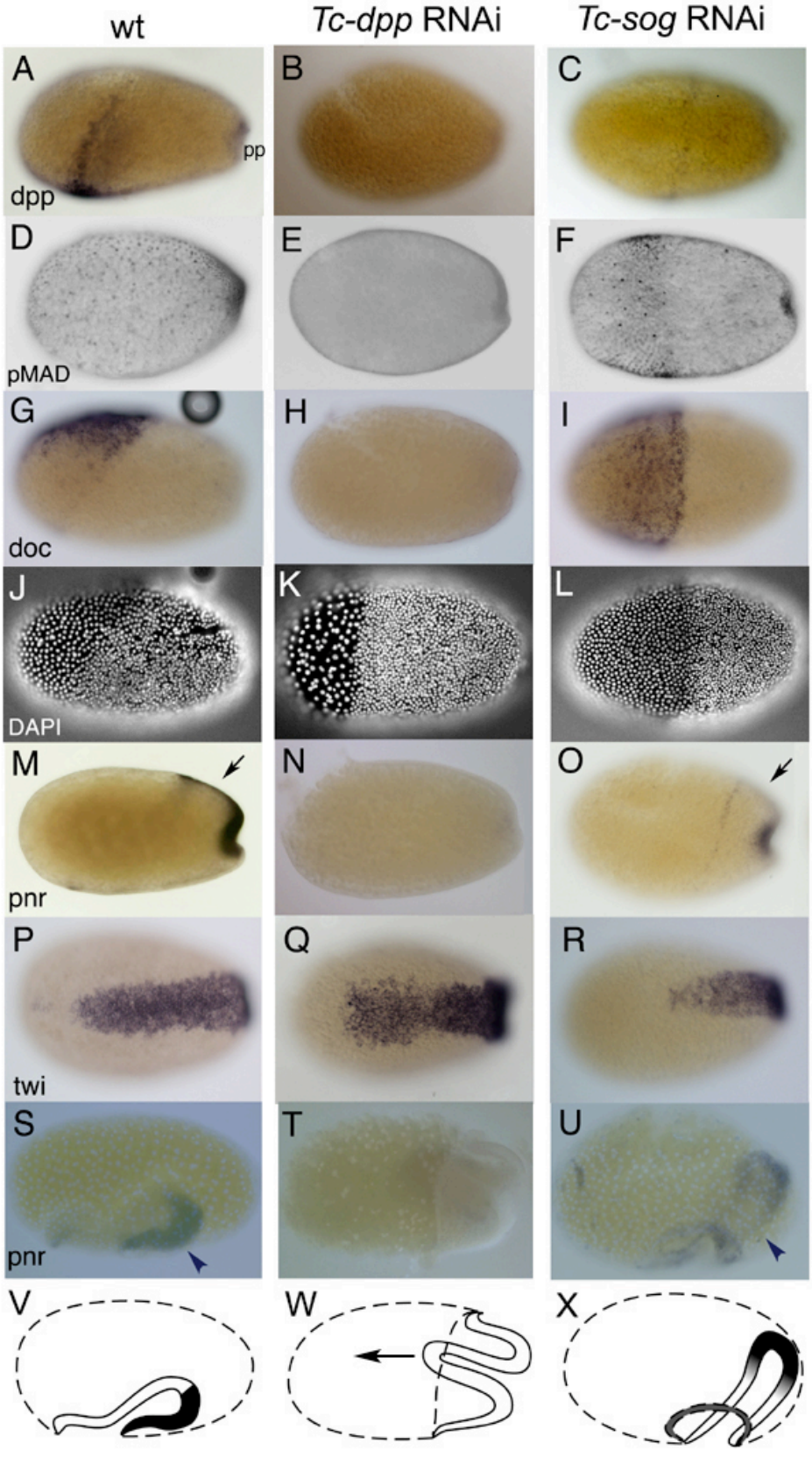
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Fig. 12. Tc-Sog transports Tc-Dpp towards the dorsal side

All photos show lateral views of embryos at the differentiated blastoderm stage, unless indicated otherwise. (A-C) In situ hybridizations with *Tc-dpp*. (A) Wildtype embryo. *Tc-dpp* is expressed in a stripe along the border of the germ rudiment and serosa and in the primitive pit (pp). (B) *Tc-dpp* RNAi embryo. No *Tc-dpp* expression is detected. (C) *Tc-sog* RNAi embryo. Tc-dpp is weakly expressed along the border of the germ rudiment and serosa. (D-F) Phosphorylated MAD (pMAD) antibody stainings. (D) Wildtype embryo. pMAD accumulates along the whole dorsal side of the embryo, but most prominently in the germ rudiment. (E) *Tc-dpp* RNAi embryo, pMAD could not be detected. (F) *Tc-sog* RNAi embryo. pMAD is present at high levels in a band along the border of the germ rudiment and the serosa, with decreasing levels towards anterior. Additional pMAD is found in the primitive pit. (G-I) In situ hybridizations with *Tc-doc*. (G) Wildtype embryo. *Tc-doc* is transcribed in a subset of dorsal cells in the serosa. (H) *Tc-dpp* RNAi embryo. *Tc-doc* transcripts could not be detected. (I) *Tc-sog* RNAi embryo. *Tc-doc* is expressed in a DV symmetrical broad band in the serosa. (J-L) DAPI counterstainings of the embryos shown in G-I. Serosal nuclei are bigger and wider-spaced than those of the germ rudiment. (J) Wildtype embryo. The border of the germ rudiment and serosa is oblique and runs from a dorsal, more posterior position to a ventral, more anterior position. (K) *Tc-dpp* RNAi embryo. The serosa/germ rudiment border is straight. The dorsal position of the border lies more to the anterior than in wildtype. (L) *Tc-sog* RNAi embryo. The germ rudiment/serosa border is straight. The ventral position of the border lies more to posterior. (M-O) *Tc-pnr* in situ hybridizations. (M) In wildtype, *Tc-pnr* is expressed at the dorsal side of the germ rudiment (arrow) and in the primitive pit. (N) After *Tc-dpp* RNAi, *Tc-pnr* transcripts could not be detected. (O) After *Tc-sog* RNAi, *Tc-pnr* is expressed in a rim along the anterior of the germ rudiment and in the primitive pit, but not at the dorsal side of the germ rudiment (arrow). (P-R) *Tc-twist* in situ hybridizations, ventral views. (P) Wildtype. (Q) After *Tc-dpp* RNAi, the *Tc-twist* domain extends to a wildtype position along the AP axis, but is slightly broader than in wildtype in the anterior half. (R) After Tc-sog RNAi, the *Tc-twi* domain is only half as long as in the wildtype. (S-U) *Tc-pnr* in situ hybridizations at early gastrulation. DAPI images from the surface of the embryos have been superimposed.
(S) In wildtype, the amnion expresses *Tc-pnr* and folds over the germ rudiment at the ventral side (arrowhead). DAPI staining reveals the serosal nuclei at the surface.
(T) *Tc-dpp* RNAi embryo. No *Tc-pnr* expression could be detected. DAPI staining reveals that a part of the germ rudiment remains at the posterior surface. The germband is more or less symmetrically folded inward at the primitive pit and extends towards anterior. (U) *Tc-sog* RNAi embryo. *Tc-pnr* is detected along the anterior margin of the germ rudiment and in the primitive pit, but not at the dorsal side, where the amnion should form (arrowhead). DAPI staining reveals the serosal nuclei at the surface. (V-X) Schematic drawings of the embryos shown in S-U with *Tc-pnr* staining in black. The arrow in W indicates the diresction of growth zone exzension.

After *Tc-dpp* RNAi (see materials and methods), no *dpp* expression could be detected in 83% of the embryos (n=65) (Fig. 12B). This closely corresponds to the fraction of older embryos exhibiting a specific phenotype (79%, n=96). In *Tc-dpp* RNAi embryos, pMad cannot be detected (Fig. 12E), demonstrating that *Tc-dpp* is responsible for the BMP signaling activity at the dorsal side and within the primitve pit. Loss of *Tc-dpp* also abolishes *Tc-doc* and *Tc-pnr* expression (Fig. 12H and Fig. 12N), confirming that *Tc-doc* and *Tc-pnr* expression depend, like in *Drosophila,* on Dpp activity. The basic cell fate decision between serosa and germ rudiment however is independent of *Tc-dpp,* as the RNAi embryos always maintained an anterior cap of serosal cells (Fig. 12K). Thus, in contrast to the amnioserosa of *Drosophila,* the serosa of *Tribolium* does not require Dpp activity. However, in the absence of Dpp activity, the border between serosa and germ rudiment becomes symmetric along the DV axis and is located at a very anterior position (Fig. 12K).

The spatial discrepancy between *Tc-dpp* expression and Dpp activity might be explained by assuming that ventrally produced Sog molecules transport Dpp towards the dorsal side, like in *Drosophila*. To test this hypothesis, *dpp* expression and pMAD distribution were analysed in *Tc-sog* RNAi embryos. *Tc-sog* RNAi was very efficient and led in 97% of the cases to a specific phenotype (n=57). Similar to the wildtype, *Tc-dpp* expression retracts from an anterior domain to a rim between the serosa and germ rudiment in *Tc-sog* RNAi embryos (Fig. 12C). Notably, the border between serosa and germ rudiment is no longer oblique, but follows a straight line and is located at a very posterior position (Fig. 12L). Thus, the stripe of *dpp* expression is symmetric along the DV axis indicating that, in contrast to *Drosophila,* the DV asymmetry of *dpp* expression itself depends on Sog activity.

In *Tc-sog* RNAi embryos, pMAD is not present in a dorsal-to-ventral gradient, but in a broad, vertical band overlapping the stripe of *dpp* expression (Fig. 12F). This shows that the dorsal localization of Dpp activity depends on Sog. The expression domains of the Dpp target genes, *Tc-doc* and *Tc-pnr*, change accordingly. *Tc-doc* expression shifts from a dorsal domain to a broad DV-symmetric band of expression within the serosa, overlapping the pMad domain (Fig. 12I, L). *Tc-pnr* expression in the primitive pit is not impeded. However, the dorsal *Tc-pnr* domain is lost (Fig. 12O, arrow). *Tc-pnr* expression rather follows the expression of *Tc-dpp* and forms a rim along the border of germ rudiment and serosa (Fig. 3O). Taken together, the dorsal expression of *Tc-doc* and *Tc-pnr* depend on *Tc-sog*. In absence of *Tc-sog*, these expression patterns loose their

dorsoventral asymmetry and occupy domains along the AP axis, following the distribution of pMAD.

The coincidence of *dpp* expression and Dpp activity after *Tc-sog* RNAi strongly suggests that, in wiltype, Tc-Sog transports Tc-Dpp molecules from their site of production to the dorsal side of the embryo, thus establishing the correct DV orientation of the Dpp gradient. Because *Tc-sog* expression retracts from the serosa at a stage shown in (Fig. 12D), the gradient look less pronounced in the serosa. The results of a double knock-down of *Tc-dpp* and *Tc-sog* are in accordance with the hypothesis that Tc-Sog inhibits and transports Tc-Dpp. Combined injections of *Tc-dpp* and *Tc-sog* dsRNA lead in only 2% to a *Tc-sog* RNAi phenotype and in 79 % to a phenotype indistinguishable from *Tc-dpp* RNAi embryos. The remaining 19% were wildtype (n=83). Thus, the *Tc-sog* phenotype depends on the presence of *Tc-dpp*, suggesting that *Tc-sog* functions largely if not entirely via *Tc-dpp*.

In summary, the observed changes in pMAD distribution and in expression of *Tc-doc* and *Tc-pnr* indicate that, like in *Drosophila, Tc-sog* does not only act as a Dpp inhibitor, but is required to enrich Dpp molecules at the dorsal side by concentration-driven ventral-to-dorsal Dpp transport.

Loss of BMP signaling severely affects gastrulation

The ventralmost 15% of the germ rudiment harbor the anlagen of the mesoderm. In this region *Tc-twist* (*Tc-twi*) is expressed (Fig. 12P). Since *Tc-twist* is likely to be regulated by Tc-Dorsal (Chen et al., 2000), its expression should be independent of Dpp signaling. Indeed, *twi* expression is not affected in the posterior of the germ rudiment after knock-down of *Tc-dpp* or *Tc-sog* (Fig. 12Q, R). However, the germ rudiment is shorter after *Tc-sog* RNAi (Fig. 12L) and the stripe of *twi* expression is correspondingly shorter in the AP dimension (Fig. 12R). After *Tc-dpp* RNAi, the germ rudiment extends to a wild type position at the ventral side (Fig. 12K). The AP dimension of the *twi* stripe appears accordingly normal, but the anterior *twi* domain expands laterally after *Tc-dpp* RNAi (Fig. 12Q). Thus, in contrast to *Drosophila,* BMP signaling has an influence on the anlagen of the mesoderm. This influence is however restricted to the anterior half of the germ rudiment.

Since *twi* expression is normal in the trunk region, one aspect of gastrulation, the invagination of the mesodermal cells (ventral furrow formation), is normal after *Tc-dpp* or *Tc-sog* knock-down. In contrast, changes in Dpp signalling severely affect the specification, and consequently the morphogenetic

movements, of the extraembryonic membranes. In wild type, *Tc-pnr* expression is believed to mark the presumptive amniotic cells at the blastoderm stage (Fig. 12M; chapter 2). During gastrulation, the dorsal *pnr* expressing cells fold over the germ rudiment at the ventral side (Fig. 12S,V) to cover the embryo as an inner extraembryonic layer. The serosa follows the amnion and soon covers the yolk and embryo as an outer extraembryonic membrane (Fig. 12V,S). The dorsal serosa cells marked by *Tc-doc* (Fig. 12G) undergo the biggest translocation in this process.

After *Tc-dpp* RNAi, *Tc-pnr* expression, and consequently the amnion, is absent. Furthermore, the size of the serosa is severely reduced (Fig. 12K), and the dorsal serosal cells are lost, as shown by the absence of *Tc-doc* expression (Fig. 12H). Thus, the small serosa only slightly expands and will cover about 2/3 of the yolk, but will never cover the complete embryo (Fig. 12T,W). Therefore, the anterior region of the embryo remains at the surface of the yolk throughout development and is positioned at the posterior pole of the egg (Fig. 12T,W). The primitive pit forms and the growth zone is established. However, germband extension does not occur towards the dorsal-anterior side of the egg. The germ band rather forms a symmetric tube which extends straight into the yolk, with the growth zone towards the anterior end of the egg. (Fig. 12W, arrow). As a result, the head is located at the posterior, while the posterior growth zone is located at the anterior of the egg. Despite this inverted orientation, all older *Tc-dpp* RNAi embryos in this paper are shown for simplicity with their heads to the left.

After *Tc-sog* RNAi, the serosa is enlarged and contains many dorsal serosal cells (Fig. 12L,I). Amniotic cells, marked by *Tc-pnr*, are still specified along the anterior border of the germ rudiment and in the primitive pit (Fig. 12O). These amniotic cells, together with the dorsal serosal cells allow a rather normal gastrulation (Fig. 12U,X). Furthermore a normal amnion arising from the growthzone will cover the abdomen of the embryo. However, the dorsal side of the blastodermal germ rudiment does not express *Tc-pnr* (Fig. 12O, arrow) and remains free of *Tc-pnr* transcripts during gastrulation (Fig. 12U; arrowhead). This part does not behave like real amnion and prevents the remaining amnion from closing completely, leaving the anterior of the germ rudiment uncovered throughout development. Thus, *Tc-sog* RNAi embryos lack the dorsal amnion derived from the blastoderm, reminiscent of *Drosophila sog* mutants lacking the amnioserosa. Nevertheless, this has only minor effects on gastrulation.

In summary, the absence of BMP signalling (*Tc-dpp* RNAi) leads to the total absence of the dorsalmost cell fates (dorsal serosal cells and amnion) and severely affects gastrulation. Knock-down of the transport mechanism (*Tc-sog* RNAi) leads to a reduction of the amnion, but only to mild effects on gastrulation.

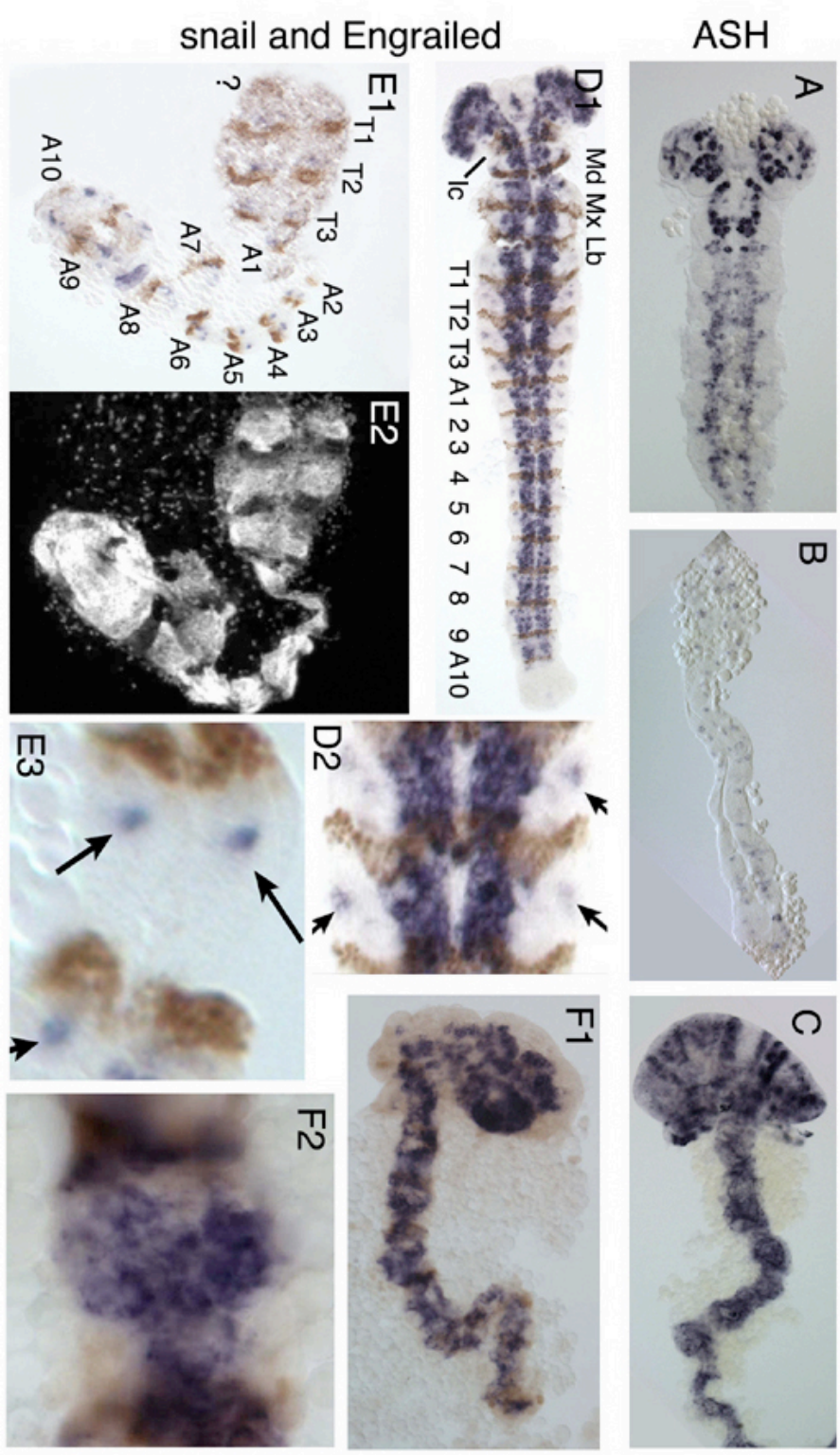
Tc-sog RNAi leads to a loss of the entire central nervous system and to a mirror image duplication of dorsal cell fates

The analysis of dorsal marker genes at the blastoderm stages (Fig. 12) suggests that the dorsoventral axis is profoundly affected by changes in Dpp signalling. To determine what DV cell fates are present after RNAi, embryos at the extended germband stage were stained with markers for the mesoderm, the ventral neurogenic, lateral and dorsal ectoderm. The mesoderm was visualized using Twi AB staining (Handel et al., 2005). As markers for the neurogenic ectoderm, *Tc-achaete-scute* (*Tc-ASH*, Wheeler et al., 2003) and *Tc-snail* (Sommer and Tautz, 1994) were used. *Tc-ASH* is expressed in cells of the central nervous system (CNS) plus in an anterior ectodermal stripe in each segment (Fig. 13A; Wheeler et al., 2003). *Tc-snail* is expressed in the CNS as well (Fig. 13D1), but also in neurons of the peripheral nervous system which mark the lateral (non-neurogenic) ectoderm (Fig. 13D2). *Tc-pnr* was used as marker for the dorsalmost ectoderm. In wildtype, *Tc-pnr* is expressed in a stripe along the dorsal rims of the embryo and marks the dorsal ectoderm at the extended germband stage (Berns, 2001; Fig. 14G, J). The presence of *Tc-dpp* transcripts and Tc-Dpp activity was monitored as well. In the wildtype germ band, the stripes of *pnr* expression correspond to the dorsal stripes of *dpp* expression and high pMAD levels (Fig. 14A, D).

As in the blastoderm, *Tc-dpp* RNAi efficiently knocked down *Tc-dpp* expression (Fig. 14B) and abolished Tc-Dpp activity (Fig. 14E), revealing that *Tc-dpp* is responsible for this pMAD activity. After loss of *Tc-dpp,* the tube shaped embryo consists only of neurogenic ectoderm, because *Tc-ASH* and *Tc-snail* expression is found throughout the embryonic circumference (Fig. 13C, F1), while *Tc-pnr* expression is absent (Fig. 14K). Since the lumen of the tube-like embryo corresponds to the extraembryonic space, the outer surface of the tube should face the inner organs. Accordingly, a layer of mesodermal cells surrounds the entire tube (Fig. 14H, a schematic drawing is presented in Fig. 18). No cuticles were obtained from these embryos. The aberrant development of *Tc-dpp* knock-down embryos apparently prevents the secretion of cuticle in late embryonic stages. Taken together, *Tc-dpp* RNAi leads to a complete loss of the dorsal ectoderm and a complementary expansion of the neurogenic ectoderm.

Fig. 13. *Tc-sog* RNAi leads to a complete loss of the neurogenic ectoderm

Extending germ bands. (A, B, C) *Tc-achaete-scute* in situ hybridizations. (D-F) *Tc-snail* in situ hybridizations with Engrailed antibody staining. (A) Wildtype embryo. *Tc-ASH* is expressed in cells of the central nervous system and in a transverse stripe at the anterior of every segment. (B) Embryo after *Tc-sog* RNAi. *Tc-ASH* can only be detected in segmental stripes. (C) Embryo after *Tc-dpp* RNAi. *Tc-ASH* transcripts can be detected throughout the embryo. (D1) Wildtype embryo. *Tc-snail* is expressed in cells of the central nervous system. 17 Engrailed stripes could be counted. Segments are labeled Ic= Intercallary, Md=Mandibular, Mx= Maxillary, Lb=Labial, T=Thoracic, A=Abdominal.
(D2) Magnification of a part of the embryo shown in D1. Additional *Tc-snail* is detected in single clusters marking the peripheral neurons of the lateral ectoderm (arrows).
(E1) Embryo after *Tc-sog* RNAi. *Tc-snail* expression is largely abolished and is only found in some single clusters. Segmentation seems to be regular, but only 13 engrailed stripes were counted. T=Thoracic, A=Abdominal. (E2) DAPI of the embryo shown in E. (E3) Magnification of a part of the embryo shown in E1. Arrows point at the periferal neurons (F1) Embryo after *Tc-dpp* RNAi. *Tc-snail* can be detected throughout the embryo. Stripes of Engrailed could be detected, but were difficult to count. Segmentation appears irregular. (F2) Close up of a part of the embryo shown in F1. Figure E1, E2, E3 from: Mikulski, 2004.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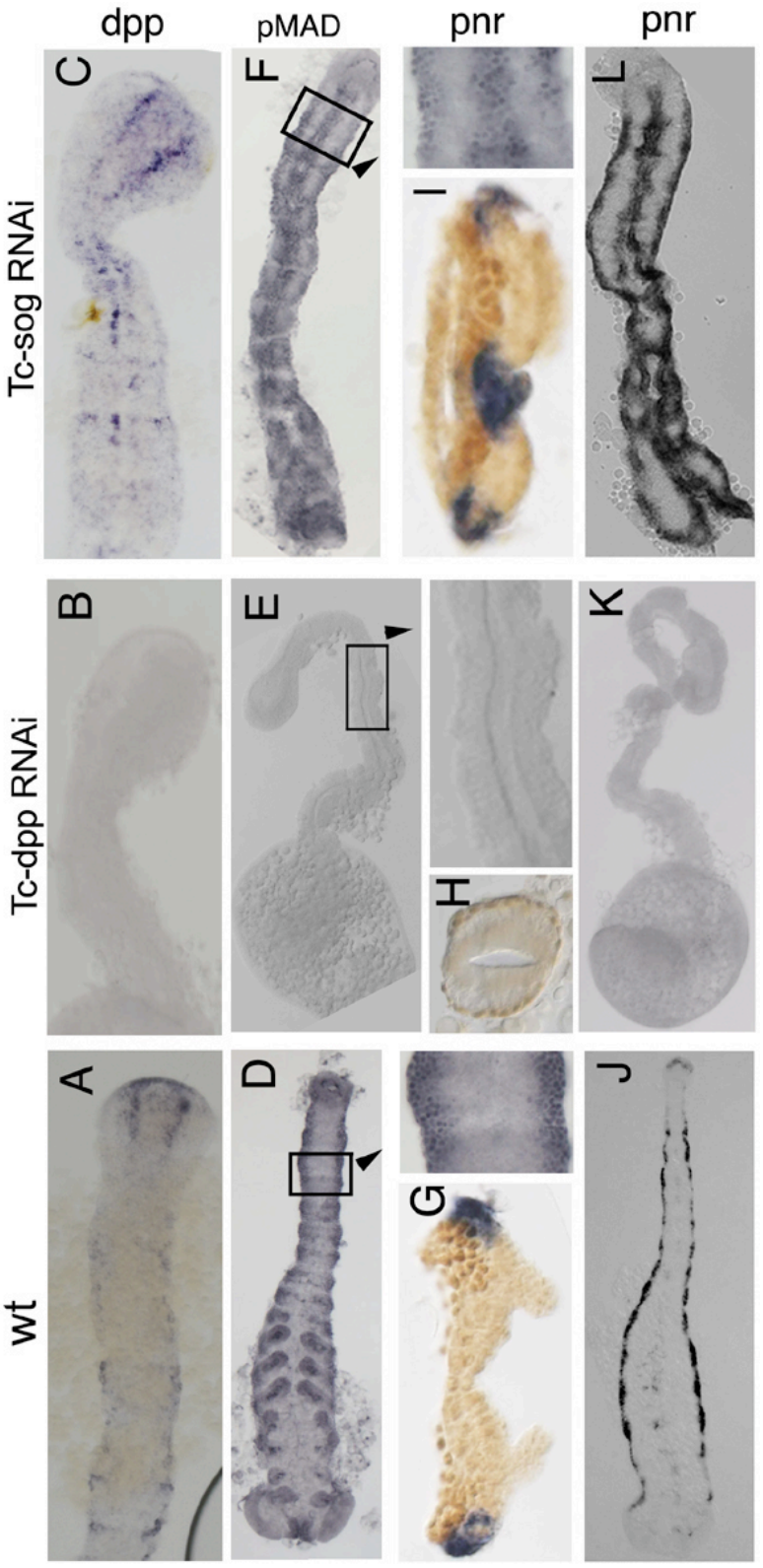
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Fig. 14. After *Tc-sog* RNAi, embryos show a "double dorsal" phenotype

(A-C) *Tc-dpp* in situ hybridizations. (A) Wildtype embryo. *Tc-dpp* is expressed along the dorsal borders of the germband and in two stripes in the growth zone. (B) *Tc-dpp* RNAi embryo. No *Tc-dpp* was detected. (C) *Tc-sog* RNAi embryo. *Tc-dpp* is weakly expressed along the dorsal margins and in two ectopic stripes along the ventral midline. The stripes are continuous with the stripes in the growth zone. (D-F) Phosphorylated MAD antibody staining. Squares indicate the area magnified below. (D) Wildtype. pMAD is detected along the dorsal margins of the germ band. (E) *Tc-dpp* RNAi embryo. pMAD could not be detected. (F) *Tc-sog* RNAi embryo. pMAD was detected along the dorsal margins of the germband and in an ectopic domain along the ventral midline. (G-I) Cross sections with *Tc-pnr* in situ hybridization and Twist antibody staining. (G) Wildtype. *Tc-pnr* is expressed at the dorsal margins. (H) *Tc-dpp* RNAi embryo. The embryo is tube-like. No *Tc-pnr* transcripts were detected. Twist is found in cells surrounding the tube. (I) *Tc-sog* RNAi embryo. *Tc-pnr* is weakly expressed along the dorsal margin and in a strong, ventral, ectopic stripe. (J-L) *Tc-pnr* in situ hybridizations, whole mount. (J) Wildtype. *Tc-pnr* is expressed along the dorsal borders. (K) Tc-dpp RNAi embryo. No *Tc-pnr* expression was detected. (L) *Tc-sog* RNAi embryo. *Tc-pnr* is expressed along the dorsal margins and in ectopic domains along the ventral midline.

After *Tc-sog* RNAi, only the segmental stripes of *Tc-ASH* could be detected (Fig. 13B). Similarly, *Tc-snail* expression was only found in peripheral neurons which can be identified as a single clusters of cells in the center of each segment (Fig. 13E). This shows that the CNS is absent after *Tc-sog* RNAi. *Tc-pnr* expression is not just expanded in *Tc-sog* RNAi embryos, but is found in two domains. In addition to the expression along the dorsal margins, an ectopic ventral domain forms at each side of the ventral midline (Fig. 14L). This "double dorsal" phenotype is strikingly revealed in cross sections (Fig. 14G,I). Corresponding to the *Tc-pnr* expression, ectopic *Tc-dpp* expression and ectopic MAD activity were found along the ventral midline of those embryos (Fig. 14C, F). Remarkably, the ectopic ventral expression of *Tc-dpp* and *Tc-pnr* was usually stronger than the normal dorsal expression domains (Fig. 14C,I). Since the peripheral neurons detected with *Tc-sna* lie in the center between the two *Tc-pnr* expression domains of each embryonic half, I assume that lateral ectoderm is present between the stripes of dorsal ectoderm. Thus, a mirror image duplication seems to have occured: the lateral ectoderm of each embryonic half is flanked by dorsal ectoderm.

This mirror image duplication of cell fates can also be seen in cuticle preparations. In wildtype, the ventral cuticle displays a typical pattern of two groups of two bristles per segment (Fig. 15B) while the dorsal side shows a pattern of eight bristles, of which two always carry a typical scale at their base (Fig. 15C). Because of the absence of the blastoderm-derived amnion, *Tc-sog* RNAi embryos do not close tirely at the dorsal side, making it difficult to score dorsal markers. However, along the ventral midline, bristles of the dorsal type are found (Fig. 15F). In the thorax area, these bristles are localized between enlarged and abnormally shaped legs indicating the presence of lateral ectoderm. This is in accordance with the "double dorsal" phenotype in which lateral ectoderm flanks the ectopic dorsal fate along the ventral midline. Thus, the loss of *sog* leads to a major re-organisation of Dpp signaling. A dorsal-to-ventral activity gradient is replaced by a bipolar activity gradient in each embryonic half.

In summary, the *Tc-sog* loss-of-function phenotype is stronger than that of *sog* in *Drosophila* or *chordin* in vertebrates, since it causes a deletion of the entire CNS and a lack of consistent DV polarity in the remaining ectoderm.

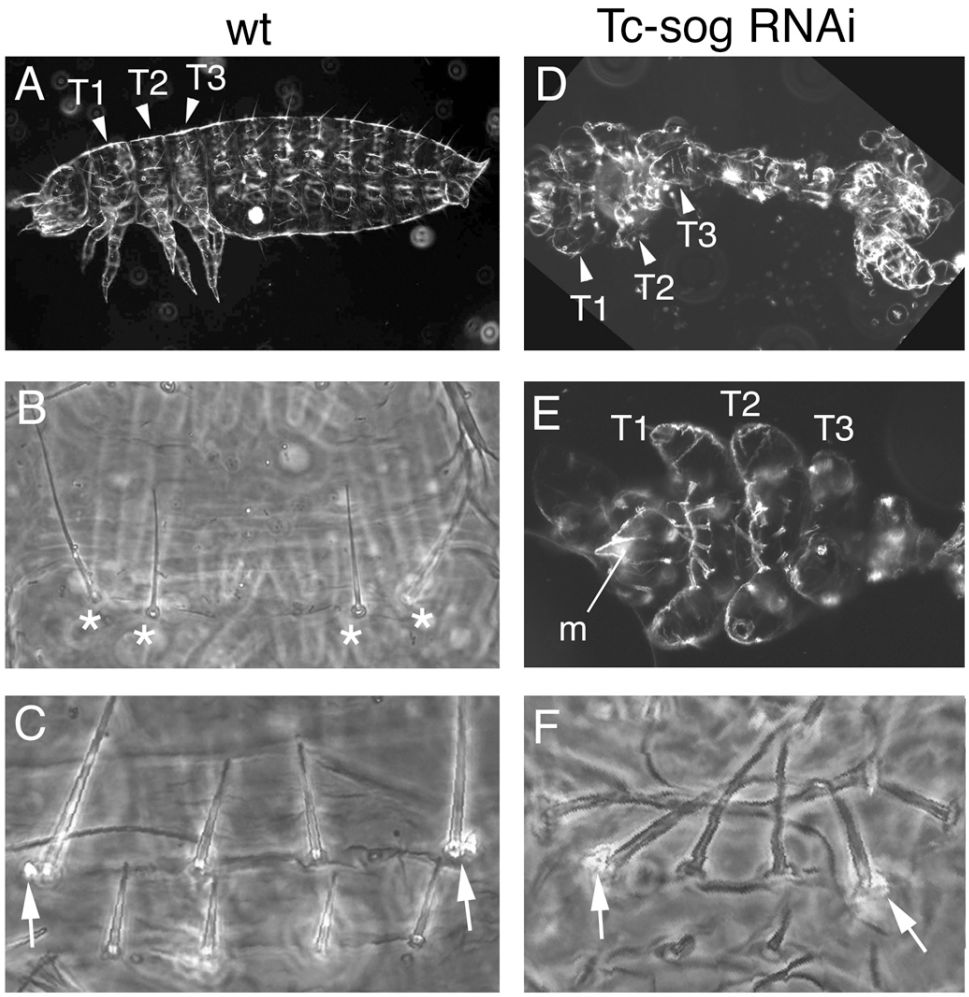
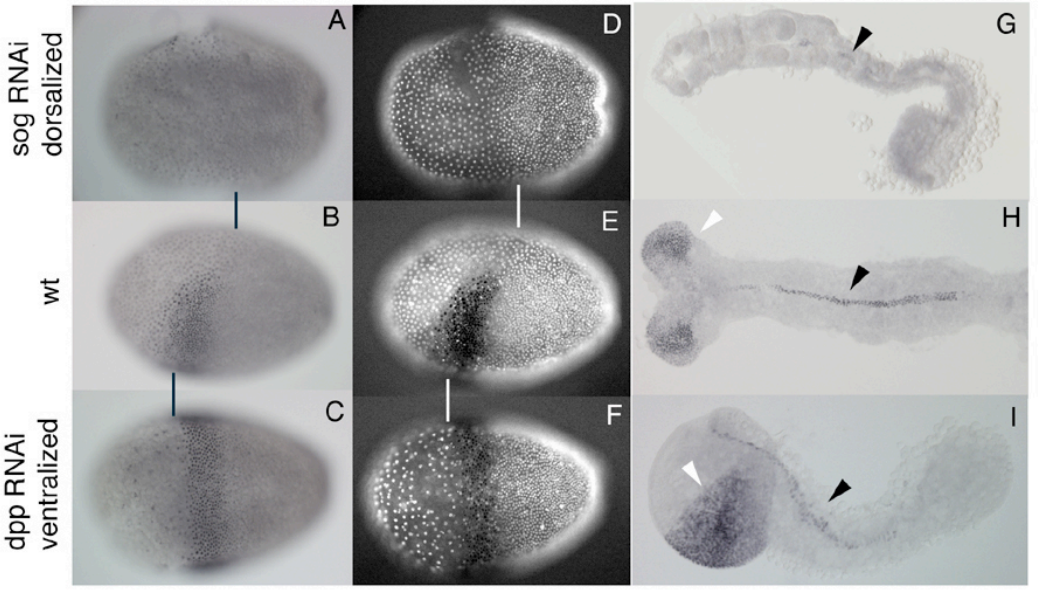


Fig. 15. Cuticles after *Tc-sog* RNAi

(A-C) Wildtype cuticles. (D-F) Cuticles after *Tc-sog* RNAi. (A) Lateral view. Thoracic segments are labelled. (B) Ventral pattern of four bristles (asterix at their base). (C) Dorsal pattern of eight bristles. Two outer bristles carry a typical scale at their base (arrows). (D) After *Tc-sog* RNAi, limb buds are present at the thoracic segments. (E) Thoracic region after *Tc-sog* RNAi. Anterior to T1 only one segment is present carrying mandible-like structures (m). (F) Bristle pattern found between the legs of *Tc-sog* RNAi embryos. Dorsal bristles with a scale (arrows) are present. Original fotos from: Stockhammer, 2003.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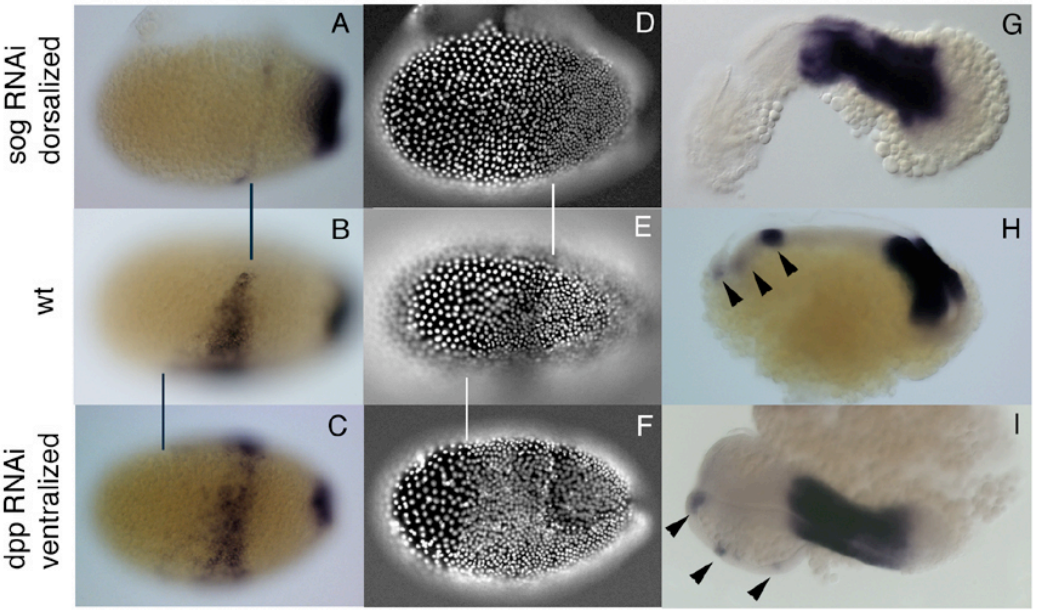
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Fig. 16. *Tc-sog* RNAi deletes the headlobes, *Tc-dpp* RNAi enlarges the head

Otd antibody stainings. (A-C) Blastoderm stages. (D-F) DAPI images of the embryos shown in A-C respectively. Nuclei of serosal cells are larger and less dense.
(G-I) Extending germ bands. (A) Embyo after *Tc-sog* RNAi. Tc-Otd could not be detected. (B) Wildtype. Tc-Otd is present in an anterior triangle. (C) Embryo after *Tc-dpp* RNAi. Tc-Otd is present in a band in the anterior germ rudiment. (D) *Tc-sog* RNAi embryo. The serosa/germ rudiment border is straight and located at a position corresponding to the wildtype dorsal border (white line). (E) In wildtype, the germ rudiment/serosa border is oblique and runs from a dorsal, more posterior point to a ventral, more anterior point (white lines). (F) After *Tc-dpp* RNAi, the germ rudiment/serosa border is straight and located at a position corresponding to the wildtype ventral position (white line). (G) After *Tc-sog* RNAi, Tc-Otd is only detected in some patches along the ventral midline (arrowhead). (H) Wildtype. Tc-Otd is found in the head lobes (white arrowhead) and along the ventral midline (black arrowhead). (I) After *Tc-dpp* RNAi, Tc-Otd is detected along the ventral midline (black arrowhead) and in an enlarged anterior domain (white arrowhead).

Fig. 17. Tc6A12 in situ hybridizations.

(A-C) Blastoderm stages. (D-F) DAPI images of the embryos shown in A-C respectively. (G-I) Extending germ bands. (A) *Tc-sog* RNAi embryo. Tc6A12 is detected in a narrow stripe in the anterior of the germ rudiment and in the primitive pit. (B) Wildtype. Tc6A12 is detected in an anterior triangle and in the primitive pit. (C) *Tc-dpp* RNAi embryo. Tc6A12 is detected in a broad anterior band and in the primitive pit. (D) After *Tc-sog* RNAi, the serosa/germ rudiment border is straight and located at a position corresponding to the wildtype dorsal border (white line). (E) In wildtype, the germ rudiment/serosa border is oblique and runs from a dorsal, more posterior point to aventral, more anterior point (white lines). (F) After *Tc-dpp* RNAi, The germ rudiment/serosa border is straight and located at a position corresponding to the wildtype ventral position (white line).
(G) After *Tc-sog* RNAi, Tc6A12 is only detected in a posterior domain. (H) Wildtype. Tc6A12 is detected in a posterior domain and in three stripes in the head (arrowheads).
(I) After *Tc-dpp* RNAi, Tc6A12 is detected in a posterior domain and in three stripes in an enlarged head (arrowheads).

BMP signaling plays a role in head formation in *Tribolium*

The border of the germ rudiment and the serosa changes after *Tc-sog* and *Tc-dpp* RNAi, as already shown in Fig. 12J-L. In wildtype, the border of the germ rudiment and serosa is oblique and runs from a dorsal, more posterior position to a ventral, more anterior position. Accordingly, the anterior of the germ rudiment can be described as a triangle. The head gap gene *Tc-orthodenticle* (*Tc-otd*) is expressed precisely within this triangle (Fig. 16B, E; Li et al., 1996). After *Tc-sog* RNAi, the germ rudiment/serosa border is straight and is located at the more posterior (dorsal) position (Fig. 16D). Consequently, the Tc-Otd domain is lost after *Tc-sog* RNAi (Fig. 16A). After *Tc-dpp* RNAi, the germ rudiment/serosa border is straight as well, but is located at the more anterior (ventral) position (Fig. 16F). As a result, the Tc-Otd domain expands towards the dorsal side and forms a broad dorsoventrally symmetric band at the anterior germ rudiment (Fig. 16C).

In the extending germband, Tc-Otd is present in the head lobes (Fig. 16H). Additional Otd can be detected along the ventral midline (Fig. 16H). After *Tc-sog* RNAi, Tc-Otd was only found in some small patches along the midline (Fig. 16G). Anterior Tc-Otd staining in the head lobes is absent (Fig. 16G). In contrast, Tc-Otd staining reveals an enlarged head after *Tc-dpp* RNAi (Fig. 16I). Similar results in blastoderm and extended germband stages are obtained when the anterior domain of Tc006A12 is used as marker for the head region (Fig. 17). The enlarged head after *Tc-dpp* RNAi and the absence of the head lobes after *Tc-sog* RNAi was consistently observed in all other performed stainings (Fig. 13C, F1, Fig. 14E, K and Fig. 13B, E1, Fig. 14F, L, respectively).

To investigate how many segments are deleted after *Tc-sog* RNAi, The number of Engrailed stripes was analyzed. Instead of the wildtype 17 stripes, I never found more than 13 Engrailed stripes plus a small anterior patch of Engrailed (Fig. 13E1). Since I found limb buds on the three anterior, complete segments of many embryos, I infer that only the 3 thoracic and 10 abdominal segments are present, plus a degenerated segment at the anterior. This was confirmed by analyzing the cuticles. Anterior to the thoracic segments marked by limb buds, one pair of mandible-like structures was found, but never antennae, maxillae or labia (Fig. 15E). This indicates that only one segment is present anterior to the thorax. In wildtype, this would be the labial segment. I assume that all segments anterior to the labium are deleted and that the labial segment undergoes a homeotic transformation to a mandibular segment. Taken together these observations demonstrate that, in contrast to *Drosophila,* the BMP-Sog system plays a major role in AP patterning in *Tribolium*. Lack of Sog leads to the absence of the head, including non-neurogenic cell fates like mesoderm.

3.5 DISCUSSION

I studied the DV patterning role of BMP/Dpp and Chordin/Sog in the short germ beetle *Tribolium* by RNAi. The results are schematized in Figure 18. The study has uncovered similarities and differences to both *Drosophila* and vertebrates. In the following I discuss these results in an evolutionary perspective.

The role of the dorsal Dpp transport by Sog

One of the main findings in this case study is the striking discrepancy between regions of *dpp* transcription and regions of high level Dpp signaling in the early *Tribolium* embryo. Dpp is differentially transcribed along the AP axis, namely in a ventral-to-dorsal stripe along the border of the germ rudiment and in a dot in the primitive pit. In contrast, Dpp activity and *dpp* target gene expression are localized to the dorsal side of the embryo. This localization is likely to be caused by Sog-mediated transport of Dpp molecules towards the dorsal side, because I demonstrate that this localization depends on Sog. In absence of Sog, Dpp activity follows the dpp expression and occupies a ventral-to-dorsal band. Unaided diffusion of Dpp (Mizutani et al., 2005; Wang and Ferguson, 2005) probably causes this band of Dpp activity to be broader than the stripe of dpp expression. However, Dpp activity seems to expand more towards the anterior than to the posterior with regard to the dpp expression (Fig. 12C, F). This could be due to receptor-mediated degradation of Dpp (Mizutani et al., 2005) in the posteriorly localized germ rudiment which expresses higher levels of Thickveins than the anteriorly localized serosa (Fig. 19). Nevertheless, in absence of Tc-Sog, the Dpp gradient fails to acquire a dorsoventral orientation and rather maintains an anteroposterior one. *Tc-dpp* target genes consequently acquire expression domains along the AP axis in the blastoderm.

This stands in stark contrast to *Drosophila*. There, the maternal NFκB/Dorsal already imposes a dorsoventral prepattern on the embryo by repressing *Dm-dpp* and the *Dm-dpp* target *Dm-zen* at the ventral side. Thus, the pattern of highest Dm-Dpp activity in a stripe along the dorsal midline only moderately differs from the *Dm-dpp* expression pattern. The dorsal transport mechanism in *Tribolium* appears more dramatic than in *Drosophila*.

However, the dorsal transport mechanism in *Drosophila* is not less powerful than in *Tribolium*. By elegant experiments in *Drosophila* it has been shown that the site of *Dm-dpp* transcription is irrelevant for the correct establishment of the narrow dorsalmost stripe of Dm-Dpp activity. If *dpp* is expressed under the control of the *even skipped* stripe 2 enhancer in a *dpp* minus background, Sog-dependent transport still correctly localizes dpp activity to a dorsal stripe. In *Tribolium,* the stripe of *Tc-dpp* expression between serosa and germ rudiment appears to be the major source for Tc-Dpp protein transported to the dorsal side. Thus, the formation of the Dpp activity gradient in the *Tribolium* embryo has similarity to the artificially generated situation in *Drosophila* where *dpp* is expressed in the *even-skipped* stripe. In the *Drosophila* experiment, a slight broadening in the dorsal band of Dpp activity is observed close to the Dpp source in eve stripe 2 (Wang and Ferguson, 2005). However, a widening of the dorsal domain of Tc-Dpp activity close the stripe of *Tc-dpp* expression is not observed in *Tribolium* and might be prevented by a corresponding widening of *Tc-sog* expression at the ventral side, locally producing more Tc-Sog (Fig. 11C). But taken together, the transport mechanism of *Drosophila* can achieve the same as that of *Tribolium*: transforming an anteroposterior *dpp* expression domain into a dorsoventral Dpp activity gradient.

One can rather argue that the transport mechanism in *Drosophila* is more crucial to development than in *Tribolium.* In *Drosophila*, highest levels of Dpp signaling can only be achieved in the presence of Sog. These signaling levels are required to activate *Dm-zen* and specify the dorsalmost cell fate, the amnioserosa. Its absence causes severe disruption of morphogenetic movements. In contrast, *Tribolium-zen* is initially expressed under control of the AP patterning system (chapter 2). A serosa is established in absence of *Tc-sog* and does not seem to be the dorsalmost cell fate (Fig. 12L). The only cells that are absent after *Tc-sog* RNAi are the dorsally located amniotic cells. However, loss of *Tc-sog* still allows peak levels of Dpp signaling along the anterior of the germ rudiment and in the primitive pit (Fig. 12F). There, amniotic cells are specified which compensate for the loss of the dorsal amnion and allow a more or less normal gastrulation. (Fig. 12O,U,X). Thus, in contrast to *Drosophila*, the disruption of the dorsal Dpp transport mechanism in *Tribolium* does not lead to the absence of an entire cell fate, neither to a severe disruption of gastrulation.

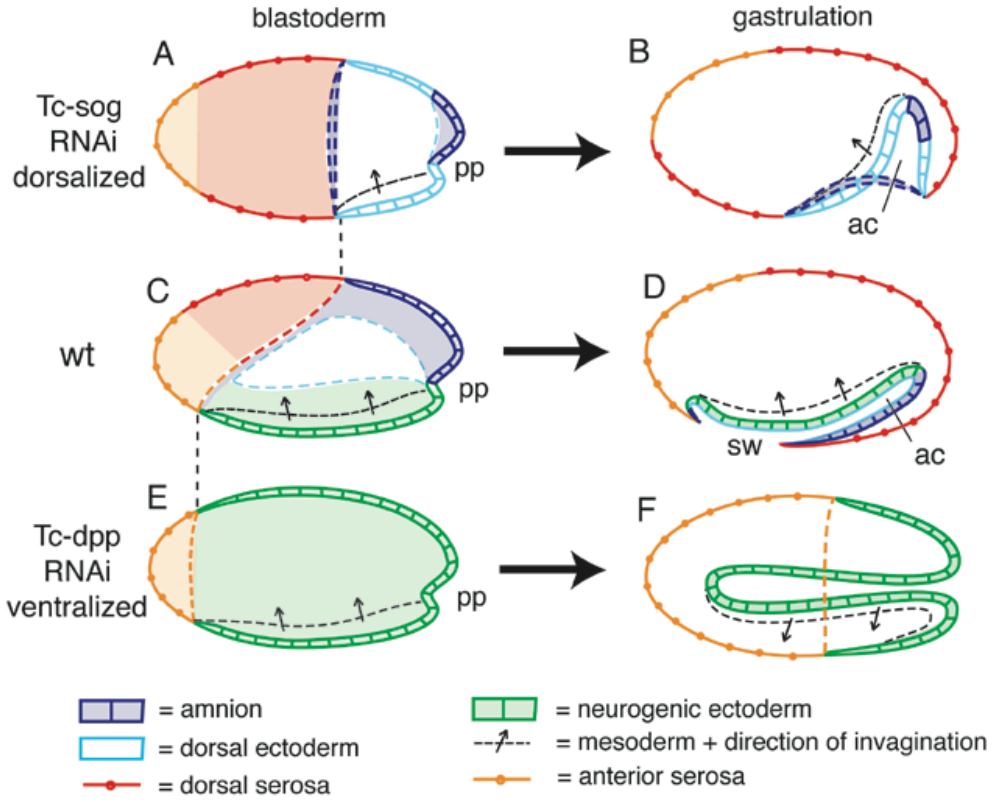

Fig. 18. Schematic drawings of the *Tc-sog* and *Tc-dpp* RNAi phenotypes
Orange: anterior serosa. Red: dorsal serosal cells. Dark blue: amnion. Bright blue: dorsal ectoderm. Green: neurogenic ectoderm. Dotted black lines with arrows: mesoderm. (A-B) *Tc-sog* RNAi. (A) The neurogenic ectoderm is absent. Dorsal cell fates occupy domains along the AP axis: dorsal serosal cells are present in a broad band anterior to the germ rudiment, amniotic cells are present along the anterior margin of the germ rudiment and in the primitive pit. Dorsal amnion is absent. The serosa/germ rudiment border is straight and is positioned at a point corresponding to the dorsal border in wildtype. The presumptive mesoderm is correspondingly shorter along the AP axis. (B) The amniotic cells in the primitive pit allow almost normal gastrulation. Because there is no continuous amnion at the dorsal side, the amniotic cavity (ac) never closes. The mesoderm invaginates normally. (C-D) Wildtype. (C) Dorsal serosal cells and amniotic cells are localized to the dorsal side. Neurogenic ectoderm and mesoderm are present. The germ rudiment/serosa border is oblique and runs from a dorsal, posterior position to a ventral, anterior position. (D) The amnion folds over the embryo at the ventral side forming the amniotic cavity (ac). The serosal window (sw) closes. (E-F) *Tc-dpp* RNAi. (E) Dorsal serosa and amnion are absent. Mesoderm is present. The germ rudiment/serosa border is straight and located at a position corresponding to the wildtype ventral border. The rest of the embryo consists of neurogenic ectoderm. (F) The embryo folds symmetrically inwards at the primitive pit and extends with the growth zone towards the anterior. The small serosa covers only 2/3 of the yolk; the anterior regions of the embryo proper remain at the posterior surface of the egg. The mesoderm invaginates normally towards the interior of the embryo, i.e. towards the exterior of the tube-like embryo (arrows in E and F). pp= primitive pit.

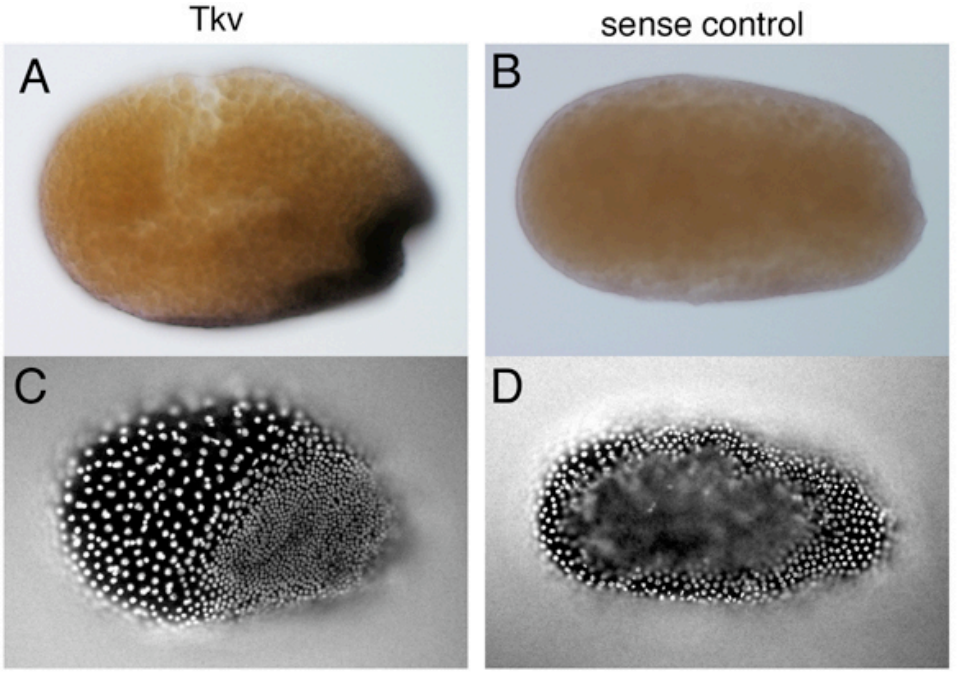

Fig. 19. *Thickveins* in situ hybridization

Tkv, the Dpp receptor, was predicted in silico and a fragment was cloned with the forward primer CAGGAGCAGTCCTGGTTTAG and the reverse primer TGCACGAGTCT GTGTCCAGC. (A) Wildtype blastoderm stage. *Tkv* is stronger expressed in the germ rudiment than in the presumptive serosa. (B) Control with a sense probe of *Tkv*. (C) DAPI of the embryo shown in A. (D) DAPI of the embryo shown in B.

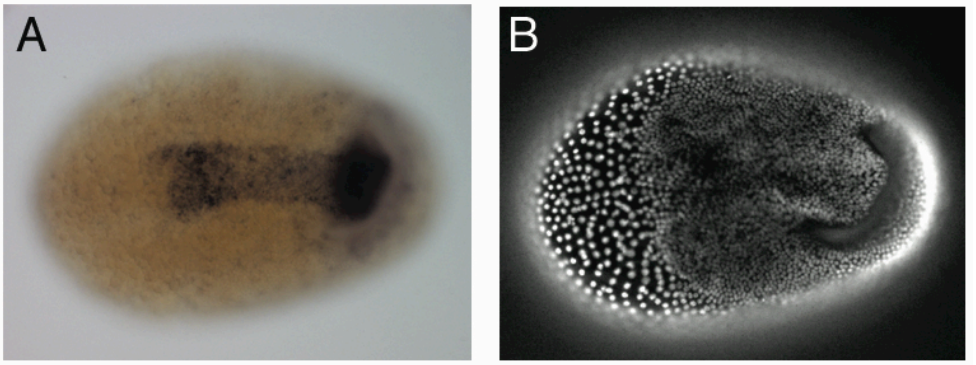

Fig. 20. *Rhomboid* in situ hybridization

Rhomboid was predicted in silico and a fragment was cloned with the forward primer TATTATAGTGTCAGCATTGGT and the revesre primer GTTGAGGAGGAC GTTGGCGA. (A) Wildtype gastrulation. *Rho* is expressed in the mesoderm. (B) DAPI of the embryo shown in A.

Finally, the *Drosophila* transport system is more elaborate than in *Tribolium*. High Dpp activity is present in a narrow stripe which is only a few cell diameters wide. The activity drops sharply at the border of the stripe, enabling an accurate demarcation of the amnioserosa. In other words, there is rather a bistable pattern of Dpp activity than a gradient (Wang and Ferguson, 2005). A second BMP, *screw*, is required for this bistable signaling pattern as well. In *Tribolium* the Dpp acticity domain is broader and the activity profile lacks sharp borders. No *screw* homologue is present in the *Tribolium* genome. Thus, *Drosophila* has probably elaborated and refined a transport mechanism already present in more primitive insects.

The evolution of this elaboration of the Drosophila BMP transport mechanism was possibly enabled by the emergence of redundancy. In *Tribolium*, Tc-Sog is the sole BMP antagonist. It prevents Dpp signaling at the ventral side and at the same time transports Dpp towards the dorsal region. Loss of *Tribolium*-Sog does not only lead to the loss of neurogenic ectoderm, but also to the incorrect localization of dorsal cell fates. In *Drosophila*, Sog mainly plays a role in dorsal transport and its absence only results in the loss of the dorsalmost cell fate, because Brinker represses expression of *dpp* and *dpp* target genes in ventral, neurogenic regions. A *brinker* homologue has been found in *Tribolium* as well, but is not expressed in the early embryo (R. Fonseca and S. Roth unpublished results). This suggests that *brinker* was not ancestrally involved in dorsoventral patterning and was recruited for this process in the lineage leading to *Drosophila*. This uncoupling of BMP transport from the repression of Dpp signaling possibly allowed *Dm-sog* to specialize on the transport of BMPs and allowed the coevolution of Sog and Screw in the intricate bistable signaling dynamics in *Drosophila*.

The origin of the double dorsal phenotype

Despite the rather normal gastrulation of *Tc-sog* RNAi embryos, their ectoderm completely lacks neurogenic tissue and is patterned in an interesting bipolar way: the dorsalmost margins as well as the ventralmost area is specified as dorsal ectoderm (double dorsal phenotype). In between, lateral ectoderm is present. How could such a pattern duplication arise?

The origin might lie in the transcription pattern of *Tc-dpp* in the growth zone. Besides a weak expression in the entire amnion, *dpp* is expressed in two stripes directly flanking the Inner Layer (IL) of the growth zone (Fig. 11L). This IL is continuous with the mesoderm of the emerging segments and probably

contributes to it. *Tc-sog* is expressed in this IL (Fig. 11F). Analogous to the situation in the blastoderm, the site of *dpp* expression does not necessarily coincide with the site of Dpp activity. Tc-Sog could bind to Dpp close to the IL and transport Dpp to the dorsal margins. A positive feedback activation of *dpp* would initiate transcription at the dorsal margins. Indeed, during segment formation, the inner ventral stripes of dpp expression disappear and new stripes of *dpp* expression form along the dorsal margins (Fig. 14A). In absence of Tc-sog, the ventral stripes of *Tc-dpp* expression persist (Fig. 14C). The weaker *Tc-dpp* expression along the dorsal margins could be induced by *dpp* expression in the amnion. In this scenario, a main function of Tc-Sog would be to inhibit growth zone specific *dpp* expression and to direct *dpp* expression to the dorsal margins.

However, the invoked positive feedback mechanism would probably cause the *dpp* expression to spread over the entire ectoderm, even if unaided diffusion of Dpp is only limited. This spreading would be prevented if the positive feedback activation of *dpp* is linked to the production of a diffusible, long-range inhibitor. Theoretical modeling has shown that such a system is able to form two activation peaks at the margins of a developmental field (Meinhardt and Gierer, 2000). In this way, the ectoderm could acquire dorsal fates along its dorsalmost border with the amnion and along its ventralmost border with the ventral midline. In wildtype, Tc-Sog would prevent the formation of the ventral stripe by inhibiting Dpp signaling there, thus imposing polarity on the system. Indeed, a *Tribolium* BMP homologue exists which appears to inhibit Dpp signaling and plays a role in embryonic patterning (M Klingler, personal communication). Furthermore, several examples have recently been described in which local TGFβ activity is linked to the production of long-range inhibitors such as lefty and sizzled (Lee et al., 2006; Schier, 2003)

I suggest that both the expression of *dpp* in the gowth zone and the proposed long range inhibitor contribute to the double dorsal phenotype in absence of *Tc-sog*.

Comparisons to vertebrates and the function of BMP signaling in head formation

A BMP transport mechanism by BMP antagonists is not only present in *Tribolium* and *Drosophila*, but is also believed to exist in zebrafish (Hammerschmidt and Mullins, 2002). There, the ventralmost cell fate, the ventral tail fin, is absent in *chordin* mutants. Thus, it could be that such a transport system was already present in a common ancestor of vertebrates and insects. A feature in

which *Tribolium* differs from *Drosophila* and vertebrates, is the presence of redundancy. Although *Tribolium* might possess a long range diffusible BMP inhibitor (see above), Tc-Sog is the main inhibitor of BMP and its loss leads to the complete absence of neurogenic tissue. In contrast, *Drosophila brinker* rescues the neurogenic ectoderm in *Dm-sog* mutants. It does this by transcriptional repression of Dpp and Dpp target genes. In vertebrates, redundant excreted BMP antagonists like Noggin and Follistatin prevent the loss of neuronal tissue in *chordin* mutants. It is plausible that the different types of redundancy independently evolved from a common, simple mechanism. Since *chordin/sog* is common to all systems and because Tc-Sog is the main BMP inhibitor in *Tribolium*, I suggest that a common ancestor of vertebrates and insects possessed a BMP signaling system in which Sog/chordin is the sole BMP antagonist. Consistently, a primitive cnidarian possesses Chordin as a BMP antagonist, whereas *noggin* is not early expressed (Matus et al., 2006).

In vertebrates, BMP signaling plays an important role in the development of the head. Depletion of *chordin* and other BMP antagonists results in reduction of head and forebrain (e.g. Bachiller et al., 2000; Anderson et al., 2002 for mouse), whereas BMP knock down enlarges the head and forebrain (Reversade et al., 2005 for *Xenopus*). The most surprising finding of this study is a similar effect of BMP signaling on *Tribolium* head formation. *Tc-dpp* RNAi enlarges the head, whereas *Tc-sog* RNAi deletes all but one head segments including mesoderm. This is not the case in *Drosophila*. Even completely dorsalized *Drosophila* embryos with uniform Dpp activity along the DV axis specify anteriormost head structures and head segments (S.Roth unpublished data). It could well be that *Tribolum* is more representative for arthropod head development than *Drosophila*, because the *Drosophila* head is specified in an exceptional way involving the maternal *bicoid* gene which is only present in a group of derived Diptera (Stauber et al., 2002). This calls for an exploration of the relationship of the vertebrate and *Tribolium* head phenotypes after changes in BMP signaling.

The effect of BMP signaling on head development in *Tribolium* could be a simple consequence of the blastoderm fate map; the head fates are DV asymmetrically located at the ventral side. Completely dorsalized embryos lack the head. In vertebrates, the head anlage is also asymmetrically positioned along the DV axis, but to the dorsal side. Ventralized zebrafish embryos lack the head. This could be coincidental, but could also reveal a developmental constraint: if the head is DV asymmetrically localized in the fate map, brain formation and CNS development would require the head to be positioned at the neuronal side. This is ventral in arthropods and dorsal in vertebrates. Changing dorsoventral coordinates

by manipulating BMP signaling would then affect the head in vertebrates and arthropods in the same way, but would simply be a result of the DV asymmetrically localized head.

The similarity of head loss in vertebrate and *Tribolium* embryos lacking BMP antagonism might also be more profound. It could reflect an ancestral involvement of BMP signaling in head formation in all Bilateria. It is plausible that cephalization in the evolving urbilaterian required increased anterior inhibition of BMP signaling to allow brain formation. The anteriorly present BMP antagonists might subsequently have acquired a role in specifying or inducing head structures in general, including non-neurogenic tissue. This could have happened independently in vertebrates and insects, considering their highly divergent embryological development. However, it could also have occurred in the urbilaterian ancestor. In this case, the loss of the head after *Tc-sog* RNAi and its enlargement after *Tc-dpp* RNAi would reveal an ancestral involvement of BMP signaling in head formation in Bilateria.

3.6 ACNKOWLEDGMENTS

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CHAPTER 4

General discussion

From the case studies in chapter 2 and 3, inferences can be made about evolutionary changes, especially about changes that have occurred in the lineage leading to *Drosophila*. Most of these inferred evolutionary modifications have already been treated in the discussions of chapter 2 and 3. In this chapter, I explore to what extent the concepts of Evo-Devo apply to the case studies and their evolutionary implications.

Modules

Schlosser and Wagner define a module as "a component of a system that operates largely independently of other components" or "a component of a system that is repeatedly used" (Schlosser and Wagner, 2004). The BMP signaling pathway from chapter 3 is clearly a module. It is not only used in early dorsoventral axis formation in vertebrates and insects (chapter 3), but for example also in arthropod appendage formation (Diaz-Benjumea et al., 1994; Jockusch et al., 2000; Niwa et al., 2000; Prpic, 2004a; Prpic, 2004b; Prpic et al., 2003; Yamamoto et al., 2004), insect wing patterning (Carroll et al., 1994; O'Connor M et al., 2006), vertebrate bone formation (Cao and Chen, 2005; Wan and Cao, 2005), *Drosophila* egg shell patterning (Deng and Bownes, 1997) and in the migration of a neuroblast in nematodes (Hishida et al., 1996). In all these cases, completely different genes are activated upon signaling. In other words, the exact output of BMP signaling is highly context dependent. The state of the cell, i.e. the other transcription factors present in the cell, determine what genes will be transcribed or not when the cell receives a BMP signal. The only function of the BMP signaling module is to provide positional information. BMP signaling is highly suited for this task. Locally produced BMPs form a gradient by diffusion. (although gradient formation usually not only depends on diffusion (Lander et al., 2002)). Cells close to the source are exposed to high levels of BMP signaling and will transcribe the target genes. The target genes in cells far from the source will remain silent, because those cells receive few BMP signals. Other signaling pathways have other properties. Notch/Delta signaling, for example, mediates

lateral inhibition in pattern formation (Celis, 2004) and hedgehog signaling is used for establishing boundaries of a developmental field (Borycki, 2004). Evolution has employed these signaling modules over and over again in different contexts. BMP signaling is one of those modules.

As introduced in chapter 1, morphological structures can also be modules. For example, the fins of fishes or the digits of vertebrate appendages can be considered modules (Shubin and Davis, 2004). To what extent is the serosa a module? On the one hand, the serosa does seem to be an independent 'tinkering unit', because *Drosophila* displaced the serosa to the dorsal side and reduced it to a small amnioserosa (chapter 2). The *Tc-zen1* RNAi experiment has shown that such a design is allowed: a small extraembryonic membrane at the dorsal side still allows germband extension, germband retraction and dorsal closure. The fact that *Tribolium* embryos can develop without a serosa suggests that the serosa is a component of a system that functions largely independent of other components. Thus, the serosa might be considered a module. On the other hand, perhaps not the modularity of the serosa, but the flexibility of the anmion allows normal later development of *Tribolium* embryos in absence of the serosa. Furthermore, the dorsal placement of the amnioserosa might be the only possible design with a reduced extraembryonic membrane. Tinkering is probably greatly restricted. Taken together, it remains unclear whether the serosa really fits the definition of a module. Nevertheless, the *Tc-zen1* RNAi experiment has shown that the evolutionary transition from a *Tribolium*-like development (with a small germ rudiment and two extraembryonic membranes) to a *Drosophila*-like development (with a big germ rudiment and a single amnioserosa) was probably easier than one would initially expect.

Gene duplication, redundancy and subfunctionalization

Around 15% of the genes in the human genome are believed to derive from duplication events, and gene duplicates account for 8-20% of the *Drosophila melanogaster*, *Caenorhabditis elegans* and *Saccharomyces cervisiae* genomes (Conery and Lynch, 2001; Lynch and Conery, 2000; Moore and Purugganan, 2003). Gene duplication can be followed by non-functionalization (i.e. one of the copies degenerates and looses its function (Nei and Roychoudhury, 1973; Takahata and Maruyama, 1979)), neofunctionalization (one of the copies acquires a new function (Cooke et al., 1997; Hughes, 1994; Ohno, 1970; Sidow, 1996; Walsh, 1995)) or subfunctionalization (i.e. the duplicates complementary

specialize on subfunctions of the ancestral gene (Force et al., 1999; Lynch and Force, 2000; Stoltzfus, 1999)).

Phylogenetic analysis has shown that *Tc-zen1* and *Tc-zen2* are more closely related to each other than to any other known *zen*-gene (Brown et al., 2002). This means that the duplication event giving rise to *Tc-zen1* and *Tc-zen2* occurred in a rather recent ancestor of *Tribolium*. This ancestor is neither shared by more primitive insects, nor by *Drosophila*. The duplication of *zen* in the lineage leading to *Tribolium* was probably followed by subfunctionalization. Non-functionalization can be excluded, because both *Tc-zen1* and *Tc-zen2* have a function (chapter 2). To decide between neofunctionalization or subfunctionalization, the function of the ancestral, single *zen* gene in the *Tribolium* ancestor has to be inferred. Serosa specification was probably a function of this gene, because both *Tribolium*- and *Drosophila-zen* have a function in extraembryonic membrane specification (chapter 2; Rushlow et al., 1987b). It would be unlikely that this property evolved twice. It could be that serosa specification was the only function of the single *zen* gene in the *Tribolium* ancestor and that *Tc-zen2* acquired a new function in dorsal closure after the duplication (=neofunctionalization). However, a recent study has shown that *zen* in the hemipteran *Oncopeltus fasciatus* has a function in dorsal closure, like in *Tribolium* (Panfilio et al., 2006). Again, it would be unlikely that this property evolved twice. Thus, this was probably an ancestral function of the single *zen* gene too. Therefore, I suggest that the ancestral, single *zen* gene in an ancestor of *Tribolium* had a function in both serosa specification and in dorsal closure. The duplication of *Tc-zen* was followed by subfunctionalization: *Tc-zen1* has specialized on serosa specification, whereas *Tc-zen2* has specialized on dorsal closure.

Molecularly, subfunctionalization is usually caused by loss of certain ancestral enhancer elements in the duplicated genes (Force et al., 2004). This was probably the case in the duplication of the *zen* genes too. *Tc-zen1* is early expressed in the serosa. *Tc-zen2* is also expressed in the early serosa, but only when the serosa is already specified under influence of *Tc-zen1*. Both genes are expressed in the later serosa, but *Tc-zen2* is additionally expressed in the late amnion. Thus, *Tc-zen2* probably lost a regulatory element for very early expression, whereas *Tc-zen1* lost a regulatory element for the amniotic expression. The genes might still be able to substitute for each other when expressed under the promoter of each other. However, since the proteins display only 38% aminoacid identity, they could also already have acquired adaptive aminoacid changes for their specialization.

Immediately after a gene duplication, the duplicate genes are of course identical and thus redundant. Because of this redundancy, one of the genes can acquire mutations and obtain a new function, while the other copy still can fulfil the ancestral, probably essential, function (neofunctionalization). In absence of redundancy, it is probably difficult for a gene to mutate without deleterious effects on the organism. However, the redundancy does not necessarily need to be provided by a copy of the mutating gene (gene A). If a completely other gene (gene B) has evolved the properties to substitute for the gene A, gene A is also free to change. An example might be the *sog* and *brinker* genes in *Drosophila*. An ancestor of the *Drosophila sog* gene was, like in *Tribolium*, probably both involved in transporting BMPs towards the dorsal side and in repressing BMP signaling ventrally. *Tribolium* does possess a *brinker* gene, but this gene does not seem to be expressed in the embryo (R. Fonseca and S. Roth, unpublished results). In the lineage leading to *Drosophila*, *brinker* apparently acquired a role in repressing *dpp* target genes at the ventral side (Jazwinska et al., 1999). Although *brinker* functions completely different from *sog* (transcriptional repression of Dpp target genes versus preventing Dpp signaling), the net result is that *brinker* substitutes for the BMP-inhibiting function of *Tc-sog* and completely rescues the neurogenic ectoderm in *Drosophila sog* mutants. This "redundancy" possibly allowed *sog* to entirely specialize on BMP transport in the lineage leading to *Drosophila* and enabled the evolution of the intricate, bistable signaling dynamics in *Drosophila* (chapter 3).

Co-option

Carroll, Grenier and Weatherbee define co-option as the recruitment of genes into new developmental or biochemical functions (Carroll et al., 2001). The Hox3 gene, the ancestor of *zen*, shows a striking series of co-option events (Brown et al., 2001; Stauber et al., 1999). Hughes and coworkers called this gene not inappropriately "the rogue Hox3 gene" (Hughes et al., 2004). In primitive arthropods like the spider *Cupiennius salei* and the mite *Archegozetes longisetosus*, Hox3 is expressed in a typical Hox-like pattern, i.e. in a domain along the anteroposterior axis (Damen and Tautz, 1998; Telford and Thomas, 1998). Presumably, Hox3 functions like a canonical homeotic gene in these organisms. In the lineage leading to the winged insects, Hox3 was co-opted in the morphogenesis of extraembryonic membranes: *Schistocerca-zen* is expressed in the serosa, amnion and in the neclace cells between amnion and serosa. Hox3/*zen* from *Thermobia* provides an interesting intermediate case. In this primitive

wingless insect, Hox3/*zen* is expressed in a domain along the anteroposterior axis and in the amnion (Hughes et al., 2004). Functional data on *zen* of the bug *Oncopeltus fasciatus* keep an interesting evolutionary scenario open. *Of-zen* is expressed in the serosa and plays a major role in dorsal closure, but has apparently no early function in serosa specification (Panfilio et al., 2006). The expression data on *Schistocerca-zen* do not doubtlessly prove a role of *zen* in serosa specification. Thus, it could be that *zen* in insects first acquired a role in late morphogenetic processes of the extraembryonic membranes, like dorsal closure, and was only later co-opted (for example in the holometabolous insects) to also specify the serosa in the early blastoderm. Until now, evidence for a role of *zen* in extraembryonic membrane specification only exists in *Drosophila* and *Tribolium* (Rushlow, 1987b; chapter 2). Taken together, Hox3 could first have been co-opted in the morphogenesis of extraembryonic membranes and later in their specification.

In the lineage leading to *Drosophila*, a copy of *zen* was co-opted as the maternal gene *bicoid* to pattern the anterior of the *Drosophila* embryo by a morphogen gradient (Stauber et al., 1999; Stauber et al., 2002; Stauber et al., 2000). In chapter 2, I suggest that an anterior and early function of such a *zen* gene (like in *Tribolium*) was a favorable starting point for this co-option. However, *Drosophila bicoid* activates head genes like *orthodenticle* (Gao and Finkelstein, 1998), whereas *Tc-zen1* probably represses *orthodenticle* and other head genes (chapter 2). Dearden and Akam provide an interesting explanation for this dramatic change in the target genes of *zen* (Dearden and Akam, 1999). In probably most insects, *orthodenticle* itself plays a role in anterior patterning (Lynch et al., 2006; Schroder, 2003). The homeodomain of Orthodenticle has a Lysine at aminoacid position 50. *Zen* carries a Glutamine at this place. Strikingly, Bicoid has a Lysine at homeodomain position 50 too. Thus, a simple mutation (AAA or AAG to GAA or GAG respectively) gives the *zen* gene the same DNA-recognition helix as the protein coded by *orthodenticle*. This mutation could have caused a duplicate *zen* gene to take over targets of *orthodenticle* and activate head development, as bicoid does. If this scenario were true, it would mean that Goldschmidt might not have been so wrong with his “hopeful moster” theory: a single mutation can generate novel protein function in evolution. However, it probably is an exceptional case. In summary, the Hox3 gene has not only been co-opted for the morphogenesis and specification of extraembryonic membranes in insects, but also for anterior patterning of the embryo proper in higher Diptera.

In chapter 2 it is suggested that the serosa plays a role in the immune defense, because it expresses Dorsal (Chen et al., 2000). This Rel/NFκB homologue has a main function in innate immunity (Anderson, 2000; Hoffmann, 2003; Hoffmann et al., 1999). Damaging the *Tribolium* serosa leads to nuclear import of Dorsal, probably activating the immune response (Chen et al., 2000). In *Tribolium* and *Drosophila*, Dorsal does play a role in dorsoventral patterning too (Kalscheuer, 2004; Roth et al., 1989). The role of Dorsal in immune response is probably ancestral, because vertebrates also use the Rel/NFκB in response to pathogens (Anderson, 2000; Hoffmann et al., 1999). Why was Dorsal co-opted for dorsoventral patterning?

The transcription factor Rel/NFκB enters the nucleus upon signalling of the receptor Toll (Silverman and Maniatis, 2001). In the innate immune response, secreted Peptidoglycan Recognition Proteins (PGRPs) and βGlucan Recognition Proteins (βGRPs) sense microbial infection and activate specific proteases that in turn activate Spätzle, the ligand for Toll (Ferrandon et al., 2004; Gobert et al., 2003; Levashina et al., 1999; Ligoxygakis et al., 2002). In *Drosophila* dorsoventral patterning, a protease cascade activating Spätzle is directed by pipe, which is expressed in the follicular epithelium of *Drosophila* egg chambers (reviewed in: Moussian and Roth, 2005). Because *pipe* is only transcribed in the ventral follicular epithelium, a ventral-to-dorsal nuclear Dorsal gradient is formed in the *Drosophila* embryo (Moussian and Roth, 2005). It is possible that such a maternal system activating Toll signalling originated to activate the immune pathway. It might be evolutionary advantageous to shortly activate the innate immune response in the egg, before it is laid. Notably, the biggest part of the blastoderm of primitive insects will give rise to the serosa (Roth, 2004). It is plausible that a boost of the immune system in this extraembryonic membrane provides a protection against pathogens. The maternally controlled cascade activating Spätzle could have fulfilled this role of activating Toll in the serosa. Because this pathway acts very early, it is a likely target of co-option for providing positional information at such early stages. A local activation of Toll would provide local nuclear import of NFκB/Dorsal. This occurs at the ventral side in *Drosophila* and leads to a ventral-to-dorsal gradient of nuclear NFκB/Dorsal (Moussian and Roth, 2005; Roth et al., 1989). This gradient is used to specify fates along the dorsoventral axis. Thus, maternal activation of the immune system in the embryo might have preceded the maternal determination of the dorsoventral axis.

The last example of co-option is the recruitment of Sog/Chordin in the specification of the head in *Tribolium*. In chapter 3 it was suggested that the loss of the *Tribolium* head after *Tc-sog* RNAi is a simple consequence of its asymmetric position along the dorsoventral axis. However, it could also be that *Tc-sog* plays a true role in AP patterning in *Tribolium*. When did this function arise? In cnidaria, BMP/chordin is employed in germ layer demarcation (Matus et al., 2006). This might represent the most ancestral function of Sog/Chordin. It could be that Sog/Chordin was directly co-opted from this role in germ layer demarcation to a function in head specification. Indeed, cnidaria already differentially express chordin along their primary axis, namely at the blastoporal pole (Matus et al., 2006), which is thought to correspond to the anterior pole of Bilateria (Finnerty et al., 2004; Martindale, 2005). In this case, the similar effect on the head, after knock down of BMP antagonism in vertebrates (Anderson et al., 2002; Bachiller et al., 2000) and *Tribolium*, would reflect an ancient co-option event. It could also be that the co-option was more indirect. It has been suggested that BMP signalling was required for defining the neuronal-abneuronal axis in an ancestor of all Bilateria (Arendt and Nubler-Jung, 1994). BMP antagonism is required to allow the specification of neuronal fates. It seems plausible that cephalization and brain formation in a Bilaterian ancestor required high levels of BMP antagonists at the anterior. Subsequently, *chordin* would have been co-opted to specify head structures in general, including non-neurogenic tissue. This co-option could either have happened independently in vertebrates and arthropods or already in the urbilaterian.

Cis-regulatory evolution, gene networks and hubs

Changes in regulatory sequences are thought to be the basis of many evolutionary innovations (Carroll, 2005a; Carroll, 2005b). From the case studies, inferences can be made about changes and conservation in the regulatory sequences of some genes.

From chapter 2 it is clear that *Tc-zen1* expression in *Tribolium* has a primary input from the AP patterning system. Indeed, the reduction of the serosa after *torso* RNAi suggests that *Tc-zen* is under control of the terminal system in *Tribolium* (Schoppmeier and Schroder, 2005). Hence, *Tc-zen1* probably has binding sites for terminal or AP patterning genes in its *cis*-regulatory sequences. Altough *Drosophila-zen* has no function along the AP axis, it might have retained some of these binding sites, because there is some DV independent *zen*-expression at both poles (Ray et al., 1991). In chapter 3, it was demonstrated that the serosa tilts towards dorsal under influence of Tc-Dpp activity. In absence of a

dorsoventral Dpp gradient, the serosa and *zen* expression do not acquire the dorsal tilt and remain DV symmetrical at the anterior pole (chapter 3) (Mikulski, 2004). Thus, the *Tc-zen1* enhancer probably also contains binding sites for SMAD, the transcription factor which enters the nucleus upon Dpp signaling (Parker et al., 2004). Such binding sites are also present in *Drosophila* (Rushlow et al., 2001), visible by the restriction of *Drosophila-zen* expression to areas with highest Dpp signaling (Raftery and Sutherland, 2003). In the lineage leading to *Drosophila*, *zen* has additionally acquired binding sites for Dorsal (Jiang et al., 1993; Kirov et al., 1993; Markstein et al., 2002; Stathopoulos et al., 2002), because Dorsal represses *Dm-zen* expression in the ventral 60% of the early blastoderm (Ray et al., 1991). There is no reason to assume that *Tribolium-zen* has binding sites for Tc-Dorsal in its *cis*-regulatory sequences.

A similar scenario can be sketched for *Tc-dpp*. In *Tribolium*, *Tc-dpp* is initially expressed under control of the AP patterning system (chapter 3). Under influence of Sog, *Tc-dpp* acquires a dorsoventrally asymmetric expression pattern. Since Sog is an secreted molecule and can only function by preventing Dpp from signalling, it only modulates nuclear SMAD (Parker et al., 2004). Thus, the *Tc-dpp* enhancer should have binding sites for SMAD too. This is probably conserved in evolution, because cnidarian, fly and vertebrate Dpp homologues can all upregulate their own expression (Juan and Hamada, 2001; Matus et al., 2006; Reversade et al., 2005). In the lineage leading to *Drosophila*, *dpp* might have retained binding sites for AP patterning genes, because, like in the case of *zen*, there is DV independent *dpp* expression at the termini, although *dpp* does not have any function there (Ray et al., 1991). Finally *dpp* must have gained binding sites for Dorsal (Huang et al., 1993; Stathopoulos et al., 2002), because Dorsal represses *Dm-dpp* in the ventral 60% of the early blastoderm of *Drosophila* (Ray et al., 1991). This is not the case in *Tribolium*.

Taken together, both *dpp* and *zen* came under repressional control of Dorsal in *Drosophila*. In *Tribolium*, Sog imposes dorsoventral asymmetry on the expression of these genes (chapter 3). In *Drosophila,* Dorsal has already pre-localized the expression of *Dm-dpp* and *Dm-zen* along the dorsoventral axis before Dm-Sog refines this pattern. There are no indications that Dorsal is a repressor in *Tribolium*. Dorsal only acts as a repressor in combination with other transcription factors (Huang et al., 1993; Juan and Hamada, 2001; Kirov et al., 1993). Indeed, the protein interaction properties of Dorsal might have undergone substantial change: *Tribolium*-Dorsal lacks whole polyglutamine, polyasparagine and polyalanine stretches in its C-terminal domain, when compared to *Drosophila*-Dorsal (Chen et al., 2000).

Dorsal did not only acquire targets of repression in the lineage leading to *Drosophila*, it probably also obtained more targets of activation. For example, the gene *intermediate nerve cord defective* (*ind*) is not activated in *Tribolium* until after gastrulation (Wheeler et al., 2005), when the Dorsal gradient has already disappeared (Chen et al., 2000). In contrast, *Drosphila*-Dorsal activates this gene early (Stathopoulos et al., 2002; Weiss et al., 1998). Possibly, the fast development of *Drosophila* favoured changes that brought genes required for dorsoventral patterning under control of Dorsal.

Like in *Drosophila*, the *Tribolium* genes *short gastrulation* or *rhomboid (rho)*, are probably targets of activation by Dorsal (Fig. 11, Fig. 20). However, their expression pattern differs from that of their orthologues in *Drosophila*. In *Tribolium*, those genes are activated in a broad, continuous, ventral domain overlapping the mesoderm. In *Drosophila*, *snail* represses *short-gastrulation* and *rhomboid* in the mesoderm (Markstein et al., 2002). The result is that *sog* and *rho* are both expressed in two domains flanking the mesoderm (Francois et al., 1994; Ip et al., 1992). Because the gene network in *Tribolium* is thought to represent a more ancestral one, early repression by *snail* probably evolved in the lineage leading to *Drosophila*. In *Drosophila*, more spatial resolution along the dorsoventral axis appears to exist at very early stages.

Taken together, the dorsoventral patterning system of *Drosophila* already very early restricts the expression of several genes to accurate domains along the dorsoventral axis. This is likely to be required for the fast development of *Drosophila*. Dorsal mediates many of these early spatial restrictions and became a hub in the *Drosophila* dorsoventral patterning gene network (Markstein et al., 2002; Stathopoulos et al., 2002).

Concluding remarks

The case studies on *zerknüllt, decapentalegic* and *short gastrulation* in *Tribolium*, and the evolutionary implications of these case studies, provided examples of important concepts in Evo-Devo. Decapentaplegic belongs to a pivotal signalling **module** that has been employed in many different contexts. The duplication of the *Tribolium zen*-gene was followed by **subfunctionalization**. Hox3 has been **co-opted** for the morphogenesis and specification of extraembryonic membranes in insects, and, ultimately, as the anterior determinant *bicoid* in higher Diptera. Finally, ***cis*-regulatory evolution** dramatically changed the **gene network** for dorsoventral patterning in the lineage leading to *Drosophila*. Dorsal became a **hub** in this network.

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Abstract

Evolutionary developmental biology (Evo-Devo) attempts to trace modifications in development that have lead to evolutionary novelty. Some important concepts in Evo-Devo are GENE DUPLICATION, SUBFUNCTIONALIZATION, MODULARITY, CO-OPTION, CIS-REGULATORY EVOLUTION, GENE NETWORKS and HUBS. This thesis consists of two case studies on genes in the beetle *Tribolium castaneum* which is thought to represent a more ancestral mode of development than the well-studied fruit fly *Drosophila melanogaster*.

In the first case study, the expression and function of the two *zerknüllt* genes (*Tc-zen1* and *Tc-zen2*) were investigated. *Drosophila-zen* is, initially under control of Dorsal, expressed at the dorsal side and specifies a single extraembryonic membrane, the amnioserosa. *Tribolium* possesses two extraembryonic membranes: an outer serosa and an inner amnion. *Tc-zen1* displays early, anterior expression and specifies the serosa. *Tc-zen1* knock-down enlarges the germ rudiment and deletes the serosa, but the remaining dorsal amnion allows relatively normal further development. Thus, in absence of *Tc-zen1*, *Tribolium* acquires features of *Drosophila* development. *Tc-zen2* is expressed later and mediates the amnion-serosa fusion necessary for dorsal closure. *Tc-zen2* depletion prevents this fusion and leads to completely everted (inside-out) larvae.

In the second case study, Decapentaplegic (Dpp, a BMP ligand) and Short gastrulation (Sog, a BMP inhibitor) were examined. In *Drosophila* and vertebrates, BMP signaling plays a major role in dorsoventral patterning. In contrast to *Drosophila*, where *Dm-dpp* expression is restricted to the dorsal side by Dorsal, *Tribolium-dpp* shows differential expression along the anteroposterior axis. However, Tc-Sog is expressed in a ventral domain and establishes a dorsoventral Dpp activity gradient by transporting Dpp towards the dorsal side. *Tc-sog* RNAi abolishes neurogenesis and normal dorsoventral polarity in the ectoderm. *Tc-dpp* RNAi leads to the loss of dorsal cell fates. Surprisingly, similar to vertebrates but in contrast to *Drosophila*, *Tribolium* BMP knock-down enlarges the head, while knock-down of BMP antagonism deletes the head. Possibly, Sog/Chordin was already CO-OPTED for head formation in an ancestor of all bilateria.

Besides CO-OPTION, the evolutionary implications of these case studies illustrate other concepts of Evo-Devo. For example, the DUPLICATION of *zen* in an ancestor of *Tribolium* was followed by SUBFUNCTIONALIZATION. Dpp belongs to a signaling MODULE that is repeatedly employed in different contexts. The CIS-REGULATORY SEQUENCES of *dpp* and *zen* acquired binding sites for Dorsal which became a HUB in the dorsoventral regulatory GENE NETWORK of *Drosophila*.

Zusammenfassung

Die evolutionäre Entwicklungsbiologie versucht Veränderungen der Entwicklung aufzufinden, die zur Entstehung von Neuheiten in der Evolution geführt haben. Wichtige Konzepte der evolutionären Entwicklungsbiologie sind GENDUPLIKATION, SUBFUNKTIONALISIERUNG, MODULARITÄT, KOOPTION, CIS-REGULATORISCHE EVOLUTION und GENNETZWERKE. Die vorliegende Arbeit besteht aus zwei Fallstudien an dem Käfer *Tribolium castaneum*, der verglichen mit dem gut untersuchten Modellorganismus *Drosophila melanogaster* eine ursprünglichere Form der Entwicklung zeigt.

In der ersten Fallstudie werden die Expression und Funktion der zwei *zerknüllt* Gene (*Tc-zen1* und *Tc-zen2*) von *Tribolium* untersucht. *Drosophila-zen* wird anfänglich unter der Kontrolle des maternalen Dorsalgradienten auf der dorsalen Seite des Embryos exprimiert und spezifiziert hier eine einzige Extraembryonalhülle, die Amnioserosa. *Tribolium* hingegen besitzt zwei Extraembryonalhüllen: eine äußere Serosa und ein inneres Amnion. *Tc-zen1* wird früh in einer anterioren Domäne exprimiert und spezifiziert die Serosa. Der Verlust der *Tc-zen1* Genfunktion vergrößert das Keimrudiment und deletiert die Serosa. Das verbleibende dorsale Amnion erlaubt trotzdem eine weitgehend normale Embryonalentwicklung. In Abwesendheit von *Tc-zen1* gewinnt *Tribolium* somit Eigenschaften der *Drosophila* Entwicklung, die durch eine dorsale Extraembryonalhülle charakterisiert ist. *Tc-zen2* wird spät exprimiert und vermittelt die Fusion von Amnion und Serosa, die eine Voraussetzung für den dorsalen Rückenschluss bildet. Ein Verlust der *Tc-zen2* Genfunktion verhindert diese Fusion und führt zu komplett invertierten Larven.

In der zweiten Fallstudie wurde die Funktion von Decapentaplegic (Dpp), einem Liganden der Bone Morphogenetic Protein (BMP) Familie, und Short gastrulation (Sog), einem extrazellulären BMP-Inhibitor der Chordin Familie, in *Tribolium* untersucht. In *Drosophila* und Vertebraten spielen BMP Signale eine zentrale Rolle bei der dorsoventralen Musterbildung. Im Unterschied zu *Drosophila*, wo die *Dm-dpp* Expression auf die dorsale Hälfte des Embryos beschränkt ist, variiert in *Tribolium* die *Tc-dpp* Expression anfänglich nur entlang der anteriorposterioren Achse. Tc-Sog wird aber in einer ventralen Domäne gebildet und etabliert einen dorsoventralen Aktivitätsgradienten von Tc-Dpp, indem es Tc-Dpp zur dorsalen Seite transportiert. Ein Verlust der *Tc-sog* Genfunktion führt zu einer kompletten Unterdrückung der Neurogenese und einer Dorsalisierung des Ektoderms, die von einer Musterverdopplung der dorsalsten Zellschicksale begleitet ist. Dieser Phänotyp ist wesentlich gravierender als der *sog*-minus Phänotyp von *Drosophila*. Der Verlust der *Tc-dpp* Genfunktion führt hingegen, ähnlich wie in *Drosophila,* zu einer Ausdehnung der neurogenen Region auf Kosten des dorsalen Ektoderms. Überraschender Weise treten in der

Kopfregion Unterschiede zu *Drosophila*, aber Übereinstimmungen mit Vertebraten auf. Der Verlust von *Tc-dpp* führt zu einer Vergrößerung, derjenige von *Tc-sog* hingegen zu einer drastischen Reduktion der Kopfregion. Dies könnte darauf hinweisen dass der BMP Inhibitor Sog/Chordin bereits in einem Vorfahren aller Bilateria für die Kopfbildung KOOPTIERT wurde.

Zusätzlich zur KOOPTION illustrieren diese Fallstudien andere wichtige Konzepte der evolutionären Entwicklungsbiologie. Der DUPLIKATION der *zen* Gene in den Vorfahren von *Tribolium* folgte eine SUBFUNKTIONALISIERUNG. Des Weiteren gehört Decapentaplegic zu einem MODUL, welches mehrmals in verschiedenen Entwicklungsprozessen eingesetzt wurde. Außerdem deuten die unterschiedlichen Expressionsmuster von *zen* und *dpp* in *Tribolium* und *Drosophila* auf CIS-REGULATORISCHE EVOLUTION hin, wobei Dorsal in den Vorfahren von *Drosophila* zu einem zentralen Regulator in dem NETZWERK der zygotischen Dorsoventralgene wurde.

Talks and conferences:

Invited speaker:

August 2004:	Department of Zoology, University of Cambridge, UK. Host: Prof. Dr. M. Akam
Febuary 2005:	Institut de Biologie Moléculaire et Cellulaire, Strasbourg, France. Host: Dr. D. Ferrandon

Speaker:

June 2004:	Sonderforschungsbereich 572 Meeting, Walberberg, Germany
March 2005:	Regional Tribolium Conference, Tübingen, Germany
August 2005:	2nd International Tribolium Conference, Göttingen, Germany.

Poster presentations:

October 2003:	18th European Drosophila Research Conference, Göttingen, Germany
April 2005:	Meeting of the German Society for Developmental Biology, Münster
July 2005:	64th Meeting of the Society for Developmental Biology, San Francisco, CA, USA

Publications of others about my work:

Schmidt-Ott, U. (2005): Insect Serosa: a Head Line in Comparative Developmental Genetics. *Current Biology* 15: R245-47

Alfonso Martinez Arias: Faculty of 1000, 20 April 2005:
http://www.f1000biology.com/article/15823534/evaluation

Popular scientific publications (in Dutch):

"Evolutionaire stambomen zijn niets waard"
Bionieuws 9, jaargang 12, 18 mei 2002, pagina 6

"De muis is een omgekeerde slak",
Bionieuws zomer-editite, jaargang 12, 20 juli 2002, pagina 3

"Thomas Morgan had beter een spin kunnen kiezen"
Bionieuws 12, jaargang 13, 20 juni 2003, pagina 6

"De doodgeboren robot"
Natuurwetenschap en Techniek 2, jaargang 72, februari 2004 (ingezonden brief)

"Embryo's beperken de lange giraffenek",
Bionieuws 20, jaargang 15, 9 december 2005, pagina 7

Opinion (in Dutch):

"Verwerpen gentech is kortzichtig",
Milieudefensie Magazine 10, jaargang 33, Oktober 2004, pagina 24-25

"De Schepper in het onderwijs?"
Democraat 3, jaargang 38, Juni 2005, pagina 5

Erklärung:

Ich versichere, dass ich die von mir vorgelegte Dissertation selbständig angefertigt, die benutzten Quellen und Hilfsmittel vollständig angegeben und die Stellen der Arbeit – einschließlich Tabellen, Karten und Abbildungen -, die anderen werken im Wortlaut oder dem Sinn nach entnommen sind, in jedem Einzelfall als Entlehnung kenntlich gemacht habe; dass diese Dissertation noch keiner anderen Fakultät oder Universität zur Prüfung vorgelegen hat; dass sie - abgesehen von unten angegebenen Teilpublikationen – noch nicht veröffentlicht worden ist sowie, dass ich eine solche Veröffentlichung vor Abschluss des Promotionsverfahrens nicht vornehmen werde. Die Bestimmungen der Promotionsordung sind mir bekannt. Die von mir vorgelegte Dissertation ist von Herrn Prof. Dr. Siegfried Roth betreut worden.

Teilpublikationen:

Van der Zee, M., Berns, N. and Roth, S. (2005)
Distinct functions of the *Tribolium zerknüllt* genes in serosa specification and dorsal closure.
Current Biology 15: 624-636

Van der Zee, M., Stockhammer, O., Mikulski, C., Fonseca, R.N., and Roth, S.
Sog/Chordin is required for ventral-to-dorsal Dpp/BMP transport and head formation in a short germ insect.
In preparation.

Köln, den 5. April 2006

Maurijn van der Zee

Lebenslauf

Persönliche Daten

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Schulbildung

1980 – 1989	Besuch der Vor- und Grundschule Westwoud in Zoeterwoude (NL)
1989 – 1995	Besuch des ‚Atheneums' des Schulverbundes ‚Het Vlietland College' in Leiden (NL) (vergleichbar dem deutschen Gymnasium).
Aug. 1995	Schulabschluß mit VWO-Diploma (entsprechend dem dt. Abitur)

Hochschulbildung

Sept. 1995-Aug. 2000	Studium der Biologie an der Universität Utrecht (NL)
Aug. 1996	Abschluß des Grundstudiums (‚Propedeuse')
1998 – 2000	‚Afstudeerstages' (vergleichbar mit der dt. Diplomarbeit):

1. in der Experimentellen Embryologie der Universität Utrecht bei Prof. Dr. Van den Biggelaar zum Thema „Einsetzen der dorsoventralen Polarität und bilateralen Symmetrie bei dem Mollusken *Patella vulgata* und dem Anneliden *Arctonoe vittata*." Ein Teil dieser Arbeit wurde in den Marine Biology Laboratories Friday Harbor der Universität von Washington (USA) durchgeführt.

2. am Nationalen Herbarium in der Universität Utrecht bei Prof. Dr. Maas zum Thema „Revision der Gattung Guatteria (Annonaceae) der Länder Guyana, französisch Guyana und Surinam". Ein Teil der Arbeit war eine sechswochige Expedition nach Guyana (Süd-Amerika).

3. in der Evolutionsbiologie der Katholischen Universität Nijmegen (NL) unter Anleitung von Prof. Dr. Schwarz zum Thema „Probiotische Synthese von RNA-Molekülen und ihrer Analoge".

Aug. 2000	Doctoraalexamen Biologie (entsprechend der dt. Diplomprüfung)

Arbeitserfahrung

Okt. 2000 - Aug. 2001	VSB-Stipendium zum wissenschaftlichen Arbeiten am Institut für Molekulare Biologie und Biotechnologie in Heraklion (Kreta, Griechenland) in der Gruppe von Dr. Averof zum Thema „Vergleichende Analyse der Genexpressionsmuster in Insektenflügeln, Annelidenparapodien und Molluskenkiemen".
Sept. 2001 - Juli. 2002	Mitarbeit im Jungenbund für Natur- und Umweltstudien
April 2002 -Juli 2002	Lehrtätigkeit im Fach Biologie am „Dr. F.H. de Bruijne Lyceum" in Utrecht (NL)

Promotionsstudium

Sept. 2002 –	Promotionsstudium in der „International Graduate School for Genetics and Functional Genomics" der Universität zu Köln
Sept 2002 – März 2003	Drei Rotationsperioden, u.a. im Labor von Herrn Dr. Damen.
April. 2003 –	Arbeit am Promotionsthema im Institut für Entwicklungsbiologie der Universität zu Köln in der Arbeitsgruppe von Prof. Dr. Roth.