The function of the TGF-β and Toll signalling pathways in *Tribolium* dorsoventral patterning

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“The developmental mechanics of organisms is divided in two parts, one ontogenetical, the other phylogenetical. In a distant future, both will be essential components of a more exact concept of common descent based on them. To begin with, and for a long time, attention will have to be restricted to the ontogenetical part alone. Once this is developed quite well, some enlightening shimmer of the causes of phylogeny will emerge from it, and thereby phylogeny will gain some fundament, albeit still be a very hypothetical one” (Wilhelm Roux, 1892) .

Contents:

Summary

Dorsoventral (DV) patterning in *Drosophila melanogaster* is one of the most well-known gene regulatory networks (GRN) in biology. To investigate if this GRN is conserved during insect evolution, functional analysis of TGF-β and Toll pathways in the short-germ beetle *Tribolium castaneum* was performed.

In the first part, the function of several BMP/Dpp extracellular modulators, including the products of Tolloid (Tld) and Twisted-gastrulation/Crossveinless (Tsg-Cv), was investigated in *Tribolium* via parental RNAi (pRNAi). While *Tc-tld* pRNAi knock-down decreases embryonic BMP activity, *Tc-tsg(cv)* knock-down completely abolishes it. These observations are strikingly different from those in *Drosophila*, where *tsg* is only required for a subset of Dpp activity. These results suggest that Tsg/Cv-like proteins are essential for BMP signalling in *Tribolium*. Since duplicated copies of *tsg(cv)*- and *tld*-related genes are present in the *Drosophila melanogaster* genome, duplication followed by sub-functionalization of these modulators might have changed the BMP/Dpp gradient during evolution of the dipteran *Drosophila* lineage.

In the second part, a functional analysis of the Toll pathway was performed. This analysis addressed the question of why the *Tribolium* Dorsal nuclear gradient is not stable, but rapidly shrinks and disappears, in contrast to the stable *Drosophila* gradient. Negative feedback accounts for this dynamic behavior: Tc-Dorsal and one of its target genes (Tc-Twist*)* activate transcription of the I-κB homolog *Tc-cactus*, which in turn terminates Dorsal function. Despite its transient role, Tc-Dorsal is strictly required to initiate DV polarity, as in *Drosophila*. However, unlike *Drosophila*, embryos lacking Tc-Dorsal display a periodic pattern of DV cell fates along the AP axis, indicating that a self-organizing ectodermal patterning system operates independently of mesoderm or maternal DV polarity cues. The presence of self-organizing patterning systems in short-germ insects like *Tribolium* is in agreement with a regulative type of embryogenesis proposed by classical fragmentation studies on hemimetabolous insects. These results also elucidate how extraembryonic tissues are organized in short-germ embryos, and how patterning information is transmitted from the early embryo to the growth zone. Altogether, the functional analysis of the TGF-β and Toll pathways in *Tribolium* dorsoventral patterning suggests that extensive changes in this GRN have occurred during insect evolution.

Zusammenfassung

Die dorsoventrale (DV) Musterbildung in *Drosophila melanogaster* (D.m.) gehört zu den am besten verstandenen Gen-regulatorischen Netzwerken (GRN) in der Biologie. Um zu untersuchen, ob dieses GRN während der Insektenevolution konserviert ist, wurden funktionale Analysen der TGF-β und Toll-Signaltransduktionwege im Kurzkeiminsekt *Tribolium castaneum* durchgeführt.

Im ersten Teil dieser Arbeit wurden verschiedene extrazelluläre BMP/Dpp-Modulatoren, wie z.B. Tolloid (Tld) und Twisted-gastrulation/Crossveinless (Tsg-Cv), mittels parentaler RNAi (pRNAi) in *Tribolium* analysiert. Während *Tc-tld* pRNAi zu einer reduzierten BMP-Aktivität im Embryo führt, verhindert *Tc-tsg(cv)* pRNAi diese vollständig. Diese Beobachtungen zeigen deutliche Veränderungen zu den Mechanismen in *Drosophila*, wo *Dm-tsg* nur teilweise für die Dpp-Aktivität benötigt wird. Die Ergebnisse deuten auf eine essentiellere Funktion Tsg/Cv-ähnlicher Proteine im BMP-Signalweg in *Tribolium* hin. Im Drosophilagenom befinden sich duplizierte Kopien von *tsg(cv)*- und *tld*-verwandten Genen. Die Duplikation gefolgt von einer Sub-Funktionalisierung dieser Mediatoren könnte im Laufe der Evolution zu Veränderungen des BMP/Dpp-Gradienten geführt haben.

Im zweiten Teil dieser Arbeit wurde die funktionale Analyse des Toll-Signaltransduktionsweges durchgeführt. Dabei sollte die Frage beantwortet werden, weshalb der Dorsal-Gradient in *Tribolium* nicht stabil ist und schnell verschwindet, wohingegen Dorsal in *Drosophila* einen stabilen Gradienten bildet. In *Tribolium* ist ein negativer Rückkopplungsmechanismus für das dynamische Verhalten des Dorsal-Gradienten verantwortlich: Tc-Dorsal aktiviert zusammen mit einem seiner Zielgene (*Tc-twist*) die Transkription des I-κB Homolog *Tc-cactus*. *Tc-cactus* wiederum ist für die Termination der Dorsal Funktion verantwortlich. Anders als in *Drosophila* führt der Verlust von Tc-Dorsal zu einer periodischen DV-Musterbildung entlang der AP-Achse. Dies deutet auf ein selbstorganisierendes ektodermales Musterbildungssystem hin, welches unabhängig vom Mesoderm und maternalen DV-Polaritätsinformationen agiert.

Die Präsenz selbstorganisierender Musterbildungssysteme in Kurzkeiminsekten wie *Tribolium castaneum* stimmt mit dem regulativen Entwicklungstypus hemimetaboler Insekten überein, welcher in klassischen Fragmentierungs Studien vorgeschlagen wurde.

Die gewonnen Ergebnisse erklären außerdem die Organisation extraembryonalen Gewebes in Kurzkeiminsekten und machen deutlich wie Musterbildungsinformation vom frühen Embryo auf die Wachstumszone übertragen wird.

Zusammenfassend führte die funktionale Analyse der TGF-β und Toll-Signaltransduktionswege in *Tribolium* zu der Annahme, dass während der Insektenevolution intensive Veränderungen im DV-GRN stattgefunden haben.

Chapter 1 - General Background, specific objectives and methods

1.1 - The emergence of a concept: Evolution and development

In 1859, in the book "Origin of Species" Charles Darwin declared on p. 449 that common descent is "the hidden bond of connection which naturalists have been seeking under the term of the natural system….the structure of the embryo is even more important for classification than that of the adult. For the embryo is the animal in its less modified state; and in so far it reveals the structure of its progenitor" (Darwin 1859). From the middle till the end of the 19th century, the link between embryology and evolution were propagated mainly by Ernst Haeckel. For Haeckel not only all animals would share a common ancestor, but also embryological events in extant animals would reflect their evolutionary path (recapitulation theory). In other words, animals from distinct groups would appear very similar at a specific embryological stage, the so called phylotypic state; and would then progressively differ. Specific morphological traits of each group would only appear after the phylotypic stage (Haeckel 1866). Although the great similarity of vertebrate embryos at the phylotypic stage has been questioned (Richardson et al. 1997), it´s widely assumed that at the germ-band stage, insect embryos show a remarkable morphological similarity, a clear phylotypic stage (reviewed in Sander and Schmidt-Ott 2004).

In the beginning of the 20th century genetics and embryology had split in different disciplines. First, geneticists led by Thomas Hunt Morgan focused on the mechanisms involved in heredity. Although Morgan was initially sceptical about Mendel´s laws, he showed later that the chromosomes carry the genetic information. Morgan is considered the father of genetics and his research initiated the *Drosophila melanogaster* escalade as the golden bug of genetics. A second group, the experimental embryologists led by Wilhelm Roux and Hans Driesch, started the developmental mechanics studies ("*Entwicklungsmechanik*"), followed by Hans Spemann and Mangold in their seminal transplant experiments (Spemann and Mangold 1923). These are considered the precursor studies of the modern developmental biology. By that time, the ultimate goal of these developmental studies was to explain their results based on "the simple components of physics and chemistry" (reviewed in Gould 1977). Haeckel recapitulation theory was by that time rejected by the Thomas Morgan School of Geneticists and by the developmental mechanicists. These two areas stayed largely separate till the end of 80s, with some notable exceptions in the works of Waddington and De Beer (De Beer 1958; Waddington 1966).

Stephen Jay Gould in this seminal Ontogeny and Phylogeny (Gould 1977) provided the theoretical basis for the recent fusion of genetics and developmental mechanics. Intriguingly, the first framework for comparative studies was obtained from the characterization of embryonic mutants in *Drosophila* (Lewis 1978). The observation that molecules with a similar structural domain, the HOX genes, are involved in anteroposterior patterning in flies and vertebrates is probably the starting point of this new era: The Evolution of Development (Carroll et al. 2001).

Following studies led to the astonishing observations that the same set of molecules e.g. transcription factors and secreted molecules are involved in pattern formation in distantly related organisms, such as flies and mammals. For instance, Bone-Morphogenetic Protein (BMP) in vertebrates and its respective homolog Decapentaplegic (Dpp) in flies are secreted molecules that act in dorsoventral pattern formation in both groups, and probably in all animals with bilateral symmetry (Bilaterians) (Ferguson 1996). Several other examples of conserved sets of transcription factors and signalling molecules have been shown to act in pattern formation on several groups over the last two decades (Carroll et al. 2001).

During late 80s and 90s Evo-Devo started to emerge as a discipline divided in two sub-areas: the micro and macro-evolutionary studies. The first tackles how genotypic changes (nucleotide sequences), in related species or different populations, lead to phenotypic (trait) differences. Examples of these kinds of studies are the analysis of the evolution of bristle patterns or pigmentation among related Drosophillid species (Sucena et al. 2003, Jeong et al. 2008) or the genetic basis of pelvic reduction in threespine stickleback fishes (Shapiro et al. 2004).

The second major area of Evo-Devo involves comparisons of distantly related groups, the so called macroevolution. For instance, recently comparisons among the extant choanoflagellates plants, fungi and animals (Metazoa) provided convincing molecular evidence that choanoflagellates are more related to Metazoa than to fungi (a sister group to all Metazoa) (Philippe et al. 2004). Macroevolutionary as well as microevolutionary studies are based on phylogenetic trees, which are hypothesis about relationships among groups. The knowledge of the last common ancestor among related groups or species is fundamental for the interpretation of Evo-Devo studies (Jenner and Wills 2007).

In the pre-genome era these phylogenetic hypothesis were based on very scarce information such as only one or fewer genes per group of organisms studied, but this has changed in the past few years. The use of several concatenated genes to build a phylogenetic tree is called a phylogenomic approach (Telford 2007). This latter approach has recently

strengthened Evo-Devo, because 1) One can determine the direction in which developmental features are evolving; 2) the knowledge of the divergence times of branches in a tree, allows evolution rates to be inferred; and 3) Phylogenetic trees allow homologies to be inferred or, conversely, show that apparently homologous features are not so (Raff 2000; Raff 2007). The three points above are important to discuss the results in an Evo-Devo framework, as well as influencing the choice of new model organisms (Jenner and Wills 2007).

Studies using ribosomal RNA and phylogenomic approaches have changed the topology of the Metazoan Tree of Life. Before the end of 90s, the Bilateria Tree of Life was based on the view that animal evolution occured from simple to complex forms through gradual steps. This view was mainly based on the possession of a body cavity, the coelom. In this traditional view, acoelomates (e.g. Platyhelminthes), Pseudo-coelomates (e.g. Nematodes) and Coelomates (arthropods and chordates) appeared successively after the split from the last common Bilaterian ancestor, the UrBilateria. In contrast, in the new Metazoan Tree of Life Nematodes and Arthropods are grouped together in the Ecdysozoan clade, animals characterized by the presence of moulting cuticle. Annelids, Molluscs and acoel flatworms belong to another group, the Lophotrochozoans (Adoutte et al. 1999; de Rosa et al. 1999). Lophotrochozoans share a spiral mode of cleavage and ciliated larval forms that may be considered as modified trochophores. Both groups are protostomes (the first opening forms the mouth) while a third group contains the deuterostomes (the first opening during gastrulation forms the anus and mouth is formed from a second opening). In this new phylogeny hemichordates, echinoderms, cephalochordates, urochordates and vertebrates constititute the monophyletic group of deuterostomes. What has this new Tree of Life changed in Evo-Devo? Basically the two major invertebrate genetic model systems, *C. elegans* and *D. melanogaster*, belong to the same group, the Ecdysozoans and no Lophotrochozoa model system has been established so far. This situation is starting to change with the establishment of the the polychaete annelid *Platynereis* as a model system (Arendt et al. 2008). Additionally this new tree of Life also indicates that the deuterostomes, including chordates, are closer to the putative Urbilaterian organism, than previously believed. Important to mention is that although this new Tree of Animal Life is widely propagated, some controversy still exist (see e.g. Rokas and Carroll 2006).

The example above is just one among many of how the availability of sequenced genomes and ESTs of several species has changed remarkably our understanding of Metazoa evolution and the relationships among their groups. Recently the genomes of twelve

Drosophillids species have been sequenced (Stark et al. 2007), and more are expected to come.

A striking observation from the genome sequencing and developmental studies in *D. melanogaster* and vertebrates is that the same basic set of molecules is involved in similar pattern mechanisms of all animals investigated so far (Carroll et al. 2001). An immediate question then came up: How can complex vertebrates like us be different from a fruit fly, if both groups share a comparable amount of developmental genes in their genomes? Again studies on the developmental genetics of *D. melanogaster* helped Evo-Devo researchers to build a "working hypothesis", centred on gene regulation.

In the beginning of 90s, studies on gene regulation of *even-skipped (eve),* a gene expressed in seven stripes in the *D. melanogaster* early embryo, provided the first hint that gene regulation would play a major role in these differences (Small et al. 1992; Arnosti et al. 1996). Mike Levine and colleagues discovered that the expression of every *eve* stripe in the *D. melanogaster* embryo is regulated by a different stretch of DNA (Module) present in the non-coding part of the DNA. From this example, it was inferred that every developmentally regulated gene contains independent DNA modules (cis-regulatory modules- CRMs), typically around 500 base pairs, that control its expression during development. From the combinatorial action of several CRMs, one can observe the seven stripes of *eve*. This example highlights the modulatory nature of the control of developmental genes. The employment and variation among different CRMs has been invoked to explain the disparate morphological variation among species. At least two pieces of evidence favor that view: 1) developmentally regulated genes, i.e., genes involved in several processes of development such as transcription factors or signalling molecules were shown to contain larger intergenic regions comparatively to structural genes in *D. melanogaster* and *C. elegans* (Nelson et al. 2004a). Structural genes, by Sean Carroll´s definition are primarily ubiquitous or 'housekeeping' genes, general transcription factors, ribosomal constituents or genes acting in metabolism (Carroll 2005b). This larger amount of non-coding DNA (intergenic) region in developmentally regulated genes would be related to the existence of several CRMs. This allows the same gene to act independently in several developmental contexts due to its several independent CRMs. A direct consequence from these results is that the modular nature of the CRMs may trigger evolutionary innovations, since mutations or appearance of new CRMs do not affect the coding region of a given gene. Coding regions of genes important for embryonic development are normally under negative selection due to its several roles in different processes (pleiotropy) (reviewed in Prud'homme et al. 2007). 2) The ratio of non-coding/coding DNA in

the human genome is much higher than in *Drosophila*, so it was proposed that most of the differences among these two groups rely on the non-coding part of the DNA (Carroll 2005a).

This concept of regulatory evolution is a central theme in Evo-Devo studies, although only recently clear examples of phenotypic variation due to CRM changes have been demonstrated (Jeong et al. 2008; Marcellini and Simpson 2006; McGregor et al. 2007). A global model to understand how changes in CRM may contribute to evolution is being generated by the analysis of entire gene regulatory networks (GRNs). Examples of well understood GRNs are the endomesodermal specification in sea urchin (Davidson et al. 2002), the *Ciona intestinalis* (Urochordate) heart differentiation (Satou and Satoh 2006), the C-Lineage Specification in *C. elegans* (Maduro et al. 2001; Baugh et al. 2005) and the dorsoventral (DV) axis formation in *Drosophila melanogaster* (Stathopoulos and Levine 2005) .

Altogether, Evo-Devo is a new discipline that aims to bring together knowledge from cell, molecular and developmental biology in an evolutionary framework. Consequently, as any evolutionary hypothesis, Evo-Devo is based on the evolutionary relationships among organisms (phylogenetic trees). The current thesis focuses on the macroevolutionary area of Evo-Devo, by studying the dorsoventral axis formation in *Tribolium castaneum* (beetle, Coleoptera) in comparison with the genetic model *Drosophila melanogaster* (fly, Diptera).

Beetles and flies share a last common ancestor about 274-285 million years ago (Savard et al. 2006b, Zdobnov and Bork 2007). The vast knowledge of the dorsoventral GRN in *D. melanogaster* (described below), enables a direct comparison of these two genetic networks. As the whole Evo-Devo area is now in the “genome sequencing era”, this thesis has largely profited from the genome sequencing of *Tribolium castaneum*. Before the description of any molecular comparative analysis, it is important to describe the major embryological events occurring in insects.

1.2 - Insect embryology and key questions in insect Evo-Devo

1.2.1 - Oogenesis modes and their correlation with germ types

Insect embryogenesis is highly variable among the different groups. On the other hand a few general trends can be observed. Among the winged insects the holometabolous insects, containing larval and pupal stages, have generally faster embryonic development than the

hemimetabolous, which hatch as nymphs (mini-adults). This embryonic "acceleration" is correlated with changes in oogenesis mode during evolution.

The panoistic oogenesis mode found in many hemimetabolous insects is believed to be the ancestral type (Bier 1970). This oogenesis type has oocytes lacking nurse cells, a common situation in the Metazoans. Irrespective of its oogenesis type, each ovary consists of several ovarioles. Each ovariole contains a germarium located anteriorly (or proximally) followed by the vittelarium where an array of oocytes in progressively stages of development can be observed (Figure 1.1).

Telotrophic meroistic and polytrophic meroistic ovaries are found mainly in holometabolous insects. In both types nurse cells exist, which are absent in panoistic ovarioles. In telotrophic meroistic ovarioles the nurse cells do not accompany the oocytes on their way through the vitellarium. This type of ovary is found for instance in beetles such as *Tribolium castaneum* (Trauner and Buning 2007). In contrast, polytrophic meroistic ovarioles contain a cluster of nurse cells, derived from the germline, and a unique posteriorly located oocyte, which migrate together along the vittelarium, surrounded by a somatic monolayer epithelium, the follicle cells. Nurse cells are responsible for synthesis of RNAs, ribossomes and other molecules, which are required during embryonic development. Polytrophic ovaries are found in hymenopteras, such as *Nasonia vitripennis* (Olesnicky and Desplan 2007), lepidopterans and dipterans such as *Drosophila melanogaster* (Roth 2003). In general, insects containing polytrophic meroistic ovaries have a faster embryonic development when compared to insects with panoistic ovaries, due to the "pre-fabrication" of organelles performed by the nurse cells.

Importantly, a somatic follicle cell layer surrounding the oocyte is a common feature of insect oogenesis. In addition, in all insect species observed so far, the oocyte nucleus moves to an asymmetric position in later stages of oogenesis (Roth 2003). In *Drosophila* this final asymmetric position of the nucleus defines the dorso-anterior position of the future embryo. This nuclear migration is important for the establishment of the dorsoventral polarity in *Drosophila*, and may be a conserved feature of insect embryogenesis (reviewed in Roth 2003).

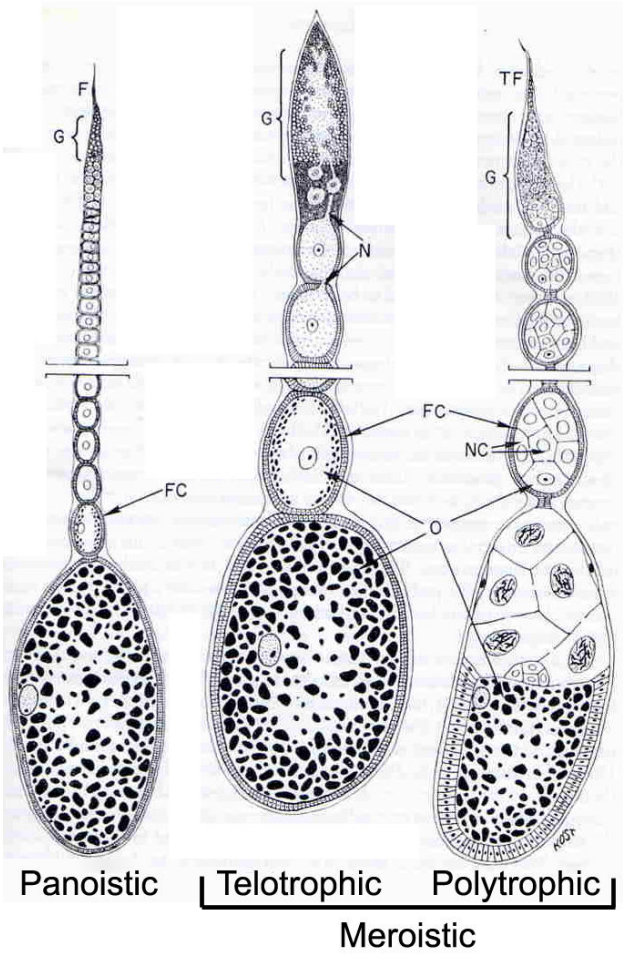


Figure 1.1: The three ovary types present in insects.
Panoistic ovaries are found in the cricket *Gryllus bimaculatus*, meroistic telotrophic, the beetle *Tribolium castaneum*, and meroistic polytrophic in the fly *Drosophila melanogaster*.Adapted from Anderson 1972. (TF) Terminal filament, (FC) Follicle cells, (NC) nurse cells, (G) germarium, (NC) nurse cells, (N) nutritive cords, (O) oocyte.

Bier, in a seminal paper (Bier 1970), was the first to propose a correlation among the type of oogenesis and the size of the germ anlage, the region of the egg that gives rise to the embryo. Before establishing this correlation, it is important to explain the differences in germ anlagen among insects. Thirty years before Bier, Gerhard Krause divided insects in short, semi-long (intermediate) and long-germ type according to the size of the germ anlage (Krause 1939). In one extreme, the short-germ type, where the germ anlage contains essentially the procephalon (head) and the remaining metameric part of the body emerges from the budding zone (growth zone) by a secondary process. In the other extreme, the long-germ type, where the different body regions in the germ anlage are represented proportionally to their relative position in the germ band. Intermediate germ types lie in between, since not only head but

also gnathal segments are present before gastrulation, but the abdominal segments are generated later during development from the growth zone.

Nowadays, two different approaches are used in the distinction among insects with short, intermediate or long-germ-types of development. The first approach is based on the timing of segment specification relative to ventral furrow formation. Segment specification would be, in this first view, defined by the appearance of *engrailed* expression, which marks the irreversible segment determination in *Drosophila* (Davis and Patel 2002). Since *engrailed* expression is variable in relation to gastrulation movements in many Diptera species, which are presumably all long-germ type, this approach has been recently questioned (Roth, 2004). The alternative approach proposed by Roth, 2004 involves fate-map establishment after local irradiation of small parts of the germ anlage. Using this latter approach, no convincing evidence of germ anlage containing only the head segments (short-germ type) has been so far observed (Roth, 2004). In this thesis the term short-germ development will be adopted for all insects which generate segments from the posterior growth zone and long-germ type of development for embryos where all presumptive segments are specificied early along the AP axis and no secondary growth zone exists. Examples of long-germ type are *Apis mellifera* (Hymenoptera) and *Drosophila melanogaster* (Diptera), and of short-germ the beetles *Tribolium castaneum* and *Atrachya menetriesi*. In addition, beetles also contain species undergoing long-germ development such as *Bruchidius villosus*.

Long-germ type of development occurs mainly in holometabolous insects, which present meroistic type of oogenesis. Apparently, a "pre-fabrication" of organelles by the nurse cells in meroistic types can provide the oocyte with molecules required for early embryogenesis, leading to faster development. Until recently it was widely accepted by morphological and embryological evidence that the emergence of polytrotrophic ovaries in Hymenopterans and Dipterans would have a common origin (Figure 2A). In this "traditional" version of insect phylogeny, beetles (Coleoptera) including *Tribolium,* would occupy a basal position in relation to Hymenoptera and Diptera. Recently, this traditional phylogenetic tree has been questioned by a phylogenomic approach (Figure 2B, Savard et al. 2006b; Zdobnov and Bork 2007). According to this new tree Hymenoptera would be basal to Coleoptera and Diptera. What are the possible implications of this new phylogenetic tree? It could imply that the meroistic polytrophic ovaries and the long-germ development of Hymenoptera and Diptera have emerged twice independently. Either in the "modern" or in the "classical" phylogenetic trees, the Eumetabola ancestor is believed to be a short-germ type insect. On the other hand, in the "modern" phylogeny, the holometabolous ancestor might have been a long-

germ insect (Figure 2B). So, although widely propagated as an example of a primitive short-germ insect, *Tribolium* short-germ type of development might have arisen secondarily from a long-germ ancestor.

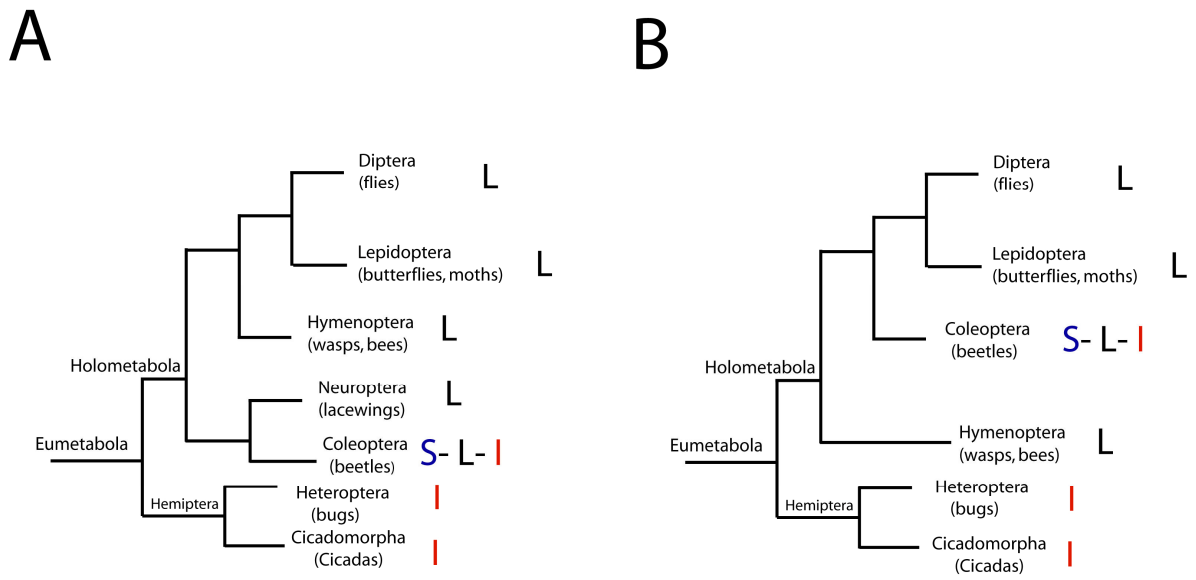


Figure 1.2: Schematic drawings of classical phylogenetic relationships among insect groups (A) and the new molecular phylogeny (B). L - long-germ type of development, S-short-germ type of development, I - intermediate-germ type of development. No species belonging to the Neuroptera order was used for the molecular phylogeny, so this group is omitted from B.

1.2.2 - From classical embryology to the molecular era

Classical embryological experiments involving transplantions, centrifugation and UV or X-ray irradiation have been extensively performed by several researchers until the end of the first-half of the 20th century. These studies suggested the existence of two separate systems involved in patterning the embryo (reviewed in Sander 1976). The longitudinal axis, nowadays called anteroposterior (rostro-caudal), and the transversal axis, the current dorsoventral axis, were described much before any molecular study was carried out. Although a complete review on these topics is out of the scope of the present thesis (see for instance Sander 1976), it is important to mention the conceptual framework which arose from those experiments.

Eggs of the leaf hopper *Eucelis plebejus* were fragmented along the longitudinal and transversal axis (Fig 1.3A) during early cleavage stage, and observed during several successive days (Sander 1971). Interestingly, eggs fragmented along the longitudinal axis, which disrupts the dorsoventral axis, displayed a remarkable capacity of recovery (Fig 1.3B).

Complete twinning was observed in several cases (Figure 1.3C), suggesting that “Embryonic Regulation” occurred. “Embryonic Regulation” was previously defined by Hans Driesch (1909) as “an event or group of events which occur in the living organism after disturbances and result in restitution of the normal functional state or lead toward this restitution” (Driesch 1909). This type of complete germ-band regeneration was never observed after transversal sectioning (Sander 1959; Sander 1971). After transversal sectioning, which disturbs the anteroposterior axis, the fragments observed after recovery contained just parts of germ bands (Fig 1.3B). Normally, these partial germ-bands generated fewer segments than expected from their location in the egg before the fragmentation. In conclusion, these experiments clearly indicated that two different systems operate in the embryonic pattern of the leaf hopper, one of them clearly displays “Embryonic Regulation” (dorsoventral), while the other does not (anteroposterior).

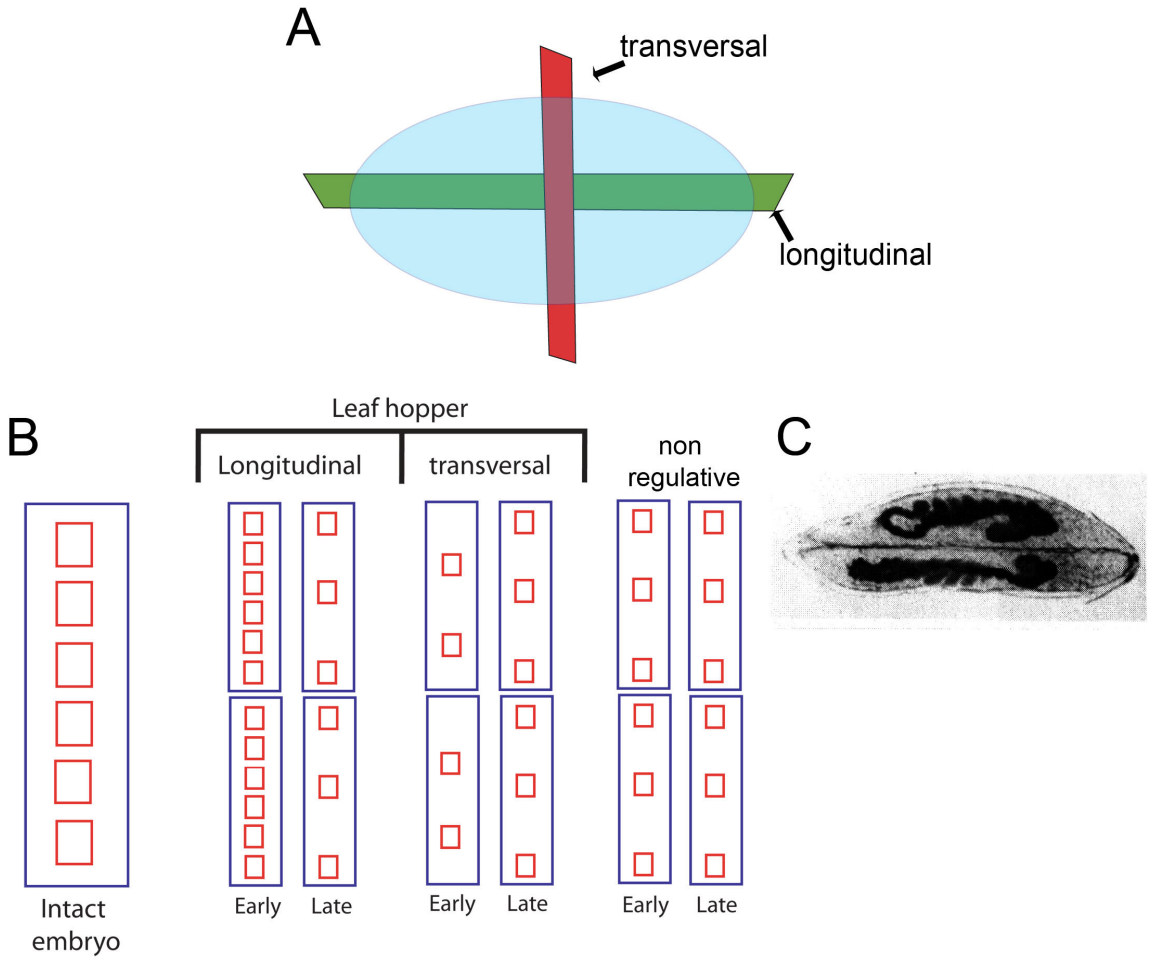


Figure 1.3: Summary of experimental results obtained by classical fragmentation studies
(A) Using leaf hopper eggs, Klaus Sander performed transverse and longitudinal fragmentation studies (Sander 1959; Sander 1971). Longitudinal sections disrupt the dorsoventral axis while transversal sections disrupt the anteroposterior axis. (B) Summary of the results obtained with leafhoppers and with non-regulative (originally called "mosaic") embryos (e.g. *Drosophila*). The height of the frames represents the size of the system or fragment. After early longitudinal fragmentation, two separate embryos containing a complete pattern can be generated. So, more pattern elements than expected are generated. If the same longitudinal fragmentation is performed later in development, this phenomenon does not occur. After transversal fragmentation, the fragments produce less pattern elements than expected. Non-regulative embryos after longitudinal fragmentation generate pattern elements according to their fates, so the expected amounts of pattern elements are generated in early or late fragmentation. Taken together, these results suggest more flexibility in the mechanism responsible for dorsoventral axis (longitudinal sections) than in anteroposterior axis patterning (transversal sections) in the leaf hopper. (C) An example of complete twinning formation after longitudinal fragmentation.

The existence of extensive embryonic regulation along the dorsoventral axis has also been reported in the beetle *Atrachya menetriesi*, where up to four embryos may be formed from one egg after cold-shock treatment (Miya and Kobayashi 1974). Occurrence of two embryos in a single egg (dorsoventral duplication) is also observed in spiders, basal arthropods under natural conditions (Holm 1952, Oda and Akiyama-Oda 2008).

Similar experiments using long-germ embryos such as *Drosophila melanogaster* did not provide similar results in relation to regeneration. After sectioning, UV irradiation or transplantion almost no embryonic regulation takes place (reviewed in Sander 1976) . For this reason these embryos are called non-regulative (mosaic in Sander, 1971) embryos, where their cells are determined early in development and cannot acquire a new function after injury (Sander 1971). It is noteworthy to mention that transplantion and centrifugation experiments also provided evidence for the existence of an anterior determinant (Sander 1976, Kalthoff 1983) before its molecular nature was uncovered (Driever and Nusslein-Volhard 1988; St Johnston and Nusslein-Volhard 1992). Importantly, regulation of the dorsoventral axis after injury as described above in short-germ embryos does not occur in long-germ embryos like *Drosophila.* This indicates that changes on the events responsible for the DV axis patterning axis must have occurred during insect evolution.

1.3 - *Drosophila melanogaster* molecular findings and their consequences for understanding the evolution of insect embryogenesis

The molecular characterization of numerous mutants affecting early patterning has been carried out during the last 25 years. These studies led to the conclusion that four maternal genetic systems, containing several genes each, are involved in setting up the polarity along the anteroposterior and the dorsoventral axis. First, the anterior system is involved in the anterior localization of *bicoid,* a transcription factor that activates zygotic downstream genes involved in the anteroposterior pattern. Second, posterior group genes (e.g. *oskar, nanos, vasa*) are involved in establishing the pole plasm, which is required for germ cell development, besides their requirement in posterior pattern. Third, the terminal class genes ensure that the receptor Torso is activated both at anterior and posterior poles of the embryo. Fourth, the DV system sets up the dorsoventral polarity of the egg (St Johnston and Nusslein-Volhard 1992). Downstream of these maternal systems zygotic genes such as gap, pair-rules, segment polarity and Hox genes are involved in the establishment of the anteroposterior (AP) axis. The fact that Hox genes are expressed in similar regions in several insect species during the phylotypic stage, the late germ band in insects, suggested that a conserved molecular mechanism would act at that stage (reviewed in Sander and Schmidt-Ott 2004).

On the other hand an overall variation in the role of these four maternal systems has been observed in insects. Duplications followed by subfunctionalization of key factors played

an important role in evolutionary changes. A classic example of an important duplication during insect evolution was described for the AP patterning system. *bicoid*, a core gene in this system, has arisen by a duplication event from a derived *Hox3* gene (*zerknüllt, zen*) in the common ancestor of the cyclorrhaphan flies (Stauber et al. 1999; Stauber et al. 2002). Since *bicoid* activates most of the anterior zygotic genes such as gap and pair-rule genes, the emergence of this gene was probably a key evolutionary innovation for anterior patterning. In beetles and wasps, *orthodenticle* (*otd*) and *hunchback* (*hb*), two *bicoid* target genes in flies, are responsible for anterior pattern, constituting the putative ancestral system in insect evolution (Schroder 2003; Lynch et al. 2006a).

The role of terminal genes was also investigated in other insects. Interestingly, the function of the terminal system, e.g. torso signalling, is apparently conserved in both poles among short-germ insects like *Tribolium* and long-germ *Drosophila* (Schoppmeier and Schroder 2005). Conversely, the function and regulation of *tailless*, a transcription factor involved in the establishment of the terminal regions in the fly seems not to be conserved in the wasp *Nasonia vitripennis* (Lynch et al. 2006b).

The role of posterior genes such as *vasa* and *nanos* in specifying germ cell development is apparently conserved not only in insects (Lall et al. 2003), but also in Lophotrochozoans and non-bilaterian animals (Extavour et al. 2005, Tsuda et al. 2003; Sato et al. 2006). On the other hand, *oskar* which is also involved in the determination of germ cell fate, besides the determination of posterior polarity, is not conserved at the sequence level outside Drosophillids.

1.3.1 - Understanding the dorsoventral (DV) system: the establishment of polarity during oogenesis

The last maternal system, the DV system is the main topic of the current thesis. 17 maternally provided factors were identified by genetic screenings and are involved in the formation of a ventralizing signal in the fly embryo. This ventralizing signal and embryonic dorsoventral polarity are dependent on events taking place during oogenesis (reviewed in Roth 2003). The *Drosophila melanogaster* oocyte at mid-oogenesis (stage 8-10) is surrounded by a somatic layer of follicle cells (Fig 1.4). At stage 9 the first visible sign of symmetry breaking along the dorsoventral axis occurs. The oocyte nucleus migrates from a posterior position towards an anterior asymmetric position along the DV axis. The oocyte nucleus final destination determines the dorso-anterior location of the oocyte (Micklem et al. 1997; Peri and

Roth 2000). At that region, the oocyte nucleus acts as a signalling center, since the mRNA of the secreted TGF-α-like ligand Gurken (Grk) accumulates around it in an anterior-dorsal cap (Neuman-Silberberg and Schupbach 1993). This localization of Grk mRNA in the anterior-dorsal cap is required for the correct orientation along the future DV axis, but not for the establishment of the different cell fates. This conclusion was obtained from the analysis of mutants where Grk mRNA is mis-localized, which display all DV fates along the AP axis (Roth and Schupbach 1994).

The secretion of Grk activates the *Drosophila* Epidermal Growth Factor receptor (Egfr) present in the overlying dorsal follicle cells, generating DV polarity in the follicle epithelium. This Egfr activation in the dorsal region leads to the restriction of *pipe* expression in ventral follicle cells by an unknown mechanism (Nilson and Schupbach 1998). *pipe* encodes a heparan sulphate 2-*O*-sulphotransferase, so it is generally assumed that *pipe* is involved in the modification of an unknown glycoprotein or glycolipid-associated carbohydrate in the perivitelline space. Recent evidence have discarded all glycosaminoglicans carbohydrates (heparin sulfate, heparin, chondroitin sulfate and dermatan sulfate) as *pipe* substrates (Sen et al. 1998; Sen et al. 2000; Zhu et al. 2005; Zhu et al. 2007), so the glycoprotein which acts as a substrate for Pipe remains unknown. The *pipe* domain extends around 40% of the ventral embryonic circumference, a similar width as the final Dorsal nuclear gradient (figure 1.4).

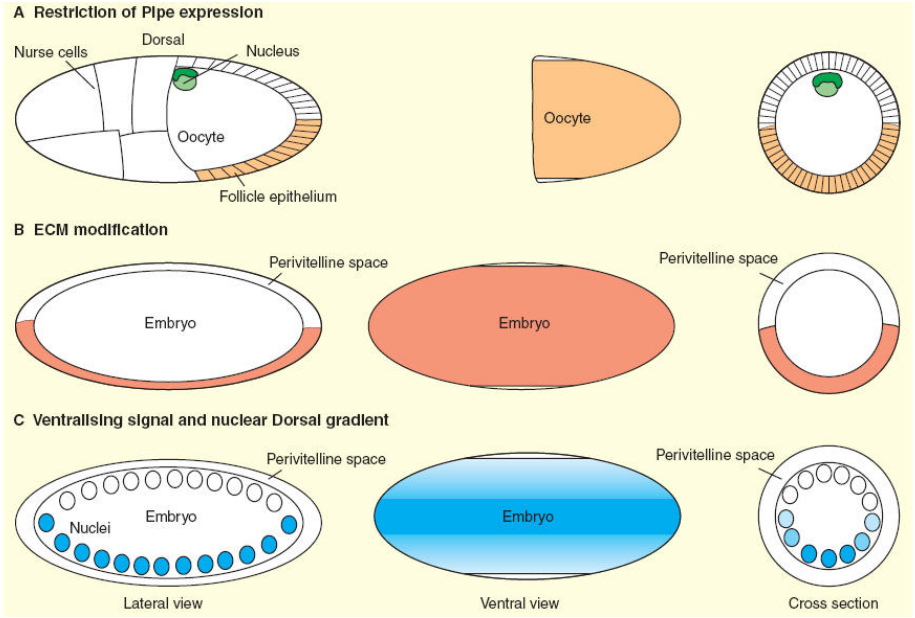


Figure 1.4: Major events during dorsoventral axis formation in *Drosophila*.
(A) Gurken (green), a TGFα protein emanating from a dorsal source in the oocyte, represses *pipe* (orange) in the follicular epithelium, restricting its expression to a ventral stripe. (B) Pipe leads to the modification of an unknown ECM component (red) which is secreted by the follicle cells and maintained within the extracellular space surrounding the late oocyte and early embryo. (C) Subsequently, an extracellular signal is generated within the region defined by the modified ECM component. This signal induces the nuclear transport of the transcription factor Dorsal inside the embryo. The signal has peak levels within a narrow stripe straddling the ventral midline (dark blue). Picture from Moussian and Roth, 2005 Curr Biol.

Importantly, this *pipe* expression domain cannot explain why the graded nuclear Dorsal gradient is achieved, since the ventral most 15% region of the embryonic circumference contain the highest amounts of nuclear Dorsal. Apparently, the *pipe* expression domain exerts a permissive role, but downstream events in the perivitelline space are required for the establishment of the correct graded nuclear Dorsal gradient inside the embryo. These downstream events are mediated in the perivitelline space by a cascade of four proteases (*gastrulation defective, snake, easter, nudel*), the serine protease inhibitor *serpin27A* and *spätzle*, a Nerve Growth Factor-like (NGF-like) molecule, which is the ligand of the receptor Toll (reviewed in Moussian and Roth 2005). Pairs of activators and inhibitors act in the perivitelline space, showing that extensive feedback regulation occurs in this region. The carboxy- and amino-terminal fragments of *gastrulation defective* (GD), the protease Easter and its inhibitor Serpin27A, and the carboxy and amino-terminal fragments of Spätzle (reviewed in Moussian and Roth 2005) are these pairs of activators-inhibitors, which are required for the formation of a unique peak of Dorsal activity in the ventral embryonic region.

Spätzle mRNA injection may generate embryos with duplicated axis, as judged by two opposite *twist* expression domains. Such axis duplication phenomenon also occurs in *egfr* and *grk* mutant eggs, where an expanded *pipe* expression domain in the follicle cells is observed (Morisato 2001). These axis duplications might arise from perturbation of the balance between the ligand (Spätzle) and its inhibitor (Spätzle amino-terminal fragment). Higher concentrations of unprocessed Spätzle or an expansion of the ventral domain where Spätzle is processed (in *egfr* or *grk* mutants) are likely to cause increased inhibitor concentrations at ventralmost positions. It is clear that the molecular events responsible for axis duplication occurs outside the embryo, since embryos with uniform high amounts of Toll signalling, show *twist* expression along the whole dorsoventral axis, but do not exhibit a duplicated axis (Roth et al. 1989; Roth and Schupbach 1994, Morisato 2001).

1.3.2 – Downstream events inside the embryo

Upon Spätzle signalling in the ventral region, a cascade of downstream events takes place inside the embryo. A complex involving Toll, the adaptor molecules Tube and Krapfen/Myd88, and the serine/threonine kinase Pelle is formed upon signalling. This leads to a local increase of Pelle concentration, resulting in autophosphorylation of this kinase. Remarkably, an activated form of Pelle is able to generate all known Toll-Dorsal patterning

thresholds when ectopically expressed along the AP axis. This implies that all required functions downstream of the Toll pathway can be mediated by the kinase Pelle (Stathopoulos and Levine 2002b).

This kinase phosphorylates Toll and Tube, thereby being released from the receptor complex (Fig 1.5). Pelle can then act directly on Cactus and Dorsal. The phosphorylation of Cactus, followed by its ubiquitination and degradation by the proteasome, releases Dorsal in the cytoplasm. Phosphorylated Dorsal then dimerises and enters the nucleus (Fig 1.5). Tamo, Drosophila Nuclear Transport Factor-2 (DNTF-2) and Members-only (Mbo), a nuclear pore protein, also interacts specifically with Dorsal and are required for nuclear transport (Fig 1.5). This nuclear transport is independent of Toll signalling. Importantly, Cactus degradation only occurs in the ventral regions where Spätzle binds to Toll, so the dorsal region of the *Drosophila* egg is free of nuclear Dorsal (reviewed in Moussian and Roth, 2005).

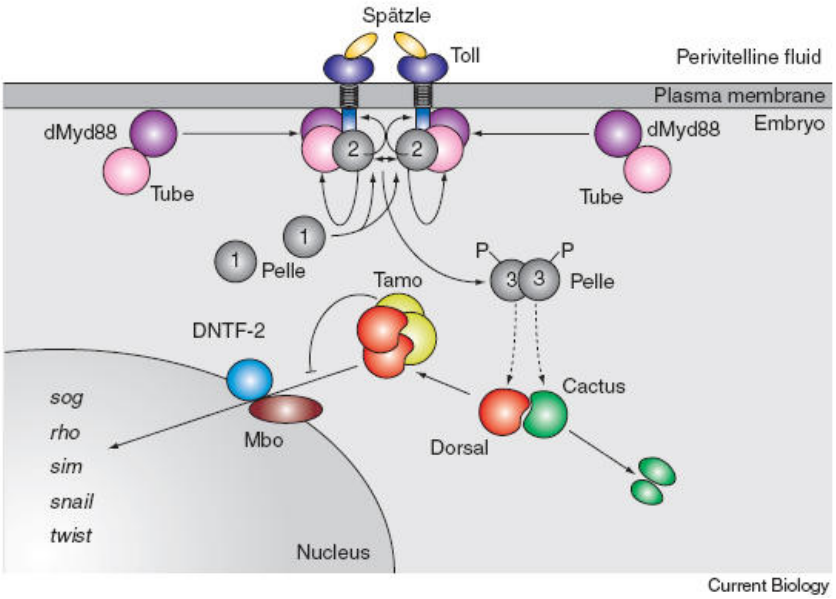


Figure 1.5: The cytoplasmic events downstream of the Toll receptor.
The activated Toll receptor recruits the membrane-localised dMyd88–Tube heterodimer to the intracellular domain of the activated Toll receptor. Subsequently, the kinase Pelle is recruited to the heterotrimeric Toll–dMyd88–Tube complex and undergoes autophosphorylation, thereby enhancing its kinase activity. By phosphorylation of Toll and Tube, Pelle is released from the heterotetrameric complex. At this step, signalling is disrupted, and the Toll–Spätzle complex is presumably internalised. Next, Pelle transduces the signal to downstream factors, resulting in the degradation of Cactus. This allows binding of Dorsal to Tamo which regulates nuclear entry of Dorsal through a nuclear pore containing DNTF-2 and Mbo. From Moussian and Roth, 2005 Curr Biol.

1.3.3 - Dorsal has a central role in the Gene Regulatory Network (GRN) responsible for DV axis formation

Once Dorsal is transported to the nuclei after Cactus degradation in ventral regions, this transcription factor activates several genes along the *Drosophila* DV axis. High amounts of Dorsal activate *snail (sna)* and *twist (twi)* in the prospective mesoderm, progressive lower amounts activate *short-gastrulation (sog*), *brinker (brk)* and *rhomboid (rho)* in the neurogenic ectoderm and even lower amounts repress *zerknüllt (zen)* and *decappentaplegic (dpp)* from the non-neurogenic ectoderm (dorsal ectoderm) (Fig 1.6, Stathopoulos and Levine 2002a). The above described genes are just examples of Dorsal target genes that were discovered by classical genetic screening (around 30 direct targets).

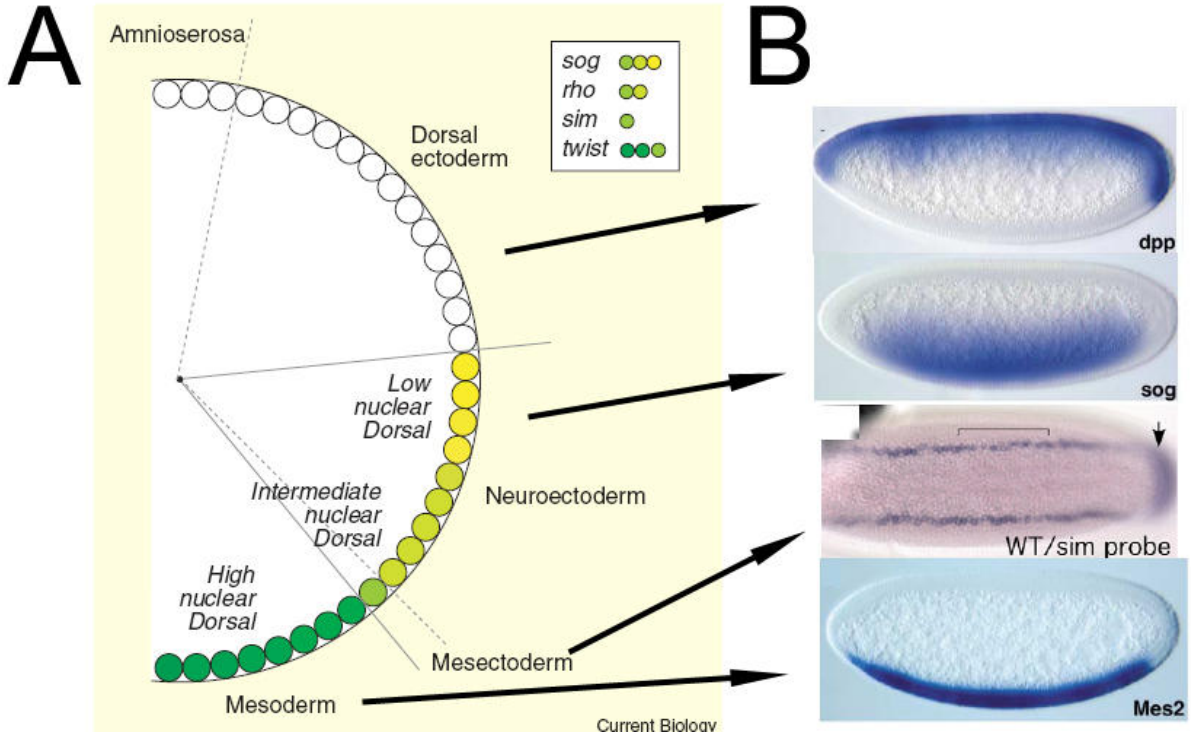


Figure 1.6: Positional information generated by the Toll pathway.
(A) A schematic half cross-section of an early *Drosophila* embryo; the nuclear Dorsal gradient leads to the activation of target genes in different regions along the dorsoventral axis. (B) Lateral views with the exception of *sim* which is a ventral view. Genes are activated in the presumptive mesoderm (*Mes2* in B), mesectoderm (*single-minded* - *sim* in B) and neuroectoderm (*sog* in B). Genes such as *dpp* are repressed from the non-neurogenic (dorsal ectoderm) region.

cDNA microarrays uncovered additional 20-40 direct Dorsal target genes (Stathopoulos et al. 2002). Recently, tilling array screenings (Biemar et al. 2006) and chromatin immunoprecipitation (ChIP)-on-chip (Sandmann et al. 2007; Zeitlinger et al. 2007) approaches added more 30 genes to this GRN. Since this latter approach covers the vast majority of the *Drosophila melanogaster* genome, 80-100 Dorsal target genes might be closer

to the actual number of Dorsal targets (Fig 1.7b). As can be observed in Figure 1.7a, not only Dorsal has a central role in this GRN, but also Dorsal target genes can be separated in different categories (colors in the picture) depending on the region of the embryo they are expressed. In blue one can observe genes expressed in the mesoderm, in yellow genes expressed in the neurogenic ectoderm and in green genes expressed in non-neurogenic ectoderm and in the amnioserosa (Fig 1.7a). Importantly, these genes are expressed in different embryonic territories due to the different characteristics of their DV enhancers (Fig 1.8).

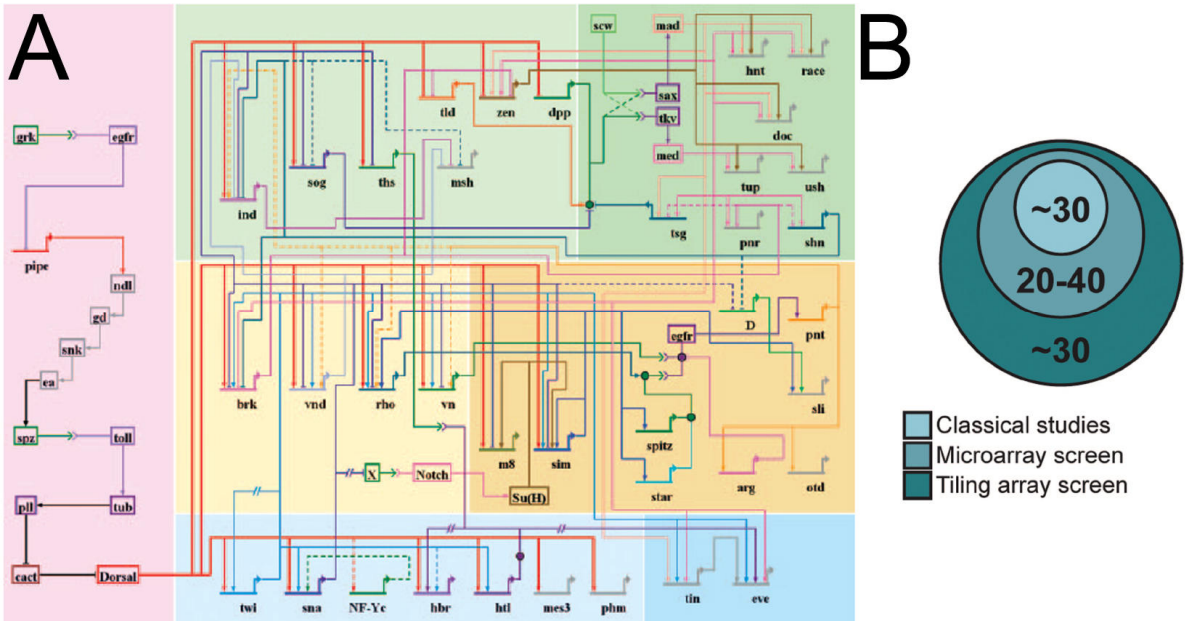


Figure 1.7: The Gene Regulatory Network responsible for DV axis formation in *Drosophila.*
(A) Dorsal directly regulates several enhancers in the mesoderm (light blue), in the mesectoderm (dark yellow), in the neurogenic ectoderm (light yellow), and in the dorsal ectoderm (light green). So, Dorsal is a central molecule in this GRN. (B) Between 80-100 enhancers are Dorsal direct targets in *Drosophila* genome, as judged by whole genome studies like, tilling arrays.

Genes responding to the Dorsal gradient can be divided in at least three different types, I, II and III. This distinction is based on the individual binding sites present on their enhancers and the region along the DV axis where the gene is expressed (Fig 1.8, reviewed in Stathopoulos and Levine 2004). Genes expressed in the ventralmost region, the presumptive mesoderm, contain the so called type I Dorsal responsive enhancer. Correlated to the highest amounts of Dorsal and Twist present in the mesoderm, enhancers of genes expressed in this

region, e.g. *twist*, *snail*, *mir-1*, *htl*, contain binding sites with the lowest affinity for the transcription factor Dorsal (Fig 1.8). In addition these enhancers also contain binding sites for Twist (Ebox). Genes containing type II enhancers such as *brk*, *ventral neuroblasts defective (vnd)*, *rho*, and *vein (vn)* contain binding sites for the repressor Snail, so they are excluded from the mesoderm (Fig 1.8, reviewed in Stathopoulos and Levine 2004). These genes also contain binding sites for the transcription factor involved in the Notch signalling pathway, Suppressor of Hairless (*SuH*), as well as Twist and Dorsal. Interestingly, although Twist is known to be a mesodermal determinant, Twist antibody stainings have shown that the Twist domain extends around 5 cells onto the prospective neurogenic ectoderm (Kosman et al. 1991, Leptin 1991), so the aforementioned neurogenic ectodermal genes depend on Twist expression and contain high affinity binding sites for Twist (Papatsenko and Levine 2005). Genes containing type III enhancers contain optimal binding sites for Dorsal and are expressed in regions where the amount of this transcription factor is the lowest. *sog* and *thisbe (ths)* are activated by Dorsal, while other genes such as *tolloid (tld)*, *dpp* and *zen* are repressed by it and by the co-repressors Groucho and CtBP (reviewed in Stathopoulos and Levine 2004). Taken together, the different amounts of Dorsal along the DV axis correlate to distinct enhancer organizations along this axis. So, Dorsal can be considered as a *bona fide* morphogen in *Drosophila* (Wolpert 1969).

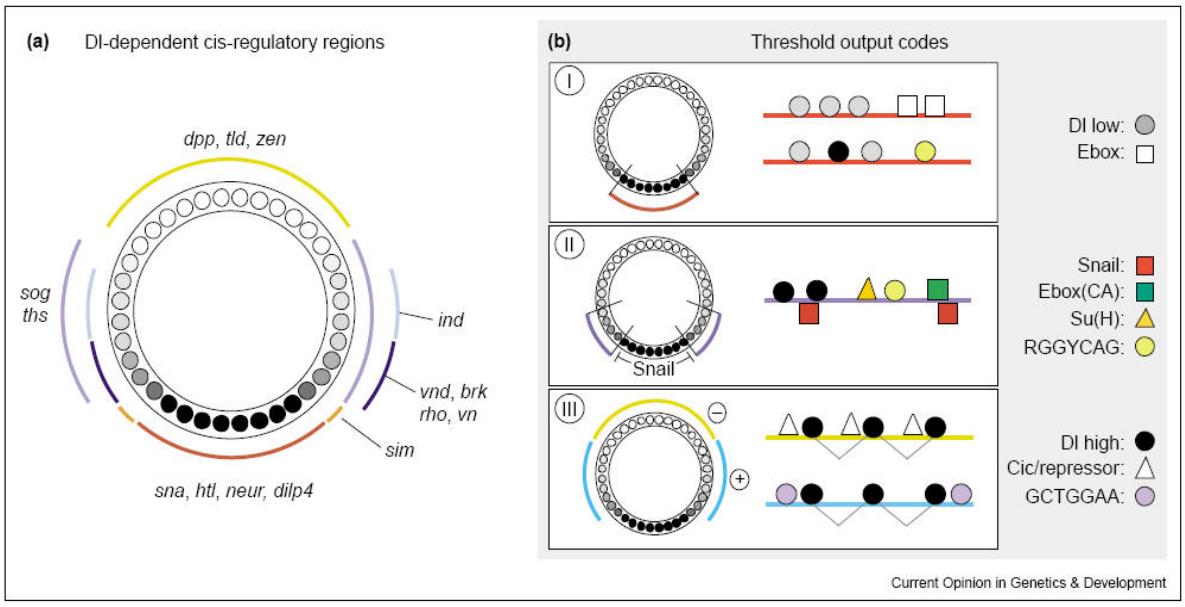


Figure 1.8: Dorsal-dependent cis-regulatory regions in Drosophila DV patterning
(A) Genes regulated by Dorsal along the *Drosophila* DV axis. (B) The known Dorsal-target gene enhancers can be categorized into three different classes. Type I enhancers contain low-affinity dorsal binding sites and may also contain E-box sites, or binding sites for Twist. Type II enhancers have binding sites for Dorsal, Twist (E-boxes with a CA core), Snail, Su(H), and a transcription factor that recognizes the RGGYCAG motif, possibly Dip3. Type III enhancers support expression in a broad lateral domain and contain high-affinity Dorsal binding sites that are closely spaced. Adapted from Stathopoulos and Levine 2004.

1.3.4 - Decapentaplegic (BMP/Dpp) and its modulators constitute a conserved network involved in DV patterning

dpp is directly repressed by Dorsal in fly embryos, so its expression is confined to the 40% most dorsal region. In addition, two other mechanisms repress Dpp activity in ventral regions, one involving *sog* and the other *brk*, which are described in detail in Chapter 3. Dpp itself is a morphogen since high amounts of Dpp activate genes in the amnioserosa and progressive lower amounts control non-neurogenic ectodermal genes (Ashe et al. 2000).

The role of BMP/Dpp4 and its modulators, *e.g* Sog/Chordin, Twisted-gastrulation (Tsg), Tolloid (Tld), in DV patterning is conserved during Bilateria evolution (Ferguson 1996, De Robertis 2008, Chapter 3). Studies in *Xenopus laevis* and *D. melanogaster* have shown that a similar set of molecules is involved in this process. Briefly, the conserved role of these molecules is the major argument favoring the revival of the old hypothesis of the inversion of the DV axis between protostomes and deuterostomes. A detailed historical description of this topic is available in Arendt 2004.

Although much attention has been drawn to the conserved aspects of the BMP/Dpp4 in DV axis formation, several aspects differ between BMP/Dpp4 modulation in *Drosophila* and *Xenopus*. First, a subset of BMP/Dpp4 modulators is not present in *Drosophila melanogaster* genome, although present in vertebrate genomes. This point will be addressed in detail in the Chapter 2 (Umulis et al. 2006; De Robertis 2008).

Second, the control of BMP/Dpp4 antagonists by maternal NF-κB related molecules might be be restricted to insects (reviewed in Bier 2000). Two BMP/Dpp4 antagonists, *sog* and *brk*, are directly regulated by the NF-κB related molecule, Dorsal, in *Drosophila*, while this regulation might be absent in vertebrates. Since it is not clear if Dorsal/NF-κB-related molecules participate in early DV polarity during vertebrate embryogenesis (Armstrong et al. 1998; Prothmann et al. 2006), it is possible that the major maternal role of Dorsal/NF-κB in *Drosophila* early patterning is a late acquisition in insect evolution. This hypothesis is analysed in detail on Chapter 4.

Third, while in *Drosophila* the molecular system involved in DV and AP axis formation is considered completely independent one from each other, this is not the case for vertebrates. It has been long realized that zebrafish and mouse mutants affecting the DV axis formation are also affected along their AP axis (reviewed in Bier, 2000), but molecular links between these molecular systems have only recently been uncovered in *Xenopus* (Fuentealba et al. 2007, Fig 1.9). It was recently shown that *Xenopus* DV and AP axis converge at the level of Smad1,5,8 phosphorylation. While the Wnt gradient, responsible for AP axis in

vertebrates, is responsible for the duration of Smad1,5,8 signal, the BMP4 gradient provides the intensity of this signal (Fuentealba et al. 2007, De Robertis 2008, Fig 1.9). This mechanism was suggested to explain the overlapping mechanisms between AP and DV axis.

In the long-germ development of *Drosophila* AP and DV axis patterning are largely independent. In insects of short-germ type, which generate segments from a posterior secondary process, similarly to the somite generation during vertebrate segmentation (Tautz 2004), it is not clear if AP and DV axis are independently patterned. To sum up, insects with short-germ development might be suitable to answer at least three important questions related to the evolution of DV axis formation: 1) Did the loss of several Dpp modulators occur early in insect evolution, or did it occur later, e.g. in higher Diptera like *Drosophila* (analysed in Chapter 2 and 3) ?; 2) Is the major role of NF-κB related molecules controlling DV axis an specific feature of *Drosophila*, or is it a general insect feature, being present in more primitive insects (analysed in Chapter 4)?; 3) Does the AP and DV crosstalk, as demonstrated for vertebrates, occur in primitive insects and was lost in higher Diptera like *Drosophila* (analysed in Chapter 4)? In order to answer these questions a suitable insect model system with short-germ development should be used for a comparative approach.

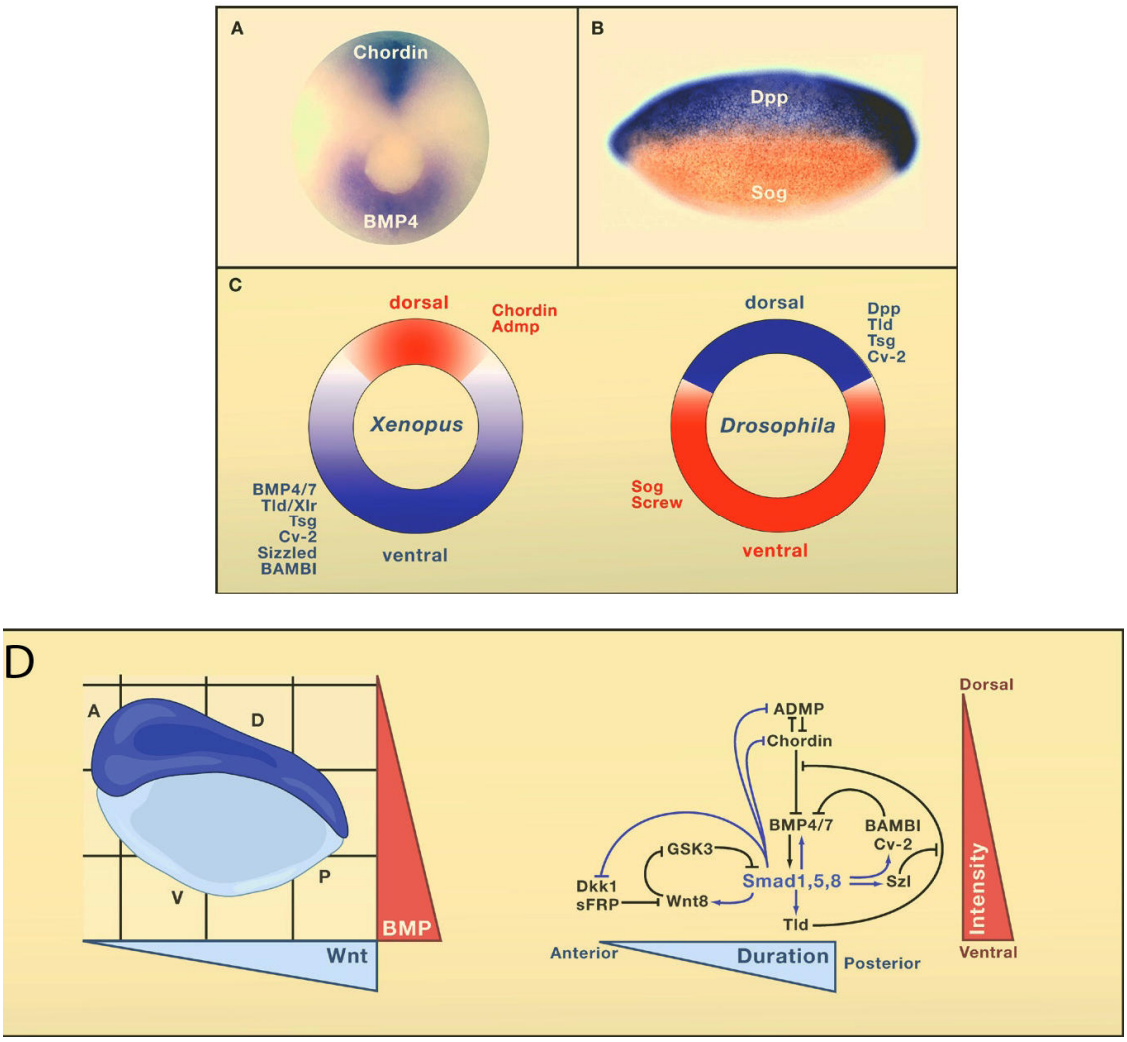


Figure 1.9: The Conserved Chordin-BMP Signalling Network and AP-DV integration at the level of Smad phosphorylation
The Chordin-BMP signalling network is conserved between *Drosophila* and *Xenopus*, (A) In *Xenopus*, *Chordin* is expressed on the dorsal side and *BMP4* at the opposite ventral pole. (B) In *Drosophila*, *dpp* is dorsal (blue) and *sog* is ventral (in brown) in the ectoderm. (C) A network of conserved secreted proteins mediates DV axis patterning in *Xenopus* and *Drosophila*, although some molecules do not act in both organisms. (D) AP (Wnt) and DV (BMP) gradients are integrated at the level of phosphorylation of the transcription factors Smads 1, 5, and 8. Note that in this self-regulating model the BMP gradient provides the intensity, but the Wnt gradient controls the duration of the Smad 1, 5, 8 signal. Direct protein-protein interactions are shown in black, and transcriptional activity of Smads 1, 5, and 8 are in blue. Adapted from De Robertis 2008.

1.4 - Establishing a comparative approach: the beetle *Tribolium castaneum* and its DV axis formation

Tribolium castaneum (Tenebrionidae, Rust Red Flour Beetle) is an organism that has been used for more than 50 years in laboratory studies (Sokoloff 1975). In the past few years *Tribolium* research has been boosted by the development of several molecular techniques such as gene knock-down by systemic RNAi and transgenesis (Bucher et al. 2002; Klingler 2004). Lethal and enhancer trap screenings are being currently performed (Richards and Genome

Consortium, 2008). Nowadays, *Tribolium* is the second most used insect model system only behind *Drosophila*.

Several developmental processes common to extant insects can be investigated in *Tribolium*, since they were largely reduced or modified in higher Diptera like *Drosophila.* Examples are head, leg formation during embryogenesis and the short-germ mode of embryonic development. During *Tribolium* embryonic development, only the head and thoracic segments are specified before gastrulation, and the remaining segments emerge from the posterior region, the growth zone. In the past few years, molecular studies have been carried out to characterize AP and DV patterning mechanisms in this beetle, which are summarized in the following paragraphs.

One of the key differences between short and long-germ insect eggs relates to extra-embryonic membranes. While in *Tribolium* the serosa is located anteriorly, in *Drosophila* the largely reduced extraembryonic membrane, the amnioserosa, is located dorsally. *zen* is required for the development of these extraembryonic membranes in both insects (Fig 1.10). Interestingly, *Tc-zen1* knock-down leads to an anterior expansion of the embryonic region at the expense of the serosa (van der Zee et al. 2005). Importantly, while the amnioserosa is a structure controlled only by the DV system in *Drosophila*, the *Tribolium* serosa specification is controlled mainly by the AP system. So, changes in the GRN responsible for extraembryonic membranes must have occurred during insect evolution.

The GRN network responsible for DV axis formation in *Tribolium* may rely less on maternal cues than the *Drosophila* system. It is possible that Dorsal has a less direct role in *Tribolium* DV patterning than its *Drosophila* ortholog and that zygotic components have a more pronounced role in beetles than in flies, as judged by at least four pieces of evidence. First, a nuclear Dorsal gradient exists in *Tribolium*, but this gradient is not stable and disappears fast during embryonic development, much before gastrulation starts (Chen et al. 2000, Fig 1.10), in contrast to the stable gradient in *Drosophila* (Roth et al. 1989). Second, the unique Toll receptor known so far in *Tribolium* seems to be zygotically expressed, possibly being activated by Dorsal. In *Drosophila*, conversely, Toll maternal mRNA is absolutely required for early patterning. Third, some genes repressed by Dorsal in *Drosophila* are not so in *Tribolium* such as *Tc-dpp* and *Tc-zen* (Fig 1.10). For instance, *Tc-dpp* is expressed in an oblique stripe and in a posterior domain not being repressed by Dorsal (Fig 1.10H). Fourth, there is no nuclear Tc-Dorsal in the growth zone, suggesting that different DV mechanisms operate at this region (Chen et al. 2000).

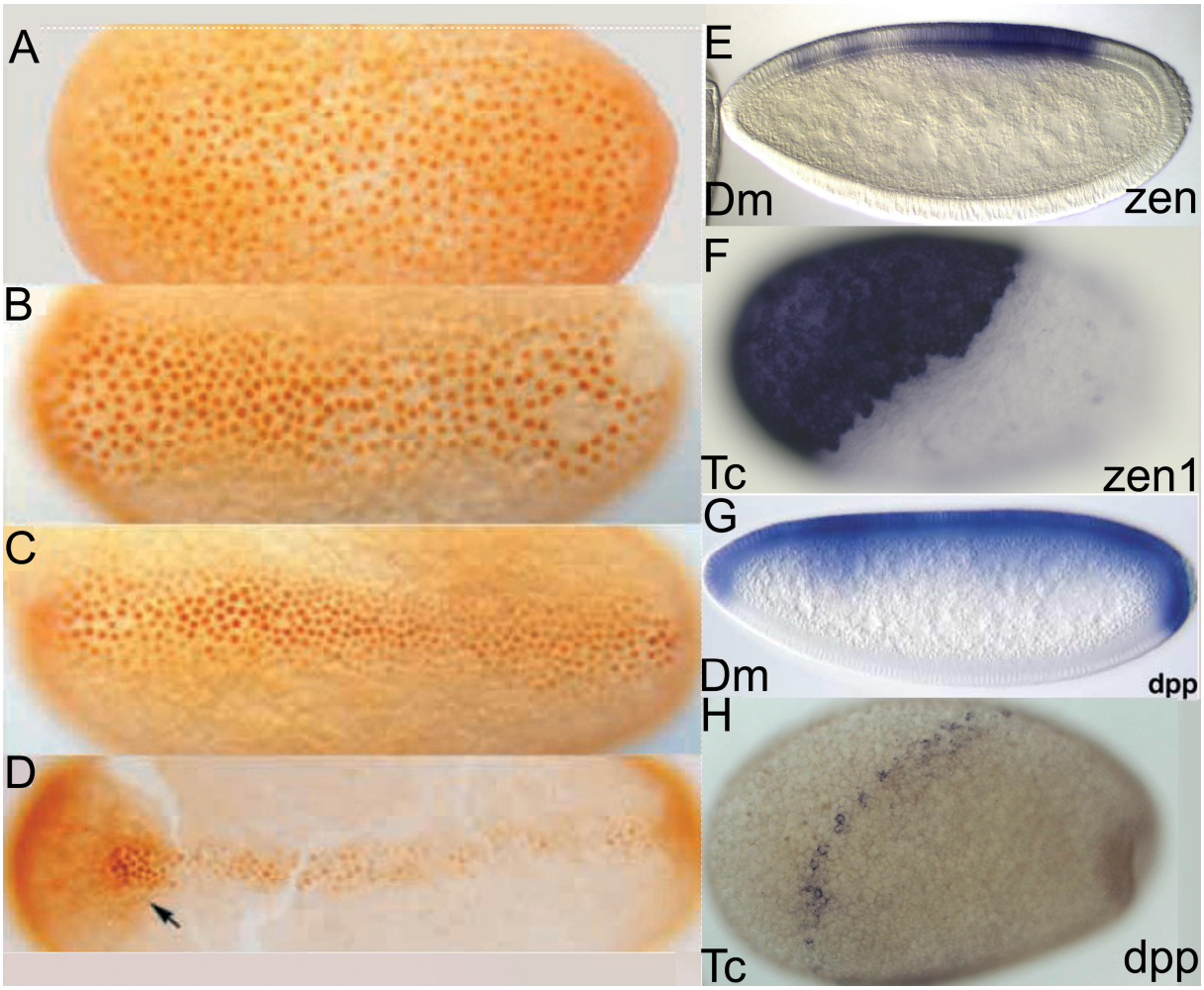


Figure 1.10: *Tribolium* DV axis formation and the comparison of beetle and fly gene expression patterns
(A-D) A transient nuclear Dorsal gradient exists in *Tribolium*, in contrast to the stable Dorsal gradient present in *Drosophila* (Roth el al. 1989, Chen et al. 2000). In D the arrow points to the anterior 'cap' expression of Tc-dorsal which coincides with the presumptive serosa. (E-F) Differences in expression of *zen* in *Drosophila* (E) and *Tribolium* (F). Zen marks extraembryonic tissue in both insects. Note the anterior *Tc-zen* expression when compared to the dorsal location of *Dm-zen*. (G) *dpp* is expressed in the dorsal side of the *Drosophila* embryo, being repressed by Dorsal. (H) In contrast *Tc-dpp* is transcribed in an oblique stripe of cells (blue) between the embryo proper and the anterior serosa.

Mesoderm formation in *Tribolium* also displays several different features from *Drosophila*. First, the ventral furrow in *Tribolium* is multilayered in the posterior region (Handel et al. 2005, Fig 1.11A,B), in contrast to *Drosophila* where a monolayer epithelium starts to invaginate. Second, in *Drosophila*, mitosis are inhibited in the mesoderm before ventral furrow formation, but cell divisions occurs specifically in mesodermal precursors in *Tribolium.* Third, invagination occurs differentially along the AP axis in *Tribolium*, so that embryonic posterior regions from the same embryo retain a more pronounced ventral furrow invagination than respective anterior regions (Handel et al. 2005, Fig 1.11C). In the long-germ

Drosophila embryogenesis, not only mesoderm invagination occurs simultaneously along the AP axis, but also the cellular organization is constant along it.

Besides the aforementioned differences in mesoderm formation, a key question is the origin of the mesoderm in segments arising from the growth zone. The *Tribolium* growth zone is composed of an outer layer that is continuous with the ectoderm, a thin layer constituting the amnion and an inner layer that is continuous with the mesoderm, besides a cell mass that were suggested to be the germ cells (Handel et al. 2005, Fig 1.11). Although Tc-Twist protein is detected in anterior regions of the inner layer and in the posterior tip of the growth zone, Tc-Twist is absent in the huge inner cell mass anterior to the posterior tip (Handel et al. 2005, Fig 1.11D). Since this cell mass is continuous with the inner layer cells that express *Tc-twist* in anterior regions, it was suggested that these cells might comprise an undifferentiated pool of mesodermal cells (Handel et al. 2005). On the other hand, it is also possible that gastrulation-like mechanisms involving cell migration between different layers might occur in the growth zone (this hypothesis is evaluated in the Chapter 4). Altogether, it is not clear how the growth zone DV pattern occurs and what is the fate of the different cell populations in this region. The question of how DV pattern from the growth zone is dependent on early cues will be described in Chapter 4.

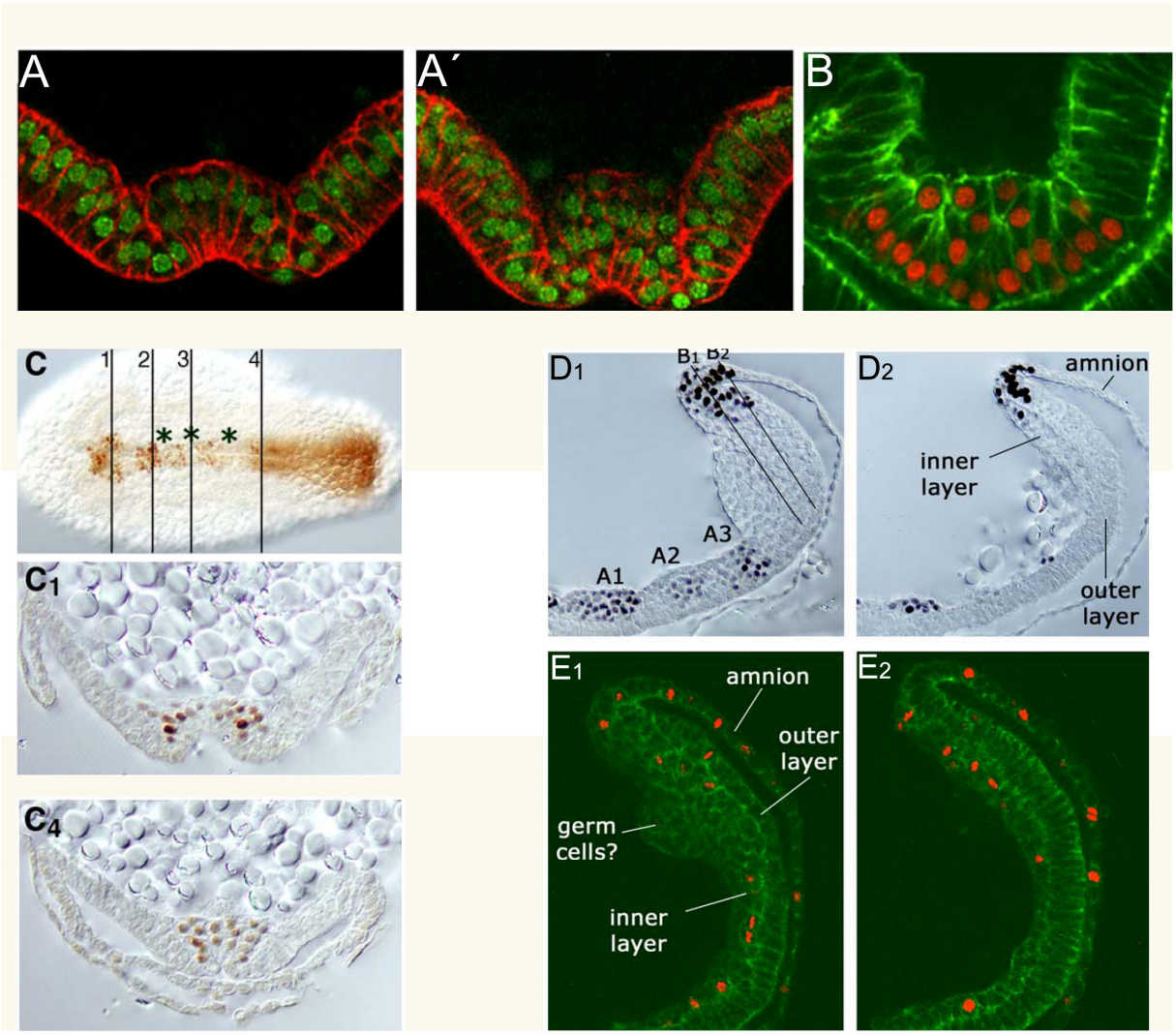


Figure 1.11: Mesoderm invagination and growth zone morphology in *Tribolium*
(A,A´) F-actin (cell membranes - red) and Yoyo (DNA-green) staining of an embryo at a similar stage to the one in C. A´ represents a posterior section and A an anterior one, displaying the progressive mesodermal internalization and the multilayered character of the ventral furrow in the posterior region. (B) Detection of Twist protein (red) in the ventral furrow. (C) An antibody staining for Twist in a representative embryo. (C1,C4) Regions of respective cross sections. The amnion and the serosa have closed ventrally in the posterior part (C4), but not in the anterior region (C1), where the extraembryonic cells are still moving ventrally. Moreover, almost the whole mesodermal cells have invaginated in C4, in contrast to C1. (D1,D2) Two longitudinal sections in an embryo undergoing germ band elongation, stained for Twist. The anterior Twist expressing cells are continuous with the inner layer of the growth zone, although inner layer cells do not express Twist. The outer layer is continuous with the ectoderm and the formation of the amniotic cavity is evident. (E1,E2) Anti-Phospho-histone3 (cell proliferation - red) and F-actin (cell membrane - green). Two optical cross sections from an embryo undergoing germ band elongation, displaying the cellular organization of the growth zone. Note the globular cell mass constituting the putative germ cells (E1). Adapted from Handel et al. 2005.

2 - Specific Objectives

The recent sequencing of *Tribolium castaneum* genome has enabled us to address the following questions related to the GRN responsible for DV patterning in this beetle:

1) Investigation of the evolution of TGF-β signalling pathway in insects (Chapters 2 and 3). I will first describe the role of several genes involved in the TGF-β signalling pathway found in the recently sequenced *Tribolium* genome (Chapter 2). Then the role and evolution of two BMP/Dpp modulators, *twisted-gastrulation/crossveinless* and *tolloid*, will be analysed in more detail (Chapter 3).

2) Investigation of the role of Dorsal/Toll pathway components in the formation of the DV axis in the short-germ *Tribolium* (Chapter 4). Particular emphasis will be given to: 1) why the Tc-Dorsal gradient is less stable and rapidly shrinks, in contrast to the stable nuclear Dorsal in *Drosophila*; 2) Whether there are in *Tribolium* genes responding to different thresholds of Dorsal activity (type I, type II) like in *Drosophila*?; 3) Is the growth zone DV pattern dependent on the early Toll signalling established during early blastoderm differentiation? These studies may generate insights into the molecular mechanisms involved in the experimental duplications along the DV axis observed in short-germ insects more than 30 years ago.

3 - Methods

In the following section the new developed methods are described and the available protocols are properly cited. Together with this thesis a CD is provided containing a step-by-step description of new methods established. Information on *Tribolium castaneum* culture, rearing, egg collection and fixation is available in the beetle book, written by Gregor Bücher, Göttingen (**http://wwwuser.gwdg.de/~gbucher1/tribolium-castaneum-beetle-book1.pdf**). For a specific question on chapter 3, the wasp *Nasonia vitripennis* is used as a model system. For similar information on *Nasonia* as a developmental biology insect model the reader should follow Lynch and Desplan 2006.

3.1 - Cloning *Tribolium castaneum* and *Nasonia vitripennis* genes and phylogenetic analysis

A BLAST survey for possible orthologous sequences was performed in the *Tribolium castaneum* (http://www.hgsc.bcm.tmc.edu/projects/tribolium/) or in *Nasonia vitripennis* (http://www.hgsc.bcm.tmc.edu/projects/nasonia/) genome website. Gene prediction models based on EST and homology evidence, the GLEAN dataset, was available for *Tribolium castaneum* sequences, which in most of the cases contains the complete coding sequence of the gene of interest. Primer design was performed with Oligo (www.oligo.net) to generate fragments ranging from 400-1000 bp for each gene. A table of cloned genes and markers used during this thesis is provided in a CD that accompanies this thesis, with respective restriction sites and enzymes used for anti-sense probe synthesis and dsRNA templates.

For phylogenetic analyses, similar aminoacid sequences to *Tribolium* or *Nasonia* genes were obtained by BLAST, alignments performed using Bioedit (CLUSTALW interface available), and sequences loaded into MEGA Version 3.1 software (Kumar et al. 2004). Neighbor-joining and Minimum Evolution were performed and 1000 replicates were used for bootstraps.

RNA extraction using 100-200 embryos (0-72 hours of development, from freshly laid eggs until completely extended germ bands) was done using TRIZOL (Invitrogen). 1 µg of RNA was used for cDNA synthesis using the SuperScript II RT (Invitrogen). The obtained PCR fragments were cloned into the pCRII TOPO, which contains SP6 and T7 promoter regions flanking the cloned product. For the completeness of the mRNA sequences 3´- and 5´ RACE

reactions were done using the SMART RACE cDNA Amplification Kit (Clontech) following the manufacturer instructions.

3.2 – RNA Probe preparation, *in situ* hybridization and immunohistochemistry

RNA probes were synthesized from purified (Quiagen QIAquick PCR purification kit) dsDNA PCR products (0,2-0,5 μg/probe). Modified Ribonucleotides containing Digoxigenin or Biotin were purchased from Roche and used to synthesize ssRNA probes. Fluorescein-labelled probes led to low signal/background ratio in *Tribolium* embryos. Briefly, the one-color colorimetric staining for *Tribolium* was essentially the same as described by Tautz and Pfeifle 1989 for *Drosophila* embryos, without the proteinase K treatment step.

For two-color colorimetric staining, the method described by Liu and Kaufman 2004 for *Oncopeltus fasciatus* was adapted for *Tribolium*. NBT/BCIP solution (Roche) and Fast Red (Roche) or Vector Red (Vector Labs) were used as substrates for two successive alkaline phosphatate enzyme reactions. For fluorescent in situ hybridizations, the method described by Kosman et al. 2004 was adapted to *Tribolium* embryos. Amplification steps using tyramide reactions (TSA) are required to obtain intense staining in *Tribolium* embryos. The immunohistochemistry was done essentially as previously described (Roth et al. 1989). Detailed *in situ*, immuno-histochemistry and fluorescence protocols with respective pictures as examples are supplied in the CD which comes together with this thesis. Protocols can also be downloaded from http://fonsecarn.50webs.com/.

Antibodies used during this study and their concentrations:

For *in situ* hybridization

Anti-DIG-AP (Roche, 1:5000)

Anti-DIG-POD (Roche, 1:100)

Anti-BIO-AP (Roche, 1:2000-1:5000)

Mouse-Anti-BIO (Roche, 1:200)

Anti-MOUSE-HRP (Roche, 1:200)

Anti-BIO-POD (Roche, 1:200)

For immunofluorescence or immunohistochemistry, primary antibodies

Rabbit-Anti-OTD (Reinhard Schröder, 1:100)

Rat-Anti-TWIST (Jessica Cande & Mike Levine, UC Berkeley, 1:100)

Guinea Pig-Anti-DORSAL (Jessica Cande & Mike Levine, UC Berkeley, 1:50 -1:100)

mouse-Anti-MAPK (Sigma, 1:2000)

mouse-Anti-ENGRAILED- 4D9 (Developmental Studies Hybridoma Bank, 1:5)

rabbit-anti-Phospo-MAD (1:2000, gift from Alfonso Martinez-Arias, Cambridge, UK)

mouse-anti-phospho-TYROSINE (Sigma; 1:500)

Rhodamine Phalloidin (R415-Invitrogen; 1:500)

For immunofluorescence or immunohistochemistry, secondary antibodies

Anti-GUINEA-PIG-Alexa555 (Molecular Probes, 1:400)

Anti-RAT-Alexa555 (Molecular Probes, 1:400)

Anti- MOUSE-Alexa555 (Molecular Probes, 1:400)

sheep-Anti-RABBIT-HRP (Roche, 1:500)

sheep-Anti-RABBIT-BIO (Roche, 1:1000)

3.3 - dsRNA synthesis, egg, pupae and beetle injections

In order to obtain a PCR product containing T7-promoter sites on both ends, PCRs from template plasmids (10-100ng template) were performed using a T7 primer and a SP6 primer containing a T7 tail (added during the first PCR cycles). These PCR fragments were then used as templates for Ambion Megascript RNAi Kit, with both RNA strands being synthesised and annealed during synthesis reaction (3 hours-37ºC). For at least one gene, *Tc-cv/Tc-tsg* (described in Chapter 3), two non-overlapping dsRNA constructs were used for injections and led to identical phenotypes. GFP dsRNA was also injected as a control for unspecific effects.

For simultaneous injection of two different dsRNA, primers leading to similar size amplicons were designed and used as templates for dsRNA synthesis reaction. A mixture of two dsRNA was performed using equal concentrations (1 μg/μl). Pupae injection was described in Bucher et al. 2002 and adult injection in van der Zee et al. 2006.

For egg injections, egg lay was performed for one hour (30ºC), when the flour was sieved and eggs collected. These eggs developed for one further hour, before their dechorionation (20% commercial bleach for 40 seconds). Eggs were aligned on a glass slide and dried for 7-10 minutes with the help of a dissecator. Voltalef oil (Atochem) was then added and injection

proceeded on the anterior part, the region where the serosa is located, although injection in posterior regions led to similar knock-down phenotypes. For analysis of phenotypes during blastoderm differentiation the embryos were let to develop further 8-10 hours, and for observation of segmented germ bands further 18-24 hours. During every egg injection experiment GFP dsRNA was injected in a similar number of embryos, which were fixed at the same time of the experimental dsRNA injected eggs. After fixation, methanol step was omitted and the vitelline membrane was carefully removed with the help of a needle (0,3mm).

3.4 - Confocal sections

Tribolium eggs were fixed as previously described (Chen et al., 2000, *Tribolium* Beetle book). A primary mouse-anti-phosphotyrosine (Sigma, 1:500) antibody was used followed by a secondary anti-mouse-Alexa-conjugated-555 (Molecular Probes, 1:400). The embryos were hand-sectioned, vecta-shield mounting medium containing DAPI (H1000) was added, and optical sections with a Leica SP2 Confocal (40x objective, immersion) were performed.

Chapter 2 - Evolution of TGF-β modulators in insects

Introduction:

TGF-βs are evolutionary conserved molecules involved in most processes where cell communication takes place. The evolutionary origin of TGF-βs traces back to the origin of multicellular organisms. Sponges, considered the sister-group of Eumetazoans contain TGF-β in their genomes, but choanoflagellates, the sister-grop of Metazoans and Fungi do not (Nichols et al. 2006; King et al. 2008). From an evolutionary point of view it is possible that very early in Metazoa evolution, TGF-βs, in particular BMPs, were required to establish different cell fates along the DV axis, or at least to distinguish different germ-layers (Matus et al. 2006b.). Many BMP modulators have arisen early in Metazoa evolution. Noggin, Chordin, secreted frizzled-related proteins (SFRPs), Gremlin, Tolloid and Follistatin-like genes are examples of secreted molecules present in the Anthozoa *Nematostella vectensis* and vertebrates (Putnam et al. 2007, Fig 2.1). These modulators are involved in establishing a gradient of BMP activity in vertebrates, since they directly bind to BMPs in the extracellular space. This binding prevents BMP interaction with cell surface receptors (reviewed in De Robertis 2008, Fig 2.1). This modulation also involves extracellular matrix components (Belenkaya et al. 2004).

Strinkingly, many of these BMP modulators indeed are absent from genomes of invertebrate Ecdysozoa model systems like *Drosophila melanogaster* and *C. elegans* (De Robertis 2008), indicating that gene loss has occurred during Ecdysozoa evolution. The fast-evolving character of these two species, has been discussed elsewhere (Dopazo and Dopazo 2005, Savard et al. 2006b). On the other hand, proteins acting in the BMP intracellular signal transduction such as mothers-against decapentaplegic (SMAD) genes are present in all extant Metazoa analysed so far.

The important open questions in the evolution of BMP/Dpp modulators in Ecdysozoa are: 1) When has specific gene losses occurred in Ecdysozoa evolution? Did it occur independently in higher Diptera and nematodes, or did the Ur-Ecdysozoa already lack many BMP/Dpp modulators? 2) If the cocktail of BMP/Dpp modulators present in Cnidaria and vertebrates also exists in ancestral insects like *Tribolium*, what is their function?

To answer these questions, I searched for BMP/Dpp modulators in the recently sequenced *Tribolium* genome (Richards and Genome consortium 2008), and whenever possible performed functional analysis with these modulators.

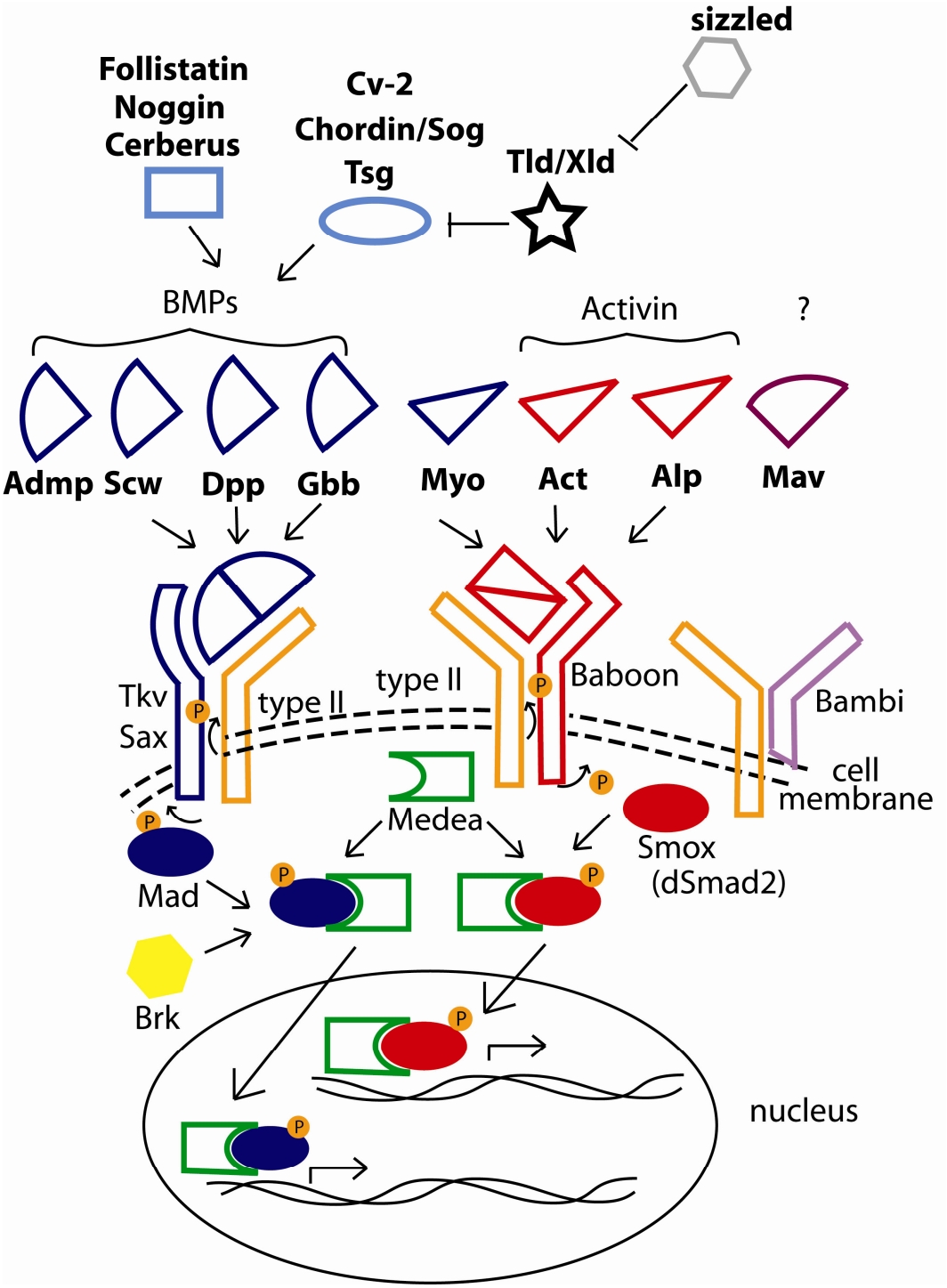


Figure 2.1: TGF-β extracellular modulation and intracellular cascade
TGF-β, in particular BMP activity is modulated by several factors in the extracellular space, such as Follistatin, Cerberus, Noggin, Chordin/Sog, Cv/Tsg, Tld and Sizzled. Gremlin and DAN proteins are proteins similar to Cerberus, so they are not displayed in the pictured. Activins and BMPs belong to the TGF-β superfamily of secreted factors that interact as homo or heterodimers with a heterodimeric receptor complex. While Tkv and Sax are BMP receptors, Baboon is an Activin receptor. The system can also be modulated by BAMBI, a pseudo-receptor lacking the intracellular tyrosine kinase domain. Inside the cell, phosphorylation of pMad or Smox leads to nuclear transport and activation of specific target genes. In *Drosophila*, the Dpp negative regulator, Brk, competes with pMad for transcriptional regulation. Modified from Van der Zee et al. 2008.

Results and Discussion:

To analyse the distribution of TGF-β pathway members in the *Tribolium castaneum* genome, I performed BLAST searches in the *Tribolium* genome using as query sequences not only fly (*D. melanogaster),* but also mouse (*Mus musculus*) coding sequences. All TGF-β ligands, receptors and extracellular or intracellular modulators present in the *Tribolium* genome were manually annotated. Phylogenetic trees (maximum likehood) performed by Maurjin van der Zee are available in Van der Zee et al. 2008. A table summarizing BMP/Dpp modulators present in the beetle genome is provided below.

Gene name	*Drosophila* ortholog	Vertebrate ortholog	GLEAN number	Embryonic expression	pRNAi phenotype
Tc-brinker	Yes	No	Tc_00748	No	No*
Tc-bambi	No	Yes	Tc-12274	Yes	No*[1]
Tc-gremlin	No	Yes	Tc_07044	Yes	NC
Tc-BMP10	No	Yes	Tc-06506	ND	ND
Tc-DAN	No	Yes	Tc_14861	ND	No
Tc-tkv	Yes	Yes	Tc_06474	Yes	Yes
Tc-sax	Yes	Yes	Tc_15984	Yes	Yes
Tc-Mad	Yes	Yes	Tc-14924	Yes	Yes
Tc-Mad2	Yes	Yes*[2]	Tc-14921	ND	ND
Tc-smadX	No	No*[3]	Tc_08788	No	No
Tc-Medea	Yes	Yes	Tc_10848	Yes	Yes
Tc-cv/Tc-tsg	Yes	Yes	Tc_03620	Yes	Yes
Tc-tok/Tc-tld	Yes	Yes	Tc_11197	Yes	Yes
Tc-sog	Yes	Yes	Tc-12650	Yes	Yes*[4]
Tc-cv2	Yes	Yes	Tc-12674	No	ND
Tc-baboon	Yes	Yes	Tc_03240	ND	No
Tc-activin	Yes	Yes	Tc_15808	No	No
Tc-dSmad2	Yes	Yes	Tc_10162	ND	ND
Tc-alp	Yes	Yes	Tc_04297	No	No
Tc-wit	Yes	Yes	Tc_ 09314	ND	ND
Tc-punt	Yes	Yes	Tc_11357	ND	ND
Tc-dpp	Yes	Yes	Tc_08466	Yes	Yes*[4]
Tc-mav	Yes	Yes	Tc_04299	ND	ND
Tc-gbb1	Yes	No	Tc_14017	Yes	NC*[5]
Tc-gbb2	No	No	Tc_14018	No	No

Table TGF-β ligands, receptors and modulators

NC – not clear, ND – not done. Color code: GREY, gene present in *Tribolium* and *Drosophila*, but not in vertebrates. ORANGE - genes present in *Tribolium* and vertebrates, but lost in *Drosophila*. GREEN - putative pseudo-gene in *Tribolium*. YELLOW - Single copy genes in *Tribolium*, but two or more copies exist in *Drosophila*. BLUE - Genes of the Activin pathway which are not involved in *Tribolium* DV patterning (see results this Chapter). PINK - Gene predicted probably due to an assembly error in the region, so probably this gene does not exist (see * 2). GLEAN - automatic gene prediction method based on homology and EST evidence, which was applied to the *Tribolium castaneum* genome (for details see Richards and Genome consortium 2008).

* Although two different dsRNA constructs against *Tc-brk* were injected into *Tribolium* pupae, no phenotype was observed. In addition, dsRNA against *Tc-brk* was also injected in last instar larvae (Tomoyasu and Denell 2004; Tomoyasu et al. 2005), but no phenotype was observed. Larval RNAi against *Tc-tld* led to pupae and adults wings with increased size (data not shown).

*[1] Two different non-overlapping dsRNA constructs against *Tc-bambi* were injected into Tribolium adults and no embryonic phenotype was observed, although the gene is expressed in overlapping domains with *Tc-dpp* (Fig 2.3).

*[2] *Tc-mad2* is not an actual gene, a problem in the assembly of that chromosomal region occurred.

*[3] *Tc-smadX* is a putative pseudo-gene (see explanation on Activin or BMP: TGF-β signalling in Tribolium embryos, page 48).

*[4] *Tc-sog* and *Tc-dpp* phenotypes were published elsewhere (van der Zee et al. 2006).

*[5] Although Weber has reported knock-down phenotypes against *Tc-gbb1*, using two different non-overlapping constructs I was unable to retrieve similar phenotypes.

TGF-β ligands and receptors

Overall, most molecules involved in TGF-β signalling are conserved among *Tribolium, Drosophila* and vertebrates. TGF-β ligands such as Dpp and Gbb (BMPs), and Activin and Alp (Activin family) are conserved between *Tribolium* and *Drosophila* (Table). Interestingly, Screw, a Dpp related ligand involved in *Drosophila* DV axis formation, is absent from *Tribolium*, *Apis mellifera* and lower Diptera genomes. This indicates that the interaction of Screw with Dpp arose late in insect evolution. On the other hand all insects investigated so far have at least one *glass bottom boat (gbb)* in their genomes. A specific duplication of *gbb* related genes occurred in the lineage which gave rise to *Tribolium* since two *Tc-gbb* genes exist in *Tribolium*, *Tc-gbb1* and *Tc-gbb2*. Since both *gbb* genes are more similar one to each other than to other insect gbb, it is likely that a duplication took place recently in the lineage giving rise to *Tribolium*. While *Tc-gbb1* is expressed in the ectoderm, in the growth zone, in the midline and in the legs (Fig 2.2 and Weber 2006), *Tc-gbb2* does not show any sign of expression in early embryos (data not shown). *Tc-gbb2* might be involved in other processes as its *Drosophila* ortholog, *e.g.* wing patterning (Khalsa et al. 1998).

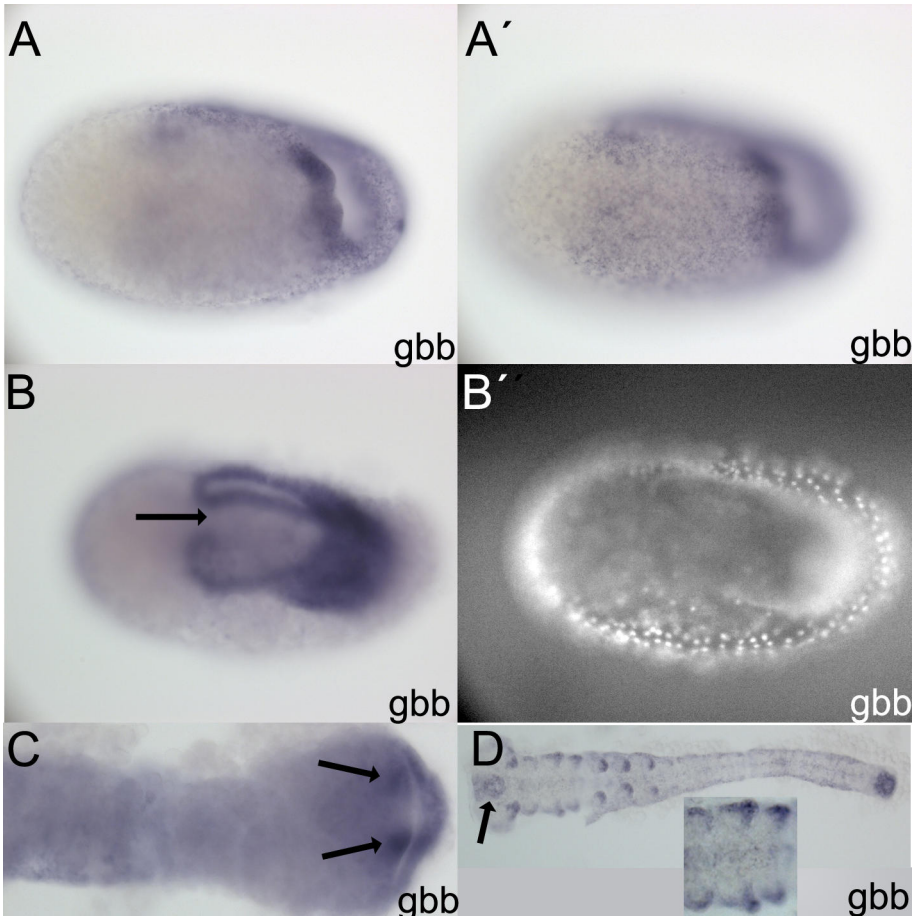


Figure 2.2: Expression of *Tc-gbb1* (A-D) *Tc-gbb1* expression. (A,A´) Gastrulating embryo in different focal planes, ventral view. *Tc-gbb1* is detected at the embryonic surface, mainly in the ectoderm. (B,B´) Shortly before serosal closure, *Tc-gbb1* is expressed in the whole embryo with the exception of the mesoderm (arrow). (C) A closer view in the growth zone shows high *Tc-gbb1* expression in two dots of the outer layer (arrows), although lower amounts are ubiquitously present. (D) A complete extended germ band embryo. *Tc-gbb1* is expressed in the dorsal borders, in the ventral midline (see high magnified thoracic region), in the legs and in the middle of the head, possibly the prospective stomodeum (arrow).

In addition, a putative BMP ligand similar to BMP9 and BMP10, which is absent from Drosophillid genomes, is found in *Tribolium* genome. In vertebrates BMP9 is involved in forebrain development (Lopez-Coviella et al. 2005) and BMP10 in cardiogenesis (Chen et al. 2004). Last, *Tribolium* lacks an ortholog of the Anti-Dorsalizing Morphogenetic Protein (ADMP), a recently described vertebrate BMP ligand expressed in regions where BMP signalling is abolished (Reversade and De Robertis 2005). Since ADMP is found in the genomes of wasps (*Nasonia vitripennis*), bees (*Apis mellifera*), hemichordates and echinoderms, and is absent in *Tribolium, Anopheles* and *Drosophila*, it is possible that this gene was present in in the Urbilateria and a single loss event occurred in insect evolution. This assumption is based on the new insect phylogeny where Hymenoptera are considered basal to Coleoptera (Savard et al. 2006b, described in Chapter 1).

An overall conservation was also observed for TGF-β receptors. TGF-β signalling occurs via a tetrameric receptor composed by two Type I receptors like Sax and Tkv and two Type II receptors like *Tc-punt.* These receptors are conserved in insects including *Tribolium*. Another molecule involved in the modulation of TGF-β and BMP signalling is BAMBI (BMP and Activin Bound Inhibitor). BAMBI behaves as a pseudo-receptor in *Xenopus laevis* (Onichtchouk et al. 1999), acting in regions where high amounts of BMPs are present. BAMBI acts as a negative feedback of BMP2/7 in *Xenopus laevis* since it lacks the intracellular tyrosine kinase domain required for downstream signalling. BAMBI is present in the *Tribolium* genome, although absent from the *Drosophila* one. As in *Xenopus*, BAMBI is also expressed in regions with high amounts of BMPs in hemichordates (Lowe et al. 2006), suggesting that this mechanism is conserved in deuterostomes. I cloned the BAMBI ortholog in *Tribolium* and analysed its expression. Interestingly, BAMBI is expressed in regions with high amounts of BMP4, as judged by stainings with the phosphorylated form of *Mothers against dpp* (pMad), a known read-out of BMP/Dpp signalling (Fig 2.3). During blastoderm stages BAMBI is expressed in dorsal regions, which overlaps with the amnion, and during germ band elongation BAMBI is detected in dorsal domains. Both domains overlap with pMad staining (Fig 2.3). Altogether, like in hemichordates and vertebrates, BAMBI is expressed in regions containing high amounts of BMP4/Dpp activity. In *Xenopus laevis*, overexpression of BAMBI leads to decrease of BMP and Activin signalling. On the other hand, knock-out mice for BAMBI are viable and fertile, indicating that this gene is dispensable for embryonic and post-natal survival (Chen et al. 2007). In aggrement with the latter observation no embryonic phenotype was observed in *Tribolium* after injection of dsRNA against *Tc-bambi*.

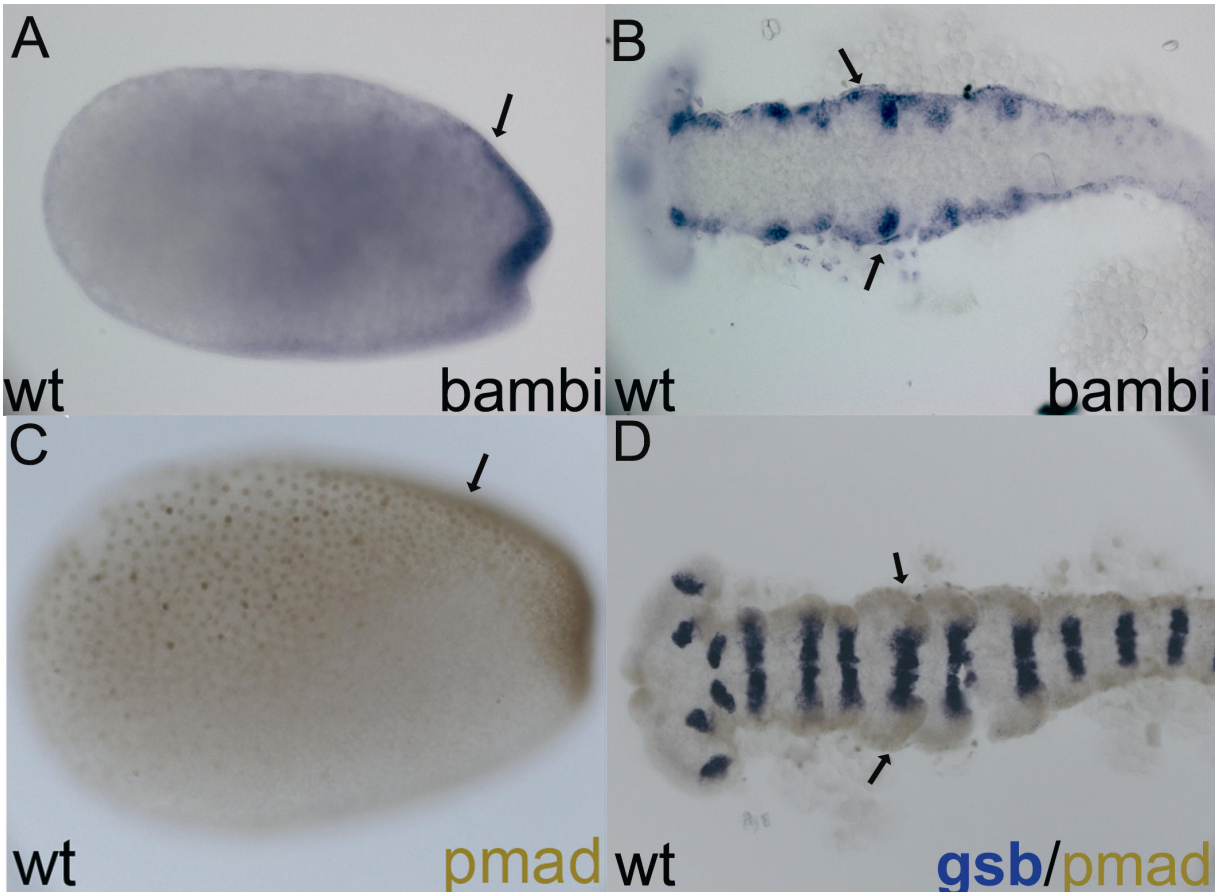


Figure 2.3: The *Tc-BAMBI* ortholog in *Tribolium* is expressed in overlapping domains with BMP/Dpp activity
(A,B) *Tc-BAMBI* in situ hybridisation. (A) *Tribolium* differentiated blastoderm embryo. *Tc-BAMBI* is expressed at the dorsal side of the embryo (arrow) but is restricted to the embryo proper, not being present in the more anterior extraembryonic serosa. (B) In the extending germ band, *Tc-BAMBI* is expressed along the dorsal borders of the embryo (arrows), which overlap with Dpp activity (pMad). (C,D) Phosphorylated Mad (pMad), readout of BMP signalling, is visualized. (C) Differentiated blastoderm. pMad is observed along the dorsal side of the embryo, in the serosa and in the dorsal ectoderm/amnion (arrow). (D) Elongating germ band. pMad antibody staining in brown. The blue in situ staining against *Tc-gooseberry* (*Tc-gsb*) is used as segmental marker. pMad is present along the dorsal borders, overlapping with the dorsal ectoderm and the amnion (arrows).

TGF-β extracellular modulators

Several molecules are involved in the modulation of BMP in the extracellular space. Gremlin, Cerberus and DAN are examples of molecules involved in antagonizing BMP signalling by direct binding to BMPs. This avoids BMP contact with cellular receptors in *Xenopus laevis* (Hsu et al. 1998; Piccolo et al. 1999). To date, no DAN related molecules were found in *Drosophila* genome, so it is generally believed that these molecules are vertebrate innovations. Two DAN related molecules are found in the *Tribolium* genome, *Tc-DAN* and *Tc-gremlin (Tc-grem). Tc-grem* is expressed in the ventral midline (Fig 2.4A,B) during germ band elongation and in a fork-like domain in the head. pRNAi experiments

against *Tc-greml* were inconclusive to determine if this gene has an embryonic role. In contrast to other RNAi phenotypes analysed so far, the penetrance of *Tc-grem* RNAi was decreased extremely fast. So only the first egg lay was used during these experiments. In addition, the observed phenotypes were variable and not consistent with a role in early Dpp modulation (Fig 2.4C-H). Indeed, the affected embryos display cell aggregates during blastoderm stages, which start to disintegrate before gastrulation (Fig 2.4C-F, arrows). In contrast to the WT *Tc-twi* expression pattern, which displays segmental modulation, *Tc-grem* RNAi embryos lack this segmental modulation. These effects were incompatible with any role of *Tc-grem* in modulating *Tc-dpp* activity, but rather suggest an involvement in a general cellular phenomenon like cell division. Whether this phenotype is specific requires further investigation.

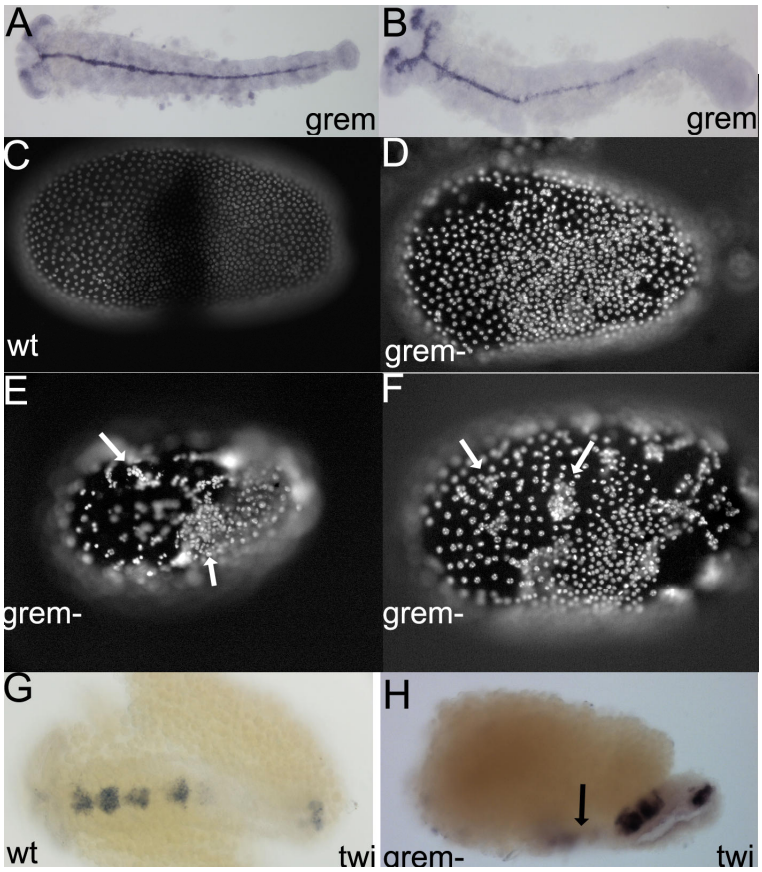


Figure 2.4: *Tc-gremlin* expression and RNAi phenotype
(A,B) *Tc-gremlin (Tc-grem)* expression. (A) A complete extended germ band shows *Tc-grem* expression in the ventral midline and in specific regions of the head, which are evident in early germ band embryos like the one in B. (C) WT embryo, *Tc-milli-pattes (Tc-mlpt*-shadow) and nuclear DAPI staining. Observe that the nuclei are spread all over the egg surface. The difference in nuclear density is caused by lack of proliferation of the anterior serosal cells, which become polyploid. (D-F) *Tc-grem* RNAi embryos display cell clumps (arrows) in several embryonic territories when compared to WT. (G) WT germ-band *Tc-twi* marks the mesoderm. Note the segmental modulation of *Tc-twi*. (H) After *Tc-grem* RNAi, the embryos start to disintegrate with disapereance of *Tc-twi* segmental modulation. Note that the *Tc-twi* anterior expression domain (arrow) disappears.

Other BMP modulators like secreted frizzled-related proteins (SFRPs e.g Sizzled) are known to be restricted to vertebrate genomes (reviewed in De Robertis, 2008). In *Xenopus laevis*, Sizzled is an inhibitor of the BMP modulator Tld (Mullins, 2006). Sizzled is not present in *Tribolium* or *Drosophila* genomes suggesting that this molecule could have emerged during vertebrate evolution or that Sizzled might have been lost in the lineage giving rise to holometabolous insects. Noggin, another extracellular inhibitor of BMP in vertebrates (reviewed in De Robertis, 2008), is also absent from the *Tribolium* genome. Since these molecules are found in Cnidaria (Matus et al. 2006a) and in Lophotrochozoans (De Robertis 2008), these molecules were probably lost in insects.

Other common BMP/Dpp modulators are cysteine-rich (CR) related molecules. Two CR containing molecules are Sog, the Chordin ortholog, and Crossveinless-2 (Cv-2), which interact with BMPs in all animals investigated so far. *Tc-sog* has been shown to act as a BMP inhibitor in *Tribolium* (van der Zee et al. 2006), *Drosophila* and vertebrates (reviewed in De Robertis, 2008). Cv-2 is present in Drosophilids, *Tribolium* and vertebrate genomes. While Cv-2 is involved in regulating the BMP gradient in *Xenopus* embryos, in *Drosophila* Cv-2 is not required for Dpp embryonic regulation, since flies lacking Cv-2 have a normal Dpp gradient and hatch (Umulis et al. 2006). Several attempts to clone *Tc-cv-2* from embryonic cDNAs have failed, so it is possible that as in *Drosophila*, *Tc-cv-2* is involved in Dpp embryonic modulation. The last protein family containing CR domains is Cv/Tsg. In contrast to Sog and Cv-2 molecules, which have several CR domains, Cv/Tsg has only two CR domains. Interestingly, the *Tribolium* genome contains only one copy of a Cv/Tsg related molecule, while the *Drosophila* genome contains three copies of these genes. A detailed functional analysis of this unique Cv/Tsg related molecule in *Tribolium* is provided in Chapter 3.

The last BMP/Dpp extracellular modulator investigated was the metalloprotease Tld. Tld related molecules are also present in all animals investigated so far. Also as observed for Cv/Tsg, the *Tribolium* genome contains only one Tld related molecule, while the *Drosophila* genome contains two Tld related metalloproteases. A detailed functional analysis of this unique Tld related molecule in *Tribolium* is provided in Chapter 3.

Activin or BMP: TGF-β signalling in *Tribolium* embryos

In vertebrates Activin and BMP2/4 participates in early embryonic patterning mechanisms. Activin is particulary important for mesoderm induction in *Xenopus* and zebrafish (Steinbeisser et al. 1993; Dyson and Gurdon 1997), while BMP2/4 is necessary for proper dorsoventral axis formation in all animals investigated so far (Ferguson 1996). In

Drosophila, Activin, its Type I receptor Baboon and Smox2 (dSmad2), an intracellular mediator that migrates to the nucleus upon signalling (Fig 2.1), are not required for embryonic patterning, but only for pupation and adult viability (Brummel et al. 1999). In contrast, Dpp is required for dorsoventral patterning in *Drosophila* and *Tribolium* (Ferguson and Anderson 1992, van der Zee et al. 2006). I asked if Activin and its downstream effectors would be involved in *Tribolium* embryonic patterning. pRNAi against *Tc-Activin* and its Type I receptor *Tc-baboon* did not cause any embryonic defect (data not shown), leading to the conclusion that Activin signalling is not required for *Tribolium* embryonic development. On the other hand, injection of dsRNA against the Dpp downstream effectors, *Tc-Medea* and *Tc-Mad*, led to knock-down phenotypes identical to *Tc-dpp* (Fig 2.5, a detailed description of this phenotype is provided in the next chapter and in Van der Zee et al. 2006). Since *Tc-Medea*, a common downstream effector of the Activin and BMP/Dpp pathway, leads to the same knock-down phenotype as *Tc-Mad*, which is only activated by the BMP/Dpp pathway (see scheme on Fig 2.1), BMP/Dpp can be considered the unique pathway involved in *Tribolium* embryonic TGF-β signalling.

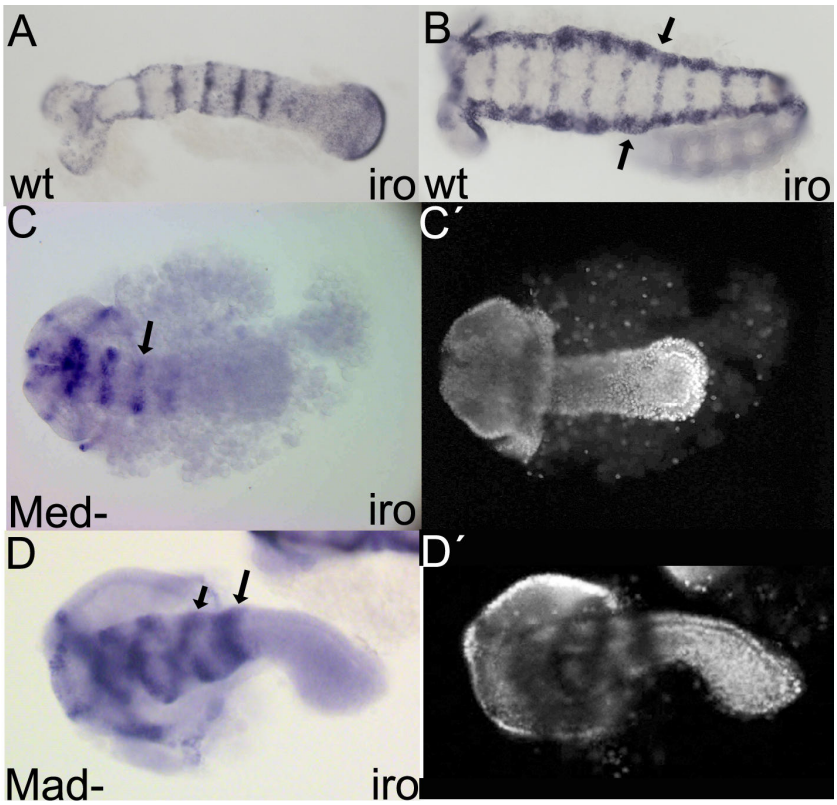


Figure 2.5: RNAi phenotypes of *Tc-Mad* and *Tc-Medea*
(A,B) WT expression of *Tc-iro* in extending germ band embryos. In early stages (B) *Tc-iro* is expressed in the dorsal borders (arrows) and in every segment (stripes along the AP axis). Arrow highlights the dorsal domains. (C) *Tc-Med* RNAi and (D) *Tc-Mad* RNAi embryos. Only the segmental stripe pattern of *Tc-iro* is detected (arrows), no expression is observed in dorsal domains. This is an indication of a ventralized knock-down phenotype. Additionally, a big head is observed, a characteristic of *Tc-dpp* RNAi (see next chapter and Van der Zee et al., 2006).

One additional Mad exists in the *Tribolium* genome, *Tc-SMADx*. At least two pieces of evidence indicate that *Tc-SMADx* is a pseudogene: 1) Tc-*SMADx* is an intronless transcript located on chromosome 6, while *Tc-mad* is located on chromosome 7 and has three introns, so it is possible that *Tc-SMADx* has arisen as a retrotransposition event from *Tc-mad;* 2) *Tc-SMADx* is a fast evolving protein, displaying long branches in phylogenetic trees; and lacking residues important for the interaction with other SMADs (Van der Zee et al. 2008).

A last BMP/Dpp intracellular modulator present in *Drosophila* and absent in vertebrate genomes is *brinker (brk)* (Jazwinska et al. 1999a). *brk* is involved in Dpp modulation during wing formation and embryonic development. Since a *brk* ortholog is present in the *Tribolium* genome, I investigated its function in this beetle. No early embryonic expression was detected; and parental or larval RNAi failed to reveal any function of *Tc-brk* in embryos or wing formation, respectively (data not shown). Since this gene is also present in other insects, like *Nasonia vitripennis*, the evolution of the function of this gene should be addressed in these other systems as well.

Taken together, TGF-β components are generally conserved among *Tribolium* and *Drosophila.* The existence of at least one ligand and three modulators (ORANGE in the table) previously considered to be restricted to vertebrates indicates that *Tribolium* retained a more ancestral composition of TGF-β components. Also interesting is the difference in numbers of modulators (YELLOW in the table) such as *crossveinless/twisted-gastrulation* and *tolloid*. A detailed functional analysis of these two modulators is provided in the next chapter.

Chapter 3 - Evolution of BMP/Dpp extracelllular modulators in insects - the study case of twisted-gastrulation/crossveinless and tolloid-like molecules

Introduction:

As mentioned in Chapter 1, Bone morphogenetic proteins (BMPs) are involved in dorsoventral patterning in flies and vertebrates. In *Drosophila*, two BMP family members *decapentaplegic* (*dpp*) and *screw* (*scw*) are required for patterning the dorsal region of the embryo (Arora et al. 1994). While *scw* is transiently and ubiquitously expressed, *dpp* mRNA is confined to the dorsal 40% of the *Drosophila* embryo (St Johnston and Nusslein-Volhard 1992). Comparably, two BMP factors, *BMP2b* and *BMP7*, are involved in dorsoventral axis formation in zebrafish and *Xenopus* (Holley et al. 1995; Ferguson 1996; Kishimoto et al. 1997; De Robertis and Kuroda 2004).

These similarities extend further to the formation of Dpp morphogenetic gradients in flies and vertebrates (Holley et al. 1995; Ferguson 1996). In *Drosophila*, high and low amounts of Dpp activity are essential for amnioserosa and non-neurogenic ectoderm specification, respectively (Ashe et al. 2000). Similarly, BMP activity is observed as a ventral-to-dorsal gradient in vertebrates (reviewed in Dale and Wardle 1999). This graded distribution of BMP4/Dpp activity is mediated by the action of at least three conserved extracellular modulators, *short-gastrulation* (*sog*/*Chordin*), *tolloid (tld)* and *twisted-gastrulation (tsg*) (Fig 3.1, Oelgeschlager et al. 2000; Ross et al. 2001; Scott et al. 2001b).

In *Drosophila, sog* has a dual role in the modulation of Dpp activity. First, Sog binds to Dpp, inhibiting its diffusion to the neurogenic ectoderm (anti-BMP function). This is achieved by the establishment of a Sog protein gradient inverse to Dpp (Srinivasan et al. 2002). Second, Sog has a long range pro-BMP function, since it is required to transport Dpp to the amnioserosa region, where Dpp activity is maximal (Ashe and Levine 1999; Decotto and Ferguson 2001). In vertebrates, first analysis indicated that Chordin/Sog would act exclusively as an antagonist of BMP (Miller-Bertoglio et al. 1997). However, epistatic analysis of *chordino/dino* and *swirl/bmp2b* double-mutants indicated a pro-BMP role of Chordin (Wagner and Mullins 2002). Further support for a conserved dual role of Sog was recently obtained in the beetle *Tribolium*, an insect with ancestral type of embryogenesis (van der Zee et al. 2006).

The second conserved BMP extracellular mediator, Tld, is a metalloprotease responsible for Sog cleavage from the Sog-Dpp inhibitory complex. This cleavage releases the Dpp ligand, both in *Drosophila* and in vertebrate embryos (Blader et al. 1997; Marques et al. 1997). In flies, null *tld* alleles show a partially ventralized phenotype similar to weak *dpp* mutants (Shimell et al. 1991; Ross et al. 2001). Furthermore, hypomorphic alleles of *tld* can be suppressed by addition of extra copies of *dpp*, indicating that *tld* acts to enhance *dpp* activity (Ferguson and Anderson 1992). In zebrafish two metalloproteases *tolloid* (*bmp1a*) and *tolloid-like (tll1)* act in early Chordin cleavage, enabling BMP signalling (pro-BMP). Additionally, the *tolloid/mini fin* mutant is dorsalized, indicating lack of BMP signalling (Connors et al. 1999; Muraoka et al. 2006). Taken together, *tld* acts as a pro-BMP molecule in all systems studied so far, although biochemical differences in cleavage between *Drosophila* and vertebrates have been recently pointed out (Canty et al. 2006).

The third conserved BMP extracellular modulator is *tsg*. In *Drosophila*, Tsg is involved, together with Sog, in the transport of Dpp towards the dorsal region (Ross et al. 2001; Wang and Ferguson 2005, Shimmi et al. 2005b, Fig 3.1A). Since *tsg* and *sog* are required for BMP signalling in the dorsal most region, both loss-of-function phenotypes are very similar in *Drosophila* (Zusman and Wieschaus 1985, Mason et al. 1994, Ross et al. 2001). In vertebrates, Tsg has been showed to inhibit (Ross et al. 2001, Chang et al. 2001; Blitz et al. 2003; Jenner and Wills 2007) and to promote BMP signalling (Oelgeschlager et al. 2000, Oelgeschlager et al. 2003, Little and Mullins 2004, Zakin and De Robertis 2004; Xie and Fisher 2005).

Since Tsg can biochemically interact with BMP, Sog and Tld, a dual role of these interactions has been used to explain these different loss-of-function phenotypes (Oelgeschlager et al. 2000; Larrain et al. 2001; Ross et al. 2001, Figure 3.1). First, Tsg can synergistically inhibit BMP/Dpp action by forming a tripartite complex between itself, SOG/chordin and a BMP ligand (Fig 3.1B). Second, Tsg seems to enhance the Tld/BMP-1-mediated cleavage rate of SOG/chordin, enabling BMP signalling (Oelgeschlager et al. 2000; Ross et al. 2001, Fig 3.1B). Thus, Tsg can stimulate or inhibit BMP signalling depending on the activity of Tld metalloproteases.

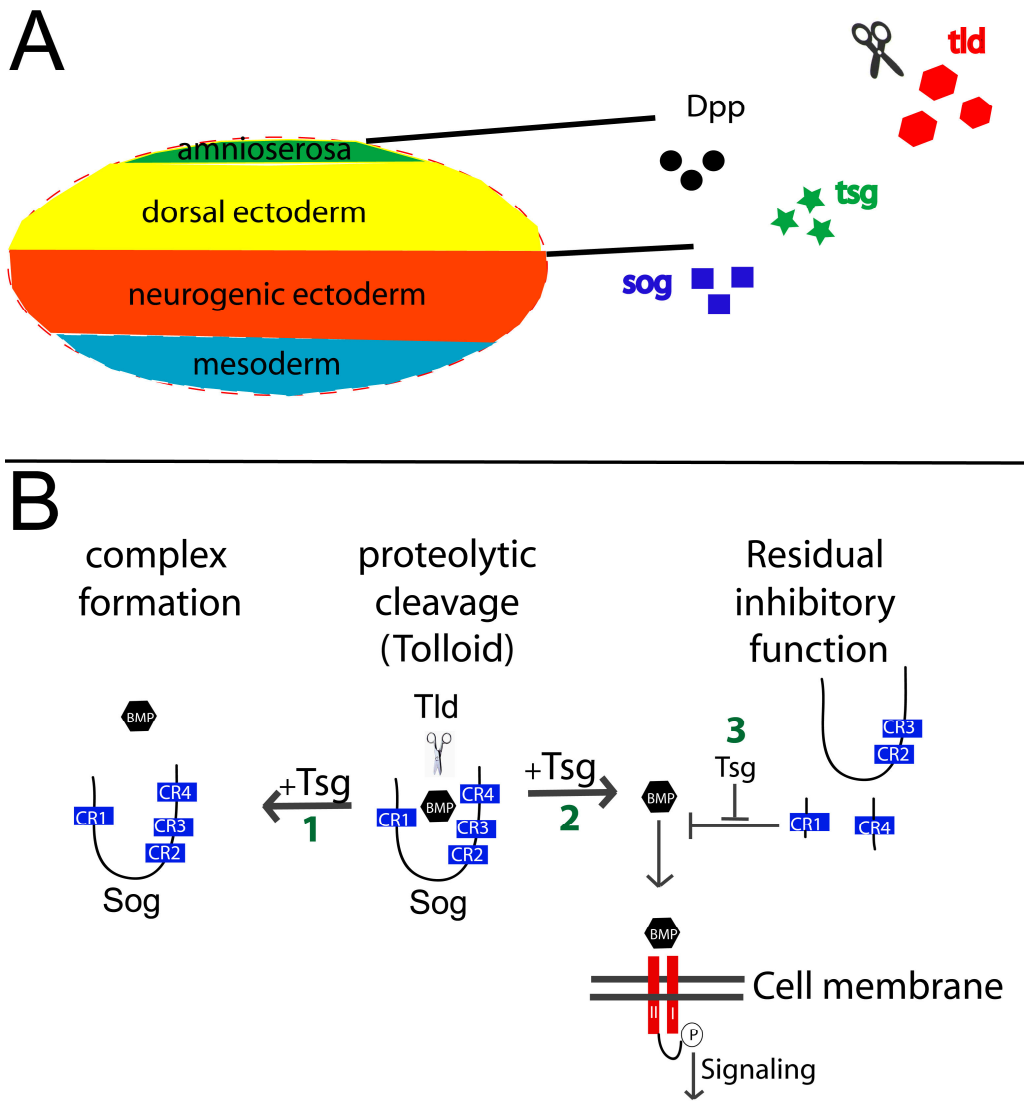


Figure 3.1: Mechanism of BMP/Dpp regulation in *Drosophila* embryos (A) and the known biochemical interactions among BMP/Tsg/Tld/Sog (B).
(A) Sog binds to Dpp transporting it to the dorsal region of the egg. Tsg enhances Sog binding to Dpp. In the dorsal region, Tld cleaves Sog, enabling Dpp signal in this region. (B) Tsg and its proposed roles in BMP/Dpp modulation. (1) Tsg enhances the inhibitory complex formation, but also (2) enhances the proteolytic cleavage of Sog via Tld, enabling signal. (3) Tsg avoids any residual inhibitory activities from cleaved Sog fragments. Adapted from Yamamoto, 2004 #502

In the recently sequenced *Tribolium castaneum* genome, I observed that a unique homolog of *tld* and a unique homolog of *tsg/cv* exist in the beetle genome. To understand the evolution of these extracellular modulators in insects, I investigated *tsg/cv* and *tld* function during *Tribolium* embryonic development.

Results:

Only one Twisted gastrulation/Crossveinless and one Tolloid-like molecule exist in the beetle genome

Two families of Twisted-gastrulation (Tsg) related molecules exist in insect genomes (Vilmos et al. 2005). The first is the Crossveinless (Cv) protein family and the second is the Tsg protein family (Vilmos et al. 2005, Figure 3.2A). While Cv proteins exist in all insect genomes sequenced to date, Tsg proteins are restricted to Drosophillids (Figure 3.2A, Vilmos et al. 2005). Two Tsg proteins exist in the *Drosophila melanogaster* genome. The first, *Dm-tsg1,* was described as a dorsoventral mutant (Zusman and Wieschaus 1985, Dm-tsg in Figure 3.2A). The second, *CG11582 (Dm-tsg3),* corresponds to the dorsoventrally affected mutant *shrew* (Vilmos et al. 2001, Bonds et al. 2007). *Dm-tsg-3* is not displayed in the phylogenetic tree, since it lacks the whole N-terminal region which contains one of the cysteine-rich domains used for the phylogenetic reconstruction (Figure 3.2A, Vilmos et al. 2001). Only one member of the Cv protein family exists in the *D. melanogaster* genome, *Dm-tsg2 (crossveinless). crossveinless* (*cv*) was recently shown to be required for BMP/Dpp signalling during wing formation, but is dispensable for embryonic development (Shimmi et al. 2005a; Vilmos et al. 2005).

Only one Tsg/Cv-like protein exists in the recently sequenced *Tribolium castaneum* genome (Richards and Genome consortium 2008). Phylogenetic analysis indicates that this *Tribolium* Tsg/Cv-like protein is most similar to the Crossveinless family (Figure 3.2A). The position of the *Tribolium* Cv/Tsg-like protein in the Crossveinless family is corroborated by specific aminoacid signatures in both protein families, as depicted for the first cysteine rich region (CR) (Figure 3.3 and Vilmos et al. 2005). Another indication for the split between these two families was obtained from the intron-exon organization. *Cv-like* genes, including *Tc-cv* and *Dm-cv,* contain four introns, in contrast to the intronless unit present in *Dm-tsg1* and in *Dm-tsg3* (*CG11582/shrew)* gene (Vilmos et al. 2001 and Figure 3.2A). This intron-exon organization is shared among Cv-like proteins from other insects like *Anopheles gambiae* (Diptera) and *Apis mellifera* (Hymenoptera), which contain only one Cv protein in their genomes containing four introns (Figure 3.2A). Interestingly, two *Nasonia vitripennis* (Hymenoptera) *cv* genes, *Nv-cvA* and *Nv-cvC,* seem to have arisen via an independently duplication event. These two genes contain intronless transcripts (Figure 3.2A, their functrion is dicussed later in this chapter). Altogether, we infer that the unique Cv/Tsg-like protein in

the *Tribolium* genome belongs to the *crossveinless* gene family, the *Tc-crossveinless (Tc-cv)* gene.

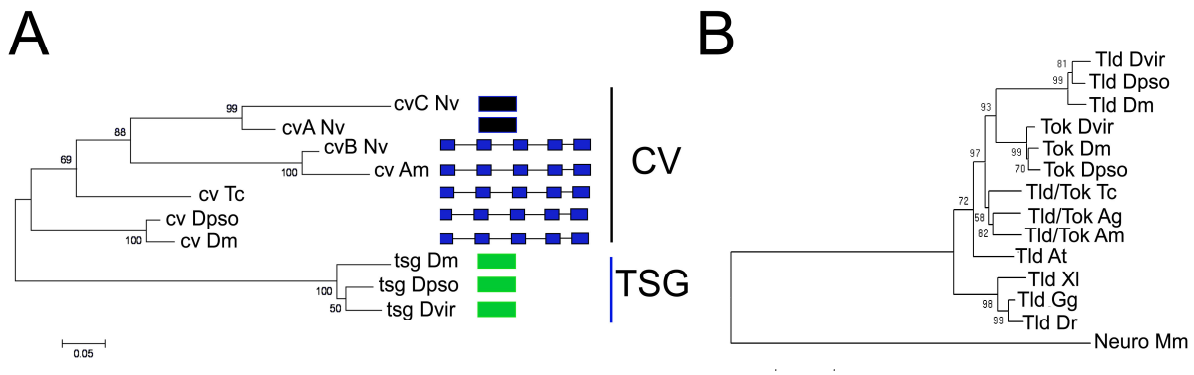


Figure 3.2: Only one *Tc-crossveinless* and one *Tc-tolloid* are found in ancestral insects. Minimum Evolution (ME) tree using Crossveinless (Cv) and Twisted-gastrulation (Tsg) proteins from insects. Only one Crossveinless/Twisted-gastrulation-like (Cv/Tsg-like) protein is present in *Apis mellifera* (Am), *Anopheles gambiae* (Ag) and *Tribolium castaneum* (Tc). In contrast, *Drosophila melanogaster* (Dm) and *Nasonia vitripennis* (Nv) contain three Cv/Tsg-like proteins, which have arisen independently. Only two *D. melanogaster* Cv/Tsg-like proteins are shown in the phylogenetic tree (CG11852/Shrew is not shown in the phylogenetic tree, since it lacks the N-terminal cysteine repeat domain). The rectangles indicate intron-exon organisation, which is identical among the proteins of each group, *tsg* and *cv*. The unique exceptions are the intronless transcripts of *Nv-cvC* and *Nv-cvA*, which are also intronless. B) ME tree using Tolloid proteins of proteostomes and deuterostomes. Two Tolloid related molecules, Tolloid and Tolkin are present in Drosophillids genomes, but only one in other arthropods. Organism abbreviation: Dpso - *Drosophila pseudobscura*, Dvir - *Drosophila virilis*, At - *Achaearanea tepidariorum*, Xl - *Xenopus laevis*, Gg - *Gallus gallus*, Dr - *Danio rerio*, Mm - *Mus musculus*. Gene Bank Access of the sequences used in the analyses: (A) *cvC-Nv* - Contig8963 in the genome, *cvA-Nv* - Contig 286, *cvB-Nv* - Contig24340, *cv Am* - XP_392649.1, *cv Tc* - Tc-03620, *cv Dpso* - GA11617-PA , *cv Dm* - CG12410-PA, *tsg Dm* - CG1502, *tsg Dpso* - GA13438, *tsg Dvir* - Dvir\GJ18736 . B) *Tld Dvir* - FBgn0026890, *Tld pso* - Dpse\GA19917, *Tld Dm* - CG6868, *Tok Dvir* - GLEANR_8989_caf1, *Tok Dm* - CG6863, *Tok Dpso* - Dpse\GA19912, *Tld/Tok Tc* - Tc-11197, *Tld/Tok Ag*- ENSANGG00000018762, *Tld/Tok Am* - ref|XP_393866.2|, *Tld At*-BAD01492, *Tld Xl*- emb|CAA70854.1|, *Tld Gg* - emb|CAC08820.1|, *Tld Dr* - ref|NP_571085.1| , *Neuropilin Mm* - *NP 032763*.

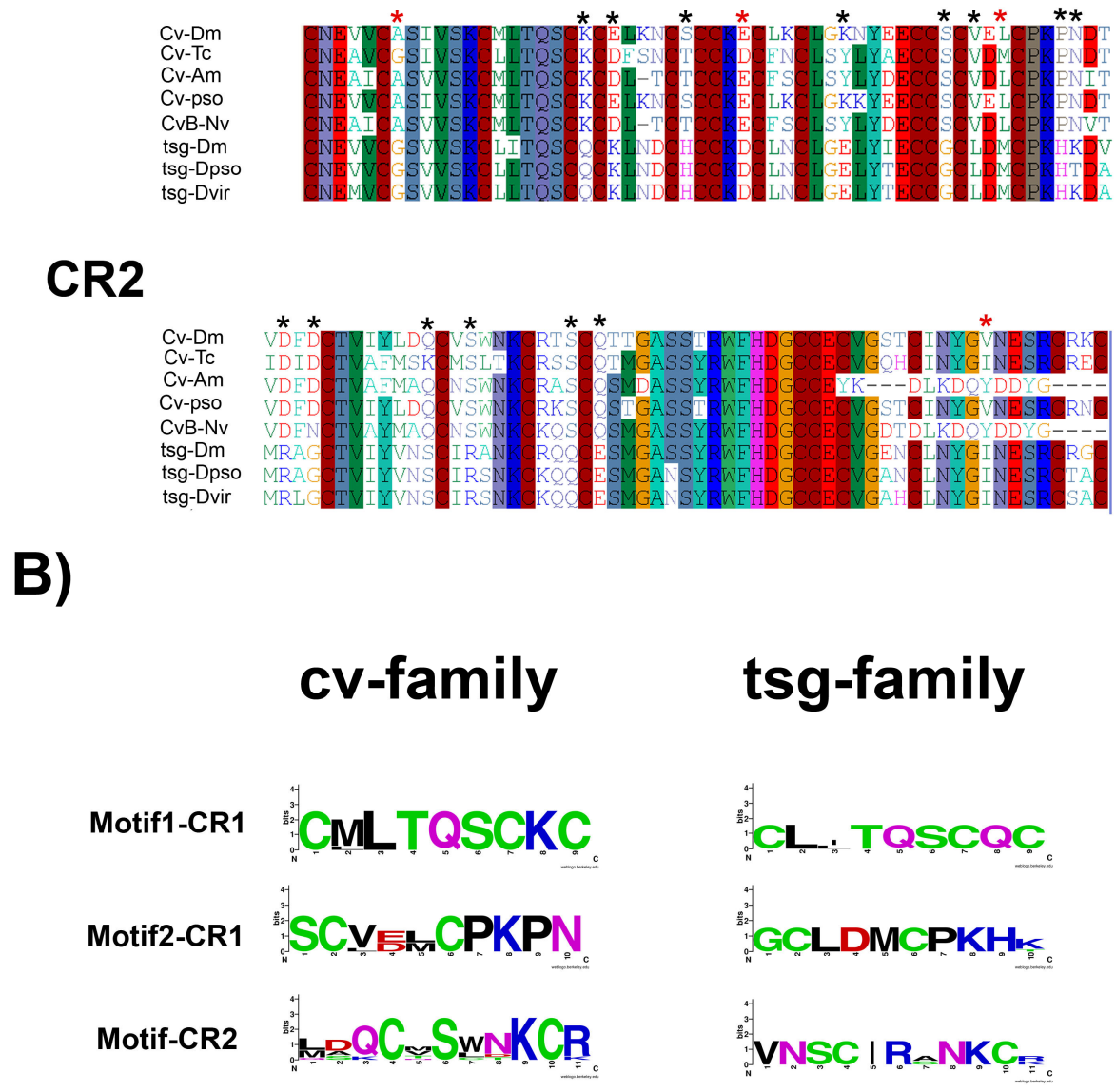


Figure 3.3: Crossveinless and Twisted-gastrulation protein families can be separated in two distinct groups

(A) Two Cysteine-Rich (CR) domains are found in Crossveinless/Twisted-gastrulation proteins in insects (CR1 and CR2). These two families can be separated by specific aminoacids signatures (asterisks). Three residues (orange asterisk) are identical between Tc-Cv and the Twisted-gastrulation protein family. All other 14 residues (in black) are identical between Tc-Cv and other Crossveinless proteins. (B) These two protein families (Cv and Tsg) have specific characteristic domains (weblogo.berkeley.edu/), that differ among them.

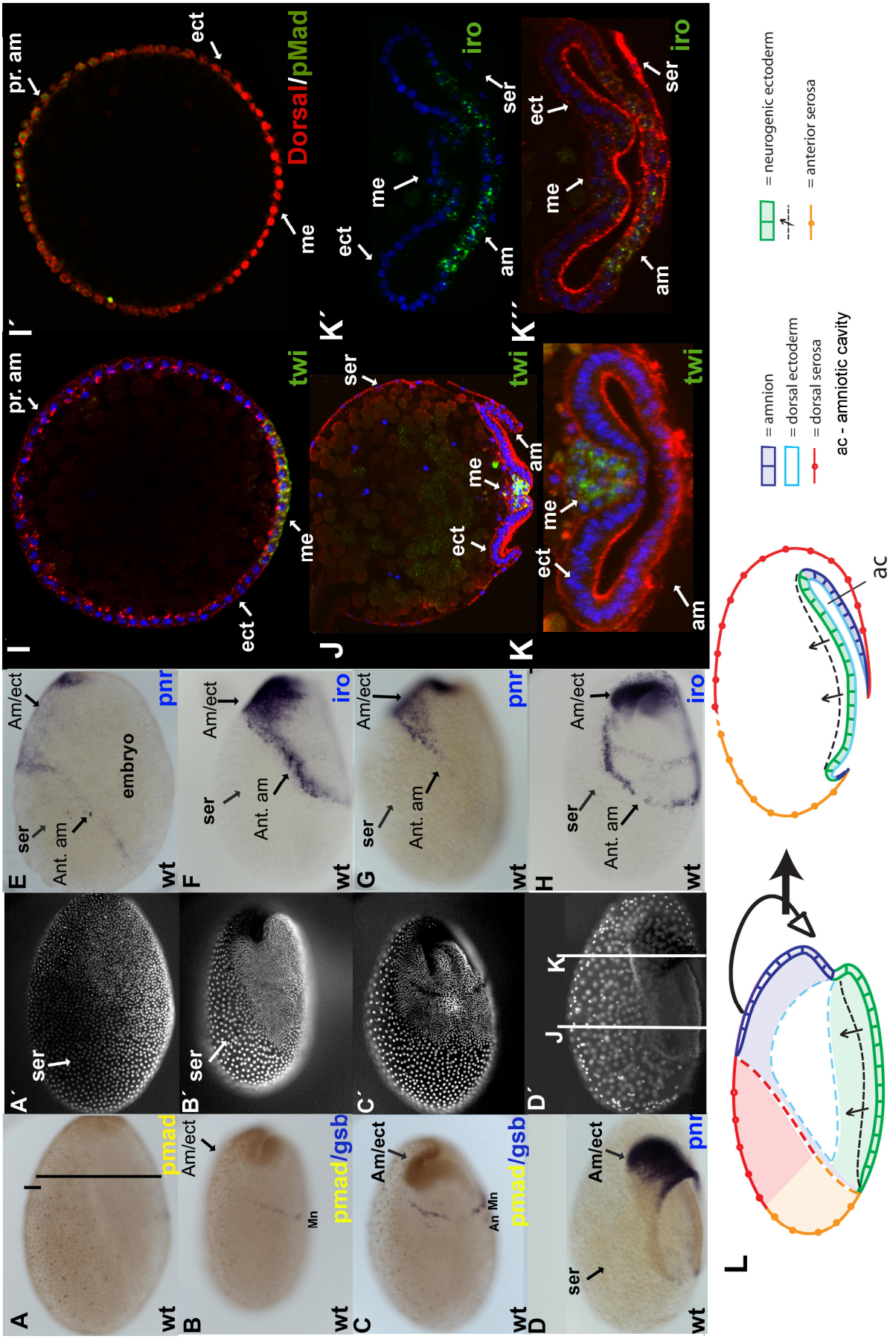
A similar strategy was applied to search for Tolloid-related molecules in the *T. castaneum* genome. Tolloid-like (Tld) molecules generally contain one metalloprotease, two EGF-like and five CUB domains, resembling the BMP1 molecule in vertebrates (Shimell et al. 1991). Besides the *tld* allele described in the screening for zygotic mutations (reviewed in Rushlow and Roth 1996), another *tld-like* molecule, *tolkin* (*tok* or *tolloid-related*), is present in the *D. melanogaster* genome. *Dm-tok is* involved in Sog processing during wing formation in *Drosophila*, a BMP/Dpp related function (Finelli et al. 1995, Serpe et al. 2005). Interestingly, *Dm-tok* is located 700bp upstream of *Dm-tld* in Drosophillids genomes, but only one Tolloid/tolkin molecule is found in syntenic chromosomal regions of *Anopheles gambiae*, *Apis mellifera* and *Tribolium castaneum* (data not shown, available at Ensembl browser). Our phylogenetic analysis supports that *Tc-tld* belongs to the Tolloid metaloprotease family, and that a duplication event *Tc-tld/Tc-tok* may have occurred in higher Diptera (Figure 3.2B). Taken together, our analyses indicate that the number of *crossveinless* and *tolloid/tolkin* molecules has changed during insect evolution.

Understanding how gastrulation, DV axis and Dpp activity are established in the *Tribolium* WT enables a proper analysis of *Tc-cv* and *Tc-tld* knock-down phenotypes

As described in Chapter 1, *Tribolium* eggs contain a huge part of their eggs constituted by extra-embryonic membranes. Two extra-embryonic membranes the serosa and the amnion are observed during *Tribolium* development. Serosal cells are recognized during blastoderm differentiation by nuclear staining (DAPI), since these cells stop dividing and become polyploid (Fig 3.4A´,B´). On the other hand, amniotic cells still divide and no clear difference in nuclear density is observed after DAPI stainings, although scanning electron microscopy (SEM) have identified cells between the serosa and the embryonic as amniotic cells (Handel et al. 2000). Investigation of two molecular markers *Tc-iroquois* (*Tc-iro*) and *Tc-pannier (Tc-pnr)* show that these amniotic cells are present in the dorsal posterior region of the egg (Am/Ect in Figure 3.4E,F,G), and in an oblique stripe between the embryo and the anteriorly located serosa (Ant. Am in Figure 3.4E,F,G). It has been recently shown that Tc-Dpp activity (pMad) is required for the pattern of the dorsal amnion via a Tc-Sog transport mechanism (Van der Zee et al. 2006). Although *Tc-dpp* is expressed as an oblique stripe overlapping with the anterior amnion (Figure 1.10H), Tc-Dpp activity is observed as a broad domain during blastoderm differentiation (Figure 3.4A). This broad domain overlaps with the dorsal amniotic

domain of *Tc-pnr* and *Tc-iro* (Figure 3.4A,E,F,G - Am/Ect) and with the dorsal serosal cells expressing *Tc-dorsocross* (Figure 3.6E).

During gastrulation, the extensive movements of the prospective extra-embryonic cells (serosa and amnion) change the topology of the *Tribolium* embryo. This is evident by the analyses of cross sections of blastoderm (Figure 3.4I,I´) and gastrulating embryos (Figure 3.4J,K). Cross sections during blastoderm stages show Dpp activity by the analysis of antibody stainings against the phosphorylated form of *mothers against dpp* (pMad), a read-out of Dpp signalling (Raftery et al. 1995; Sutherland et al. 2003). pMad is observed in a broad dorsal domain that overlaps with the amnion and the dorsal ectoderm (Figure 3.4I´ - green staining in the dorsal nuclei), while the nuclear Dorsal gradient and the mesodermal cells (me) expressing *Tc-twist* are present in the ventralmost regions (Figure 3.4I,I`). Cross sections of embryos undergoing gastrulation show the extensive cell movements which occur during this process (Figure 3.4J,K). The amniotic cells located in the dorsalposterior region, which express *Tc-iro* and *Tc-pnr* (Figure 3.4E,F,G,H – Am/Ect) fold underneath the spreading dorsal serosa and, advancing with the latter, close the amniotic cavity at the ventral region (Figure 3.4D - *Tc-pnr* during gastrulation and two cross sections in Figure 3.4J and K). Briefly, two major spatial changes have occurred during the gastrulation process. The mesoderm during gastrulation is internalized by ventral furrow formation (Handel et al. 2005 and Figure 3.4J,K), being located dorsally after this process. The serosa, the amnion and the ectoderm are, after gastrulation, located ventrally in the egg (Figure 3.4K´,K´´). Importantly, cells expressing *Tc-iro* and containing Dpp activity (pMad), which during blastoderm stages can be observed dorsally (Figure 3.4A,F - Am/Ect, and Figure 3.4I´- pr. am.), are present after gastrulation ventrally (Figure 3.4K´,K´´). During further development the serosa completely surrounds the embryo and the amnion (see schematic drawings on Figure 3.4L and Figure 3.4K´,K´´- ser). The knowledge of the gastrulation movements in WT eggs will be important to understand the consequences of the loss of the Dpp modulators *Tc-cv* and *Tc-tld* in the next section.


A
wt
pmad
A´
ser
I
B
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Am/ect
B´
ser
Mn
pmad/gsb
C
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Am/ect
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L
= amnion
= dorsal ectoderm
= dorsal serosa
= neurogenic ectoderm
= anterior serosa
ac - amniotic cavity
ac

Figure 3.4: Dpp activity and gastrulation movements in WT embryos
(A-C) Dpp activity in WT embryos (brown). *Tc-gooseberry* is used as a segmental marker (blue). (A-C) Lateral view (A) Before the visible differentiation of the embryo proper and the serosa, pMad (Dpp activity) is detected as a broad dorsal domain. Transversal cross sections of embryos at a similar stage are provided in I and I´. (B) Shortly before gastrulation a strong dorsalposterior domain, which overlaps with the amnion and the dorsal ectoderm (Am/Ect) is evident. (C) During gastrulation, the pMad domain covers the embryo through the ventral region. (D,E,G) *Tc-pnr* stainings in WT embryos. *Tc-pnr* during gastrulation is detected in the dorsal ectoderm and in the amnion. (D´) DAPI staining of the same embryo as shown in D. Transversal cross sections along different AP regions of embryos at the same stage as the one in D´are provided in J and K. (E) Blastoderm undergoing differentiation and (G) Differentiated blastoderm - *Tc-pnr* is expressed in the anterior amnion (Ant. am.) and in the dorsal ectoderm and dorsal amnion (Am/ect). (F) Differentiated blastoderm - *Tc-iro* is expressed in similar domains as *Tc-pnr*. (H) *Tc-iro* is expressed in the amnion and in the posterior ectoderm that covers the embryo through the ventral side (ventro-lateral view). (I) Transversal cross section of a syncitial blastoderm embryo stained for *Tc-twi* (*in situ*-green), phosphotyrosine (red) and DAPI (blue) (I´) Transvesal cross section of a syncitial blastoderm embryo stained for anti-Dorsal (antibody - red) and pMad (antibody - green). (J,K) Transversal cross sections at the anterior (J) and at the posterior (K,K´,K´´) region of an embryo at a similar stage to the one in D´. In J and K the yellow *Tc-twi* staining marks the mesoderm. (K´,K´´) The same cross section is shown in K´ and K´´. In K´ only DAPI (blue) and *Tc-iro* (green) are shown. In K´´ the same two stainings as K´ plus an anti-phosphotyrosine antibody (membrane staining- red) is shown. Ant. am. - anterior amnion, Am/ect - amnion/ectoderm, ser – serosa, me – mesoderm, ect – ectoderm, pr. am - prospective amnion. An - antenna, Mn - mandible , Mx – maxilla, (L) Schematic drawings from blastoderm and gastrulating embryos, the arrow denotes the movements of the amniotic cells. These drawings are a courtesy of Maurjin van der Zee.

Tc-cv and *Tc-tld* expression suggest a role in early embryo patterning

I analysed the expression pattern of *Tc-cv* and *Tc-tld* during *Tribolium* embryonic development. Maternal transcripts can be detected by RT-PCR (data not shown), but the first sign of distinct expression is observed during blastoderm differentiation. At this stage *Tc-tld* is expressed in the whole germ rudiment (Figure 3.5A), being absent from the anteriorly located serosa (Figure 3.5A,A´). In differentiated blastoderm stages, *Tc-tld* transcripts are absent in the ventral most region, where the prospective mesoderm is located (Figure 3.5B, arrow). *Tc-sog* and *Tc-twist* transcripts are expressed in this ventral region at that stage (Handel et al. 2005, van der Zee et al., 2006 and see Figure 3.5C for a double-staining *Tc-twi*/*Tc-tld*). In contrast to *Drosophila* where Dm-Dorsal directly represses *Dm-tld* expression (Kirov et al. 1994), the Tc-Dorsal gradient has largely vanished at this stage (Chen et al. 2000), suggesting that Tc-Dorsal does not directly repress *Tc-tld* transcription.

After gastrulation, during serosal closure stage, *Tc-tld* is detected in the ectoderm, but is still absent from the mesoderm (Figure 3.5D,D´, arrow). *Tc-tld* expression domain extends over the neurogenic ectoderm at that stage, similarly to the recently reported expression of *Ag-tld* in the mosquito *A. gambiae* (Goltsev et al. 2007). In contrast, *D. melanogaster tld*

expression is confined to the non-neurogenic ectoderm and the amnioserosa (Kirov et al. 1994).

During germ band elongation, a broad domain in the posterior most region, the growth zone, is observed, besides a staining between the head lobes (Figure 3.5E). In elongating germ bands a ventral midline staining about 3 cell wide can be observed (Figure 3.4F´, higher magnified). Cells expressing *Tc-tld* between the two head lobes might represent the presumptive stomodeum (Figure 3.5F, arrow). Taken together, this complex expression pattern of *Tc-tld* suggests that this gene could be involved in early *Tribolium* embryo patterning.

Early *Tc-cv* expression can be detected as an oblique stripe between the germ rudiment and the serosa, besides a staining in the posterior pit (Figure 3.5G). This expression pattern of *Tc-cv* is similar to *Tc-dpp* at a similar embryonic stage (Chen et al. 2000, van der Zee et al. 2006, Figure 1.10H). During gastrulation and in early germ band stages *Tc-cv* is expressed in the ectoderm like *Tc-tld*, being absent from the mesoderm (compare Figure 3.5B,H and 3.5D,I). Extending germ band embryos also express *Tc-cv* in the growth zone (Fig 3.5J´, higher magnified), and in a ring-shape domain between the head lobes (Fig 3.5J). This ring of expression surrounds the large dot-like expression domain of *Tc-tld* at a similar stage (Figure 3.5J, *Tc-tld* in 3.5F). In completely extended germ bands, *Tc-cv* transcripts can also be detected in the dorsal ectoderm and in the legs (data not shown). Taken together, as for *Tc-tld*, *Tc-cv* expression pattern suggests a complex role during *Tribolium* embryogenesis. We addressed if *Tc-tld* and *Tc-cv* would be involved in BMP/Dpp modulation by performing knock-down via pRNAi against these factors.

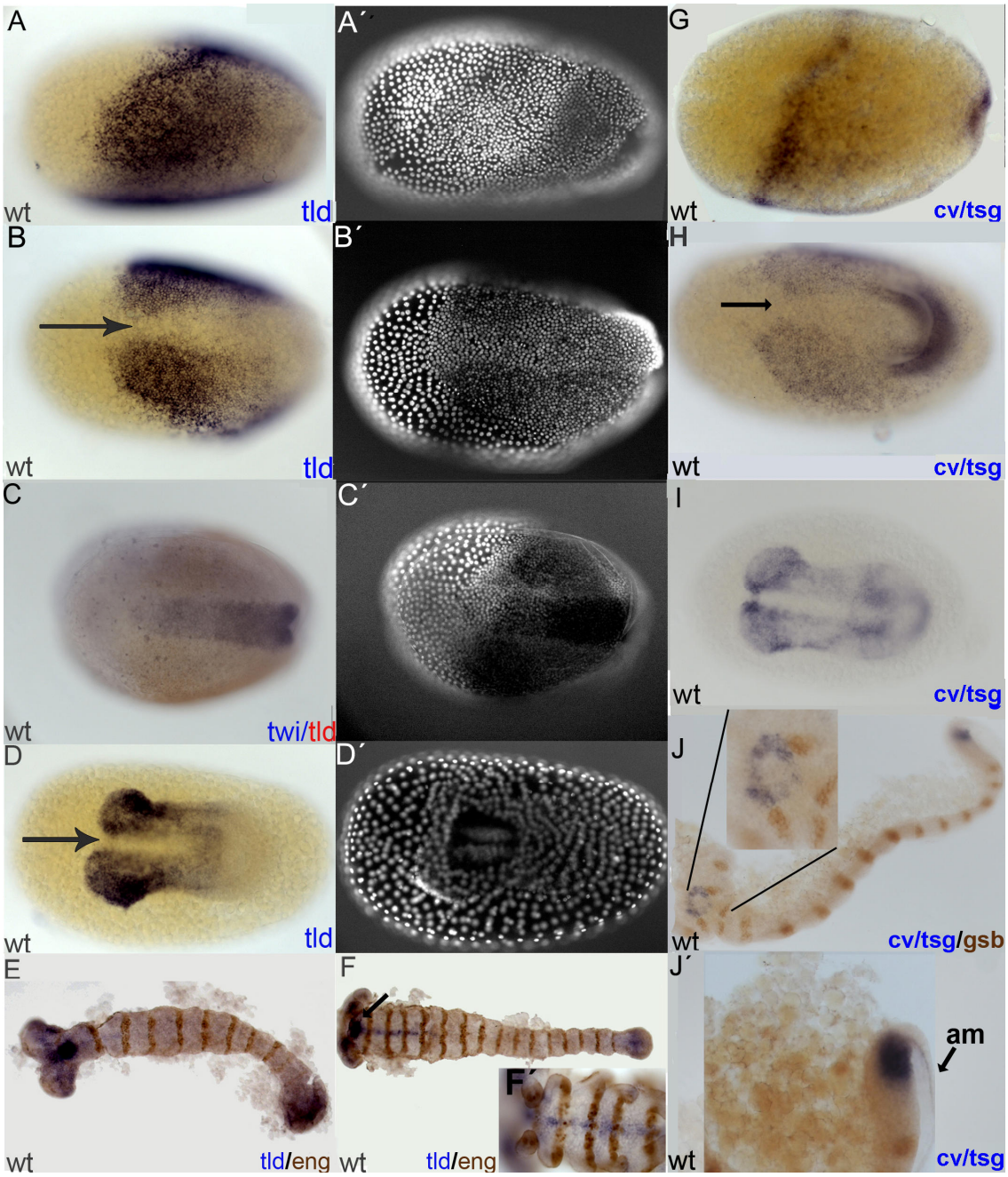


Figure 3.5: ***Tc-tolloid (Tc-tld) and Tc-crossveinless (Tc-cv)*** **expression during** ***Tribolium*** **embryogenesis.**
A-F) *Tc-tld* expression. (A) Lateral view of a blastoderm embryo undergoing differentiation. The serosa wider spaced nuclei are evident in the anterior region differing from the compact nuclei in the posterior which contain the amnion plus embryo proper. *Tc-tld* is expressed in almost the whole embryo, but not in the anterior serosa. (B) An embryo older than the one in A with the ventral region faced up. Note that *Tc-tld* is not expressed in the ventral region neither in serosa nuclei. (C) Double staining for *Tc-twist* (purple) and *Tc-tld* (orange). (D) An embryo during serosa closure stage. *Tc-tld* is expressed at the surface of the embryo but not in the middle of the head (arrow). (E) A germ band containing 10 engrailed stripes also stained for *Tc-tld*. *Tc-tld* is detected in the middle of the head lobes, possibly the stomodeum and broadly in the growth zone. (F) In an almost completely extended germ band, a ventral midline expression can be observed (F´). (G-J´) - *Tc-cv* expression (G) Early blastoderm stage embryo, *Tc-cv* is expressed in a stripe in the region between serosa and germ rudiment and in a posterior region. This pattern resembles *Tc-dpp* (van der Zee et al. 2006). (H) Shortly after gastrulation, *Tc-cv* is expressed in the ectoderm, similarly to *Tc-tld*, being absent from the mesoderm (arrow) (I) As observed for *Tc-tld*, *Tc-cv* is expressed in almost the whole surface of the embryo with an exception in the middle of the head, where the mesodermal domain of *Tc-twi* is found. (J) Double staining for *Tc-cv* (blue) and the segmental polarity gene *Tc-gsb* (orange). (J´) Closer view of *Tc-cv* expression in the growth zone. *Tc-cv* is absent from the amnion (am) (details on growth zone formation and provided in Figure 3.11).

Tc-tld and *Tc-cv* loss-of-function affect Dpp activity leading to changes in early fate map of *Tribolium* blastoderm

Similarly to double stranded RNA (dsRNA) injection of *Tc-dpp* in pupae, *Tc-cv* and *Tc-tld* pupal injection led to sterility (van der Zee et al. 2006). After adult injection (aRNAi), eggs were recovered. Larval hatching and cuticle formation were not observed, suggesting early and strong phenotypes in the absence of these transcripts. To understand to what extent the Tc-Dpp activity is dependent on *Tc-tld* and *Tc-cv*, we investigated pMad. During blastoderm differentiation, pMad overlaps with the dorsal serosa and the dorsal amnion (described in Figure 3.4 and Figure 3.6A, see also schematic drawing in Figure 3.6J).

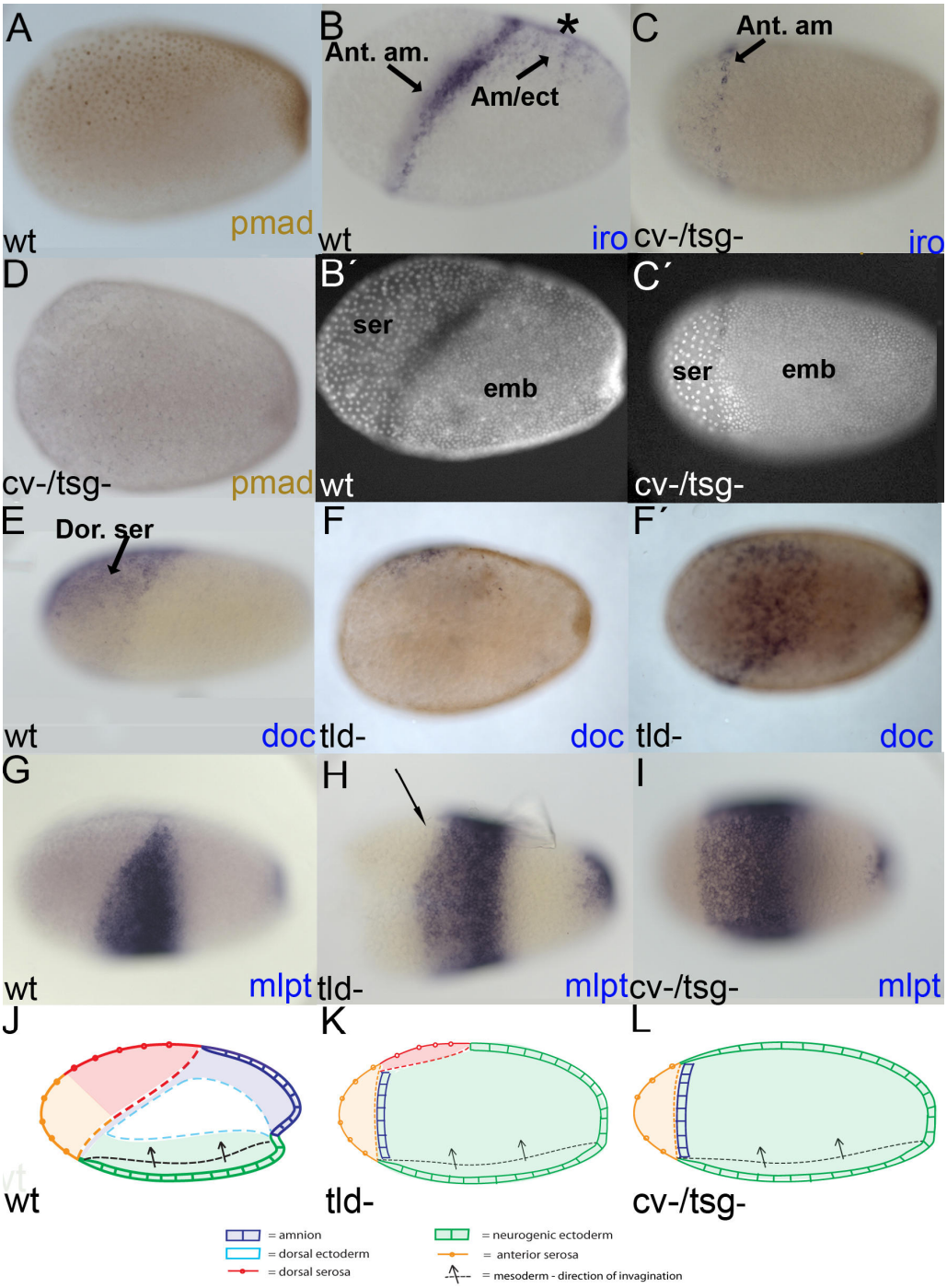


Figure 3.6: Early changes in blastodermal patterning after *Tc-cv* and *Tc-tld* RNAi
(A-I) Lateral views, with the exception of the embryo in F´ (dorsal view). (A,D) pMad antibody staining (Dpp readout). In WT (A) pMad is detected in the dorsal serosa and in the amnion, while pMad is absent after *Tc-cv/tsg* knock-down (D) (B,C). *Tc-iro* expression in differentiated blastoderm stages. In WT (B) *Tc-iro* is expressed in an oblique stripe (Ant. am.) and in a dorsal-posterior domain (asterisk, Am/ect). (C) After *Tc-cv/tsg* RNAi the anterior *Tc-iro* domain becomes symmetric along the DV axis and the dorsal domain is absent. (B´C`) DAPI stainings. After *Tc-cv/Tc-tsg* RNAi (C´) the germ rudiment (smaller nuclei) is expanded towards a dorsal-anterior position, acquiring a symmetric distribution along the DV axis. In WT (B´) an oblique distribution of nuclei is observed. (E,F) *Tc-doc* expression. (E) In WT, *Tc-doc* is expressed in the dorsal serosa, where Dpp activity is detected (red in J). After *Tc-tld* RNAi (F) a small number of cells express *Tc-tld* in the dorsal region (see text for details and F´ for a dorsal view). (G,H,I) *Tc-mlpt* expression. (G) In WT *Tc-mlpt* is expressed in the head region. After *Tc-cv* RNAi (I), the *Tc-mlpt* head domain, becomes completely symmetric along the DV axis. After *Tc-tld* RNAi (H), *Tc-mlpt* is also expanded, but does not become completely symmetric along the DV axis (the arrow shows the residual DV polarity). (J,K,L) Shematic drawings highlight the different fates along the DV axis. Note that the difference between the two knock-down phenotypes are the dorsal serosal cells present after *Tc-tld* RNAi. This residual polarity leads to different gastrulation movements when compared to *Tc-cv* RNAi embryos (see next figure).

In WT embryos *Tc-doc* marks the dorsal serosa, overlapping with pMad (Fig 3.6A,E). *Tc-iro* is expressed in one oblique anterior stripe (Figure 3.6B, anterior amnion) and in a broad dorsal posterior domain (Figure 3.6B, Am/ect, see also schematic drawing in 3.6J). After *Tc-cv* RNAi, pMad, in the dorsal region, *Tc-doc,* in the dorsal serosa, and the dorsal domain of *Tc-iro* are deleted (Figure 3.6D for pMad, Figure 3.6C for *Tc-iro* and *Tc-doc* data not shown). Only the anterior stripe (Ant. am) of *Tc-iro* remains after *Tc-cv* RNAi, which acquires a straight orientation in relation to the DV axis (Figure 3.6C, see respective DAPI stainings Figure 3.6B´,C´). Altogether, these results indicate that Dpp activity during blastodermal stages is completely absent after *Tc-cv* RNAi, since the same changes in molecular markers is observed after *Tc-dpp* RNAi (van der Zee et al. 2006).

After *Tc-tld* RNAi the dorsal domain of *Tc-iro* and pMad activity are also absent (data not shown). In contrast, a patch of *Tc-doc* expression in the dorsal serosa is still detected (Figure 3.6F, dorsal view in 3.6F´). This indicates that in the absence of *Tc-tld*, BMP/Dpp signalling still occurs in a small region of the dorsal serosa (see schematic drawing Figure 3.6K). The analysis of the expression pattern of the gap gene *Tc-mlpt* (Savard et al. 2006a) in WT, in *Tc-tld* RNAi, and *Tc-cv* RNAi embryos give further support to the aforementioned fate map shifts. In WT, *Tc-mlpt* is expressed in a broad triangular domain overlapping with the head (van der Zee et al. 2005; Savard et al. 2006a, Figure 3.6G). After *Tc-cv* RNAi, the *Tc-mlpt* domain is strongly expanded dorso-anteriorly, leading to a completely symmetric expression along the DV axis (Figure 3.6I). After *Tc-tld* RNAi, the *Tc-mlpt* domain is not completely symmetrical along the DV axis, but still retains some residual polarity (Figure 3.6H, see arrow). Taken together, our results imply that *Tc-cv* and *Tc-tld* are involved in early Dpp activity in *Tribolium,* and in the specification of the early *Tribolium* fate map.

Gastrulation is severely affected after *Tc-cv* and *Tc-tld* knock-downs

To understand how the knock-downs of *Tc-tld* and *Tc-cv* affect *Tribolium* gastrulation, we studied the expression of amniotic and serosal markers during this process. In *Tribolium* WT embryos, the prospective amnion differs from the embryo proper during early gastrulation (Handel et al. 2000). The prospective amniotic and dorso-ectodermal cells express *Tc-iro* during differentiated blastoderm stages and early gastrulation (Figure 3.6B, 3.4F). During WT gastrulation these dorsal cells expressing *Tc-iro* close the amniotic cavity at the ventral face of the germband (Figure 3.4H, Figure 3.7D Am/ect).

During WT gastrulation, pMad is also observed in the amniotic layer that covers the embryo at the ventral side (Figure 3.4B,C, Figure 3.7A). After *Tc-cv* RNAi, the lack of the dorsal amnion (*Tc-iro*, Figure 3.6C) and the dorsal serosa (*Tc-doc*, data not shown) leads to a completely symmetric gastrulation (see schematic drawing in Figure 3.7I). The anterior amniotic stripe of *Tc-iro* is still observed (Figure 3.7F – Ant. Am), but the absence of dorsal amnion and dorsal serosa leads to embryos that gastrulate in an inverted position. The growth zone is then found in an opposite anteroposterior orientation in *Tc-cv* knock-downs when compared to WT embryos (Figure 3.7F, F´ schematic drawing in 3.7I).

Different gastrulation movements occur in *Tc-tld* RNAi embryos. These embryos still retain some polarity, since they express *Tc-doc* in a small patch of the dorsal serosa during blastodermal stages (Figure 3.6F). This *Tc-doc* domain is found after gastrulation at the ventral region, overlapping with pMad (Figure 3.7B´). These *Tc-tld* RNAi embryos display a big head in the ventral side after gastrulation, indicating that the serosal closure did not take place (see schematic drawing Figure 3.7H). This is explained by the lack of the posterior amnion (Figure 3.7E, data not shown), and the absence of the majority of the dorsal serosa (Figure 3.6F). Altogether, the two knock-down phenotypes are similar but not identical, since a residual Dpp activity in *Tc-tld* knock-down exist. This leads to a residual polarity during gastrulation.

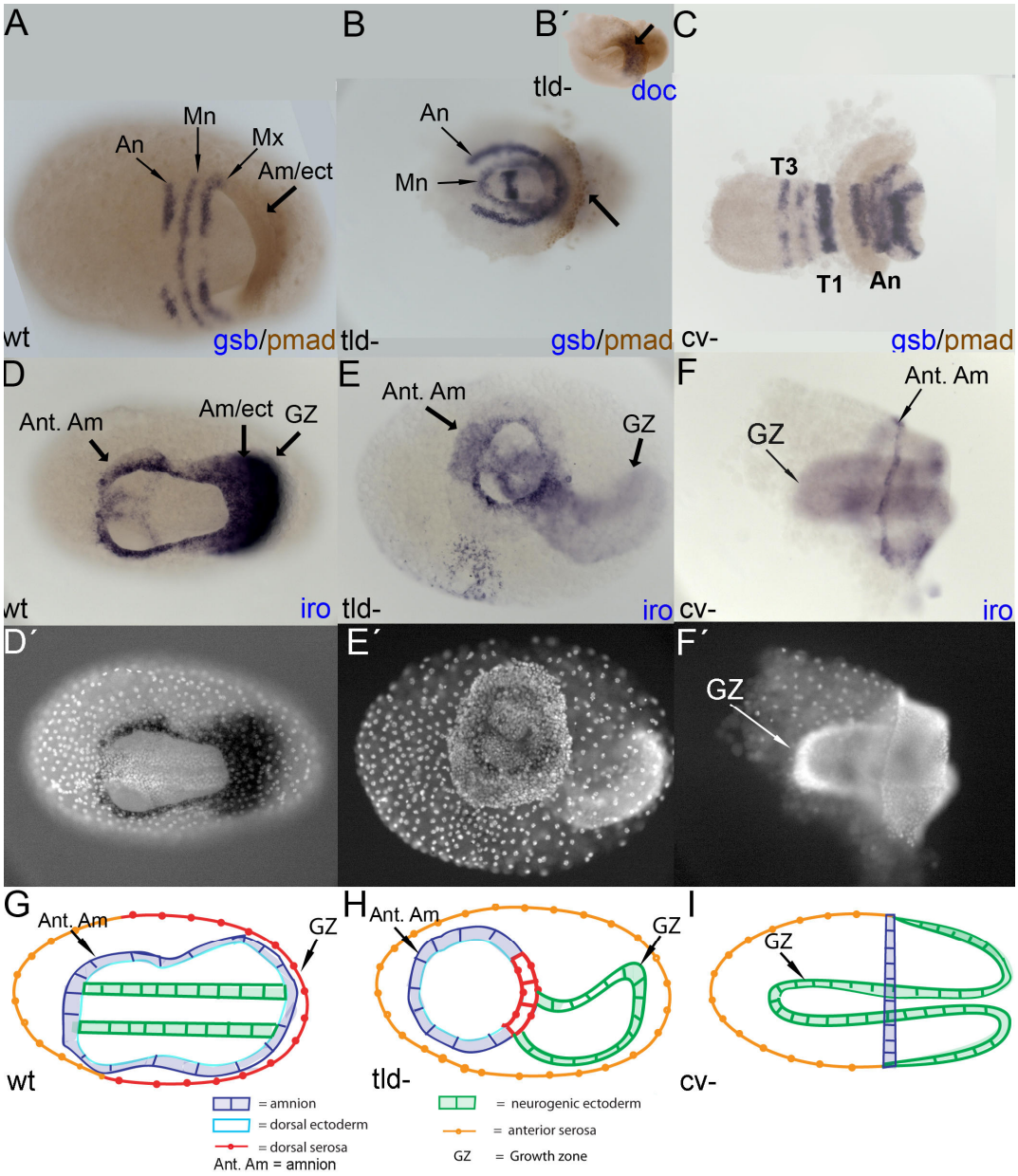


Figure 3.7 Gastrulation movements in *Tc-cv* and *Tc-tld* RNAi embryos
(A-C) Double-staining with the segmental marker *Tc-gsb* and the phosphorylated form of mad (pMad), the read-out of the Dpp gradient. (A) In WT, the dorsal serosa and the amnion cover the embryo during gastrulation. These amniotc cells contain Dpp activity, since they are positive for pMad (see also Figure 3.4). The antennal (An), mandibular (Mn) and maxillar (Mx) segments cells express *Tc-gsb*. (B) In *Tc-tld* RNAi embryos, an ectopic domain of Dpp activity in the head is observed (arrow). These cells in the head also express *Tc-doc* (B´-arrow).Note that the posterior amniotic cells are absent after *Tc-tld* RNAi. In contrast to the WT (D´), *Tc-tld* RNAi embryos display a big head in the ventral region (see E´) (C) In *Tc-cv* RNAi embryos Dpp activity is absent and embryos gastrulate in an inverted position when compared to the WT. (D-F) *Tc-iro* stainings. (D) In WT, *Tc-iro* is detected in the anterior amnion (Ant. Am.) around the head and in the posterior amnion and ectoderm (Am/ect) as well. (E) After *Tc-tld* RNAi the ectopic serosal cells in the head region which express *Tc-doc* (B´) and pMad (B), do not allow a proper serosal closure. In addition, *Tc-iro* is expressed anteriorly in the amnion but not in the posterior region (E). (F) After *Tc-cv* RNAi the anterior amniotic *Tc-iro* stripe (Ant. Am), observed during blastoderm stages (Figure 3.6C) is still present (Ant. Am) and the embryos gastrulate in an inverted position when compared to the WT (see DAPI staining in F´), since the posterior amnion is absent (Am/ect). Schematic drawings of WT (G), *Tc-tld* RNAi (H) and *Tc-cv* RNAi embryos (I).

Tc-tld and *Tc-cv* knock-down germ-band embryos are ventralized

Tc-dpp is required to establish the dorsal regions of *Tribolium* germ band embryos, since *Tc-dpp* knock-down embryos lack the dorsal (non-neurogenic) ectoderm (van der Zee et al. 2006). In WT germ band embryos, pMad is detected in dorsalmost regions along the DV axis (Figure 3.8D, blue arrows). *Tc-iro* expression is found in every segment and, similarly to pMad, in the dorsalmost regions (Figure 3.8G, arrows). I investigated possible changes in the expression domains of these genes after knock-down of *Tc-tld* and *Tc-cv*. After *Tc-cv* or *Tc-tld* RNAi, thin "tube-like" embryos, resembling *Tc-dpp* RNAi are observed (Figure 3.8B´-C´).The dorsal domains of *Tc-iro* expression are absent after these knock-downs, but the segmental pattern is still evident (*Tc-tld* Figure 3.8H, *Tc-cv* data not shown). The dorsal domains of pMad are completely deleted after *Tc-cv* or *Tc-tld* RNAi, but a patch of pMad expression in the head remains after *Tc-tld* RNAi (Figure 3.8E, arrow). This head domain in *Tc-tld* RNAi might be composed of serosal cells, since *Tc-doc* is expressed in an overlapping domain (Figure 3.8J, arrow). This *Tc-doc* domain is not observed in WT, since there the serosal cells migrate normally (Figure 3.8I, arrows); only dorsal domains of *Tc-doc* are detected. Further evidence for a difference between *Tc-cv* and *Tc-tld* RNAi embryos is provided by the analysis of expression of the gap gene *Tc-mlpt* (Savard et al. 2006). In WT *Tc-mlpt* is expressed in the head and in the maxilla, besides a broad abdominal domain (Figure 3.8A). After *Tc-tld* RNAi the head domain and maxilla appears fused (Figure 3.8B), while after *Tc-cv* RNAi the head domain expands completely along the DV axis (Figure 3.8C, asterisk). In addition, a bigger head in *Tc-cv* RNAi embryos is observed when compared to *Tc-tld* RNAi (Figure A´,B´,C´ for DAPI stainings).

Since dorsal ectodermal markers are absent in both knock-down embryos, is possible that the ventral (neurogenic) region would be expanded. In WT, *Tc-achaete-scute (Tc-ash)* is expressed in the CNS and in the peripheral neurons (Figure 3.9A). After *Tc-cv* or *Tc-tld* RNAi, neuronal cells are observed along the whole DV axis (Figure 3.9B, *Tc-tld* data not shown). Taken together, *Tc-cv* and *Tc-tld* knock-down embryos are ventralized, and differences between the two phenotypes can be associated with remaining dorsal serosal cells in *Tc-tld* knock-downs that generate residual polarity (schematic drawings Figures 3.6K, 3.7H and 3.8J).

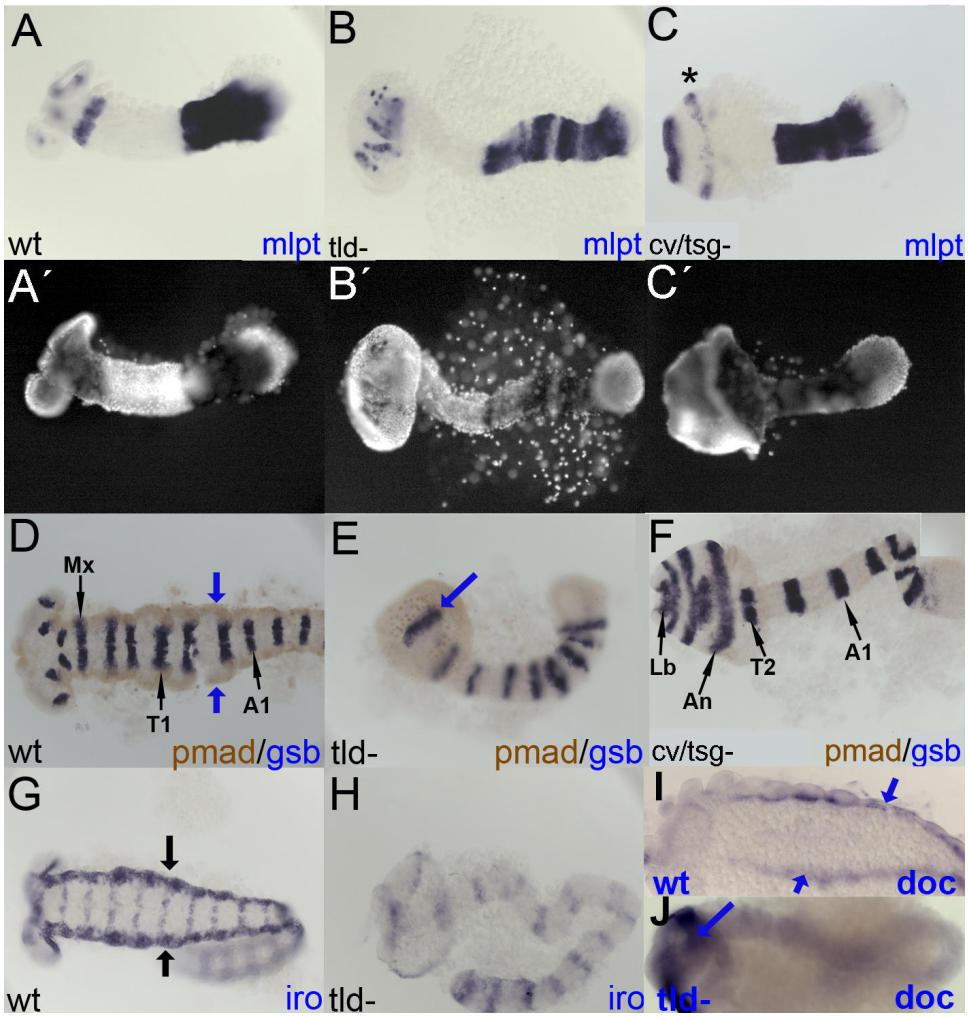


Figure 3.8: Dorsoventral patterning and Dpp activity in *Tc-cv* and *Tc-tld* RNAi embryos
(A-C) *Tc-mlpt* stainings. (A) In WT, *Tc-mlpt* is expressed in the mandibular segment and in a broad abdominal domain located posteriorly. (B) After *Tc-tld* RNAi, the mandibular segmental, and head staining of *Tc-mlpt* are less defined, although a big head is observed in the respective DAPI staining (B´). (C) A big head with a an expanded domain of *Tc-mlpt* is observed. This domain occupies the whole DV axis (asterisk and see respective DAPI). (D-F) *Tc-gsb* and Dpp activity (pMad). (D) In WT, pMad is detected in the dorsalmost cells of the embryo (blue arrow) and *Tc-gsb* is expressed in stripes (every segment). (E) After *Tc-tld* RNAi, pMad is not detected in the dorsalmost cells, but a few cells in the head still retain Dpp activity (pMad-blue arrow). These cells also express *Tc-doc* (see J - blue arrow). (F) *Tc-cv* RNAi embryos undergo normal segmentation process but do not display pMad in the dorsalmost cells. (G,H) *Tc-iro* expression pattern. (G) WT *Tc-iro* is expressed in every generated segment and in dorsal domains, that overlap with the pMad (black arrows). (H) After *Tc-tld* RNAi the segmentation component of *Tc-iro* expression is detected, but the dorsal domain of Dpp activity is absent. A similar phenomenon is observed for *Tc-cv* (data not shown). (I,J) *Tc-doc* expression pattern. (I) In WT, *Tc-doc* is expressed in dorsal domains (blue arrows). (J) After *Tc-tld* RNAi a domain of *Tc-doc* expression is detected in the head, but no dorsal domains are detected. This domain in the head overlaps with pMad (shown in E). The dorsal domain of *Tc-doc* is deleted after *Tc-tld* RNAi. Mx - maxilla, Lb – labium, An – antenna, T1, T2 – first and second thoracic segment, respectively, A1 – first abdominal segment.

Injection of two different dsRNA enables the establishment of an epistatic hierarchy among *dpp*, *cv(tsg)*, *sog* and *tld*

The results above indicate that *Tc-cv (tsg)* would be required for most, if not all, Dpp activity in *Tribolium* embryo. To evaluate if *cv(tsg)* functions are completely dependent on *Tc-sog* to promote Dpp signalling or would also act independent of *Tc-sog*, we injected *Tc-cv* and *Tc-sog* dsRNA together in *Tribolium* adult females. To investigate if the obtained embryos were ventralized (a loss of *Tc-cv* or *Tc-dpp*) or dorsalised (a loss of *Tc-sog)* we performed double stainings with the segmental marker *Tc-gooseberry (Tc-gsb)* and the pan-neuronal marker *Tc-ash.* The depletion of both dsRNAs (*Tc-cv* and *Tc-sog*) led to embryonic phenotypes identical to *Tc-cv* loss-of-function, a big head embryo with an expansion of neuronal cells (Figure 3.9B,D).

These results indicate that Tc-Cv acts downstream of Tc-Sog, in other words, Dpp is unable to signal in the absence of Tc-Cv, even when the inhibitor Tc-Sog is not present. Double injections of *Tc-dpp* and *Tc-sog* dsRNA led, as expected, to a *Tc-dpp* loss-of-function phenotype (Figure 3.9C, van der Zee et al. 2006). In these double-injection experiments, *Tc-sog* knock-down in the collected embryos was confirmed by the absence of *Tc-sog* transcripts after in situ hybridization (data not shown).

Since *Tc-tld* would be presumably only required to cleave *Tc-sog*, releasing the inhibitory complex, we performed double injections of *Tc-sog* and *Tc-tld* RNAi, expecting a *Tc-sog* RNAi phenotype. Embryos recovered from *Tc-sog/Tc-tld* double injection displayed the same phenotype as *Tc-sog* single injection (Figure 3.9E, F). These embryos are “double dorsal” displaying additional ectopic dorsal tissue along the ventral midline, similarly to *Tc-sog* RNAi (van der Zee et al. 2006, Figure 3.9E,F). These results indicate that the double knock-down *Tc-sog/Tc-tld* resembles *Tc-sog* RNAi and that all *Tc-tld* functions in *Tribolium* embryos are mediated via *Tc-sog*. Thus, Tc-Tld acts upstream of Tc-Sog, being only required to cleave this latter molecule. From these epistatic analyses a hierarchy of interactions among Tc-Dpp and its modulators is proposed (Figure 3.10B).

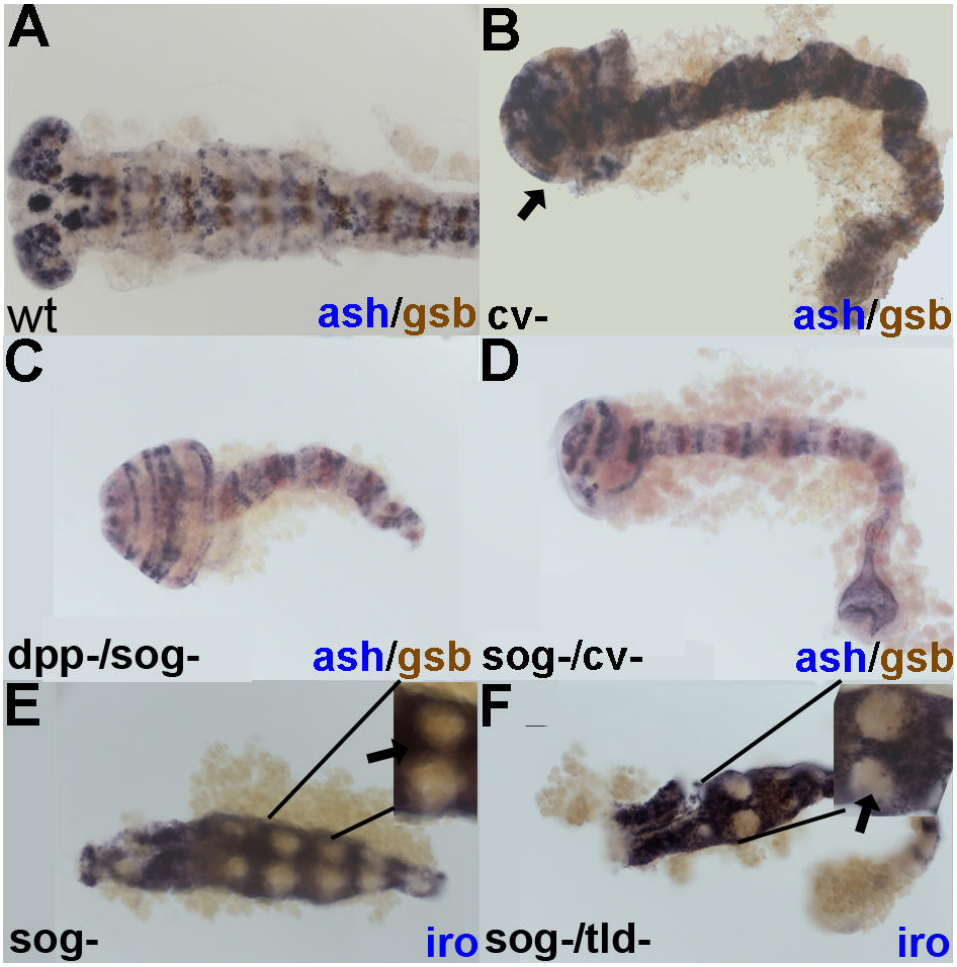


Figure 3.9: dsRNA double-injections against *Tc-dpp* and its modulators suggest that Tc-Cv acts downstream of Tc-Sog
(A-D) Double-stainings for *Tc-ash* (blue) and *Tc-gsb* (brown). (A) In WT, *Tc-ash* (blue) is expressed in all neurons and *Tc-gsb* (brown) in the ventral part of the segments. (B) *Tc-cv* RNAi embryos are ventralized, being composed by a huge amount of neuronal cells (blue) and a big head (arrow). (C) Double-injected embryos for *Tc-dpp* and *Tc-sog* are identical to *Tc-dpp* single RNAi embryos (see also van der Zee et al. 2006). (D) Double-injected embryos for *Tc-cv* and *Tc-sog*. These embryos are ventralized, being identical to *Tc-cv* knock-down (shown in B). (E-F) *Tc-iro* stainings. (E) After *Tc-sog* RNAi, the embryo becomes "double-dorsal" (Van der Zee et al. 2006), since an ectopic domain of *Tc-iro* is observed ventrally along the whole AP axis (arrow), besides the dorsal domain. A WT embryo in a comparable stage is shown in Figure 3.8G. (F) Double-injected embryos for *Tc-tld* and *Tc-sog* are identical to *Tc-sog* single RNAi embryos, with patches of mesodermal cells between the ectopic ventral domain (arrow in F, non stained cells).

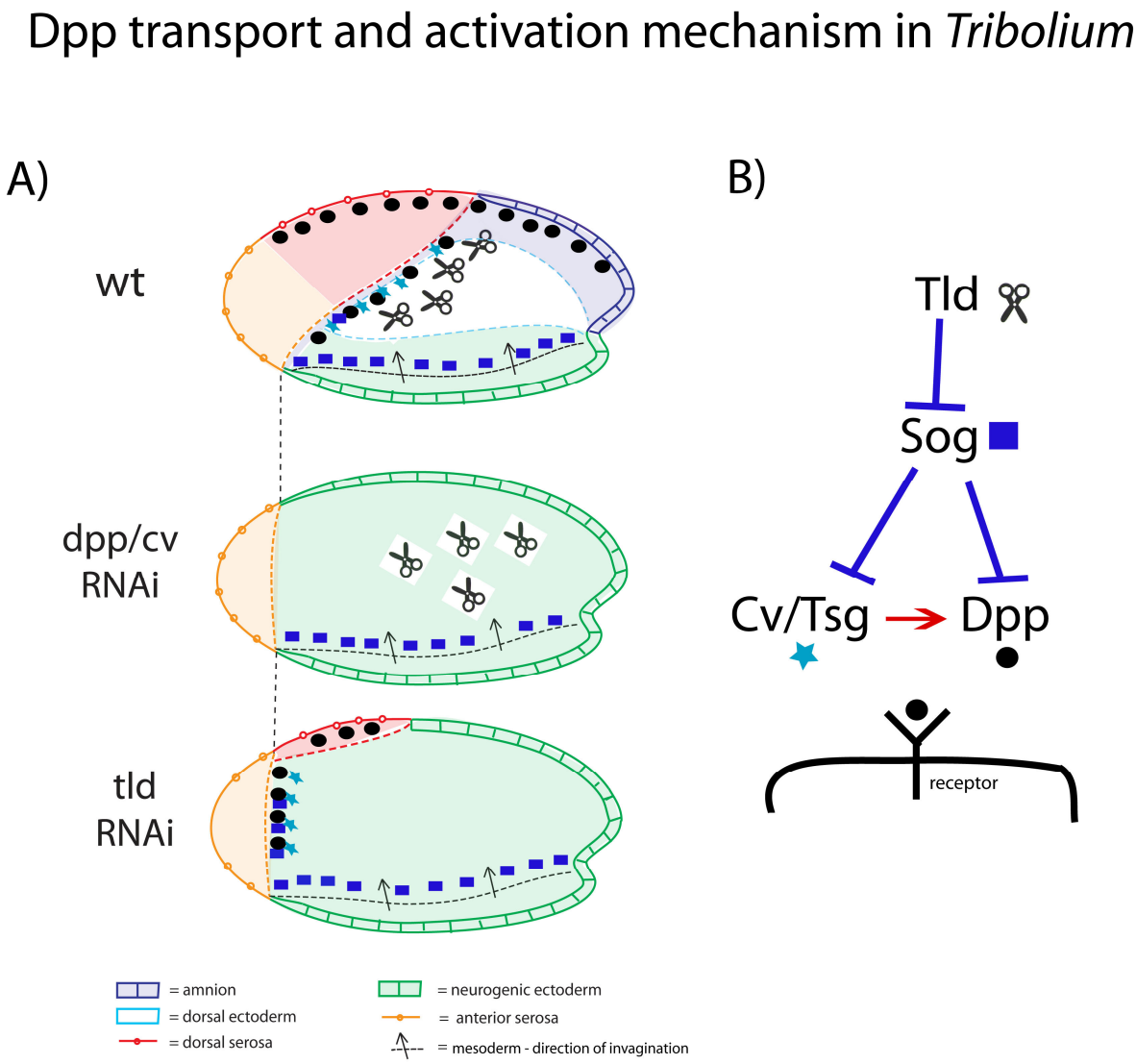


Figure 3.10: A model for Dpp transport and activation in *Tribolium* early embryo
(A) In WT embryos *Tc-cv* (star) and *Tc-dpp* (circle) are expressed in a stripe between the embryo proper and the serosa (Figure 3.4G, Figure 1.10H, and Van der Zee et al., 2006). *Tc-sog* (square) is expressed ventrally and is required to transport Tc-Dpp towards the dorsal regions (Van der Zee et al., 2006). *Tc-tld* (scissors) is broadly expressed in dorso-lateral regions of WT *Tribolium* embryos (Figure 3.4A,B). After *Tc-dpp* or *Tc-cv* RNAi, Dpp activity is completely absent in early embryos (Figure 3.6D and Van der Zee et al. 2006), implying that Tc-Cv is required for early Tc-Dpp signalling. After *Tc-tld* RNAi, Dpp activity is still detected in the dorsalmost region of the embryo.(B) Based on double-injection experiments (Figure 3.9), the epistatic relationships among the three Dpp modulators (*Tc-cv*, *Tc-tld* and *Tc-sog*) in *Tribolium* are inferred. Since *Tc-cv* and *Tc-dpp* knock-down phenotypes are identical, we conclude that Tc-Cv has a pro-BMP/Dpp function in early *Tribolium* embryos.

Cells from the inner-layer (IL) of the growth zone migrate over the outer-layer (OL) after *Tc-cv* or *Tc-dpp* RNAi

Another important feature of short-germ insects is the presence of the growth zone, the posterior region where the abdominal segments emerge by a secondary process. Not only new segments should be specified, but they should also emerge with normal DV polarity. In WT lateral views of the growth zone four distinct cell populations can be observed (see also schematic drawing on Figure 3.11F). The amnion can be identified as a thin cell layer characterized by the expression of *Tc-iro* (Figure 3.11A, F - am). The amniotic cavity (Figure 3.11F- amc) is observed between the amnion and the outer layer (Figure 3.11F - OL). The OL is continuous with the anteriorly located ectoderm (Figure 3.11F - ect). These ectodermal cells express segment polarity genes like *Tc-gooseberry* (*Tc-gsb*), *Tc-engrailed* (*Tc-en*) and *Tc-iro* (Handel et al., 2005 and data not shown). On top of the OL, the inner layer (IL) (Figure 3.11F - IL) is continuous with the anteriorly located mesoderm (Figure 3.11C,F – me). Note that this IL does not express the mesodermal gene *Tc-twist*, which is only present the anterior located mesoderm and in the posterior tip (Figure 3.11C,D, F - IL, post). So the fate of these cells is not clear (an analysis of the fate of these IL cells is provided on Chapter 4). Last, a rounded cell mass in contact with the IL corresponds to the putative germ cells (Figure 3.11B, F-gc). These cells also express the germ cell marker *Tc-vasa* (Figure 4.9, described in Chapter 4).

Tc-dpp and its modulators, *Tc-sog*, *Tc-cv* and *Tc-tld* are also expressed in the growth zone. *Tc-dpp* is expressed in the OL and in the amnion, while *Tc-sog* is mainly expressed in IL of the growth zone and in the ectoderm of the anterior segmented region (Van der Zee et al., 2006 and Figure 3.11). *Tc-cv* is expressed in the IL and OL of the growth zone (Figure 3.4J) and *Tc-tld* is broadly expressed in this region (data not shown).

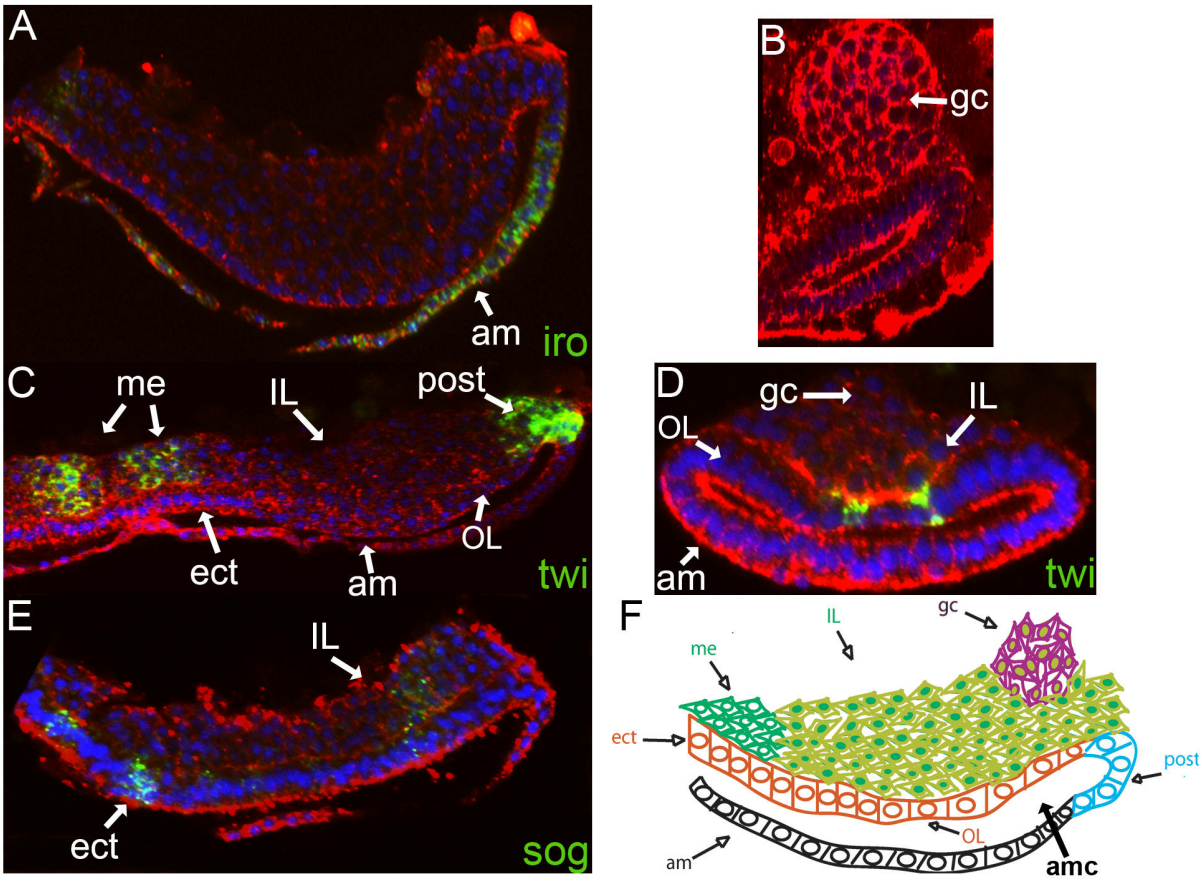


Figure 3.11: WT growth zone morphology and molecular marker expression during germ band elongation
(A,B,C,E) Lateral views of growth zones after in situ hybridizations (green), nuclear staining (DAPI -blue) and cell membrane stainings (anti-phosphotyrosine-red). (A) *Tc-iro* (green) is expressed in the amnion (am). (B) Lateral view, a rounded mass of cells constitutes the germ cells (for a *Tc-vasa* staining, a germ cell marker, see Figure 4.9). (C) *Tc-twist* is expressed in the mesoderm and in the posterior tip. (D) Optical cross section from a segmented germ band (8 segments), like the one in C, in situ hybridization against *Tc-twist* (green, overlay in yellow). (E) *Tc-sog* is expressed mainly in the inner layer of the GZ, and in the ectoderm anteriorly. (F) Schematic drawing of the growth zone cell fates. Am - amnion (black), me - mesoderm (green), IL - inner layer (pale green), OL - outer layer (red, cuboidal cells), post - posterior tip (blue), gc - germ cells (purple), ect - ectoderm (red).

Since Dpp and its modulators are expressed in this region, we investigated if *Tc-dpp*, *Tc-cv* or *Tc-tld* knock-down would lead to evident morphological defects in this region. Importantly, in WT growth zone the IL cells are located on top of the OL, asymmetrically along the DV axis (Figure 3.11F). After knock-.down of *Tc-dpp* or *Tc-cv*, the amnion is completely deleted in the whole embryo, including the GZ, being substituted by cells of the ectoderm (Figure 3.12D and 3.7I for gastrulation). In these knock-down embryos a further loss of DV polarity is observed, the IL cells migrate laterally over the OL, losing partially their DV asymmetry (Figure 3.12D). In conclusion, amniotic specification seems to be required to establish the growth zone DV polarity, maintaining a compact IL on top of the OL.

In *Tc-dpp* RNAi embryos, mesodermal cells expressing *Tc-twist* are observed all around the ectodermal cells anteriorly during germ band elongation (Van der Zee et al., 2006). It is possible that as new segments are generated from the GZ, the IL cells leave the posterior region and differentiate into mesodermal cells (see also Chapter 4 for further evidence that the mesoderm is generated from IL cells). So, after *Tc-dpp* or *Tc-cv* RNAi the DV polarity of the GZ is not maintained. Since *Tc-dpp* or *Tc-cv* RNAi embryos are identical also in relation to GZ pattern, I infer that *Tc-cv* function is as important as *Tc-dpp* during *Tribolium* embryonic development, a completely different situation when compared to the current *Drosophila* paradigm (see the discussion of this chapter).

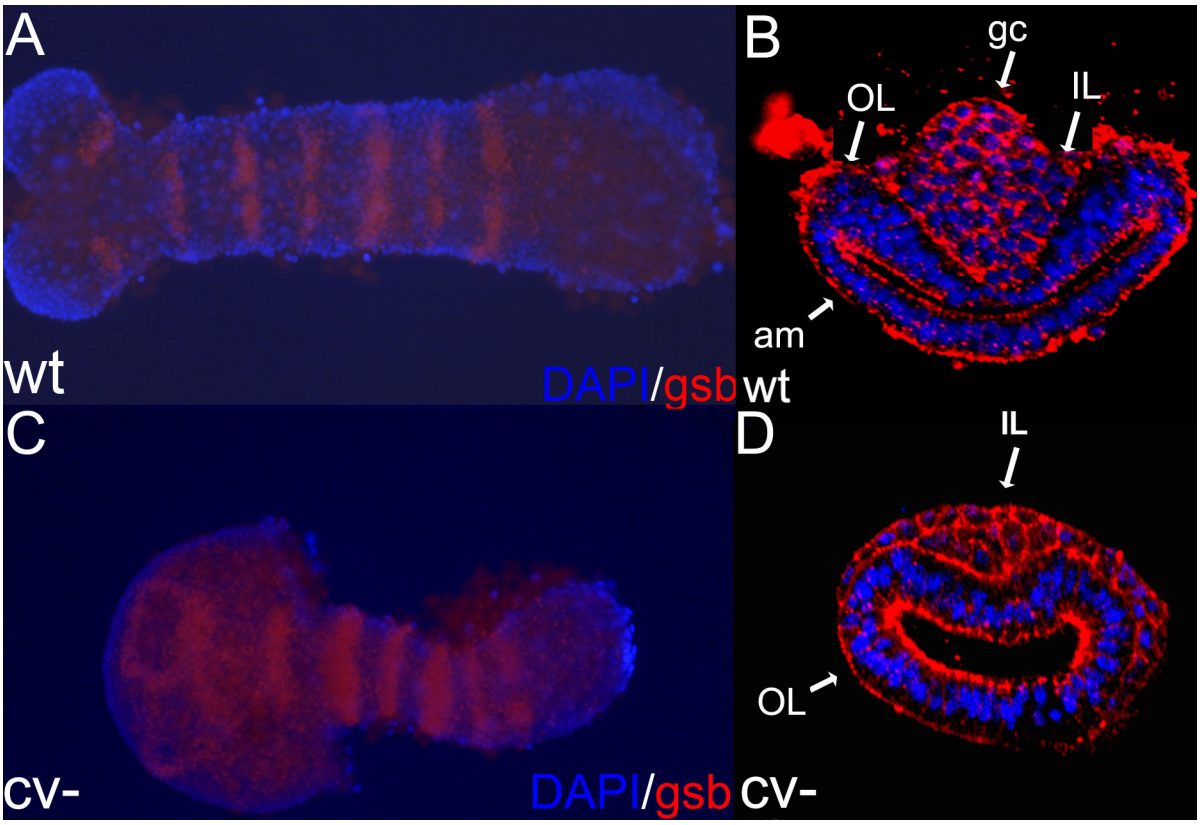


Figure 3.12: The inner layer cells of the growth zone migrates over the outer layer in *Tc-cv* RNAi embryos
(A)WT and (C) *Tc-cv* RNAi embryos stained for the segmental marker *Tc-gsb* and DAPI. (C) *Tc-cv* RNAi embryos can be recognized by the huge head when compared to the WT (A). Optical section of the growth zone of a WT (B) and *Tc-cv* RNAi embryo (D). The inner layer (IL) cells migrate over the outer layer (OL) after *Tc-cv* RNAi, in contrast to the asymmetric position of the IL in WT. gc - germ cells, am - amnion.

Cv/Tsg-related proteins also have a major role in *Nasonia vitripennis* Dpp modulation

To understand if the major role of Cv/Tsg-related proteins for Dpp modulation in *Tribolium* is a specific feature of this beetle or if it is a more general aspect in insect evolution, the function of Cv/Tsg orthologs in the wasp *Nasonia vitripennis* was analysed. This wasp has recently been reemerged as a comparative model to *Drosophila* (Beukeboom and Desplan 2003). *Nasonia* shares the long-germ type of development with *Drosophila*, although this type of development has probably arisen independently in both insects (see chapter 1). *Nasonia vitripennis* embryogenesis takes approximately the same time as *Drosophila melanogaster* embryogenesis to be completed. Importantly, pRNAi can be applied in *Nasonia* and mutant screenings have been performed based on the analysis of cuticular phenotypes (Pultz et al. 1999; Pultz et al. 2000; Lynch and Desplan 2006). Additionally, the *Nasonia vitripennis* genome has been recently proposed to be sequenced (Werren et al. 2004).

As in *Drosophila*, three related sequences to Crossveinless are present in *Nasonia vitripennis* genome (*Nv-cvA, Nv-cvB, Nv-cvC*, see also Figure 3.2). Interestingly, two of these sequences contain intronless transcripts, *Nv-cvA* and *Nv-cvC.* The third *Nasonia vitripennis cv* gene, *Nv-cvB*, has the same gene structure as *Tc-cv*, containing four introns (Figure 3.2). The duplication events that gave rise to *Nv-cvA* and *Nv-cvC* are probably specific to *Nasonia*, since the genome of the bee *Apis mellifera* only contains one *cv*-related molecule that groups with *Nv-cvB* (four introns). *Nc-cvA* and *Nv-cvB* were cloned using embryonic cDNA preparations, while *Nv-cvC* was not amplified from the same preparations. Indeed, *Nv-cvC* might be a pseudo-gene since it contains several frameshift mutations (see also the extreme long-branch in Figure 3.2).

pRNAi against *Nv-cvB* led to 90% of non-hatched larvae in the first egg lay. Most obtained cuticles from this first egg lay displayed an empty milky appereance, without any organized structure (data not shown). A small percentage of non-hatched larvae showed a cuticular organization (Figure 3.13). A small piece of cuticle in the posterior region of the egg shell is observed. In the two following egg lays the number of non-hatched larvae slowly decreased, from around 70% in the second to 50% in the third, concomitant with an increase of the small piece of cuticle phenotype. This suggests that the strongest *Nv-cvB* knock-down phenotypes do not develop proper cuticule and progressively weaker phenotypes develop it.

In contrast to *Drosophila melanogaster* cuticle preparations, where dorsoventral mutants can be identified by changes in the DV asymmetric pattern of denticle belts (e.g Arora and Nusslein-Volhard 1992), *Nasonia vitripennis* WT cuticles do not display such DV asymmetric denticle belt distribution (Figure 3.13). So, it is not possible to infer from the

cuticle analysis if this small cuticle is a DV phenotype or not. On the other hand, mutants containing these small piece of cuticle were suggested to be DV mutants in the *Nasonia vitripennis* screening, mainly based on the similarity to the *Drosophila melanogaster* DV cuticle phenotypes (Figure 3.13, Pultz et al. 2000). To further investigate if the knock-down of *Nv-cvB* leads to a DV phenotype, DV molecular markers were investigated.

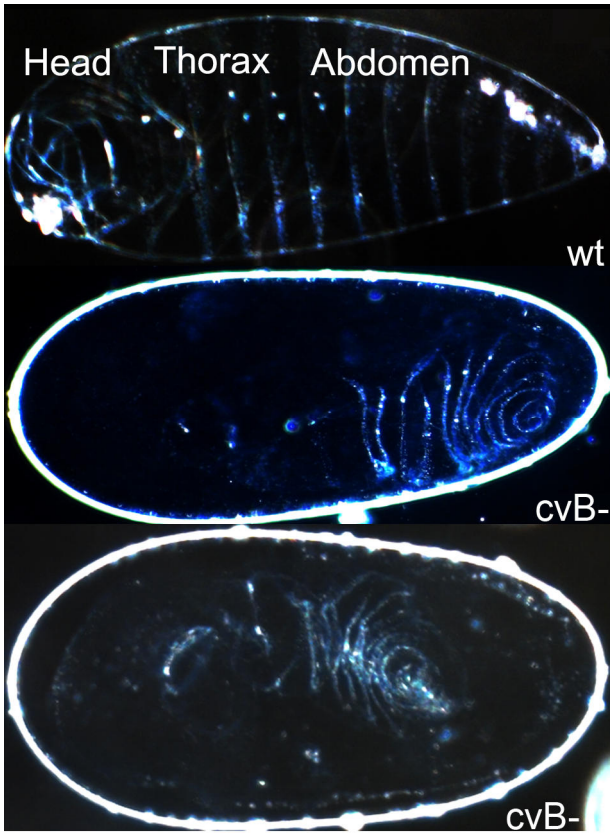


Figure 3.13: *Nasonia cvB* RNAi knock-down lead to a small piece of cuticle located at the posterior part of the egg shell.

In *Nasonia*, and in all insects investigated so far, the prospective ventral neurogenic ectodermal cells express *Nv-vnd*, which flanks the prospective mesodermal cells stained for *Nv-twi* (Figure 3.14). After *Nv-cvB* RNAi, a dorsal expansion of the ventral neurogenic ectoderm and of the mesoderm, particularly in the middle of the embryo, is observed (Figure 3.14). Note that the *Nv-vnd* domain expands towards the whole dorsal side of the embryo in the absence of *Nv-cvB.* This expansion indicates a loss of Dpp activity, since Nv-Dpp is required for patterning the dorsal region also in *Nasonia* (Jeremy Lynch, unpublished observations). Since the *Nv-dpp* knock-down phenotype is much stronger (Lynch, unpublished) than the *Nv-cvB* knock-down, it is possible that another Cv-related molecule like *Nv-cvA* would also be involved in embryonic Dpp modulation. Preliminary *Nv-cvA* RNAi experiments (data not show), showed a similar DV phenotype for this gene. It is possible that

the combined action of these two genes is required for Dpp signalling in *Nasonia vitripennis*. Double-injections (*Nv-cvA and Nv-cvB)* may answer if Dpp activity can be completely abolished after the removal of all Cv activity in early embryos. In conclusion, the loss of Dpp activity after *Nv-cvB* RNAi implies that in WT embryos, *Nv-cvB* promotes Dpp activity. This observation is similar to the role of the unique Cv (Tc-Cv) protein in *Tribolium*, and suggests an ancestral role of Cv protein in promoting BMP activity in insects.

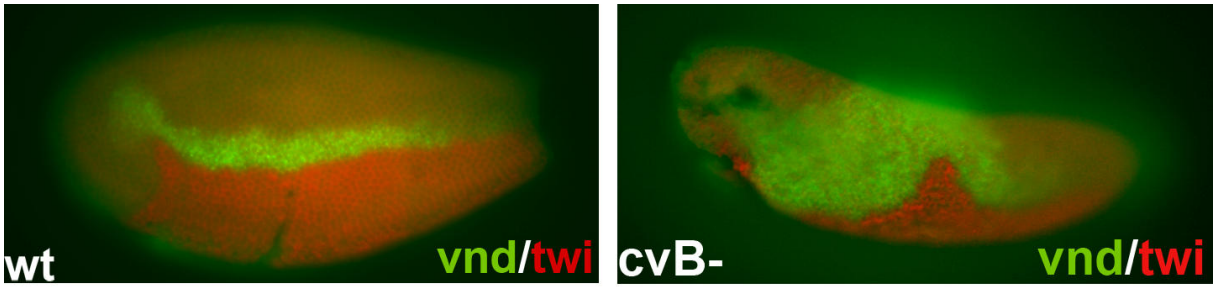


Figure 3.14: Knock-down for *Nv-cvB* leads to an expansion of the mesoderm and neurogenic ectoderm indicating that Cv promotes Dpp activity in *Nasonia*
Double-fluorescent in situ hybridization for *Nv-vnd* (green) and *Nv-twi* (red). In WT, the ventral neurogenic ectodermal cells expressing *Nv-vnd*, flank the mesodermal cells expressing *Nv-twi*. After *Nv-cvB* RNAi, the ventral neurogenic ectodermal cells are largely expanded towards the dorsal regions, particularly in the middle. In the middle region of the embryo, the mesoderm (*Nv-twi*) is also expanded.

Discussion:

Duplications in Cv/Tsg and Tld-related molecules: a possible road to fast development

Several molecules containing cysteine rich (CR) repeats have been shown to be involved in Dpp extracellular modulation in metazoans (reviewed in Garcia Abreu et al. 2002). Among them, three *cv/tsg-like* genes are present in genomes of higher Diptera. These genes are the "classical" *tsg* (*Dm-tsg1*) isolated from mutant screenings (Zusman and Wieschaus 1985), CG11582 (*tsg3)* and *crossveinless (Dm-tsg2* or *cv)*. In contrast, *Apis mellifera*, *Tribolium castaneum* and *Anopheles gambiae* genomes contain only one *cv/tsg-like* molecule. This unique *cv/tsg-like* molecule in *Tribolium castaneum* is more similar to the *Drosophila melanogaster cv* gene, which in *Drosophila melanogaster* does not possess a role in early embryonic pattern, but rather promotes Dpp modulation in the wing (Shimmi et al. 2005a; Vilmos et al. 2005). Besides the phylogenetic analysis (Figure 3.2A), further support for a group of *Tc-cv* and *Dm-cv* were also obtained by: 1) the existence of specific amino-acid residues shared by Tc-Cv and Dm-Cv that differs to Dm-Tsg (Figure 3.3); 2) a common intron-exon structure for *Tc-cv* and *Dm-cv,* in contrast to the two intronless transcripts of *Dm-tsg* and *Dm-tsg3* (Figure 3.2A).

I suggest that retro-transposition events generated these two *tsg-like* molecules present in higher flies, since these two intronless molecules are not present in the genome of the primitive mosquito Diptera *Anopheles gambiae.* In the context of fast embryonic development of higher Diptera, these small transcripts could have substituted the ancestral *cv* molecule which is here shown to be involved in Dpp extracellular modulation in *Tribolium castaneum* embryos. Other molecules involved in embryonic DV axis formation in *Drosophila melanogaster*, but not involved in *Tribolium castaneum* DV axis are *brinker* and *screw*. Both genes possess intronless transcriptional units in *Drosophila melanogaster*, reinforcing the trend of shorter transcripts acting in the fast development of the long-germ Diptera embryo.

The trend of shorter transcripts may not be unique to higher Dipterans, since in the long-germ *Nasonia vitripennis* (Hymenoptera), which has a comparable developmental time to *Drosophila melanogaster* (Pultz et al. 2005), two intronless *cv* genes have been independently generated. One of these intronless genes (*Nv-cvA*) might act during early *Nasonia vitripennis* embryogenesis since its cDNA was isolated from an embryonic cDNA preparation.

A similar duplication followed by a sub-functionalization event took place for Tolkin/tolloid metaloproteases in higher Diptera. *Dm-tolloid* (*tld*) and *Dm-tolkin (tok or tolloid-related)* are found in tandem in the same chromosomal region in *Drosophila*, but only one *tld/tok* gene is found in other holometabolous insects (Figure 3.2B and data not shown). Recently Serpe et al. 2005 showed that *Dm-tok* and *Dm-tld* have different catalytic properties in pupal wings and during embryogenenesis, respectively. *Dm-tok* and *Dm-tld* are expressed in a similar pattern in embryos, but *Dm-tok* overexpression is unable to rescue the loss of *Dm-tld* in embryos (Nguyen et al., 1994). *Dm-tld* is also not able to rescue the pupal wing phenotype caused by *Dm-tok* loss-of-function (Serpe et al. 2005). Serpe et al. 2005 proposed that the different kinetics in Dm-Sog processing between Dm-Tok (slower) and Dm-Tld (faster) would account for the failures in the reciprocal rescue situation. It is possible that ancestral insects contained a unique Tolkin/Tolloid molecule acting in the embryo and in wing pattern, and that duplications followed by sub-functionalization occurred in higher Diptera.

Taken together, our results support a model where the modulation of Dpp activitiy has changed during insect evolution. The duplication events described above may have changed the shape of the Dpp gradient in higher Diptera. In agreement with this hypothesis, recent studies in the primitive Diptera *Anopheles gambiae* demonstrated that the expression pattern of *Ag-tld* and *Ag-sog* (Goltsev et al. 2007) resembles the ones described here for *Tc-tld* and *Tc-sog* (van der Zee et al. 2006). Moreover, Dpp activity (pMad) is broader in *A. gambiae* and *T. castaneum* than in *D. melanogaster.*

Intriguingly, mosquitos and beetles have two separated extra-embryonic membranes, the amnion and the serosa (Goltsev et al. 2007, van der Zee et al. 2005 and references therein). In contrast, higher flies, including *D. melanogaster*, have undergone an extreme reduction of extra-embryonic membranes (Schmidt-Ott 2000). A correlation between extra-embryonic reduction and changes in Dpp modulation can be hypothesized.

Tc-cv and *Tc-tld* are essential for the establishment of Dpp activity and pattern the dorsal regions of *Tribolium* embryos

The patterning of the dorsal region in *Tribolium* eggs, comprising dorsal serosa (*Tc-doc*) and dorsal ectoderm/amnion (*Tc-pnr/Tc-iro*), requires at least two pro-BMP/Dpp molecules, *Tc-cv and Tc-tld* (this thesis), and *Tc-sog* mediated transport (van der Zee et al. 2006). *Tc-cv* is strictly required for Dpp activity in *Tribolium* early embryos, since in its

absence all *Tc-dpp* target genes known so far in *Tribolium* are not expressed. Further evidence comes from the observation that both knock-down phenotypes (*Tc-dpp* and *Tc-cv* RNAi) are morphologically identical (Figures 3.6, 3.7, Van der Zee et al. 2006).

In contrast, *Tc-tld* is not required for all Dpp activity in the early embryo. After *Tc-tld* knock-down, the *Tc-doc* dorsal serosa expression domain is largely reduced, but some cells still express this gene, indicating that *Tc-dpp* activity still exists there (Figure 3.6F). In WT it has been proposed that Tc-Sog transports Tc-Dpp towards dorsal regions, so Tc-Sog would inhibit Dpp signalling in ventral regions (van der Zee et al. 2006). The release of Tc-Dpp from its inhibitor Tc-Sog is provided in dorsal regions by the metalloprotease Tc-Tld (Figure 3.10A). Apparently after *Tc-tld* RNAi Tc-Sog cannot completely inhibit Tc-Dpp in the dorsalmost regions, since the dorsal region is distant from the ventral domain of *Tc-sog* transcription (Van der Zee et al., 2006, Figure 3.10A). In WT, the degradation of Tc-Sog via Tc-Tld would be required for Dpp signalling in the dorsalmost regions of the egg (Figure 3.10A).

Tc-cv major role in *Tribolium* implies that Dpp modulation has changed during insect evolution

Although the function of Cv/Tsg related molecules have been extensively studied in *Drosophila* and vertebrates, contradictory results using different model systems have been reported (Ross et al. 2001; Scott et al. 2001a; Xie and Fisher 2005; Jenner and Wills 2007). In order to reconcile these results, it has been proposed that Cv/Tsg would have a dual role in modulating BMP/Dpp signalling (Figure 3.1B). First, Cv/Tsg would act enhancing Sog binding to Dpp, an inhibitory function for Dpp signalling. Second, Cv/Tsg would enhance Tld cleavage of Sog, enabling Dpp to signal (Larrain et al. 2001; Ross et al. 2001; Scott et al. 2001b, Figure 3.1B). If the biochemical interactions among Cv/Tsg, Sog, Dpp and Tld are conserved, at least two hypothetical scenarios can be proposed to explain the major role of Tc-Cv/Tc-Tsg in *Tribolium* when compared to *Drosophila* and vertebrates.

First, assuming a dual role of Tc-Cv, our results indicate that the main role of this molecule in WT is a pro-BMP function, since *Tc-cv* knock-down phenotypes are identical to *Tc-dpp* knock-down. Importantly, Tc-Cv in *Tribolium* is not only necessary to enhance Sog/Chordin degradation via Tld, since embryos double knock-down for *Tc-cv* and *Tc-sog* display the same phenotype as *Tc-cv* RNAi, an absence of Tc-Dpp signalling. These results suggest that *Tc-cv* performs *Tc-sog* independent roles on BMP/Dpp modulation. Similar

observations of Tsg roles independent of Sog were recently obtained in *Drosophila* and vertebrates (Wang and Ferguson 2005, Little and Mullins 2004, Xie and Fisher 2005). In this hypothetical scenario Tc-Cv would interact with other BMP/Dpp modulators, besides Tc-Sog. Several proteins containing structural domains resembling Sog/Chordin are present in *Drosophila, Tribolium* and vertebrate genomes (Garcia Abreu et al. 2002). At least one of them, Crossveinless-2 (Cv-2) is involved in BMP/Dpp modulation in vertebrate embryonic development (Rentzsch et al. 2006, Ikeya et al. 2006). In *Drosophila* and *Tribolium* Cv-2 seems not to be required for early embryogenesis (Umulis et al. 2006, described in chapter 2).

From the aforementioned results, a model where Tc-Cv acts downstream of Tc-Sog, and is essential to Tc-Dpp signalling is supported (Figure 3.10B). A second hypothetical scenario is that Tc-Dpp would not be able to signal when Tc-Cv related molecules are not present. Favoring this hypothesis it was recently suggested that *Drosophila* Tsg could facilitate binding of BMPs to cell surface (Wang and Ferguson 2005). Since *Tc-cv* is expressed in a similar domain as *Tc-dpp* in *Tribolium* (Figure 3.4G), it is possible that most of early Tc-Dpp protein is immediately bound to Tc-Cv in the extracellular space. In the absence of Tc-Cv, Tc-Dpp could be rapidly degraded by a receptor mediated mechanism in *Tribolium,* as recently proposed for *Drosophila* (Mizutani et al. 2005). If Tc-Dpp would not be able to signal in the absence of Tc-Cv in *Tribolium*, why would BMP/Dpp4 be able to signal in the absence of Cv/Tsg in *Drosophila* and Xenopus? A possible explanation would be that the multiple copies of Cv/Tsg related molecules present in *Drosophila melanogaster* (*Dm-tsg3* and *Dm-cv*) and Xenopus, Tsg1 and Tsg2 (Oelgeschlager et al. 2004) could account for the residual Dpp activity in Cv/Tsg single mutants in these two model systems. Only the analysis of double-mutants for Cv/Tsg related molecules in *Drosophila* and zebrafish would be able to confirm or refute this hypothesis.

If the scenario involving duplications and sub-functionalizations in vertebrates and *Drosophila* is true, the situation observed in *Tribolium* could be the ancestral case of BMP/Dpp modulation. Alternatively, the *Tribolium* situation could be a derived state, where Cv/Tsg could have acquired independently a more pronounced role for Dpp signalling in the lineage leading to *Tribolium*. The RNAi experiments against one *cv* gene (*Nv-cvB)* in the Hymenoptera *Nasonia* argue against a specific *Tribolium* requirement. We have observed a major role for *crossveinless* related molecules in DV patterning probably in Dpp modulation also in this insect (Figure 3.12 and 3.13).

Taken together, this study suggests that duplication, followed by sub-functionalization involving Dpp modulators occurred in the higher Diptera lineage which gave rise to

Drosophila. I propose that different organisms use the multiple existing BMP/Dpp modulators in distinct morphogenetic processes. It is possible that the number of Dpp modulators acting in *Tribolium* DV axis formation is reduced when compared to *Drosophila* and vertebrates, since *Tc-cv* and *Tc-dpp* knock-downs are identical. *Tribolium* might have retained a more ancestral type of DV axis formation, with a less complex system involved in BMP/Dpp modulation (see also Van der Zee et al. 2006). Further functional analysis in other insects or primitive arthropods will shed new light into this question.

Chapter 4 - Self-regulatory circuits in dorsoventral axis formation of the short-germ beetle *Tribolium castaneum*

Introduction

In Chapter 1 a detailed description of the events required for DV axis formation in insects is provided with a particular emphasis on the evolutionary aspects. The reader is particularly encouraged to follow the fragmentation experiments performed by Klaus Sander described in Chapter 1 and the description of how DV polarity is achieved in *Drosophila*. In *Drosophila* the nuclear concentration gradient of the rel/NF-kB transcription factor Dorsal is established early in development (Stathopoulos and Levine 2002a). This nuclear gradient forms under control of maternally expressed genes in the early (syncytial) blastoderm embryo; highest Dorsal concentrations are observed in ventral nuclei and progressively lower concentrations in nuclei of lateral and dorsal regions (reviewed in Moussian and Roth 2005 and detailed in Chapter I). Dorsal initiates the differentiation of the mesoderm (ventral) and the neurogenic ectoderm (lateral) through the differential activation and repression of zygotic target genes. Comprehensive genomic approaches, including whole genome microarrays, tiling arrays and ChIP-on-chip experiments led to the identification of almost all Dorsal targets genes (ca. 50) (Stathopoulos et al. 2002; Biemar et al. 2006; Zeitlinger et al. 2007), making the system to one of the best understood complex gene regulatory networks for early embryonic patterning (Stathopoulos and Levine 2005).

Not only the events downstream of Dorsal, but also the upstream processes leading to the formation of the gradient are well understood. The formation of the Dorsal gradient depends on spatial cues which emerge in the follicular epithelium during oogenesis and subsequently are transmitted to the fluid-filled perivitelline space that surrounds the embryo (reviewed in Moussian and Roth 2005 and Chapter 1). In ventral regions of the perivitelline space a proteolytic cascade is initiated, generating the ligand of the transmembrane receptor Toll. Upon Toll activation, the inhibitory protein Cactus is degraded allowing the nuclear accumulation of Dorsal. This leads to the formation of a nuclear Dorsal gradient with peak levels at the ventral side (reviewed in Moussian and Roth 2005 and Chapter 1). Taken together, the comprehensive knowledge about the formation and action of the Dorsal gradient provides an excellent basis for evolutionary comparisons.

Results

The Toll pathway is required for DV axis establishment in *Tribolium*

The analysis of the Toll pathway was initially approched via BLAST searches on the recently sequenced *Tribolium* genome (Richards et al. 2008) for orthologs of genes known to act in *Drosophila* DV axis formation. The beetle genome contains 4 Toll receptor homologues closely related to *Drosophila Toll*[1]. However, only one of these homologues, the previously described *Tc-Toll* (Maxton-Kuchenmeister et al. 1999, data not shown), is expressed in the early embryo. I will refer to this homologue as *Tc-Toll*[1]. The adaptor protein Myd88, the kinase Pelle acting downstream of the receptor and the cytoplasmic inhibitor Cactus are represented each by a single homologue in the genome. Finally, two NF-κB homologues closely related to *Drosophila dorsal* exist in *Tribolium*. However, only one of these, the previously described *Tc-dorsal* is maternally expressed and present in the early embryo (Chen et al., 2000, for a phylogenetic analysis of these genes see Figure 4.1 and Zou et al. 2007).

To study the function of these genes, I used parental and embryonic RNAi techniques. While the injection of double stranded RNA (dsRNA) into female pupae and female adults (pRNAi) targets ovarian and embryonic mRNAs, injection of dsRNA into early embryos (eRNAi) targets only embryonic mRNAs either of maternal or zygotic origin. Comparing the outcome of both types of injections is of particular importance if the gene in question is expressed maternally and stored as maternal protein.

pRNAi for *Tc-Toll*[1], *Tc-pelle, Tc-myd88* or *Tc-dorsal* led to adult beetles producing normally shaped eggs which supported embryonic development. However, with high penetrance (90-95% for *Tc-Toll*[1] pRNAi), embryonic development was abnormal. The embryos showed severe defects in DV patterning. No phenotypic differences were observed among pRNAi experiments for the four genes (data not shown). This suggests that, like in *Drosophila*, they act in the same pathway and are required for the establishment of the embryonic DV axis. Since the knock-down phenotypes of all four genes are identical, the following figures on this chapter show only the results of *Tc-Toll*[1] RNAi experiments.

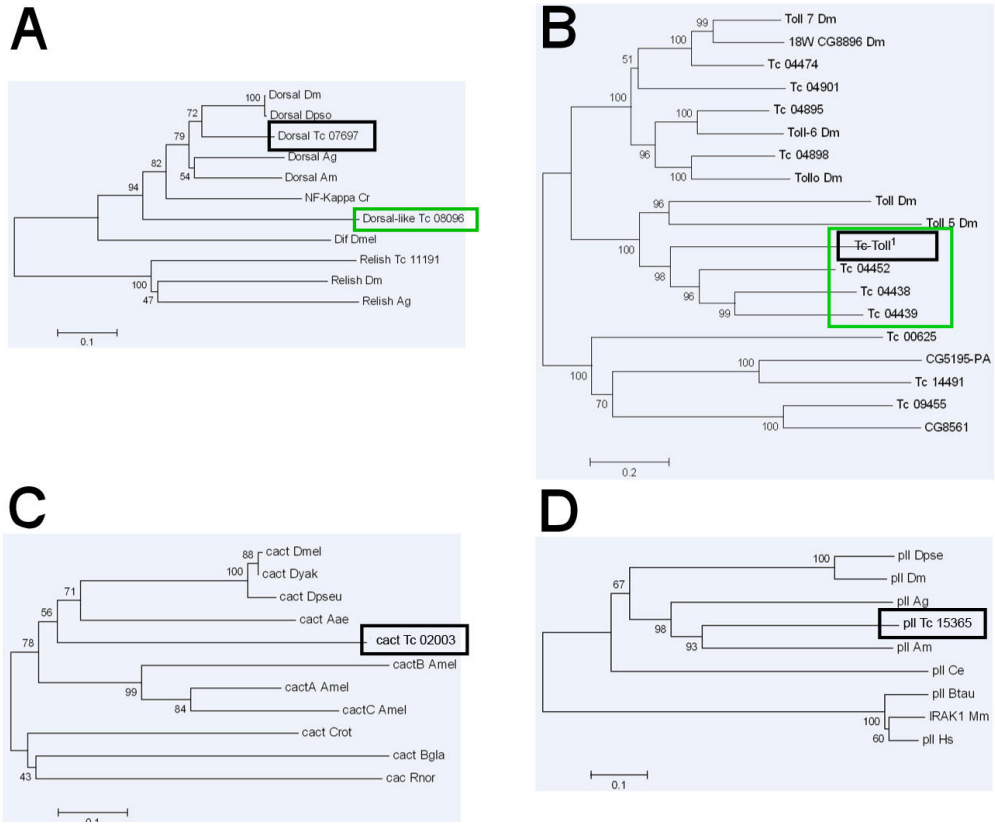


Figure 4.1: Phylogenetic trees for Dorsal, Toll, Cactus, and Pelle homologues

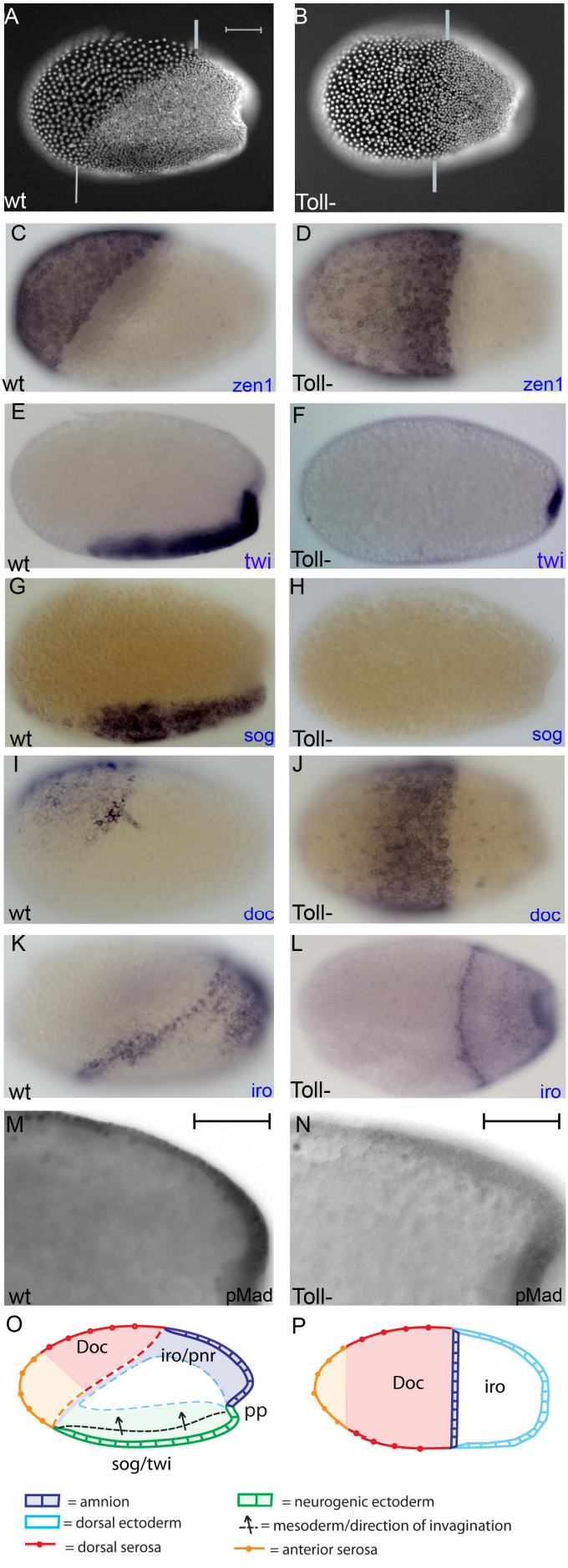
TBLASTN was used in order to obtain sequences similar to *Tc-Toll, Tc-dorsal, Tc-pll* and *Tc-cact* in several insect genomes. After alignments using CLUSTALW, the data was manually curated, and the sequences loaded into MEGA Version 3.1 software (Kumar et al. 2004). Neighbour-joining analysis was performed using 1000 replicates for bootstraps. The species abbreviations are as follows: (Aae) *Aedes aegypti* (Ag) *Anopheles gambiae* (Am) *Apis mellifera* (Bg) *Biomphalaria glabrata* (Bt) *Bos taurus* (Ce) *Caenorhabditis elegans* (Cr) *Carcinoscorpius rotundicauda* (Dy) *Drosophila yakuba* (Dm) *Drosophila melanogaster* (Dpso) *Drosophila pseudobscura* (Hs) *Homo sapiens* (Mm) *Mus musculus* (Rn) *Rattus norvegicus* (Tc)- *Tribolium castaneum.* (A) NF-κB homologues. The most similar molecule to Dm-Dorsal in the *Tribolium castaneum* genome is the previously published Tc-Dorsal molecule (Chen et al. 2000). (B) Toll homologues. A specific expansion of the Toll receptors related to Dm-Toll (involved in DV axis formation) seems to have occurred in *Tribolium* (green box). Three of these receptors are found in tandem on chromosome X of *Tribolium* and one on 1st chromosome. This latter gene was the previously described as *Tc-Toll* (Maxton-Kuchenmeister et al. 1999) and is renamed *Tc-Toll*^{1} (Glean_00176) in this study. (C) I-κB homologues. Only one *Tc-cact* related molecule is found in the *Tribolium* genome. At least three paralogs can be found in the genome of the Hymenoptera *Apis mellifera.* (D) Pelle homologues. Only one Pelle homologue was detected in the *Tribolium* genome. All *T. castaneum* gene numbers refer to GLEAN models as reported in Richards and Genome consortium 2008. Gene Bank or EMBL-Bank accession numbers: (A) *Dorsal Dm– CG-6667 , Dorsal Dpso–GA19765-PA; Dorsal Ag- gb|AAW67214.1|, Dorsal Am- gb|AAP23055.1|, NF-kappa Cr - gb|AAZ40333.1|,Dif Dm –CG-6794 ,Relish Dm - CG11992-PA, Relish Ag -|gb|AAQ57599.1|* . (B) *Toll 7 Dm – CG-8595, Toll-6 Dm– CG-7250 , Tollo Dm–CG-6890 , Toll Dm– CG-5490 , Toll 5 Dm- CG-7121.* (C) *cact Dm -CG5848-PB, cact Dy-gb|AAQ65041.1, cact Dpso- GA19176-PA ,cact Aae-EAT48251.1, cactA Am -XP_001121009.1, cactB Am -XP_001121575.1, cactC Am -XP_394485.2, cact Cr- gb|AAZ40334.1|, cact Bg - gb|ABL74452.1|, cac Rn -XP_343066.2* . (D) *pll Dpso- GA19272-PA, pll Dm- CG5974-PA, pll Ag- XP_311931.3, pll Am- XP_624002.2, pll Ce-NP_502587.2, pll Bt-gi:95006987, IRAK1 Mm- gb|AAO63014.1|, pll Hs -AAK62888.1*

Knock-down of *Tc-Toll*[1] leads to embryos which lack all signs of DV polarity during early development. This phenotype can be readily seen in differentiated blastoderm embryos by staining the cell nuclei (DAPI). In contrast to *Drosophila* embryos, which show a uniform distribution of blastoderm nuclei, *Tribolium* wild-type (WT) embryos display a pronounced DV asymmetry in nuclear density: the blastoderm is subdivided into an anterior-dorsal region with widely spaced cells giving rise to the extraembryonic serosa and a posterior-ventral germ rudiment with densely packed cells giving rise to the embryo proper (Figure 4.2A). This DV asymmetry disappears after *Tc-Toll*[1] pRNAi. The border between presumptive serosa and germ rudiment becomes straight and the embryo is subdivided into a large anterior serosa and a small posterior germ rudiment (Figure 4.2B). Accordingly, *Tc-zerknüllt1* (*Tc-zen1*), a gene expressed exclusively in the presumptive serosa and required to establish the serosal fate (van der Zee et al. 2005), shifts from a dorsally tilted expression domain in WT to a broad symmetric domain (Figure 4.2C,D). This phenotype can be explained by an expansion of dorsal regions at the expense of ventral regions of the blastoderm and thus provides a first indication that *Tc-Toll*[1] RNAi leads to a dorsalisation of the embryo. Since only the DV asymmetry of the border between serosa and germ rudiment, but not the border itself is lost, the distinction between extraembryonic and embryonic tissue is not the result of DV, but rather of AP patterning. In *Drosophila*, on the contrary, the extraembryonic tissue (the amnioserosa) represents the dorsal-most structure and expands uniformly along the DV axis in dorsalized embryos.

Further support for a dorsalisation of the fate map upon *Tc-Toll*[1] RNAi comes from the expression of genes specific for ventral (mesoderm) and ventrolateral (neuroectodermal) regions of WT embryos. *Tc-twist* and *Tc-sog* are expressed in nested domains with *Tc-twi* marking the future mesoderm and *Tc-sog* a wider domain including a part of the neuroectoderm (van der Zee et al. 2006, Figure 4.2E,G). After *Tc-Toll*[1] RNAi the expression of *Tc-sog* was completely abolished and that of *Tc-twi* was restricted to a small symmetric domain at the posterior pole (Figure 4.2F,H). This suggests a loss of ventral and ventrolateral fates. A corresponding expansion of dorsal fates can be inferred from the expression of genes restricted to the dorsal regions in WT, *e.g., Tc-doc*, a marker for the dorsal serosa and *Tc-iro* (both markers are described in Chapter 3), a marker for the dorsal fates of the germ rudiment (amnion and dorsal ectoderm) (Figure 4.2I,K). Both *Tc-doc* and *Tc-iro* expression expand uniformly around the circumference of the embryo after *Tc-Toll*[1] pRNAi (Figure 4.2J,L), indicating an expansion of dorsal at the expense of ventral fates within the serosa and the germ rudiment, respectively. In WT, *Tc-iro* is not only expressed within a dorsal domain, but

also in a narrow stripe marking the anterior border of the germ rudiment (this region gives rise to the anterior amnion described in Chapter 3). This stripe of *Tc-iro* expression is maintained, but becomes straight after *Tc-Toll*^{1} RNAi, in accordance with the changes observed for DAPI stainings, *Tc-zen* and *Tc-doc* expression. The uniform levels of *Tc-iro* within the germ rudiment of *Tc-Toll*^{1} RNAi embryos could correspond to an expansion of the dorsal-most embryonic fate, the dorsal amnion, or of dorsolateral fates (dorsal ectoderm). To distinguish between these two cell fates, I monitored Dpp signalling via the distribution of phosphorylated MAD (pMad). Our group has previously shown that in WT dorsal amnion formation requires high levels of Dpp signalling (high pMad), which depends on Tc-Sog mediated ventral-to-dorsal transport of Dpp (Figure 4.2M, van der Zee et al. 2006). In contrast, *Tc-Toll*^{1} RNAi embryos lack peak levels of pMad and instead have low levels of pMad at all positions of the circumference (Figure 4.2N and Figure 4.3A,B). I assume that the loss of *Tc-sog* expression in *Tc-Toll*^{1} RNAi embryos (Fig 4.2G,H) prevents Dpp transport and formation of Dpp peak levels at the dorsal side. A detailed explanation on how Dpp transport and modulation mechanism occurs is provided in Chapter 3 and in Van der Zee et al., 2006.

Another phenotypic consequence of the dorsalisation of the fate map observed after *Tc-Toll*^{1} RNAi is the deletion of the head anlagen (Figure 4.3). In WT *Tc-otd* and Tc-*msh2* are expressed in the prospective head lobes and in the prospective antennal segment, respectively. After *Tc-Toll*^{1} RNAi both domains are deleted (Figure 4.3E,F). Further support for the loss of the anterior region of the germ rudiment is provided by the analysis of the head gap gene *Tc-mlpt,* which in WT is expressed in an anterior triangular shape domain (Figure 4.3 C). After *Tc-Toll*^{1} RNAi, this domain is reduced to a symmetric stripe along the DV axis (Figure 4.3D). This stripe overlaps with the anterior amnion (Figure 4.2L). The lack of the head domain has been previously observed after *Tc-sog* RNAi (Van der Zee et al. 2006). Since *Tc-sog* expression was absent after *Tc-Toll*^{1} RNAi (Figure 4.2G), it is assumed that the head deletion in these RNAi embryos is caused by the lack of *Tc-sog.*


A
wt
B
Toll-
C
wt
zen1
D
Toll-
zen1
E
wt
twi
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Toll-
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iro/pnr
pp
sog/twi
P
Doc
iro
= amnion
= dorsal ectoderm
= dorsal serosa
= neurogenic ectoderm
= mesoderm/direction of invagination
= anterior serosa

Figure 4.2: *Tc-Toll*^{1} RNAi embryos lack DV asymmetry and are dorsalised
Lateral views of embryos at late differentiated blastoderm/primitive pit stage (Handel et al., 2000). Anterior is to the left, dorsal is up. Scale bar: 100µm. (A,C,E,G,I,K,M) WT. (B,D,F,H,J,L,N) *Tc-Toll*^{1} RNAi. (A,B) DAPI staining. Regions of low nuclear density correspond to the serosa, regions of high nuclear density correspond to the germ rudiment. (C-L) In situ hybridisations. (C,D) *Tc-zen1* expression marking the presumptive serosa. (E,F) *Tc-twi* expression marking the presumptive mesoderm. (G,H) *Tc-sog* expression marking the mesoderm and ventral parts of the presumptive neuroectoderm. (I,J) *Tc-doc* expression marking the dorsal serosa. (K,L) *Tc-iro* expression marking the anterior amnion (stripe at the anterior border of the germ rudiment), the dorsal amnion (high expression levels) and the dorsal ectoderm (low expression levels). (M,N) Anti-pMad antibody staining marking regions of high and intermediate levels of Dpp signalling. (M) Dorsal region of the germ rudiment of a WT embryo. (N) An arbitrary region of the germ rudiment of a *Tc-Toll* RNAi embryo. (O,P) Schematic drawings of the fate map of WT (O) and *Tc-Tol1*^{1} RNAi (P) embryos at the posterior pit stage. A-D and J courtesy from Maurjin van der Zee.

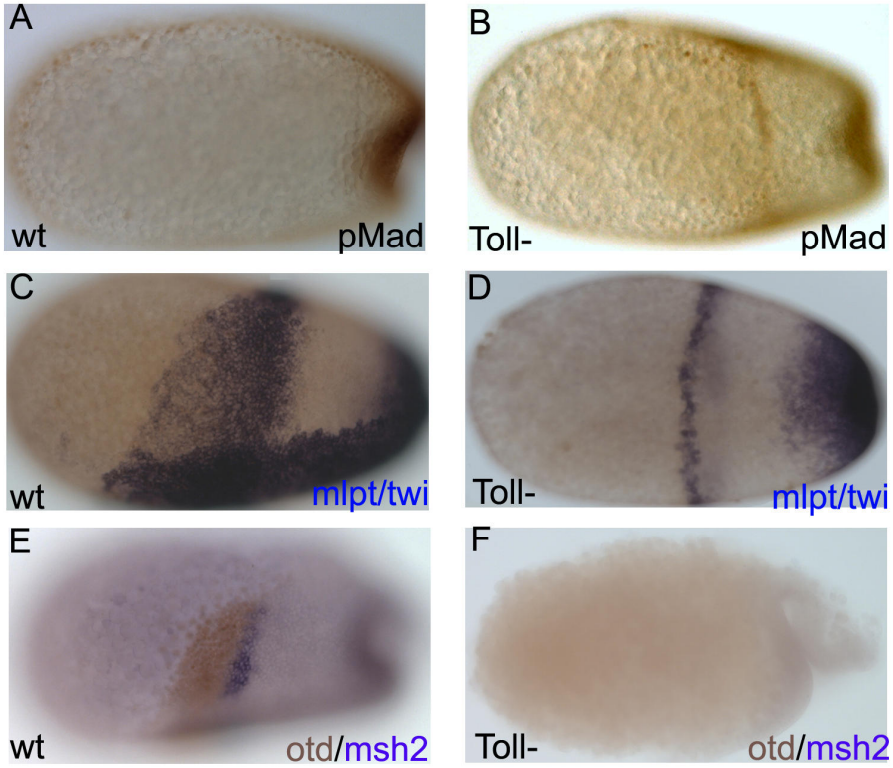

Figure 4.3: Deletion of ventral and anterior regions of the germ anlage after *Tc-Toll*^{1} RNAi. Lateral views of embryos. Anterior is to the left, dorsal is up. (A-D) Differentiated blastoderm/primitive pit stage. (A,B) pMad stainings of WT (A) and *Tc-Toll*^{1} RNAi embryos (B). pMad (brown) is detected in the dorsal region of the embryo (the future amnion) and in the primitive pit. An oblique stripe of Dpp activity in the region between the serosa and the germ rudiment also exists in the WT (not shown in the picture, but the domain is similar to *Tc-iro*, Figure 4.2) (B) After *Tc-Toll*^{1} RNAi nuclear pMad is only detected in a stripe between the germ rudiment and the serosa giving rise to the anterior amnion and in the posterior pit. (C,D) Differentiated blastoderm/primitive pit stage. (C,D) Double in situ hibridisation for *Tc-mlpt* and *Tc-twist*. The ventral expression of *Tc-twi* is absent and anterior expression of *Tc-mlpt* is reduced to a stripe, which is symmetric along the DV axis after *Tc-Toll*^{1} RNAi. This stripe overlaps with the anterior amnion (see Fig 4.2L and schematic drawings Fig 4.2O,P).(E,F) *Tc-msh2* in situ hybridisation (blue) and Tc-Otd antibody staining (brown). (E,F) Gastrulating embryos. (E) In WT, Tc-Otd marks the prospective head lobes and *Tc-msh2* the prospective antennal segment (F) In *Tc-Toll*^{1} RNAi embryos both expression domains are deleted. The head deletion phenotype is identical to that of *Tc-sog* RNAi embryos (van der Zee et al. 2006).

In summary, as in *Drosophila*, the Toll pathway is responsible for all aspects of early DV polarity in *Tribolium*. The loss of pathway activity results in a dorsalisation of the blastoderm embryo. However, in contrast to dorsalised *Drosophila* embryos, which display extraembryonic (amnioserosal) cells around the circumference (Konrad et al. 1988; Wharton et al. 1993), dorsalised *Tribolium* embryos possess separate regions of extraembryonic and ectodermal anlagen along the AP axis. The anterior two thirds of the embryo consist of an enlarged dorsalised serosa followed by a narrow stripe of anterior amnion and a broad domain of dorsal ectoderm (schematic drawings Figure 4.2O,P). Thus, cell fates organized along the DV axis in *Drosophila* are organized along the AP axis in *Tribolium*.

In *Drosophila,* Toll signalling initiates the nuclear uptake of Dorsal at the ventral side of the embryo. The total amount of Dorsal protein does not change along the DV axis. At the dorsal side Dorsal protein remains in the cytoplasm while in ventral regions it is transferred to the nuclei. If this mechanism would also apply to *Tribolium,* Dorsal protein should remain in the cytoplasm at all positions of the embryonic circumference upon *Tc-Toll*^{1} RNAi. Indeed, in *Tc-Toll*^{1} RNAi blastoderm embryos no nuclear uptake of the Tc-Dorsal is observed, and the Tc-Dorsal distribution at all positions along of the embryonic circumference equals that of the dorsal side of WT embryos (Figure 4.4). This suggests that the nuclear Tc-Dorsal gradient forms, as in *Drosophila,* by redistribution of Tc-Dorsal protein.

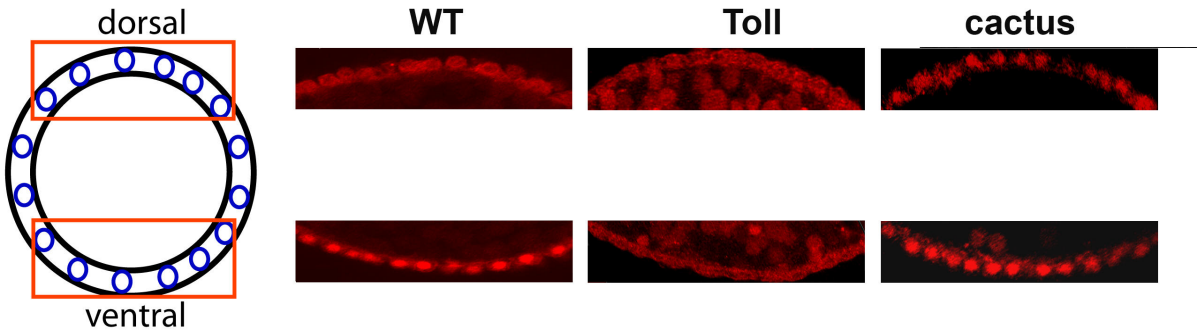

Figure 4.4: The nuclear Dorsal gradient in *Tc-Toll*^{1} and *Tc-cact* RNAi embryos
Cross sections of embryos stained with anti-Tc-Dorsal antibodies. Only partial sections of the circumference are shown. In WT and *Tc-cact* RNAi embryos dorsal and ventral regions can be distinguished. In *Tc-Tol1*^{l} RNAi embryos all positions of the along circumference have identical Tc-Dorsal distribution.

Feedback loops regulate the Tc-Dorsal gradient

The *Drosophila* Dorsal nuclear gradient remains largely static with regard to its DV extent. In contrast, the shape of the nuclear Tc-Dorsal gradient is highly dynamic. Nuclear import is initiated around the entire DV circumference and is subsequently restricted to the ventral side where a nuclear gradient forms. This gradient rapidly shrinks and disappears before gastrulation (Chen at al., 2000; Figure 1.10, Figure 4.7B,E). A first hint to explain this dynamic behaviour came from the observation that *Tc-Toll*[1] was not stored as maternal mRNA in the deposited egg like *Toll* in *Drosophila*, but rather appeared to be a target of the Tc-Dorsal gradient, being up-regulated in ventral regions with high Tc-Dorsal concentrations (Maxton-Kuchenmeister et al. 1999, Figure 4.5B).

To test whether *Tc-Toll*[1] might be stored as maternal protein instead of maternal mRNA, we performed an embryonic RNAi knock-down experiment with *Tc-Toll*[1]. After dsRNA injection into preblastoderm embryos a complete loss of DV polarity was observed, and the resulting phenotypes were indistinguishable from those produced by parental RNAi (Figure 4.6). This strongly supports the notion that *Tc-Toll* expression is entirely zygotic and highlights the importance of feedback regulation by Tc-Dorsal.

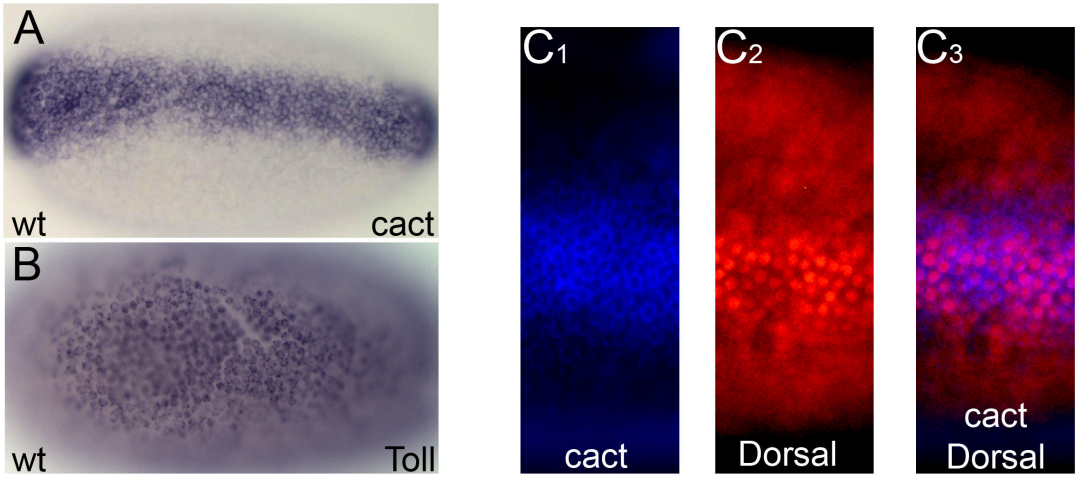

Figure 4.5 Dorsal, the receptor Toll and the inhibitor Cactus are expressed in overlapping domains in blastoderm stages of *Tribolium*.
Ventral views of *Tribolium* embryos at uniform blastoderm stage. Anterior to the left.(A,B) In situ hybridization for *Tc-cact* (A) and *Tc-Toll*[1] (B). The earliest detectable *Tc-cact* expression is narrower than the early broad domain of *Tc-Toll*[1]. This broad domain of *Tc-Toll*[1] overlaps with Tc-Dorsal (Chen et al. 2000). (C) In situ hybridisation (*Tc-cact*, C1; blue) and antibody staining (Tc-Dorsal, C2; red).C3 Overlay shows the co-localization of Tc-Dorsal and *Tc-cact* transcripts.

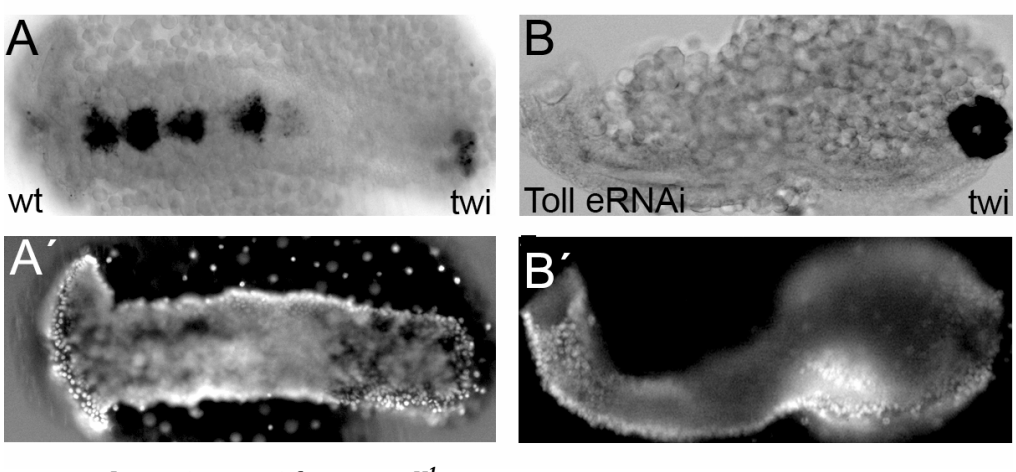

Figure 4.6: Embryonic RNAi for *Tc-Toll*^{1}
Embryos at germ band stage. Anterior points to the left. WT (A) and *Tc-Toll*^{1} eRNAi embryos (B) stained for the mesodermal marker *Tc-twi.* The respective DAPI nuclear stainings are shown (A´,B´). (A) In WT *Tc-twi* is detected in the segmented mesoderm and in a posterior domain in the growth zone. (B) After *Tc-Toll*^{1} eRNAi, *Tc-twi* is only detected in the growth zone. This *Tc-Toll*^{1} knock-down phenotype obtained with embryonic injection is identical to the one obtained with parental RNAi (see Fig 5D).(A´B´) The head is missing after *Tc-Toll*^{1} RNAi (B´), a characteristic of this knock-down phenotype (see also Figure 4.3).

Positive feedback regulation is frequently counter-balanced by inhibitory processes to provide the basis for spatial patterning. In particular, I expected an inhibitory mechanism to account for early shrinkage and disappearance of the nuclear Tc-Dorsal gradient. Therefore, I analysed the regulation of the Dorsal-inhibitor Cactus. As in the case of *Tc-Toll*^{1}, *Tc-cact* transcripts are absent in pre-blastoderm embryos, indicating that in contrast to *Drosophila cact*, *Tc-cact* is not expressed from a store of maternal mRNA. However, the syncytial blastoderm stage, *Tc-cact* is expressed ventrally, overlapping with the Tc-Dorsal gradient (Figure 4.7A). Double in situ hybridisations show that *Tc-cact* expression at this stage is narrower than *Tc-sog* and *Tc-twi* expression (Figure 4.7I,J). Carefully staged single in situ hybridisations suggest that the *Tc-cact* expression domain is also narrower than *Tc-Toll*^{1} expression at this stage (Figure 4.5A,B). Like *Tc-sog* and *Tc-twi, Tc-cact* expression depends on *Tc-Toll*^{1} signalling since it is largely abolished after *Tc-Toll*^{1} RNAi; only a spot of *Tc-cact* expression is detected at the posterior pole (Figure 4.7C). When the Dorsal gradient begins to disappear in the ventral region of *Tribolium* embryos, *Tc-cact* expression also shrinks (Figure 4.7D,E for double staining see Figure 4.5C). In differentiated blastoderm stages the Tc-Dorsal gradient vanishes from the ventral region (Chen et al. 2000), however, *Tc-cact* is up-regulated and acquires a *Tc-twi*-like expression profile (Figure 4.7F). This indicates that *Tc-cact* expression is maintained independently from Tc-Dorsal at this stage. Similar to *Tc-twi, Tc-cact* expression is seen within the mesoderm in early germ band embryos (Figure 4.7K). To

test whether this mesodermal expression depends on *Tc-twi* we performed *Tc-twi* pRNAi experiments. In embryos lacking *Tc-twi* function, *Tc-cact* expression starts to vanish at the differentiated blastoderm stage (data not shown) and is strongly reduced when compared to WT at the beginning of gastrulation (Figure 4.7G,H). Together these data suggest that *Tc-cact* is a target gene of both Tc-Dorsal and of the Dorsal target gene *Tc-twi*.

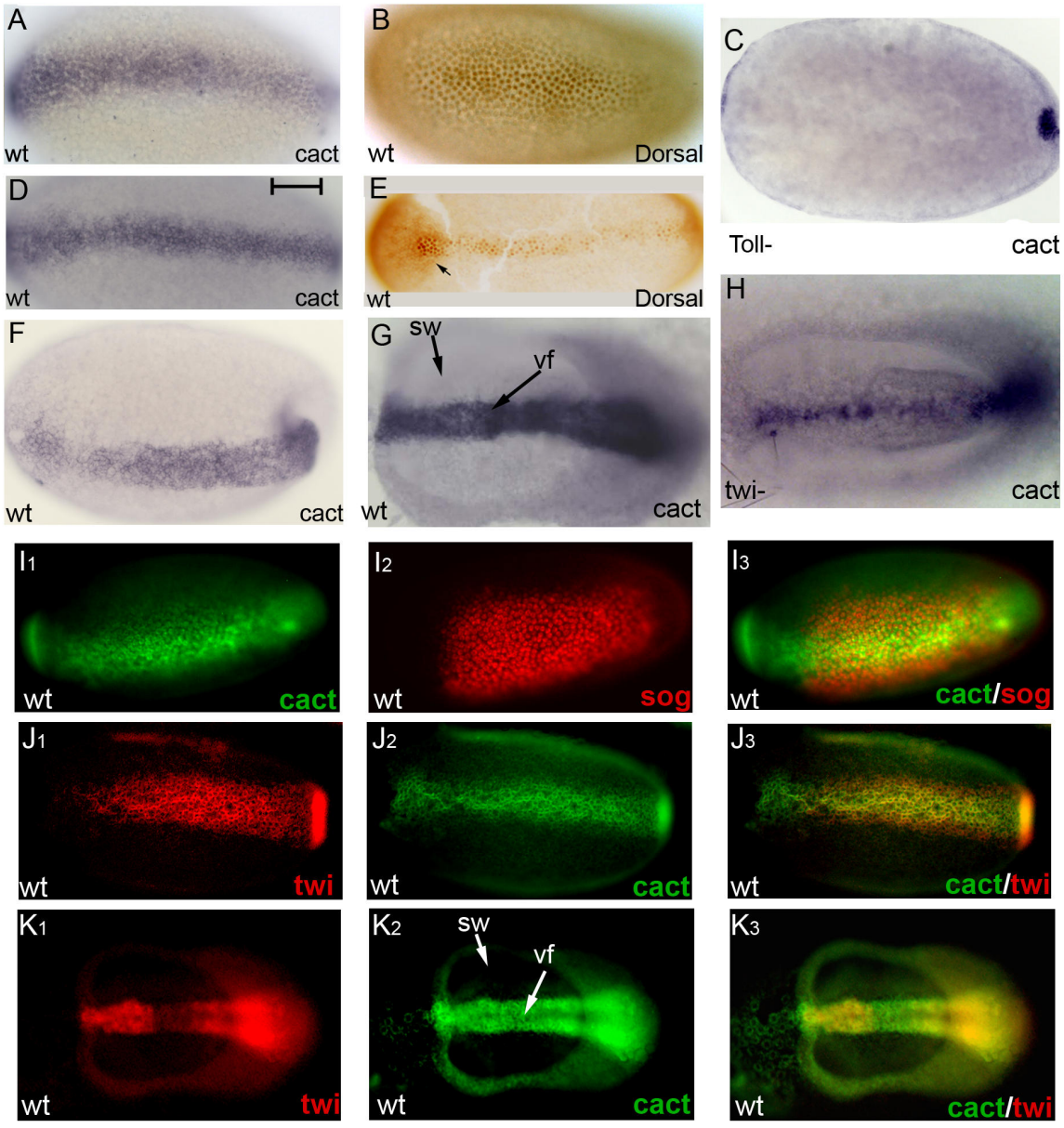

Figure 4.7: *Tc-cact* is activated by Tc-Dorsal and *Tc-twi*
Ventral surface views of embryos with the anterior pole pointing to the left. Scale bar: 100µm. (A,B,I,J) Uniform blastoderm stage. (C,D) Differentiated blastoderm stage. (E,F) Posterior pit stage. (G,H,K) Gastrulation. (A,C,D,F-H) In situ hybridisation for *Tc-cact* of WT (A,D,F,G), *Tc-Toll*^{1} RNAi (C) and *Tc-twi* RNAi embryos (H). (B,E) Anti-Tc-Dorsal antibody staining of embryos corresponding in age to the embryos shown in A and D. (I-K) Fluorescent double in situ hybridisations of WT embryos. The first two columns show single channels, the third the overlap. (I) *Tc-cact* and *Tc-sog* double in situ hybridisations. (J,K) *Tc-cact* and *Tc-twi* double in situ hybridisations. sw: serosal window. vf: ventral furrow. (E) From Chen et al. 2000.

In *Drosophila*, loss of *cactus* function results in the increased uptake of Dorsal protein in lateral and dorsal positions of the embryonic circumference. This causes the expansion of ventral at the expense of dorsal fates (Roth et al. 1991). I asked if *Tc-cact* knock-down in *Tribolium* would generate a similar phenotype. Parental and embryonic *Tc-cact* RNAi produced identical phenotypes indicating that *Tc-cact* is probably not provided as maternal protein and that *Tc-cact* function largely depends on zygotic expression. The RNAi embryos showed an expansion of the nuclear Tc-Dorsal gradient and increased uptake of Tc-Dorsal into nuclei of the dorsal side (Figure 4.4). However, I did not observe embryos in which all nuclei around the circumference took up the same high amounts of nuclear Tc-Dorsal. Thus, like in *Drosophila*, a graded, albeit flatter distribution of a nuclear Dorsal was maintained. Accordingly, *Tc-twi*, a ventral target gene of the Tc-Dorsal, showed an expanded expression domain, but was not uniformly expressed along the DV axis (Figure 4.8A,B).

Since *Tc-cact* expression is maintained by *Tc-twi* during differentiated blastoderm and early gastrulation, I wondered whether *Tc-twi* controls the nuclear uptake of Tc-Dorsal via activation of *Tc-cact*. To approach this question I analysed the Tc-Dorsal protein distribution in *Tc-twi* RNAi embryos. In contrast to WT embryos, where Tc-Dorsal is lost from the ventral nuclei prior to gastrulation (Figure 4.8C,E), Tc-Dorsal accumulates in ventral nuclei of gastrulating embryos in *Tc-twi* RNAi embryos (Figure 4.8D,F).

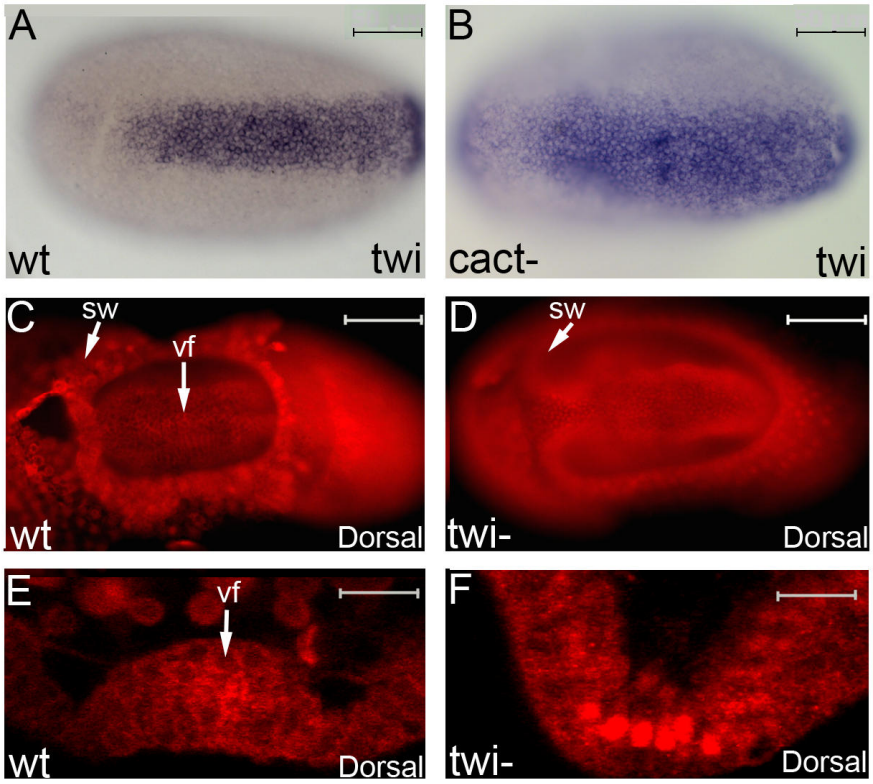

Figure 4.8: *Tc-cact* act as an inhibitor of Tc-Dorsal nuclear uptake
(A-D) Ventral surface views of embryos with the anterior pole pointing to the left. (E,F) Magnified cross sections from ventral regions of the embryos shown in (C) and (D). (A,B) Uniform blastoderm stage. Scale bar: 50µm (C-F) Gastrulation. (C,D) Scale bar: 100µm (E,F) Scale bar: 20µm (A,C,E) WT. (B) *Tc-cact* RNAi. (D,F) *Tc-twi* RNAi. (A,B) *Tc-twi* in situ hybridisations. (C-F) Anti-Tc-Dorsal antibody stainings. (C,D) The serosal window (sw) has not closed. Thus, the ventral side of embryo proper is visible. In WT (C,E) the ventralmost cells (mesoderm) start to be internalized by ventral furrow formation (vf). In *Tc-twi* RNAi embryos (D,F) the ventral furrow does not form. In contrast to WT, the ventralmost cells accumulate high levels of Tc-Dorsal in their nuclei.

In conclusion Cactus, as in *Drosophila*, is crucial in controlling the formation of the nuclear Dorsal gradient in *Tribolium*. However, *Tc-cact* regulation is strikingly different from that of *Drosophila cact*. First, at early stages *Tc-cact* itself is a target of Tc-Dorsal and is strongly activated at the ventral side like the mesodermal target gene, *Tc-twi*. Second, at later stages *Tc-cact* is a target of *Tc-twi*. Activation of *Tc-cact* by *Tc-twi* is required to terminate the nuclear uptake of Tc-Dorsal in late blastoderm and gastrulating embryos. Thus, a combination of Dorsal and Twi-dependent activation of *Tc-cact* appears to be responsible for the rapid shrinking and disappearance of the Dorsal gradient in the beetle. This implies that in *Tribolium* the role of *twi* is not restricted to mesoderm formation, but appears also to be involved in terminating Dorsal function and initiating the transition from maternal to zygotic control of DV patterning.

The later development of *Tc-Toll* RNAi embryos provides insights into growth zone function

While *Drosophila* embryos specify all segments prior to gastrulation (long-germ type) *Tribolium* embryos form the abdominal segments during later embryonic development from a posterior growth zone (short-germ type). An important question related to short-germ type development is whether formation and function of the growth zone require DV polarity in the early embryo (discussed in Chapter 1). With the help of *Tc-Toll*^{1} RNAi embryos this question can be addressed.

To approach this question I first analysed segmentation in *Tc-Toll*^{1} RNAi embryos. As markers for segment formation I used *Tc-gooseberry* (*Tc-gsb*), *Tc-engrailed* (*Tc-en*) and *Tc-iro* (Figure 4.9A and data not shown). *Tc-gsb* and *Tc-iro* are expressed in non-overlapping stripes in each segment. Moreover, *Tc-gsb* is restricted to ventral regions of the mature (more anterior) segments. After *Tc-Toll*^{1} both genes showed, like in WT, a periodic expression (Figure 4.9B), suggesting that dorsalized embryos form segment anlagen with normal segment polarity. In more anterior segments *Tc-gsb* was gradually lost presumably because its expression becomes restricted to ventral regions, which are absent in *Tc-Toll*^{1} RNAi embryos. Strikingly, the number of stripes indicates that a full set of abdominal segments is present (Figure 4.9B and data not shown) suggesting that embryos lacking DV polarity possess an active growth zone.

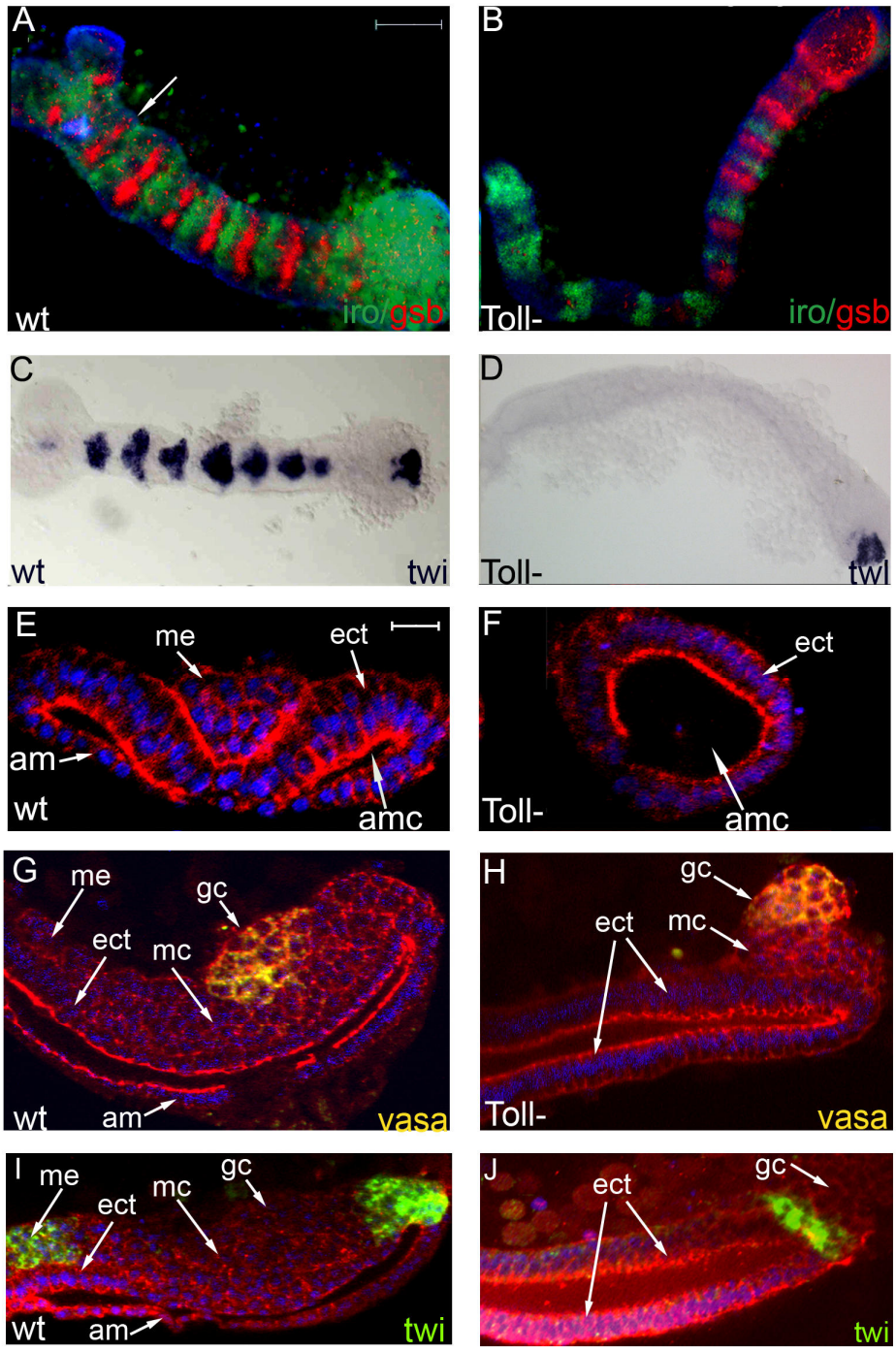

Figure 4.9: Segment formation and the growth zone of *Tc-Toll*^{1} RNAi embryos.
(A,C,E,G,I) WT. (B,D,F,H,J) *Tc-Toll*^{1} RNAi. (A,B) Germ band stage embryos. Anterior is to the left. Double in situ hybridisations with the segmental markers *Tc-iroquois (Tc-iro,* green*)* and *Tc-gooseberry (Tc-gsb,* red*)* and DAPI staining (blue). Scale bar: (A) 100µm (E) 20µm. In WT (A) *Tc-iro* is expressed in the amnion covering the growth zone and in every segment. *Tc-gsb* is expressed complementary to *Tc-iro* in every segment. During segment differentiation (i.e. in more anterior segments), *Tc-gsb* becomes restricted to the ventral ectoderm (arrow). In *Tc-Toll*^{1} RNAi embryos (B) no *Tc-iro* expression is seen in the region of the growth zone indicating a loss of the amnion. (C,D) *Tc-twi* in situ hybridisation (E,F). Phalloidin and DAPI staining to visualize F-actin (cell outlines) and DNA (nuclei), respectively. Cross sections through germ band stage embryos (7 segments) at approximately 70% embryonic length. (G-J) Lateral views of growth zones. Anti-Phosphotyrosine antibody staining marking the cell outlines (red) and *Tc-vasa* fluorescent in situ hybridisation marking the germ cells (green in G,H) or *Tc-twi* fluorescent in situ hybridisation marking cells in the posterior tip and the mesoderm in the anterior region (green in I,J). am: amnion, amc: amniotic cavity, ect: ectoderm, gc: germ cells, me: mesoderm, mc: mesenchymal cells of growth zone. (E) Courtesy from Cornelia von Levetzow

If the growth zone remains active in *Tc-Toll*^{1} RNAi embryos it might also be able to produce different DV cell types including mesodermal cells. However, *Tc-twi* expression marking the mesodermal cells of each segment in WT was absent from all segments, including those derived from the growth zone after *Tc-Toll*^{1} RNAi. *Tc-twi* was only maintained, like in WT, at the posterior tip of the growth zone, indicating that this domain is specified independently of the Tc-Toll signalling (Figure 4.9C,D). The absence of mesodermal cells was also confirmed by phalloiding staining of cross sections. The segmented region of WT embryos is composed of three cell layers (Figure 4.9E). (1) A thin outer epithelial cell sheet represents the amnion (am). (2) A layer of high columnar epithelial cells which is continuous with the amnion at its borders represents the embryonic ectoderm (ect). (3) Mesenchymal cells facing the yolk that express *Tc-twi* represent the mesoderm (me). Within the growth zone, the same cell layers exist, but *Tc-twi* is only found at the very posterior tip (Figure 4.9E,I, Handel et al. 2005); thus the majority of mesenchymal cells (mc) within the growth zone lack *Tc-twi* expression. On top of these mesenchymal cells (mc), the germ cells (gc), which express *Tc-vasa*, reside as a globular mass (Figure 4.9G).

The segmented region of *Tc-Toll* RNAi embryos is composed of a hollow tube of high columnar cells, identical in shape to the ectodermal cells of WT embryos (Figure 4.9F, for explanation of how this morphology arises during gastrulation see Figure 4.10). Thin epithelial cells representing the amnion and mesodermal cells are absent (Figure 4.9F) suggesting that the segmented region consists only of ectodermal cells. This morphology extends into the growth zone region. However, at the posterior tip of the growth zone a small population of mesenchymal cells is found, which is associated with the posterior *Tc-twi* expressing cells and the germ cells (Figure 4.9D,J). The more anterior mesenchymal cells (mc) lacking *Tc-twi* expression in WT are absent after *Tc-Toll*^{1} RNAi (Figure 4.9H,J).

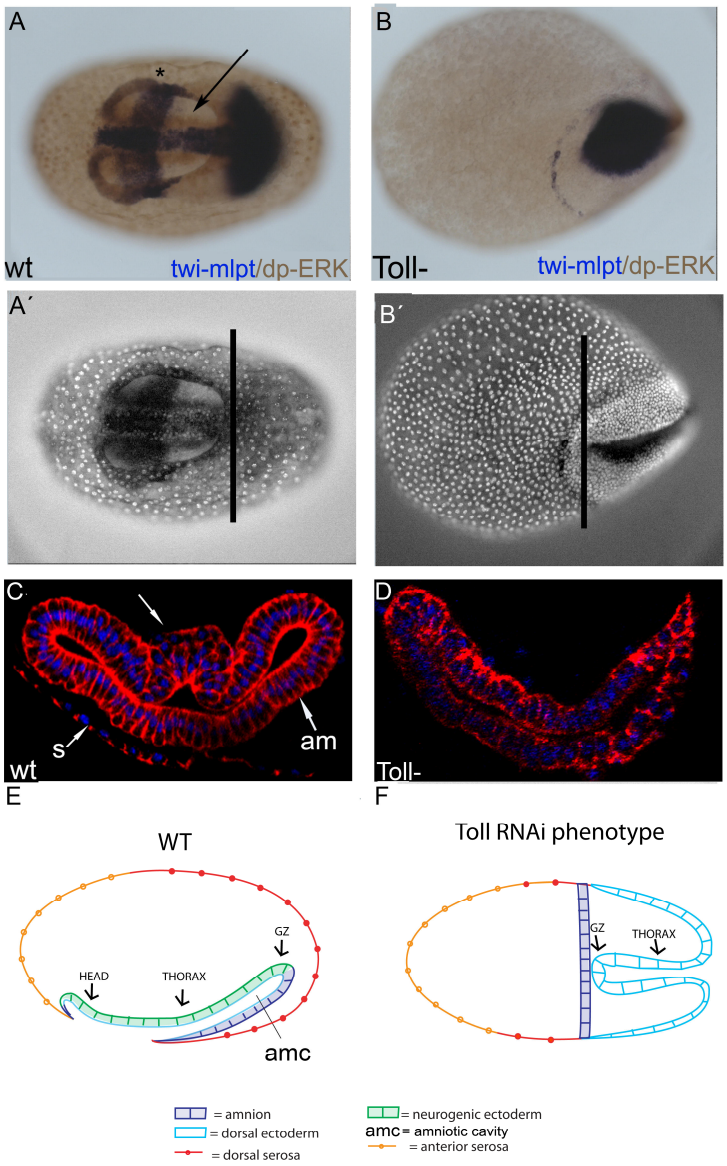

Figure 4.10: Gastrulation is DV symmetric after *Tc-Toll*^{1} RNAi.
(A-D) Gastrulating embryos. (A,B) Gastrulating embryos at the serosal window stage. *Tc-mlpt* and *Tc-twi* are detected by in situ hybridisation (blue), activated MAPK by antibodies against dp-ERK (brown). (A) Ventral surface view of WT embryo. *Tc-mlpt* is expressed in the mandibular segment (asterisk) and in a posterior domain. *Tc-twi* marks the mesoderm (blue) which invaginates by ventral furrow formation. Activated MAPK marks the ventral ectoderm (brown - arrow). (A') DAPI staining of the same embryo shows the closing serosal window. (B) *Tc-Toll*^{1} RNAi embryos gastrulate largely symmetric along the DV axis. Random asymmetries are likely to be due to the imbalance of forces produced by morphogenetic movements. Ventral *Tc-twi* expression and MAPK (dp-ERK) activation are absent. *Tc-mlpt* is reduced to a faint anterior ring and a posterior domain. (B') DAPI staining of the same embryo shows a symmetric posterior invagination. (C,D) Cross sections with phalloidin and DAPI staining to visualize F-actin (cell outlines) and DNA (nuclei), respectively. The positions of the cross sections are indicated in A´ and B´, respectively. (C) In WT embryos the mesoderm invaginates through ventral furrow formation. Parallel to ventral furrow formation the posterior amniotic fold expands over the ventral side of the embryo. The ventral furrow is visible (arrow); the amnion (am) and the serosa (s) covers the embryo at the ventral side. (D) After *Tc-Toll*^{1} RNAi ventral furrow formation is abolished. The embryo consists of two epithelial cell monolayers which have identical morphology. These layers are formed by a posterior slit-like invagination, which is largely symmetric along the DV axis (see B,B´and the schematic drawings E,F). (E,F) Schematic drawings of the gastrulation in WT (E) and *Tc-Toll*^{1} RNAi embryos (F). Compared to WT embryos, *Tc-Toll*^{1} RNAi embryos have a completely symmetric gastrulation. The head and gnathal segments are absent and the embryo appears to be inverted after gastrulation. (C) Courtesy of Cornelia von Levetzow.

Together, these observations allow several conclusions about the growth zone of WT embryos. 1) Segment formation within the growth zone is an ectodermal process which does not require the presence of somatic mesoderm or correct ectodermal DV polarity. 2) The absence of most of the mesenchymal cells in the growth zone suggests that these cells have mesodermal identity despite their lack of *Tc-twi* expression. They are likely to be derived from cells which had expressed *Tc-twi* in the early embryo and invaginated during ventral furrow formation. Since early *Tc-twi* expression and ventral furrow formation are abolished after *Tc-Toll*^{1} RNAi (Figure 4.2 and Figure 4.10), these cells are missing within the growth zone. 3) The remaining *Tc-twi* expressing cells of the posterior tip of the growth zone do not contribute to the mesoderm of the segmented region.

Disruption of Dorsal/Toll pathway leads to a periodic pattern of DV cell fates along the AP axis

Tc-Toll^{1} RNAi embryos form hollow tubes of high columnar cells suggesting that these cells have ectodermal fates. I used molecular markers to analyse the further differentiation of these cells. The ectoderm of WT embryos can be subdivided into dorsal, dorsolateral, neurogenic and mesectodermal regions. The cells of the dorsal ectoderm express *Tc-pnr* and *Tc-dpp* (Figure 4.11A,C). They show high levels of Dpp signalling as visualized by pMad staining (Figure 4.11E,E´) and high levels of MAPK activity as visualized by dp-ERK staining (Figure 4.11C). The cells of the dorsolateral ectoderm are characterized by lower levels of Dpp signalling (reduced pMad staining) and the presence of single neurons of the peripheral nervous system (PNS) expressing *Tc-sna* (Figure 4.11E,E´, arrow). The cells of the neuroectoderm give rise to the neuroblasts of the central nervous system (CNS) which express *Tc-sna* (Figure 4.11E,E´) and *Tc-ash* (Wheeler et al. 2003, data not shown). Within the neuroectoderm further markers can be used to distinguish dorsal and ventral regions. e.g., neuroectodermal *Tc-msh2* expression is restricted to dorsal neuroblasts (Figure 4.11G; arrow). Finally, the mesectodermal cells at the ventral midline express *Tc-otd* (Figure 4.11G). We used these markers to investigate the differentiation of *Tc-Toll*^{1} RNAi embryos.

Since these embryos lack DV polarity I predicted that they would be completely dorsalised displaying uniform expression of *Tc-pnr and Tc-dpp*. Instead, they show rings of *Tc-pnr* and *Tc-dpp* expression (Figure 4.11B,D). pMad expression changes periodically along the AP axis indicating periodic changes of Dpp signalling (Figure 4.11F,F´). The regions of low pMad staining harbour narrow domains of cells expressing the neuronal markers *Tc-sna*

and Tc-ash. (Figure 4.11F,F´ arrows and data not shown). To test whether these cells belong to the PNS or CNS we used *Tc-msh2*. After *Tc-Toll*^{1} *RNAi*, *Tc-msh2* also shows periodic expression presumably in the same cells expressing *Tc-sna* and *Tc-ash* (Figure 4.11H). Thus, these cells are likely to correspond to dorsal CNS neurons of WT embryos indicating that at least the dorsal part of the neuroectoderm is established in *Tc-Toll* RNAi embryos. Among the markers investigated only *Tc-otd* was absent showing that the ventral most ectodermal cell fates are lacking (Figure 4.11H).

The Dpp inhibitor *Tc-sog* is not re-expressed in late *Tc-Toll*^{1} RNAi embryos. However, a possible explanation for the periodic change in Dpp signalling is provided by the observation that the Dpp inhibitor *Tc- bambi* (described in Chapter 2) is expressed in a periodic manner after *Tc-Toll*^{1} RNAi (Figure 4.11I, J). Taken together, *Tc-Toll*^{1} *RNAi,* embryos display a remarkable degree of ectodermal DV patterning. All major regions are established with the exception of the mesectoderm. However, the sequential alteration of cell fates does not take place along the DV, but rather along the AP axis of the embryo. This observation suggests that ectodermal patterning is an autonomous process taking place in absence of Toll signalling. Toll signalling is required to provide the orthogonal orientation of the ectodermal patterning mechanism with regard to the AP axis.

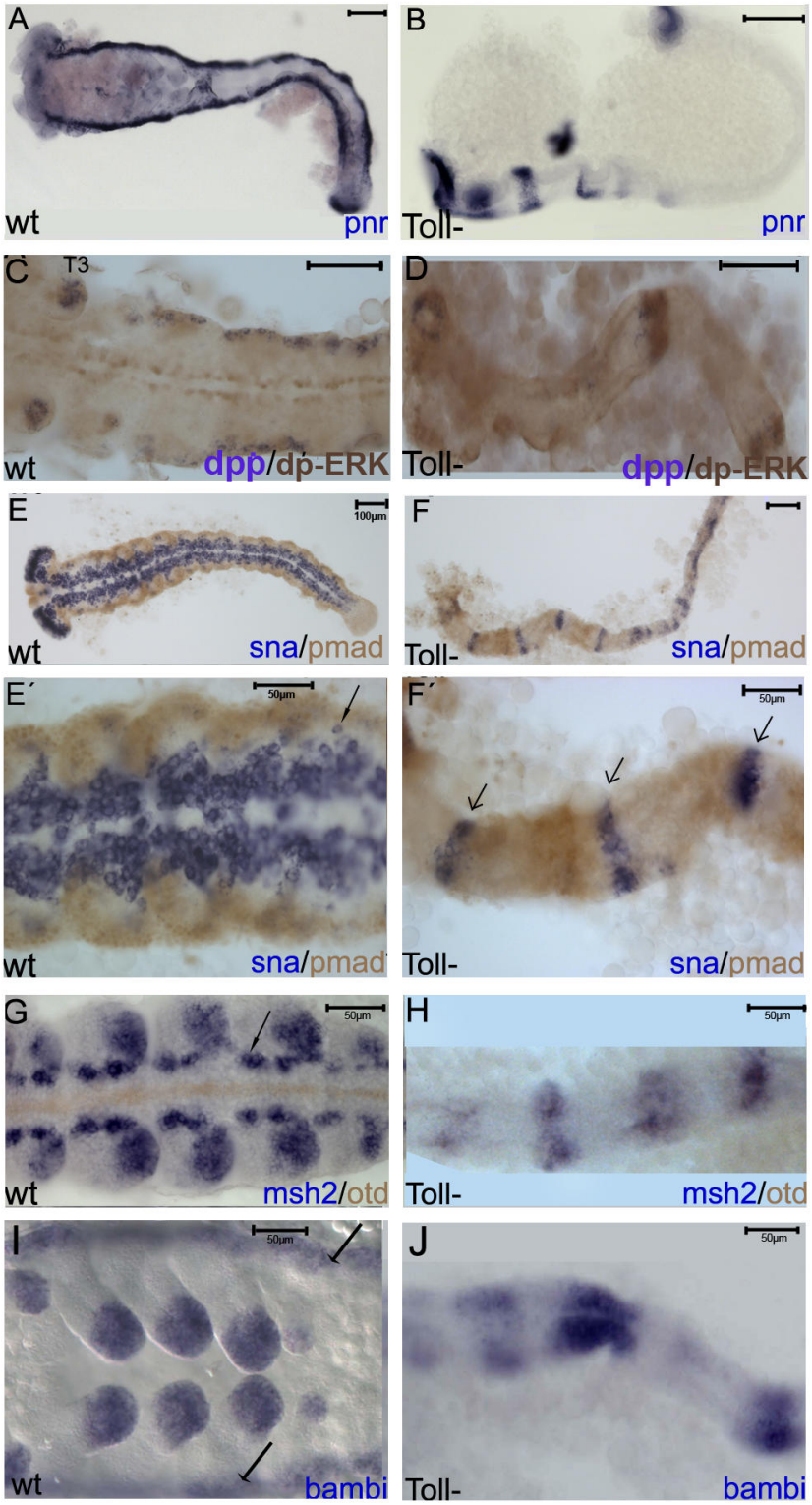

Figure 4.11: *Tc-Toll*^{1} RNAi embryos show residual DV patterning along the AP axis
Fully segmented germ band stage embryos. Anterior is to the left. (A,B) *Tc-pnr* in situ hybridisation in WT (A) and *Tc-Toll*^{1} RNAi (B). (C,D) *Tc-dpp* in situ hibridisation (blue) and activated MAPK antibody staining (dp-ERK, brown) in WT (C) and *Tc-Toll*^{1} RNAi embryos (D), (E,F) *Tc-sna* in situ hybridisation (blue) and pMad antibody staining (Dpp signalling-brown) in WT (E) and *Tc-Toll*^{1} RNAi (F). (E',F') Magnified views of the embryos shown in (E,F). The arrows highlight neuronal cells. (G,H) In situ hybridisation for *Tc-msh2* (blue) and Tc-Otd antibody staining (brown) in WT (G) and *Tc-Toll*^{1} RNAi embryos (H). The arrow highlight the dorsal most neurons (I,J). In situ hybridisation for *Tc-bambi* in WT (I) *Tc-Toll*^{1} RNAi (J) The arrows indicate the dorsal ectoderm.

Discussion:

Feedback control involving Toll signalling components and *Tc-twi*

Tc-Toll[1] transcription appears to start evenly along the DV axis at the syncytial blastoderm stage, but is rapidly enhanced at the ventral side where higher levels of nuclear Tc-Dorsal accumulate (Chen et al. 2000). This positive feedback between Toll expression and nuclear import of Dorsal could explain an initiation of DV axis formation at ectopic positions of the embryonic blastoderm, a situation which has been observed upon experimental manipulations in beetles and various hemimetabolous insects (Sander 1976). During normal development however, ectopic axis formation has to be prevented and this can be achieved by coupling positive feedback control to inhibitory processes. Linking self-enhancement to limiting mechanisms provides a general condition for pattern formation as has been shown by mathematical modelling (Meinhardt and Gierer 2000). The Tc-Dorsal dependent transcriptional activation of *Tc-cact* might provide the mechanism counter-balancing the positive feedback between *Tc-Toll* and Tc-Dorsal (Figure 4.12).

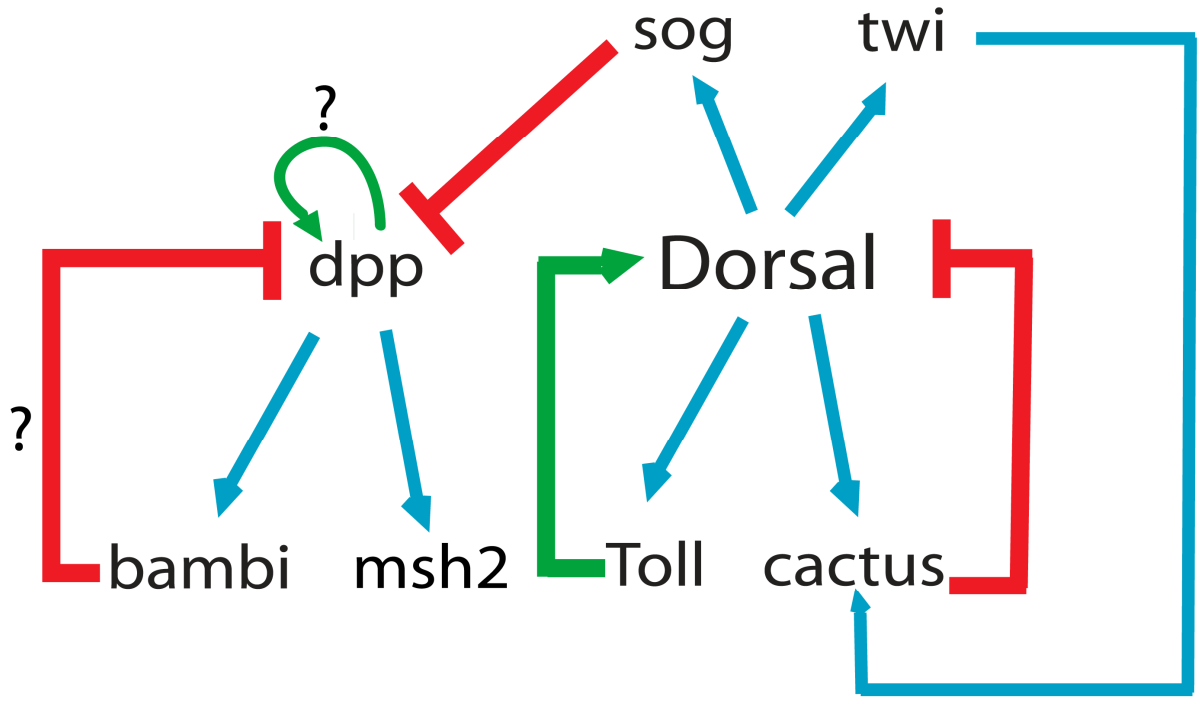

Figure 4.12: Two self-regulatory circuits are involved in *Tribolium* DV axis formation
Blue arrows represent activation, red lines inhibition and green arrows self-activation.

Within the limits of detection, *Tc-cact* expression appears to be restricted to the ventral side of the embryo. However, the knock-down of *Tc-cact* leads to nuclear import of Tc-Dorsal also at the dorsal side. To explain this long-range requirement of *Tc-cact* one might speculate

that my detection of *Tc-cact* transcripts is not sensitive enough or that Tc-Cact protein is able to diffuse within the cytoplasm from ventral towards dorsal. Irrespective of the mechanism, a long range action of Tc-Cact would meet an important prediction for pattern formation by reaction-diffusion systems, namely that the inhibitor should spread faster and thus act less local than the activator (Meinhardt and Gierer 2000, discussed in Chapter 5).

Besides its potential role for pattern formation *Tc-cact* activation seems also to be involved in the temporal control of the Dorsal gradient. During late blastoderm stages *Tc-cact* activation by *Tc-dorsal* is replaced through activation by *Tc-twi*. The *Tc-twi* knock-down phenotype shows that this shift is relevant to prevent Dorsal from accumulating in ventral nuclei during gastrulation. Thus, it seems that in *Tribolium* a Dorsal target gene is involved in terminating Dorsal function (Figure 4.12).

Collectively, these observations indicate that major evolutionary changes have occurred regarding Tc-Dorsal gradient formation and the network of downstream target genes. Nevertheless, traces of the feedback mechanisms uncovered in *Tribolium* have been preserved in the *Drosophila* lineage. Recently, zygotic enhancers of *Dm-cactus* and *Dm-Toll* were identified by ChIP-on-chip experiments and bioinformatics approaches (Sandmann et al. 2007). These enhancers contain *Dm-Dorsal* and *Dm-twist* binding sites and are active in the prospective mesoderm of *Drosophila.* However, the analysis of mutant phenotypes precludes an important function of these enhancers in DV patterning or cell type specification (Anderson et al. 1985; Roth et al. 1991). A weak stabilizing function may explain why they were retained in evolution.

On an even larger evolutionary scale it is interesting to note that negative feedback control is a hallmark of NF-κB mediated signalling. Like in *Tribolium*, the transcription of the Cactus homolog I-κB is activated by NF-κB in vertebrates both in the mesoderm and during innate immune response (Caamano and Hunter 2002, Zhang et al. 2006). The ensuing negative feedback loop can cause oscillatory signalling outputs or termination of signalling (Hoffmann et al. 2002; Nelson et al. 2004b; Covert et al. 2005). Even an involvement of *twist* in negative feedback regulation of NF-κB has been demonstrated in vertebrate mesoderm cells (Sosic et al. 2003). It has been proposed that the twi-NF-κB interactions represent an evolutionary conserved regulatory module. The Dorsal/NF-κB and *twi* dependent activation of *Tc-cact* might be a relic of mesodermal and innate immune functions the pathway had in the common ancestor of vertebrates and arthropods (Moussian and Roth 2005). According to this scenario, the ancestral feedback mechanisms were adjusted to the needs of spatial patterning after the pathway was adopted for DV axis formation.

Feedback control at the level of Dpp signalling

Classical fragmentation experiments have suggested two routes for pattern regulation along the DV axis: an early route which takes place before gastrulation and a later one which can be initiated after mesoderm internalisation (Sander 1976). Our group have previously provided evidence for late autonomous patterning within the ectoderm which depends on the Dpp/Sog system and additional inhibitory processes (van der Zee et al. 2006). *Tc-Toll*^{1} knock-down embryos add additional support for this assumption. They show pattern duplications of ectodermal DV cell fates along the AP axis. This remarkable phenotype is not just restricted to the abdominal segments derived form the growth zone, but it occurs also within the anterior (thoracic) segments (Figure 4.11). Thus, it is unlikely to reflect a specific mechanism which only operates in the growth zone.

The modulation Dpp activity underlying the periodic cell fate changes is likely to be due to periodic transcription of *Tc-dpp* and inhibition of Tc-Dpp diffusion or signalling along the AP axis (Figure 4.11F´). Since *Tc-sog* is not re-expressed in *Tc-Toll*^{1} RNAi embryos, we analysed the expression of other Dpp inhibitors. *Tc-bambi* showed periodic expression in the same domains as *Tc-dpp* and thus might provide the inhibitory function (Figure 4.11J). The fact that *Tc-dpp* is transcribed in regions of high pMad activity suggests positive feedback control which is counterbalanced by *Tc-bambi*. Thus, the interactions might be similar to that described for *Tc-Toll* and *Tc-cact* (Figure 4.12).

The unusual orientation of the ectodermal patterning process might depend on the early AP asymmetry of Dpp signalling in *Tc-Toll*^{1} RNAi embryos. After *Tc-Toll*^{1} RNAi *Tc-dpp* is expressed along the symmetric border between serosa and germ rudiment and in the posterior pit region (data not shown). These regions also have high levels of pMad (Figure 4.3). Thus, the ectodermal patterning process is initiated with AP asymmetric boundary conditions after *Tc-Toll*^{1} RNAi. In WT embryos this process is oriented along the DV axis through the Toll dependent activation of *Tc-sog* at the ventral side, which leads to a Dpp signalling gradient with peak levels along the dorsal midline (van der Zee et al., 2006).

The DV polarity of the growth zone

Our experiments clearly demonstrate that Tc-Dorsal is essential for establishing all aspects of normal DV polarity in *Tribolium,* including DV polarity of the growth zone from which the abdominal segments emerge. Thus, although DV patterning in the growth zone starts after gastrulation, when the Tc-Dorsal gradient has vanished, it is not independent of Tc-Dorsal.

I suggest that there are two ways by which early DV polarity is transmitted to the growth zone. First, distinct inner and outer cell layers are formed during gastrulation. The observation that the majority of the mesenchymal layer cells are absent in *Tc-Toll* RNAi embryos (Figure 4.9G,H, mc) strongly suggests that the mesenchymal cells in the growth zone are derived from cells internalized by ventral furrow formation in the early embryo. These cells cannot be re-supplied by a growth zone specific process of cell internalisation. Thus, gastrulation-like mechanisms do not continue in the growth zone of *Tribolium,* as has been suggested for the tail-bud of vertebrate embryos (Kane and Warga 2004). Second, DV patterning in the growth zone does not only depend on the generation of two separate cell layers. The ectoderm needs also to be patterned a process which mainly depends on Dpp signalling. To a certain degree this process takes place in a *Tc-Toll*^{1} knock-down embryo. However, the orientation of the resulting pattern is incorrect. We assume that during WT development DV polarity is first established within the anterior (gnathal and thoracic) segments. Subsequently, this pattern is used as a template for the DV pattern of the abdominal segments emerging from the growth zone. This would require a process of forward-induction from differentiated to non-differentiated tissues. Since there is no DV polarity in *Tc-Toll*^{1} RNAi early embryos, forward-induction cannot operate.

The evolution of Toll signalling

The loss of Toll signalling in *Tribolium* leads to phenotypes which are similar to those produced by the loss of the Dpp inhibitor *sog*. In both situations the ectoderm lacks normal polarity, the amnion and the CNS are largely deleted (the CNS is completely absent after *Tc-sog* RNAi and reduced to narrow periodic stripes after *Tc-Toll* RNAi) and the embryos form long tube-like structures (for another common feature see Figure 4.3). This situation is strikingly different in *Drosophila*. There, loss of Toll signalling leads to completely dorsalized embryos, while loss of *sog* causes only minor deletions in the CNS and subtle ectodermal patterning defects (Francois et al. 1994). These differences are due to the fact that Toll signalling in *Drosophila* provides functions which the Dpp/Sog system fulfils in *Tribolium*. For example, in *Drosophila* Dorsal represses *dpp* and activates *brinker*, an inhibitor of Dpp target genes, within the presumptive neuroectoderm and thereby specifies the CNS through mechanisms which act independent from and parallel to *sog* (Jazwinska et al. 1999b). These mechanisms do not exist in *Tribolium*. Apparently, the Dorsal gradient has a less direct role with regard to cell-type specification in *Tribolium* than in *Drosophila,* and DV patterning in

Tribolium relies to higher degree on the Dpp/Sog system. Since the Dpp/Sog (BMP/Chordin) system is involved in DV axis formation in all bilaterian animals investigated so far, this is likely to represent the ancestral mode of DV axis formation (De Robertis and Kuroda 2004; Akiyama-Oda and Oda 2006, Lowe et al. 2006). I suggest that the trend observed by comparing *Drosophila* and *Tribolium* applies to other insect orders and that the functional shift between Dpp and Toll signalling with regard to DV axis formation will be even more prominent in basal hemimetabolous insects. Thus, the study of more basal insects groups might reveal the evolutionary path of how Toll signalling was co-opted for DV axis formation (Roth 2003; Roth 2004).

Chapter 5 – General Discussion

The three previous chapters provided details about the evolution of BMP/Dpp modulators (Chapter 2 and 3) and the presence of self-regulatory circuits at the zygotic level in the beetle *Tribolium* (Chapter 4). In this chapter I would like to discuss the implications of these results in a broader perspective.

Self-activation coupled to a long-range inhibition: a common strategy for patterning in biology ?

The model of reaction-diffusion coupling proposed more than 50 years ago by Alan Turing and further developed by Meinhardt and Gierer is one of the best known mathematical models for biological pattern formation. Briefly, this model states that pattern formation can only occur if an auto-catalytic activator produces a long range inhibitor, which in turn, antagonizes the self-activation. This activator-inhibitor pair is the key component of the model (Fig 5.1). In Meinhardt´s model, a stable long-range pattern of morphogen activity can emerge from the interactions between these two components.

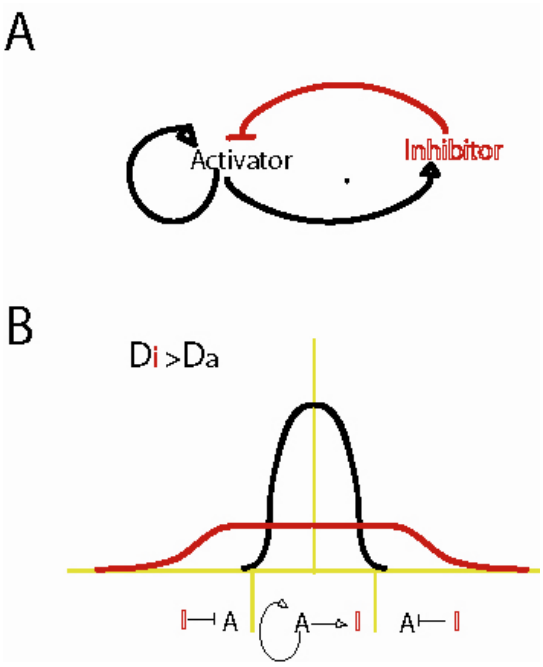

Figure 5.1: Stable pattern by an activator/inhibitor pair according to Meinhardt.
(A) The system consists of an activator and an inhibitor synthesized by the same source. The activator turns on itself and its own inhibitor. (B) A stable pattern is achieved if the inhibitor diffuses faster than the activator, so in the periphery the inhibitor completely inactivates the activator, but in the source the activator is still active and produces the inhibitor. Adapted from Plouhinec and De Robertis 2007.

This model generates a stable pattern in several nonliving physicochemical systems (Castets et al. 1990; Ouyang and Swinney 1991). Evidence has also accumulated for self-activation coupled to long range inhibition in several systems such as vertebrate axis formation (Chen and Schier 2002), breaking of left-right symmetry in vertebrate embryos (reviewed in Meinhardt 2006), in hair follicle patterning of developing murine skin (Sick et al. 2006) and in DV patterning of the frog embryo (Plouhinec and De Robertis 2007). All these processes contain at least one pair of a self-activator coupled to a long range inhibitor, and perturbations on these molecules lead to changes predicted by the aforementioned model. Importantly, Meinhardt´s model of self-activation assumes a steady state for the morphogen gradient, in other words, the dynamic interactions of the components stably maintain pattern over time.

Three key features are required for the *Tribolium* Dorsal gradient to fit into a Meinhardt model definition: (1) An inhibitor synthesized is under activator control; (2) The inhibitor diffuses faster than the activator; (3) A stable pattern in time and space is generated. Another characteristic which is often observed in biological systems is that the activator is a morphogen, a molecule that gives qualitatively different instructions to cells depending on its concentration. What ultimately determines pattern, therefore, is where morphogen gradients cross threshold values at which genes are turned on or off (reviewed in Lander, 2007).

The knock-down of *Tc-dorsal* or *Tc-Toll*[1] completely abolishes the expression of the putative inhibitor Tc-Cactus, so the auto-activation component of the system namely Tc-Dorsal-Tc-Toll is present and the inhibitor is synthesized under the activation control (Figure 4.7). *Tc-cactus* activation via Tc-Dorsal is probably a direct input, since knock-down of *Tc-twist* and other ventrally expressed genes (e.g. *twist* and *snail*) do not abolish *Tc-cactus* expression in early blastoderm stages (von Levetzow C data not shown). Tc-Cactus acts as an inhibitor of Tc-Dorsal, since after *Tc-cactus* RNAi, ectopic nuclear Tc-Dorsal is observed in nuclei located in the dorsal region (Fig 4.4). To conclude, the first condition for a self-activation coupled to long range inhibition in a Meinhardt model can be satisfied.

Whether the inhibitor (Tc-Cactus) diffuses faster than the activator (Tc-Dorsal) is an open question. The fact that nuclear Tc-Dorsal is found in dorsal regions after *Tc-cactus* RNAi is an indication that in WT, Tc-Cactus might diffuse towards dorsal embryonic regions keeping Tc-Dorsal in the cytoplasm, so Tc-Cactus might diffuse faster than Tc-Dorsal.

The unique condition which cannot be matched by *Tribolium* Dorsal in a self-activation coupled to long-range diffusion mechanism is the formation of a stable pattern in space and time. This gradient is highly dynamic and vanishes before gastrulation (Fig 1.10, Chen et al. 2000). Moreover, the expression domains of Dorsal target genes (*twi, cact, sog*) are highly dynamic when the nuclear Dorsal gradient is present (Figure 5.2), suggesting that the system do not achieve a steady-state.

Indeed, it seems logical that patterning information should be stably maintained over time in many developmental processes, since downstream responses of cells (e.g. gene expression) are relatively slow compared with the times required for diffusing molecules to move from one cell to another. In contrast, the nuclear Dorsal gradient in *Tribolium* persists for only a few hours, when compared to other morphogenetic gradients like Dpp and Wingless (Wg) in fly imaginal discs, where patterning takes several days. Irrespective of its life-time Tc-Dorsal can be considered a morphogen since it generates at least two thresholds of gene expression as judged by two-color fluorescent in situ hybridization. *Tc-sog* is expressed in a ventral broad domain, while *Tc-twi* and *Tc-cact* are expressed in narrower domains (Fig 5.2).

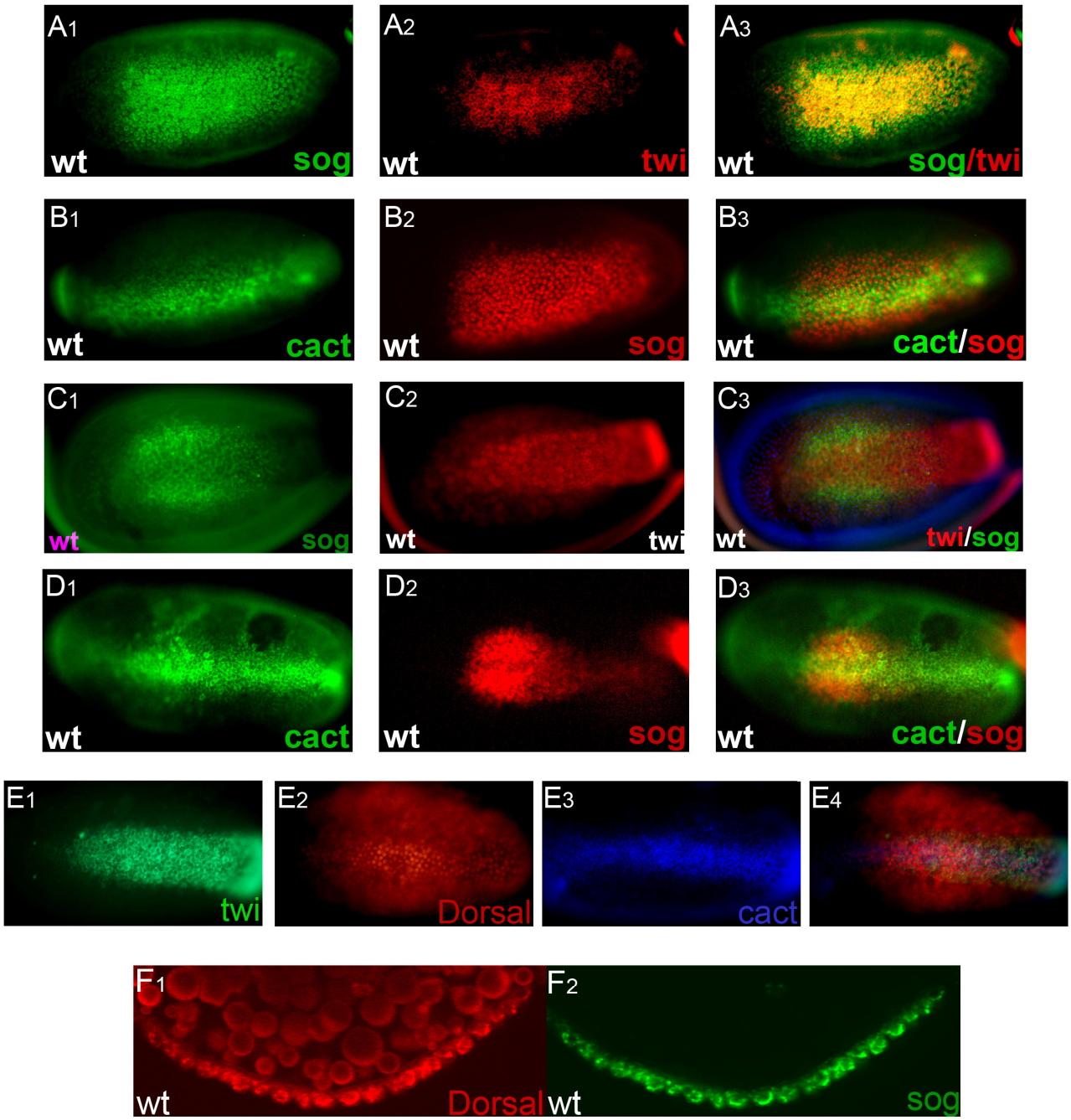

Figure 5.2: Dorsal is a morphogen, but the expression domains of its target genes are highly dynamic

(A-D) Double fluorescent *in situ* hybridizations. The two first rows show single channels and the third the overlay. (A-D) Comparable embryonic stages show that *sog* expression is broader than *twist* and *cactus*. (E) Fluorescent double in situ hybridization (E_1 - *Tc-twi* and E_3 – *Tc-cact*) plus anti-Dorsal antibody (red-E2). During blastoderm differentiation the nuclear Dorsal gradient (E_2) and its target genes become narrower (compare *sog* D_2 to B_2, *cactus* E_3 to B_1) (F) Ventral part of a transversal cross section showing Dorsal antibody staining (red) and *sog* expression (green) at a stage comparable to the one in B1,B2.

An alternative to the model of self-activation coupled to a long range inhibitor are chemical wave mechanisms in which the pattern information is transient and constantly changes its shape (Meinhardt 2004). Some of these wave mechanistic models could be used to explain the fact that the patterning information is not maintained over time. A speculative scenario can be imagined to explain the dynamics and vanishment of the *Tribolium* gradient described here. The appearance of an external factor besides the activator-inhibitor pair during development might lead to the disruption of the gradient. For instance, the generation of cell membranes could restrict the free diffusion of the inhibitor Tc-Cactus during early embryogenesis, leading the Dorsal gradient to break down. Another possibility would be the involvement of an auto-catalytic molecule, like *Tc-twist*, which would disrupt the balance between the positive feedback (Tc-Toll/Tc-Dorsal) and Tc-Cactus (negative feedback). Taken together, the Dorsal gradient may not be solely explained by models that assume a steady-state approach, but rather on models which use transient or non-steady state models.

Recently, the formation of the seven pair-rule stripes of *even-skipped* (*eve*) in fly embryos was proposed to follow a different mechanism than the self-activation coupled to long-range inhibition. This process of stripe formation was explained solely on the basis of simple repressor gradients (Clyde et al. 2003). In this well-studied system, it is unlikely that a process of self-activation/long-range inhibition takes place, since differences in the affinity of binding sites for local gap repressors in *eve* enhancers are able to explain the actual stripe patterning (Clyde et al. 2003, Jaeger et al. 2004).

In addition, in the past few years non-steady state models were developed for several morphogens. Modelling of Bicoid and Dpp gradients using non-steady state approaches, for instance, were able to reproduce their actual gradients in WT embryos (Mizutani et al. 2005; Bergmann et al. 2007). Interestingly, these non-steady state models were able to explain why overexpression of Bicoid leads to a lower shift of expression of its downstream genes than expected (Bergmann et al. 2007); and effects of some combinatorial mutations in the Dpp gradient, which were unclear by previous steady-state modelling (Mizutani et al. 2005). The question if morphogens act in steady or non-steady state conditions is being actively discussed (Lander 2007; Bergmann et al. 2008).

Genome content coupled to functional studies highlights macroevolutionary trends

Evo-devo is divided in two major branchs: macro-evolutionary studies and micro-evolutionary studies. Microevolutionary studies deal particularly with the problem of variation within species and populations. In the past few years, several traits were studied in order to investigate which regions of the genomes are involved in phenotypic differences (Sucena et al. 2003; Marcellini and Simpson 2006; Jeong et al. 2008). On the other hand, macroevolutionary studies deal with evolutionary patterns developed over millions and hundreds of millions of years, such as the differences between beetles and flies investigated in the current thesis (Carroll 1997). Although macro-evolutionary studies cannot detect the actual causes of evolution *e.g* the nucleotide sequences that have changed from the common ancestor to the extant organisms, I argue here that performing functional studies (RNAi, trangenesis) in species where the genome sequence is available can help to detect macroevolutionary trends.

The present thesis can be considered as a clear example of this approach. Since the *Tribolium castaneum* genome was available, I was able to analyse the function of almost all genes involved in the Dorsal/Toll and TGF-β pathways, the two most important pathways involved in fly DV axis formation. Importantly, the knowledge of the genome strengths this approach since: (1) The genome sequence speeds up the gene identification and helps the interpretation of the results, since possible paralogs, which can mask results due to redundancy, are evident. (2) Genes present in basal groups, which were lost in derived ones can be found in the genome and functionally investigated (Chapter 2). (3) Functional analysis of several members of a single pathway facilitates phenotypic analysis via RNAi (Chapter 2 and 4). The functional studies using members of the Toll and TGF-β pathways uncovered that several functions performed by the Dorsal/Toll pathway in *Drosophila melanogaster* are performed in *Tribolium* by the BMP/Dpp pathway (Chapters 2 to 4). A similar approach is being performed in the Hymenoptera *Nasonia vitripennis* (Jeremy Lynch, unpublished results), which suggests an even more pronounced role of the BMP/Dpp pathway when compared to Dorsal/Toll pathway in this wasp.

On the other hand, comparative studies via candidate gene approach using RNAi have two main disadvantages. First, the bias of this approach for conservation limits the finding new components acting in a given process or pathway, so one cannot find new genes based on this approach. In contrast, unbias approaches such as RNAi against genes with interesting

expression patterns (obtained by in situ screenings from EST collection) led, for instance, to the discovery of one poly-cistronic mRNAs involved in *Tribolium* segmentation (Savard et al. 2006a). Second, limitations on the identification of functional orthologs using this approach exist. For instance, proteases involved in the processing of Spätzle, the ventralizing signal present in the perivitelline space of *Drosophila melanogaster*, are a huge family of proteins in multicellular genomes. Around 190 proteins containing serine-protease like domains are present in the *Drosophila melanogaster* genome (Shah et al. 2008), which make challenging the identification of the serine-proteases acting in early DV polarity in *Tribolium*, if any of them indeed acts.

Taken together, macroevolutionary studies based on the biased choice of candidate gene approach will be exhausted in the next few years and macro-evolutionary studies must follow unbiased approaches. A very powerful technique that can be applied to any non-genetic model with an available genome is the CHIP-on-chip (Sandmann et al. 2007). Using this approach it should be possible to dissect the Gene Regulatory Network responsible for DV axis formation in *Tribolium*, and compare it with the vast amount of information from *Drosophila.*

Changes in the DV GRN involves not only cis-regulatory evolution, but also gene duplications, gene losses and arising of new protein domains

It has been widely propagated that cis-regulatory evolution is the main force driving evolution of new traits (Carroll 2005b; Carroll 2005a). The question whether cis-regulatory changes, gene losses, duplications, or the emergence of new protein domains are responsible for the changes reported for the evolution of DV axis formation is of great interest. Although we don´t have a complete understading of these changes at the moment, I describe here examples of changes involving all the aforementioned processes.

Among all the possible mechanisms described above, cis-regulatory changes are the most difficult to investigate in large evolutionary distances such as the common ancestor of *Tribolium-Drosophila*, or even within the common ancestor of Diptera like *Anopheles-Drosophila.* This is explained by the general lack of conservation in insect non-coding regions. The task of finding cis-regulatory modules (CRMs or enhancers) is further complicated by compensatory changes in different binding sites. It has been shown that enhancers undergo compensatory changes, which might underlie stabilizing selection (Ludwig et al. 2000). The recent finding that often more than one CRM is involved in the regulation of

a given gene at a specific time (*e.g.* Sandmann et al. 2007; Zeitlinger et al. 2007.) further complicates the discovery of CRMs.

Recently Papatsenko and Levine 2007 proposed that two classes of enhancers might exist in metazoan genomes. In one enhancer class the binding site arrangement would not be important since the concentration of a given transcriptional factor is not limited. Examples of this type of enhancer would be the DV type I enhancers like *Dm-twist* or *Dm-snail*, present in ventral regions of *Drosophila* embryos where Dm-Dorsal and Dm-Twist amounts are maximal. Other AP enhancers like *Dm-even-skipped* stripe 2 enhancer would also belong to this category, since quantitative analysis of Bicoid and Hunchback expression patterns, which directly bind to these enhancers, revealed that these activators are not severely limiting. The posterior border of the stripe 2 is located in a region of the *Drosophila* embryo that contains 11% and 50% of the peak levels of Bicoid and Hunchback gradients, respectively. In contrast, a second class of enhancers is present in genes where a limited amount of activators and repressors are present. The type II genes such as *vein, vnd, brk,* and *rho* contain enhancers belonging to this second class. The arrangement and the distance among Dorsal and Twist binding sites in these enhancers are conserved among 12 Drosophilid species (Papatsenko and Levine 2007). The authors propose that the limited amount of Dorsal and Twist in the neurogenic ectodermal region (2% and 1% in the dorsal border, respectively) has constrained the binding site arrangement in these enhancers during Drosophillid evolution. On the other hand, this constraint probably does not extend outside Diptera, since in *Tribolium vnd* is expressed only after the Dorsal gradient has already vanished (Wheeler et al. 2005 and unpublished observations). The last class of *Drosophila* DV enhancers, the type III, do not have a constrained arrangement of binding sites. This is explained by the presence of four binding sites for the single important transcription factor Dorsal, so synergist action among two factors seems not to exist. The distance among these binding sites is variable during Drosophillid evolution.

Although these caveats for the identification of orthologous CRMs in other insects exist, two recent studies identified sequences in *Anopheles gambiae* genome (*vnd* and *sog* loci) which are able to drive an expression pattern in *Drosophila* similar to the their expression pattern in mosquito (Zinzen et al. 2006; Goltsev et al. 2007). In both studies *Anopheles gambiae* enhancers were found based on searches in the specific gene locus for binding sites using *Drosophila melanogaster* Position Weight Matrices (PWMs). Particularly interesting is the expression pattern of *Ag-sog,* which is similar to the expression of the *Tribolium castaneum sog* ortholog, in the ventralmost region of the egg. In *Drosophila*

melanogaster, *sog* is repressed from the mesoderm (ventralmost region) by *Dm-snail*, being expressed as two broad lateral domains overlapping with the prospective neuroectoderm (Francois et al. 1994).

It was suggested that the *Ag-sog* enhancer would contain low affinity Dorsal binding sites, which would be responsible for the ventral expression in this mosquito (Goltsev et al. 2007). On the other hand, this cannot be the solely explanation of why this gene is expressed in this ventral domain, since *Ag-snail* is expressed in a ventral domain as in *Drosophila melanogaster*. So, *Ag-snail* seems not to repress *Ag-sog*, although both proteins are expressed at the same time in the same cells. Two possibilities may explain why *snail* does not repress sog in *Anopheles gambiae.* First, it is possible that *Ag-snail* protein itself cannot act as a repressor of *Ag-sog*. In agreement with this hypothesis, duplications on *snail* related genes occurred during higher Diptera evolution, since three snail-related genes: *escargot (esc), worniu (wor)* and *snail (sna)* are present in the *Drosophila melanogaster* genome, but only one *sna* related-gene is present in *Anopheles gambiae* or *Tribolium castaneum*. It has been proposed that *Dm-esc* and *Dm-wor* are less efficient than *Dm-sna* in target gene repression, in agreement with the aforementioned hypothesis (Hemavathy et al. 2004). Second, it is possible that the *sog* enhancer does not contain Sna binding sites in *Anopheles gambiae*, while these binding sites are present in its *Drosophila melanogaster* enhancer.

Interestingly, also in *Tribolium castaneum Tc-sna* does not repress *Tc-sog* (von Levetzow personal communication). Indeed *Tc-snail* is only detected at late blastoderm stages when the Dorsal gradient has largely vanished (Basal 2003 and data not shown). Since the nuclear Dorsal gradient was not investigated in *Anopheles gambiae*, it is not clear if *Ag-sog* responds to high amounts of Ag-Dorsal, as *Ag-twi* and *Ag-sna*; which are expressed only in the ventralmost region. In *Tribolium castaneum*, *Tc-sog* responds to the lowest levels of nuclear Dorsal (Figure 5.2F) and its expression pattern starts in a broad ventral domain followed by dynamic changes correlated to the vanishment of the nuclear gradient (Figure 5.2). During gastrulation, *Tc-sog* expression domain overlaps with *Tc-twi*. So, the *Tribolium castaneum Tc-sog* enhancer might contain high affinity Dorsal binding sites on its enhancer, as the *Drosophila melanogaster* ortholog.

From an evolutionary point of view, the idea that *sog* responds to low amounts of Dorsal in *Tribolium castaneum* (since *Tc-sog* is present in every nuclei which contain Dorsal, see Figure 5.2) and *Drosophila,* and to high amounts in *Anopheles gambiae* might indicate a specific mosquito feature. Only the investigation of the nuclear Dorsal gradient in *Anopheles gambiae,* its dynamics and relation to its target genes can confirm if *Anopheles gambiae sog*

actually responds to high amounts of Dorsal. Interestingly, several genes involved in *Anopheles gambiae* DV patterning have expression domains which are more similar to expression domains found in *Tribolium castaneum* than in *Drosophila melanogaster*. For instance, the gene *Ag-tld* is expressed, like in *Tribolium castaneum,* in the whole ectodermal region (showed in Chapter 3), while the *Drosophila melanogaster* ortholog is detected only in the non-neurogenic (dorsal) ectoderm. In addition, Dpp activity in *Anopheles gambiae* is broader than the sharp dorsal stripe observed in *Drosophila*. *Tribolium castaneum* Dpp activity (pMad) resembles the *Anopheles gambiae* situation, a broad region overlapping with the extraembryonic and non-neurogenic ectodermal anlagen (Chapter 3 and 4). If the *Anopheles gambiae* situation is more related to the *Tribolium castaneum* available data than with *Drosophila melanogaster*, I speculate that Dorsal do not act as a repressor in *Anopheles gambiae* as it does in *Drosophila melanogaster.* So, it is possible that many changes in the GRN responsible for DV axis formation in insects have occurred in the lineage giving rise to higher Diptera. These changes are correlated with the reduction in the extra-embryonic membranes in these latter insects (discussed in detail in Chapter 3). Other changes like the major role of Dpp pathway versus Toll pathway were discussed in Chapter 4.

A major difference between *Drosophila melanogaster* and *Tribolium castaneum* is the time required for embryogenesis, which is three to four times faster in the fly than in the beetle. Since in flies the first nuclear cycles (0-13) can be extremely fast (8 minutes), there is not enough time for the transcription of genes containing large introns. Recently, De Renzis et al. 2007 have shown that among 59 genes activated during the maternal-zygotic transition (MZT), 41 are annotated as intronless transcripts. This is a significant enrichment given that only 20% of *Drosophila melanogaster* genes are intronless. Several proteins involved in *Tribolium castaneum* early patterning contain transcripts with several introns, while their *Drosophila melanogaster* orthologs are intronless (e.g *sna*, *cv/tsg* like genes and *screw* a Dpp-like ligand). This difference in transcript length could be the result of positive selection for shorter transcripts in the MZT of fly embryos, when compared to the slower *Tribolium castaneum* embryogenesis. At least some of these genes have duplicated copies which were maintained during *Drosophila* evolution, probably due to sub-functionalization of the paralogs.

Gene loss might also have contributed for the evolution of the GRN involved in DV axis formation. In Chapter 2, using as an example the TGF-β superfamily, I describe several genes of the pathway that are present in the Ur-Holometabolous (the putative ancestor of all

holometabolous insects), which were lost in *Drosophila melanogaster* (e.g *bambi*, *gremlin*, *bmp9/10*, *admp*).

Altogether, a general trend for a direct role of Dorsal in cell patterning seems to have occurred during insect evolution. Many genes that are apparently not directly regulated by Dorsal in *Tribolium castaneum*, do so in *Drosophila melanogaster*. Gene duplication and loss of molecules involved in DV axis coupled to cis-regulatory changes have remarkably changed the GRN network involved in insect DV axis formation.

DV pattern of the growth zone: forward induction mechanism and its evolutionary implications

I have demonstrated that growth zone DV patterning depends on cues established during early embryogenesis (Chapter 4). I showed that a forward induction mechanism, where the anterior mesodermal region provides a signal to the unpatterned posterior region is involved in *Tribolium castaneum* DV axis (Figure 5.3). GZ DV polarity is not established in embryos lacking early signals. On the other hand, embryos that lack DV polarity still form the WT number of segments retaining normal polarity. The fact that the establishment of the early DV polarity is connected to the GZ DV polarity suggests that transitions between long and short-germ type of embryogenesis might have happened repeatedly during insect evolution. Different environments might be involved as selective forces shaping these transitions. Clear examples of these variations are the variable types of embryogenesis in beetles (short, intermediate and long) and the independent emergence (convergent evolution) of long-germ development in Hymenoptera and Diptera.

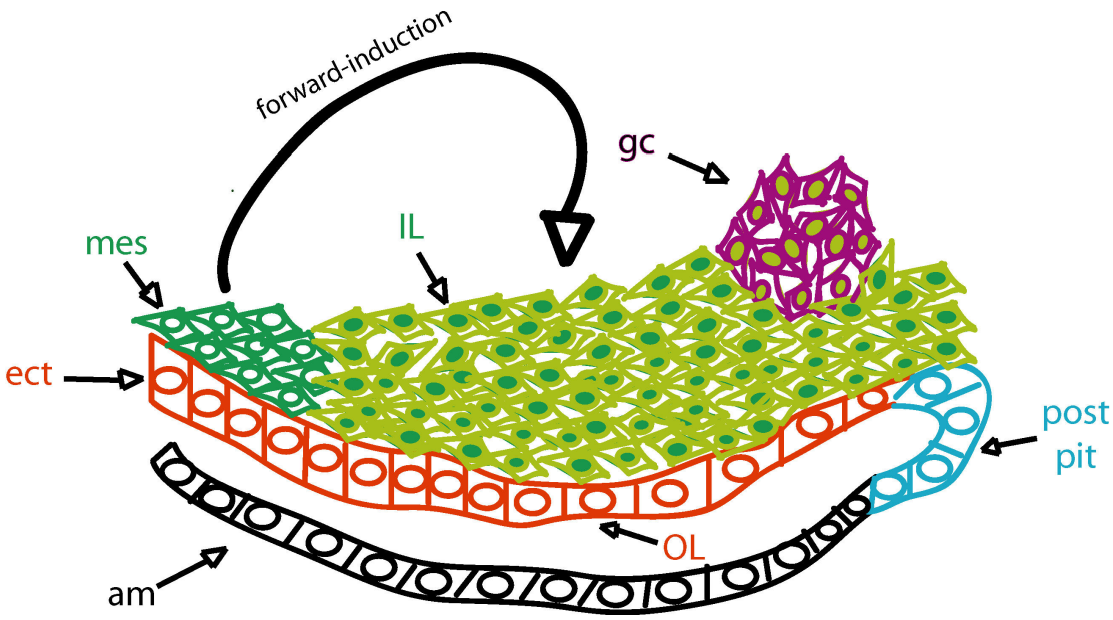

Figure 5.3: GZ forward induction mechanism is dependent on the early polarity provided by maternal components. IL - inner layer, OL - outer layer, gc - germ cells, mes – mesoderm, ect - ectoderm, am – amnion. Mesodermal cells localized anteriorly signals to the undifferentiated IL cells in a forward induction mechanism.

Pleiotropy, immune function and DV patterning: a single event in insect evolution?

It has been suggested that some genes in GRNs are more prone to changes than others. Examples of these genes are *shaven-baby/ovo* controlling trichome pattern and selector genes like *Ultrabithorax (Ubx)* in leg formation (Sucena et al. 2003; Hersh et al. 2007). These genes normally act in several processes, so they are pleiotropic. Pleiotropic genes are under purifying (negative) selection in their coding sequences, but changes in regulatory sequences might be tolerated. Changes in CRM of these genes are believed to be the major mechanism for evolutionary innovation (Carroll 2005b). On the other hand, situations where changes in coding sequences are correlated to major evolutionary transitions have also been reported, like *Ubx* aminoacid changes during Arthropod evolution (Ronshaugen et al. 2002).

On the other hand, the previously proposed co-option event of the Toll pathway (Roth, 2003; Roth,2004) from an ancestral role in immune response towards the DV axis patterning, involved the co-option of a whole pathway. This co-option event might have been facilitated by the self-regulative properties of the pathway like it was observed in *Tribolium castaneum*.

So, the connection of the transcriptional activation of I-κB and the nuclear transport of Dorsal would be a putative first event for the involvement of the Toll pathway in DV pattern. Another important step might have been the connection between the Toll pathway and the TGF-β pathway via Sog regulation by Dorsal/NF-κB. The acquisition of Dorsal/NF-κB binding sites by the *sog* enhancer was probably an important step in evolution. It is possible that in all insects that use the Toll pathway for DV patterning, *sog* expression will be wider in cell rows than mesodermal expressed genes like *twi.*

Recent work from spider suggests that DV patterning in basal arthropods can be solely explained by Dpp signalling pathway and its major antagonist Sog (Akiyama-Oda and Oda 2006; Oda and Akiyama-Oda 2008). Spider eggs do not display clear AP/DV axis during oogenesis (Foelix 1996). In contrast, in insects a clear AP axis is observed inside mother´s belly, and DV axis polarity is correlated, at least in *Drosophila melanogaster*, with the symmetry break up caused by nuclear movement at late stages of oogenesis (Roth 2003). This leads in *Drosophila melanogaster* to the ventralizing signal from the perivitelline space which will lead to the embryonic DV axis. It is possible that the symmetry break up events during oogenesis are largely absent from basal Arthropods like spiders, since DV assymmetry appears much later in embryogenesis of these organisms (Akiyama-Oda and Oda 2006; Yamazaki et al. 2005). So, the appeareance of patterning using extra-embryonic secreted signalling, and the usage of Toll pathway for DV patterning might have been important innovations for insect evolution. Only the study of basal insects and Arthropods, using functional genome-wide studies, will enable a comprehensive understanding of the macroevolutionary trends involved in the evolution of the DV axis patterning mechanisms.

Conclusions

1) The number and function of Dpp modulators acting in early embryonic pattern has changed during insect evolution.

2) Self-regulatory circuits are involved in *Tribolium* DV axis at the zygotic level.

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Additional Info:

Selected Posters:

1) Fonseca RN, van der Zee M, Roth S. *twisted gastrulation*, *tolloid* and *brinker* in the short-germ *Tribolium*. In: 2nd Tribolium International Meeting, 2005, Göttingen.

2) Fonseca RN, van der Zee M, von Levetzow, C, Roth S. European Drosophila Research Conference, 2007, Vienna/Austria.

Talks:

1) **Evolution of Dpp extracellular modulation in insects: twisted-gastrulation and tolloid in the short-germ insect Tribolium**. European society for Evolutionary Developmental biology (EED) Meeting, Prague, August 16-19, 2006.

2) **Modulation of BMP signalling in Tribolium** 3rd International *Tribolium* Meeting, Prague Meeting, 2006

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Die Bestimmungen der Promotionsordung sind mir bekannt. Die von mir vorgelegte Dissertation ist von Herrn Prof. Siegfried Roth betreut worden.

Teilpublikationen:

1. *Sog/Chordin is required for ventral-to-dorsal BMP/Dpp transport and head formation in a short-germ insect* Maurijn van der Zee, Oliver Stockhammer, Cornelia von Levetzow, **Rodrigo Nunes da Fonseca**, and Siegfried Roth, Proc Natl Acad Sci U S A. 2006 Oct 31;103(44):16307-12. 2006
2. *TGF-ß signalling in Tribolium: vertebrate-like components in a beetle.* Maurijn van der Zee, **Rodrigo Nunes da Fonseca** and Siegfried Roth, **Dev Genes Evol**, Apr;218(3-4):203-213, 2008
3. *Self-regulatory circuits in dorsoventral axis formation of the short-germ beetle Tribolium castaneum* **Rodrigo Nunes da Fonseca**, Patrick Kalscheuer, Abidin Basal, Cornelia von Levetzow, Maurijn van der Zee, Siegfried Roth. **Dev Cell** Apr;14(4):605-15, 2008
4. *The genome of the model beetle and pest Tribolium castaneum* Richards et al (including **Rodrigo Nunes da Fonseca**). **Nature** Mar 23; [Epub ahead of print] 2008.
5. *Evolution of Dpp extracellular modulation in insects: twisted-gastrulation and tolloid in the short-germ insect Tribolium.* **Rodrigo Nunes da Fonseca,** Maurijn van der Zee and Siegfried Roth, in preparation.

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