Evolution of the gene regulatory network controlling trunk segmentation in insects

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List of Abbreviations

A1-8	first to eighth abdominal segments
abd-A	*abdominal A*
Abd-B	*Abdominal B*
An (An')	*Anopheles gambiae*
Antp	*Antennapedia*
Dfd	*Deformed*
Dm (Dm')	*Drosophila melanogaster*
dsRNA	double stranded RNA
en	*engrailed*
EST	expressed sequence tag
Eve	*even-skipped*
ftz	*fushi-tarazu*
Gb (Gb')	*Gryllus bimaculatus*
gt	*giant*
hb	*hunchback*
Hox	Homeobox
kni	*knirps*
Kr	*Krüppel*
lb	labial segment
md	mandibular segment
mRNA	messenger RNA
mx	maxillary segment
Nv (Nv')	*Nasonia vitripennis*
O/N	over night
Of (Of')	*Oncopeltus fasciatus*
otd	*orthodenticle*
PCR	polymerase chain reaction
PS	Parasegment
RNAi	RNA-Interference
Scr	*Sex combs reduced*
T1	first thoracic segment
T2	second thoracic segment
T3	third thoracic segment
Tc (Tc')	*Tribolium castaneum*
Ubx	*Ultrabithorax*
wg	*wingless*
zen	*zerknüllt*

Summary

The study of pattern formation in insects is the main source of our current understanding of the genetic processes underlying the development of an organism. Ontogeny has been thoroughly studied in the model organism *Drosophila melanogaster*, where a set of transcription factors and signaling molecules pattern the fly embryo through a segmentation gene cascade. Over the past 20 years, this model has been compared to different organisms throughout the Metazoa. Here I describe the functional analysis of genes and gene regulatory network controlling segmentation in the short germ beetle *Tribolium castaneum*.

The *hunchback* gene is one of the major early determinants in the *Drosophila* segmentation cascade, where it serves an instructive role in patterning the entire body plan. In several insects, the role of *hb* in patterning body compartments (cardinal regions) is conserved. However, in hemimetabolous insects developing as short germs *hb* role has been reported to differ from the canonical gap function described in holometabolous insects. In the first chapter I describe the role of *hb* in *Tribolium*, a holometabolous insect developing as short germ. This analysis revealed that *Tc'hb* has an indirect effect in segmentation, mediated by other gap genes like *giant*, and a most likely a direct effect in the segment identity specification, by setting the anterior border of thoracic and abdominal Hox genes. This finding suggests an ancestral role of *hb* as a cardinal gene within insects and allows the reinterpretation of the canonical gap phenotype described in the fly.

The expression analysis of ESTs in *Tribolium* identified a putative non-coding RNA showing a gap-like expression pattern during segmentation. In the second chapter I describe the functional analysis of this gene, named *mille-pattes*. This analysis defined *Tc'mlpt* as a novel segmentation gene in *Tribolium*, which controlls trunk segmentation in a cross-regulatory network among gap genes and regulates the expression domains of Hox genes. Strikingly, *mlpt* does not code for a transcription factor, but instead, encodes several small peptides, which are conserved among *mlpt* homologues in various insects.

As a model, the gene regulatory network controlling segmentation in *Drosophila* has been thoroughly tested in many other organisms, revealing a surprising plasticity of the developmental mechanism controlling segmentation among insects. In order to identify the regulatory interactions among the gap genes that are controlling segmentation in *Tribolium*, I further characterized the gap gene cross-regulatory network in *Tribolium* and their interaction with pair rule genes. This analysis provided a powerful data set on the regulatory interactions among gap genes and their interactions with pair rule genes in *Tribolium*.

Finally, the concomitant characterization of segmentation genes presented in this thesis allowed the reinterpretation of the role of *hunchback* among insects, specially the canonical gap phenotype described for *Tribolium* and *Drosophila*. Furthermore, by studying the interactions between gap and Hox genes in *Tribolium* it was possible to propose a model for the regulation and function of *Tc'Antp* and for the regulation of the Hox genes along the AP axis in *Tribolium*.

Zusammenfassung

Die Studien zur Musterbildung in Insekten ist die Hauptquelle unseres heutigen Verständnisses der zugrunde liegenden genetischen Prozesse während der Entwicklung eines Organismus. Die Ontogenie des Modellorganismus *Drosophila melanogaster*, bei der der gesamten Embryo über eine hierarchische Kaskade von Transkriptionsfaktoren und Signalmoleküle „gestaltet" wird, wurde extensiv untersucht. Über die letzten 20 Jahre hinweg wurde dieses Modell mit der Entwicklung von verschiedenen Metazoen Organismen verglichen. Hier beschreibe ich die funktionelle Analyse von Genen und regulatorischen Netzwerken welche die Segmentierung der Kurzkeim Embryos von Tribolium castaneum steuern.

Das Gen *hunchback* ist eines der frühen Hauptdeterminanten in der Segmentierungskaskade von *Drosophila*, wo es eine vornehmlich leitende Rolle während der Musterbildung des gesamten Anlageplans vollführt. Die Funktion von *hunchback* während der „Musterbildung" scheint zudem in verschiedenen Insekten konserviert zu sein. In kurzkeimenden hemimetabolen Insekten, wurde jedoch herausgefunden, dass sich die Rolle von *hunchback* von der kanonischen Funktion eines Gapgens, wie es in holometabolen Insekten beschrieben wurde, unterscheidet. Im ersten Kapitel beschreibe ich die Funktion des *hunchback* Gens im kurzkeimenden Holometabolen Insekt, *Tribolium castaneum*. Die Analyse hat gezeigt dass *hunchback* einen indirekten Effekt auf die Segmentierung zeigt, der möglicherweise über andere Gapgene wie *giant* vermittelt wird und wahrscheinlich einen direkten Einfluss auf die Spezifikation der Segmentidentität besitzt, in dem es die anteriore Grenze für thorakale und abdominale Hox Gene spezifiziert. Diese Ergebnisse legen eine anzestrale Funktion für *hunchback* als Kardinal Gen innerhalb der Insekten nahe und erlaubt eine Neuinterpretation des kanonischen Gapgen Phänotypen wie er in *Drosophila* beschrieben wurde.

Die Expressionsanalyse von ESTs in Tribolium führte zu der Identifikation einer vermeintlich nicht kodierenden RNA mit einer Gapgen typischen Expression während der Entwicklung. Im zweiten Kapitel dieser Arbeit beschreibe ich die funktionelle Analyse dieses Gens, *milles-pattes*. Die Ergebnisse dieser Analyse erlauben die Klassifikation von *Tc'mlpt* als neues Segmentierungsgen in Tribolium, das als Teil eines regulatorischen Netzwerks von Gapgenen an der Segmentierung der „Rumpfsegmente" beteiligt ist und zudem die Expressionsdomänen von Hox Genen spezifiziert. Auffallend ist hierbei das *Tc'mlpt* nicht für einen Transkriptionsfaktor kodiert, sondern stattdessen für mehrere kurze Peptide kodiert die in *mlpt* Homologen aus verschiedenen Insektenspezies konserviert sind.

Das Modell des regulatorischen Netzwerkes dass die Segmentierung von *Drosophila melanogaster* leitet wurde in verschiedenen Organismen ausgiebig getestet und enthüllte eine erstaunliche Plastizität der entwicklungsbiologischen Mechanismen welche die Segmentierung in Insekten kontrollieren. Um die regulatorischen Interaktionen der Gapgene, die die Segmentierung von *Tribolium* steuern, zu identifizieren, habe ich weiterhin das regulatorische Netzwerk der Gapgene in *Tribolium* analysiert, sowie deren Interaktion mit den Paarregelgenen. Diese Analyse

bietet einen umfangreichen Datensatz zu den regulatorischen Interaktionen zwischen Gapgenen sowie deren Wechselwirkung mit den Paarregelgenen in *Tribolium*.

Die begleitende Charakterisierung der Segmentierungsgene die in dieser Arbeit vorgestellt wurden, erlaubt eine Neuinterpretation der Funktion des *hunchback* Gens in Insekten, und im besonderen des kanonischen Gapgen Phänotyps wie er für *Tribolium* und *Drosophila* zuvor beschrieben wurde. Desweiteren erlaubte die Untersuchung der Wechselwirkungen zwischen Gap- und Hox-Genen in *Tribolium* ein Modell für die Regulation und Funktion des Hox Gens *Tc'Antp*, sowie der Regulation von Hox Genen entlang der AP-Achse im Allgemeinen, abzuleiten.

Introduction

The generation of complex body plans from very simple biological structures has been one of the greatest mysteries of life on earth. How can a single cell divide and differentiate into the approximately 100 trillion cells that form our complex body?

Advances in developmental genetics and molecular biology over the past 30 years have revealed that development depends on key regulatory genes, which are surprisingly conserved among most metazoans. The wide conservation of this 'genetic toolkit' has allowed molecular comparison between close and distant related organism. This molecular comparative embryology has developed into the modern concept of Evolutionary Developmental biology (Evo-Devo), and has been proven to be a powerful approach to identify how genes and modules are differently used through evolution to control development and to generate the past and present morphological diversity.

The study of pattern formation in insects is responsible for a great deal of our current understanding of the genetic processes underlying the development of an organism. The fruit fly *Drosophila melanogaster* represents the best-characterized developmental model, where a restricted amount of transcription regulators orchestrate embryonic patterning by controlling when and where RNA molecules and proteins will be produced or inhibited. Many of the genes originally characterized as patterning genes in *Drosophila* were later found to play essential roles during vertebrate development (e.g. *hairy*, *hedgehog*, the Hox genes, etc). The comparison of these genes throughout the Metazoa has provided a valuable source of evolutionary scenarios controlling the developmental mechanisms underlying the diversity of body plans.

Most of the genes involved in segmentation in *Drosophila* have already been characterized in other insects. Yet, the mode of embryogenesis observed in *Drosophila* represents a highly derived developmental feature.

Modes of Embryogenesis

Prior to the development of genetic screens and molecular markers, embryologists already described key developmental traits essential for studying Evo-Devo in insects. By the use of ablation and transplantation techniques, the embryology of insects was classified with respect to the portion of the egg at the blastoderm stage, committed to become the germ rudiment (Krause, 1939). As a more recent and generally accepted terminology, this classification can be divided into two types: the long germ mode, where the entire egg length is occupied by a large (long) germ rudiment and all body segments are specified at the syncytial blastoderm stage (syncytial segmentation); and the short germ mode, where a small (short) germ rudiment is formed at the posterior pole of the egg and only the more anterior segments are patterned at blastoderm stage, with the remaining segments being patterned after the onset of gastrulation (syncytial/cellularized segmentation) (Roth, 2004; Tautz and Friedrich M., 1994; Tautz and Sommer, 1995).

Variations of the short germ mode, the so-called intermediate germ mode, are common among insects, in which more or less segments, compared to the short germ mode, are patterned in the blastoderm. However, the most prominent distinction between embryology modes among insects is the use of a secondary phase of development that patterns the remaining segments in a cellularized environment. This secondary segmentation phase is dispensable for long germ insects and shared in both short and intermediate germ insects. Therefore, short germ mode is used hereafter to indicate 'syncytial/cellularized segmentation' (which includes the intermediate mode) while long germ to indicate 'syncytial segmentation'.

The phylogenetic distribution of the different germ modes suggests that the last common ancestor of insects was of the short germ type (Davis and Patel, 2002). While the short mode is found in all insect orders except dipterans, the long germ mode is restricted to more derived insects displaying holometabolism (presence of metamorphosis) as well as meroistic ovaries (presence of nurse cells). Another argument for the ancestral short germ condition comes from the fact that the pattern of early embryogenesis of crustaceans bears significant resemblance to the short germ mode of development in insects (Davis and Patel, 2002). Plylogenetic studies have

suggested that crustaceans are the sister group of insects (Blaxter, 2001; Friedrich and Tautz, 1995; Giribet et al., 2001; Hwang et al., 2001).

Although shared by most of the insects, little information is available on the molecular basis of short germ embryogenesis when compared to the detailed knowledge of the long germ development of the model insect *Drosophila melanogaster*.

The segmentation cascade in the long germ insect *Drosophila*

A systematic genetic screen performed in the late 70's (Nüsslein-Volhard and Wieschaus, 1980) and the compiling of molecular data from almost 30 years of research have revealed a surprisingly restricted set of genes controlling segmentation in *Drosophila* embryos. When these genes are mutated in the fly, severe segmentation defects are observed in embryonic region where the gene is expressed in the wild type embryo. The authors divided these genes into the classes of maternal mutants (maternal lethal effect), gap mutants (deletion of adjacent segments), pair rule mutants (deletion of double segmental periodicity) and the segment polarity mutants (deletion of segment compartments). This work proposed a segmentation gene cascade, which patterns the *Drosophila* embryo from head to tail before cellularization takes place in the early blastoderm (top-down segmentation) (Figure 1) (Tautz, 2004).

By the onset of cellularization, at the stage where the segment polarity genes start to be expressed, a series of segmental units is established along the anteroposterior (AP) axis (segmentation). The regional expression of the Hox genes at this stage controls the further differentiation of these compartments into segments of distinct identity. The formation of body segments in *Drosophila* can therefore be subdivided into two separate, but not independent, patterning processes: the metamerization of repeated units along the AP axis (segmentation genes) and the segment-specific morphogenesis specifying distinct identity within this array of metameres (Hox genes) (Figure 1) (Akam, 1987).
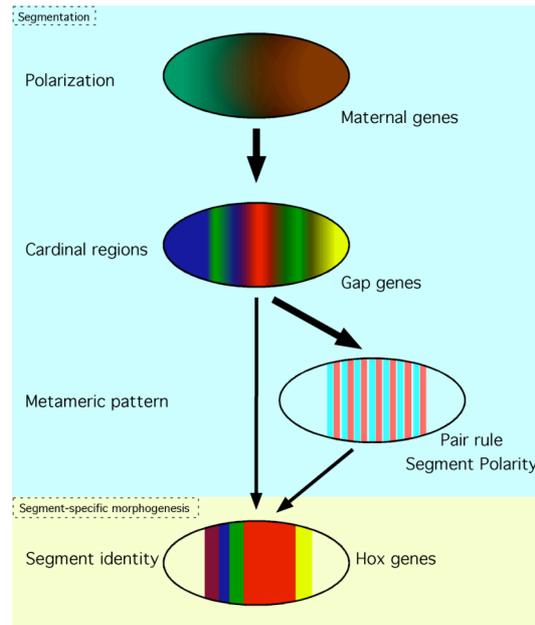
Figure 1. Schematic drawing of the *Drosophila* segmentation cascade illustrating the hierarchy of the processes (left) and the class of segmentation genes (right). Arrows indicate regulation (directional) between classes of segmentation genes. Strength of the arrows indicates strength of the regulation. For example, the major role of the gap genes in regulating the pair rule genes compared to a minor regulation of Hox genes is depicted.

Segmentation

In *Drosophila,* the segmentation cascade controlling the metamerization process is initiated with the diffusion of maternally provided transcripts located at the anterior and posterior pole of the egg. After fertilization, the translation of these transcripts generates protein gradients that provide the first positional information (morphogen) in the egg. For example, while the protein product of the *bicoid* (*Dm'bcd*) gene is translated at the anterior pole and diffuses towards posterior, the protein products of *nanos* (*Dm'nos*) are translated at the posterior pole and diffuse towards anterior (Driever and Nusslein-Volhard, 1988; St Johnston and Nüsslein-Volhard, 1992). Two additional morphogen gradients are formed by the translation of *hunchback* (*Dm'hb*) and *caudal (Dm'cad)* transcripts. In contrast to *Dm'bcd* and *Dm'nos*, the maternally provided *Dm'hb* and *Dm'cad* transcripts are uniformly distributed in the egg. Their gradients are therefore formed by differential translation along the AP axis. While Dm'Bcd represses the translation of *Dm'cad* at the anterior

pole (Dubnau and Struhl, 1996; Rivera-Pomar and Jackle, 1996), Dm'Nos prevents the translation of *Dm'hb* at the posterior pole (Hülskamp et al., 1989; Irish et al., 1989a; Tautz, 1988). Together, these morphogen gradients generate a patterning field controlling the expression of the genes belonging to the next level of the segmentation cascade, the gap genes.

As the first zygotic genes to be transcribed in the egg, the gap genes interpret the differential concentration of the maternal factors within this patterning field. When high levels of Dm'Bcd and Dm'Hb are present (anterior pole), the expression of the anterior gap genes such as orthodenticle (*Dm'otd*), giant (*Dm'gt*) and *Dm'hb* itself are activated (Gao and Finkelstein, 1998; Simpson-Brose et al., 1994). The expression of *Krüppel (Dm'Kr)* domain is activated when the levels of Dm'Bcd and Dm'Hb start to decline (central region) (Schulz and Tautz, 1994). At the posterior 1/3 of the egg, the posterior gap genes, such as *knirps (Dm'kni)* and *Dm'gt* are activated by high levels of Dm'Cad (Schulz and Tautz, 1995). The positional information from the maternal system also provides negative input in the regulation of the gap genes. Ubiquitous expression of Dm'Hb results in the repression of of *Dm'kni* and *Dm'gt* posterior domains (Hülskamp et al., 1990).

Therefore, following the positional information of the maternal genes along the AP axis, the regulation of the gap genes convert the positional information provided by the long range gradients of the maternal system into a series of overlapping short range gradients covering the entire embryonic axis. These gap gene domains are further refined and maintained by cross-interactions among the gap genes (Gaul and Jäckle, 1990; Jäckle et al., 1986; Struhl et al., 1992). For example, after the *Dm'Kr* expression is established in the central region of the egg, this domain is further maintained by the repression of Dm'Hb and Dm'Gt at the anterior border and the Dm'Kni and Tailless (Dm'Tll) at the posterior border (Harding and Levine, 1988; Jäckle et al., 1986). This differential distribution of gap genes along the AP axis regulates the next level of the segmentation cascade, the pair rule genes.

The expression of the pair rule genes are the first metameric pattern in the developing embryo and represent the transition from the aperiodic pattern of the gap genes to double-segmental periodicity of stripes. The transcription of the pair rule genes is very dynamic with some genes initially expressed in broad domains. The pair rule genes *hairy (Dm'h)*, *even-skipped (Dm'eve)* and *runt (Dm'run)* are described as

primary pair rule genes since they are regulated by maternal and gap genes. The seven stripes of the primary pair rule genes are independently patterned by characteristic combinations of gap genes. The pair rule genes *fushi tarazu* (*Dm'ftz*), *odd paired* (*Dm'opa*), *odd skipped* (*Dm'odd*), *paired* (*Dm'prd*), *sloppy-paired* (*Dm'slp*), and *Tenascin major* (*Dm'Ten-m*) are described as secondary pair rule genes, since they are regulated by the primary pair rules. Working as transcriptional repressors, the cross-regulatory interactions among the pair rule genes maintain and refine these series of seven or eight stripes with double-segmental periodicity into segmental stripes. This expression regulates the last level of the segmentation cascade, the expression of the segment polarity genes (Baker, 1988; DiNardo and O'Farrell, 1987; Ingham, 1988; Lawrence et al., 1987).

After cellularization, the expression of the segment polarity genes, as *engrailed* (*Dm'en*) and *wingless* (*Dm'wg*), determines the different compartments within each of the segments, specifying the locating and polarity of parasegment boundaries in the embryo. The parasegments is the basic developmental unit seen in the expression patterns of the pair rule and segment polarity gene as well as in the early morphological feature, parasegmental groves, observed prior germ band retraction. The parasegments are offset from the segment boundaries and disappear during germ band retractions, when the segmental grooves become obvious in the embryo (Ingolia, 2004).

By this stage, the readout of the segmentation cascade provides the patterning of 14 undifferentiated segments. The head segments are patterned by head gap genes but are independent from the pair rule gene expression. Without additional information, the embryo would consist of pre-gnathal segments followed by a set of 14 undifferentiated segments. However, the expression domains of the segmentation genes also control another set of genes responsible for specifying the identity of these segments, the homeotic genes.

Specification of segment identity

The assignment of distinct morphology to each of the undifferentiated segments is performed by a class of homeobox-containing transcription factors known as the homeotic genes (Lewis, 1978). These genes work as genetic switches along the

AP axis that turn on or off different programs of cellular differentiation in many metazoans (Carroll, 2002). They are commonly referred to as Hox genes. When a certain Hox gene is depleted in a certain segment, another Hox gene is expressed instead, leading to the specification of another body structure in this segment. A classical example is the work of Struhl (1982), that showed that when *Ultrabithorax* (*Dm'Ubx*) is ectopically expressed in the T3 in *Drosophila*, an extra pair of wings is formed in this dipteran.

An additional feature of the Hox gene is co-linearity. The arrangement of the Hox genes on the chromosome (Hox cluster) displays the same order in respect to their expression along the AP axis. Hox genes located at first positions (5') in the cluster are expressed at more anterior positions along the embryonic AP axis.

The primary expression of the Hox genes in *Drosophila* is governed by segmentation genes from different levels of the cascade hierarchy. The repressive activity of the gap genes sets the expression borders of the trunk Hox genes (e.g. *Dm'hb* regulating *Dm'Ubx*), while pair rule genes act as activators to specify peaks of expression of the Hox genes in specific segments (e.g. *Dm'ftz* regulation of *Dm'Scr*, *Dm'Antp* and *Dm'Ubx*) (Ingham and Martinez-Arias, 1986; Irish et al., 1989b). Within each segment, the segment polarity genes and unknown tissue specific factors further resolve the expression of the Hox genes (Rusch and Kaufman, 2000).

An additional source of regulation comes from the epistatic effect that Hox genes have on more anteriorly expressed ones, effect known as posterior prevalence rule in vertebrates (Duboule, 1991). The activity of a more posteriorly-acting Hox gene can not only prevent the transcription of more anteriorly-acting hox genes, but also block its activity in case both genes are co-expressed (González-Reyes et al., 1990; Mann and Hogness, 1990).

With the onset of gastrulation, the activity of the segmentation genes decays and the expression domains of the Hox genes are maintained by an additional mechanism involving the products of the Polycomb group genes (Mann and Hogness, 1990; Zhang et al., 2005).

In summary, the segmentation cascade in *Drosophila* represents a highly specific mechanism to control both the metamerization of the embryo into segments and to control segment-specific morphogenesis.

However, the long germ mode of embryogenesis found in *Drosophila*, where segments are patterned almost simultaneously, is thought to be a developmental innovation restricted to higher dipterans and hymenopterans (see below) and represents a highly derived mode of development. Nevertheless, functional analysis of the orthologs of the *Drosophila* segmentation genes in other insects with different germ band modes has contributed to our understanding of the genetic mechanisms involved in the segmentation of other animals as well as to reveal how this network has changed during the course of evolution.

The segmentation cascade in insects

Most of the genes responsible for segmenting the *Drosophila* embryo have been characterized in several other insects (reviewed in Damen, 2007; Davis and Patel, 2002; Tautz and Sommer, 1995). One of the first questions addressed was to which extent the segmentation cascade of the long germ *Drosophila* embryo would apply to the segmentation of short germ insects and non-insect arthropods (Patel et al., 1989a; Patel et al., 1989b). These works were the first demonstration of the highly conserved expression pattern of the segment polarity genes among arthropods. In general, the segmentation genes operating close to the phylotypic stage, an early stage where all arthropod embryos converge into a very similar morphology, as the segment polarity and Hox genes, tend to be highly conserved at the expression pattern and sequence levels among insects and arthropods (Anderson, 1973; Slack et al., 1993). On the other hand, this conservation is reduced for the genes located at higher positions in the segmentation hierarchy, such as gap and maternal genes.

While there are only few cases were the function of orthologs of the *Drosophila* maternal factors was analyzed in other insects, most of the genes belonging to the gap gene class have been described as essential segmentation genes among insects (Bucher and Klingler, 2004; Cerny et al., 2005; Copf et al., 2004; He et al., 2006; Liu and Kaufman, 2004a; Liu and Kaufman, 2004b; Lynch et al., 2006a; Lynch et al., 2006b; Mito et al., 2006; Mito et al., 2005; Pultz et al., 2005; Schroder, 2003; Shinmyo et al., 2005). In general, the role of the gap genes exhibits intriguing variations.

In the long germ parasitic wasp *Nasonia vitripennis*, embryos mutant for the gap genes *Nv'otd*, *Nv'hb* and *Nv'gt* display the canonical gap phenotype where adjacent segments are missing at the cuticular level (Brent et al., 2007; Lynch et al., 2006b; Pultz et al., 2005). While in *Drosophila* this phenotype is a result of the essential role of the gap genes in regulating pair rule genes (Carroll and Scott, 1986), the extent to which the gap genes regulate pair rule genes in *Nasonia* remains unknown. Intriguingly, reconstruction of the insect phylogeny based on molecular data, has recently placed the order Hymenoptera branching at the base of holometabolous insects phylogenetic tree (Savard et al., 2006c). A consequence of this finding is that the long germ development found in *Nasonia* would have evolved independently from the long germ mode of *Drosophila*. Nevertheless, the molecular fate map of segmentation genes described for both species is remarkably similar (Brent et al., 2007; Pultz et al., 2005).

In hemimetabolous insects displaying the short germ mode, the central role of the gap genes seems to be the regulation of the Hox genes. RNAi silencing experiments for *hb*, *Kr* and *gt* in several insects result in homeotic transformations of segments in the region where the silenced gene is expressed in the wild type (Liu and Kaufman, 2004a; Liu and Kaufman, 2004b; Mito et al., 2006; Mito et al., 2005). Their expression is nevertheless essential for the regulation of pair rule genes, since the knockdown embryos display defects in segmentation. Intriguingly, these effects lie outside of the expression domain of the depleted gene in the wild type, and the nature of this regulation is still largely unknown.

The pair-rule class of segmentation genes shows a wide variety of expression patterns among insects. In *Tribolium*, the orthologs of the pair rule genes *Tc'eve, Tc'run*, *Tc'odd, Tc'prd* and *Tc'slp* constitute a gene regulatory circuit crucial for the sequential formation of segments (Choe et al., 2006). Interestingly, although the orthologs of the pair rule genes *Tc'h* and *Tc'ftz* are also expressed in a pair rule fashion in *Tribolium*, their function seems to be unlinked with the segmentation process, contrasting with the canonical pair rule phenotype observed for these genes in *Drosophila*.

In hemimetabolous insects, the ortholog of the *Drosophila* secondary pair rule gene, *prd*, is expressed in a pair rule fashion in *Schistocerca* and *Oncopeltus*, while the primary pair rule gene *eve* is not expressed in a pair rule pattern neither in

Schistocerca nor in *Oncopeltus* (Davis et al., 2001; Liu and Kaufman, 2005; Patel et al., 1992). It is still not clear, however, to which extend the pair rule regulatory networks known from *Drosophila* and *Tribolium* would be functional in more basal insects.

Although no functional analysis have been done for the class of segment polarity genes outside *Drosophila*, their conserved expression patterns allow to assume similarity of the segmentation processes among animals (Patel, 1994; Patel et al., 1989a; Patel et al., 1989b). One example of a segmental polarity gene is *en*, which is expressed in the posterior compartment of every segment in every arthropod studied to date.

The segmentation cascade in the short germ insect *Tribolium*

The flour beetle *Tribolium castaneum* has emerged as a powerful model organism to study the molecular mechanisms underlying insect development (Klingler, 2004). Most of the genetic and molecular approaches developed for *Drosophila* can be directly applied to *Tribolium*, with the advantage of the powerful technique to easily knockdown gene function via systemic RNA interference (Bucher et al., 2002).

For developmental studies, *Tribolium* is thought to be a more representative model organism for insect development, since it displays the more basal short germ type of embryogenesis shared by most of the insects (see above) (Tautz, 2004). Additionally, the genome of *Tribolium* presents the lowest rates of evolution among the insects studied so far, eminent in a 3-fold reduction compared to *Drosophila* (Savard et al., 2006b). This steady rate of divergence allows a better identification of ancestral genes in the *Tribolium* genome, shared by distantly related species like our own. For example, the *Tribolium* genome possesses orthologs of several ancestral signaling molecules involved in the segmentation of vertebrates such as Wnt, Fgf, *gremlin*, *bambi*, BMP10, that were lost in the lineage leading to *Drosophila*.

All major segmentation genes described in *Drosophila* have orthologs in *Tribolium* and occupy similar positions in the segmentation gene hierarchy. Several *Drosophila* gap gene homologues, such as *hb*, *Kr*, *gt* and *tll*, have been already characterized in *Tribolium* (Bucher and Klingler, 2004; Cerny et al., 2005; Schroder

et al., 2000; Wolff et al., 1995). Their expression domains are roughly comparable to *Drosophila*. However, the borders of the expression domains in the trunk are shifted towards anterior (Bucher and Klingler, 2004). Thus, some abdominal segments in *Tribolium* are not covered by any of the known *Drosophila* gap gene orthologs analyzed in the beetle.

Functional analysis of *Tc'Kr* and *Tc'gt* in *Tribolium* has revealed that their inactivation leads to homeotic transformations with secondary effects on the metamerization process (Bucher and Klingler, 2004; Cerny et al., 2005). Analysis of Hox gene expression in *Tc'Kr* mutant embryos revealed the misregulation of Hox genes in these embryos (see General Discussion) (Cerny et al., 2005).

Although the role of *hb* in anterior patterning was already described in *Tribolium* (Schroder, 2003), its characterization in the gap gene network is still largely unknown. In the fly, *Dm'hb* acts as a canonical gap gene (see above) and regulates other gap genes such as *Dm'Kr* as well as pair rule and Hox genes. Recent discoveries in *Oncopeltus fasciatus* and *Gryllus bimaculatus* suggest, however, that the ancestral role of this gene differs from a canonical gap function described in *Drosophila* development; although the regulatory interactions of *hb* in these two short germ insects have not yet been fully characterized (Liu and Kaufman, 2004a; Mito et al., 2006).

In general, the analysis of the *Drosophila* segmentation genes in other insects has provided a valuable source of genetic information on the evolution of the segmentation process. The candidate gene approach allows the comparison of a genetic component in a distinct genetic and cellular context. This analysis provides information on the developmental machinery underlying segmentation, and also helps us to understand the evolutionary mechanism underlying the genetic variation among species that by different genetic means produces similar morphological outcomes.

The aim of this work is to shed light on the evolution of the gene regulatory network controlling trunk segmentation in insects. This work involves (1) the functional analysis of the anterior patterning gene *Tc'hb*; (2) the functional analysis of a novel segmentation gene showing a gap-like expression pattern and (3) the cross-regulatory interactions among gap genes and the regulation of target genes in the segmentation process in *Tribolium*.

Material and Methods

Rearing

Beetle stocks of the *Tribolium castaneum* strain San Bernardino were reared on white flour supplemented with brewer's yeast at 30°C (Berghammer et al., 1999). Flour stock were kept O/N at 65°C for parasitic disinfection. The pupae for injections were obtained by collecting eggs in a 9h interval and leaving them for about 25 days at 30°C to develop.

In vitro transcription of antisense RNA probes and double stranded RNA

The DNA clones of the target genes contained promoter sites (T7, T3 or Sp6) flanking the target gene sequences. The templates for the RNA probes were generated by PCR amplification of the clones using primers for the flanking promoter site (T3 and T7, or Sp6 and T7). The templates for double stranded RNA were generated by PCR amplification of the clones using the T7 primer with either a T7T3 fused primer or a T7Sp6. The template will therefore contain T7 promoter sites in both sides.

In vitro transcription of the antisense RNA probes were carried using T3, T7 or Sp6 polymerase (Roche) depending on the orientation of the target gene sequence in the vector. In vitro transcription of the double stranded RNA molecules was carried using the T7 MEGAscript RNAi Kit (Ambion) with precipitation with Lithium Chloride.

Parental RNAi

Parental RNA interference essays were performed as described in Bucher et al. (2002) with modifications. Approximately 200 female pupae were used in each experiment. The pupae were fixed (ventral up) by their posterior most abdominal segments onto microscope slides by using double sided tape (Scotch 665). Double-stranded RNA was injected into pupae at a concentration of 2 µg/µl. We found this concentration ideal to obtain maximum penetrance for most genes. After injection, the pupae were taken off of the slides and transferred to "culture vials" containing full grain flour in order to facilitate eclosion and reared under standard conditions (see

above). Wild type males pupae were add to the vials in a ration of 1 male to 3-5 females. The first egg collection was performed 5 days after injection and the eggs were kept at 30°C to monitor RNAi penetrance at the cuticular level. The strengh of the phenotype decays with the age of the female after injection, providing thus a phenotypic series. Knockdown embryos were collected every second day and one collection per week was kept at 30°C to monitor the cuticular phenotype. The collections were performed until the phenotypic effect had decreased significantly. Embryos for in situ hybridizations were taken from females showing a very high penetrance as judged by the parallel analysis of cuticle phenotypes.

Embryonic RNAi

Double-stranded RNA was injected into 2 to 4 hours after laying Tribolium eggs at a concentration of 2 µg/µl. The adult stock was cleaned from laid eggs by sieving procedure and let at 30ºC for egg lay. After one hour the eggs were collected and washed for one min in 10% and for 2 min in running water to soften the chorion. The eggs were then lined up onto the longer edge of microscopic slides with the anterior pole pointed towards the outside of the slide. Prior injection, the embryos were covered by a thin layer of Halo Carbon oil. The injection solution (10% phenol red, 2 µg/µl of dsRNA diluted in water) was centrifuged for at least 30 min at full speed and kept in ice during the injection procedure. The embryos were injected in their anterior pole with a standard microscope and a Eppendorf FemtoJet injection device set to manual in order to optimize the injection volume. The injected embryos were kept in a closed plastic box with moist paper towels to prevent desiccation. Hatched larvae were collected for after 5 days and processed for cuticle preparation.

Cuticle preparation

The eggs were sieved from the flour and washed for 1 min in 50% bleach solution and 2 min in running water to remove the chorion. The eggs were transferred to an embryo dish containing 1:1 Hoyer's medium and Lactic Acid solution and incubated overnight at 65°C (Berghammer et al., 1999).

The cuticles were mounted onto microscope slides with two cover slips each side of the samples and one cover slip on top of it. This way the samples maintained their three-dimensional shape. Pictures were taken with a confocal microscope.

Embryo fixation

The eggs were sieved from the flour and washed for 1 min in 50% bleach solution and 2 min in running water to remove the chorion. The fixation was performed in a scintillation vial with 3 ml PBS, 6 ml Heptane and 4% formaldehyde for 30 min. The eggs were then devitellinized by replacing the aqueous phase with 8 ml of Methanol and by shaking thoroughly for 30 sec. The eggs that lose the vitelline membrane become hydrophilic and move from the interphase to the hydrophilic phase (MeOH). After several washes with MeOH they were transferred to Eppendorf vials. The remaining eggs were passed through a 0.9 mm needle until all vitelline membranes were removed.

In situ hybridization

Gene expression profiles were obtained by whole mount in situ hybridization as previously described (Tautz and Pfeifle, 1989). For double staining essays, digoxygenin- or fluorescein-labeled probes were detected using alkaline phosphatase-coupled antibodies and INT/BCIP (red) or NBT/BCIP (blue) substrates.

Chapter I – A cardinal role of hunchback in Tribolium

Introduction

The *hb* gene was first characterized as a gap gene in the fly *Drosophila melanogaster* (Bender et al., 1988; Lehmann and Nusslein-Volhard, 1987; Tautz, 1988; Tautz, 1987; White and Lehmann, 1986). Loss of zygotic *Dm'hb* in *Drosophila* results in a "gap" phenotype that includes the loss of labial and thoracic segments in addition to the fusion of the abdominal segments 7 and 8 (Figure 2).

The activity of *Dm'hb* is firstly established during early oogenesis when the translation of *Dm'hb* maternal transcripts is prevented by Dm'Nos at the posterior pole of the egg (see above). Dm'Hb protein therefore forms an anterior to posterior gradient in the egg with higher levels in the anterior and lower to undetectable levels in the posterior (Dahanukar and Wharton, 1996; Payre et al., 1994; Wang and Lehmann, 1991). A second anterior Dm'Hb gradient is formed by the activation of its zygotic expression by the anterior morphogen *Dm'bcd* (Driever and Nusslein-Volhard, 1989; Driever et al., 1989; Struhl et al., 1989). The activities of both Dm'Hb gradients seem to have a partially redundant function (Hülskamp et al., 1990). Additionally, Hb is also expressed in the extraembryonic epithelial tissues and the developing nervous system in the zygote (Patel et al., 2001).

Notably, for at least one decade after the original study, the magnitude of the function of *Dm'hb* was mis-interpreted due to the difficulty of uncoupling the maternal (hb^{mat}) and zygotic (hb^{zyg}) effects (Simpson-Brose et al., 1994). The possibility of removing both, $Dm'hb^{mat}$ and $Dm'hb^{zyg}$ activities revealed that Dm'Hb is essential for the activation of all known target genes of the anterior morphogen *Dm'bcd*. Additionally, the expression of the anterior domain that generates the Dm'Hb gradient is also dependent on its maternal product. When $Dm'hb^{mat}$ is removed, the $Dm'hb^{zyg}$ expression is drastically reduced and shifted anteriorly towards regions of higher levels of Dm'Bcd activity (Simpson-Brose et al., 1994).

Accordingly, it has also been shown that the role of *Dm'bcd* is to provide the correct long-range polarity to the embryo by regulating $Dm'hb^{zyg}$ activity (Hülskamp et al., 1990; Simpson-Brose et al., 1994; Struhl et al., 1992). Without *Dm'hb* activity, *Dm'bcd* is only able to specify the most terminal head structures formed under high

concentrations of *Dm'bcd* in the wild type. Therefore *Dm'bcd* does not act autonomously in the anterior half of the embryo in the absence of *Dm'hb*. On the other hand, *Dm'hb* has an instructive role in patterning the entire *Drosophila* body plan.

Taken together, these findings demonstrate that *Dm'hb* is an important morphogen in the *Drosophila* segmentation cascade and that it is crucial for organizing the embryonic AP axis. Together with *Dm'bcd*, the primary role of *Dm'hb* is performed by (1) providing the primary positional information via its maternal activity and (2) by transferring the positional information of the maternal patterning systems to the subsequent levels of the segmentation cascade. Following the decay of Dm'Hb levels along the AP axis, the domains of expression of anterior, central and posterior gap genes are established.

The expression of *Dm'hb* is further refined into three stripes, two lying in thoracic segments and one in the seventh and eighth abdominal segments. These stripes of expression are derived from separate promoters and regulated by other gap genes (Tautz, 1987). These secondary stripes are essential for the metamerization of the second thoracic (Parasegment 4 – PS4) as well as the seventh and eighth abdominal segments (PS13) (Lehmann and Nusslein-Volhard, 1987; Tautz, 1987).

The primary effect of removing $Dm'hb^{zyg}$ activity is the incorrect interpretation of the underlying signals provided by the maternal systems, causing strong disarrangements of gap gene expression (Gaul and Jäckle, 1990). Given that $Dm'hb^{zyg}$ activates anterior and represses posterior downstream targets, the lack of $Dm'hb^{zyg}$ leads to loss of gap gene expression in the head and anterior thoracic regions while posterior thoracic and abdominal gap gene expression expands towards anterior (Hulskamp et al., 1994; Hülskamp et al., 1990; Schulz and Tautz, 1994). This misexpression of gap genes affects the expression of the pair rule genes, which in turn leads to metamerization defects in the gnathal, thoracic and first abdominal segment primordia (Hulskamp et al., 1994; White and Lehmann, 1986). These effects are intensified when *Dm'bcd* and the $Dm'hb^{mat}$ product are also mutated, since *Dm'bcd* acts synergistically with *Dm'hb* during anterior patterning (Hülskamp et al., 1990; Simpson-Brose et al., 1994). In such a mutant, only three segments displaying a mirror image and abdominal identity (A7/A8, A6, A7/A8) are formed.

In summary, the wild type function of *Dm'* is to sets the primary borders of gap gene expression that are further refined by their cross-regulatory interactions. The gap genes regulate the primary expression of the pair rule genes that is also refined via cross-regulatory interactions among pair rule genes. The refined patterns of pair rule genes define the 14 stripes of the segment polarity genes at the onset of the gastrulation.

In addition to the role of *Dm'hb* during metamerization, a secondary effect caused by the lack of *Dm'hb* activity is the misregulation of abdominal Hox genes (e.g. Qian et al., 1991; White and Lehmann, 1986; Zhang and Bienz, 1992). The anterior domain of *Dm'hb* limits the anterior expression borders of the *Dm'Ubx* and *Dm'Antp* domains (Irish et al., 1989b; Lehmann and Nusslein-Volhard, 1987; Qian et al., 1991; White and Lehmann, 1986; Zhang and Bienz, 1992). When *Dm'hb* activity is removed, the expansion of *Dm'Ubx* and *Dm'Antp* expression towards more anterior segments leads to homeotic transformations of head and thoracic segments towards abdominal ones (Irish et al., 1989b; Lehmann and Nusslein-Volhard, 1987; White and Lehmann, 1986). This phenotype is, however, only observed for three neomorphic alleles, which carry a mutation in either the C or the D box domains (Hulskamp et al., 1994). The C and D box are functional domains originally defined by their conservation between *Drosophila* and *Musca domestica* (Hulskamp et al., 1994). In amorphic *Dm'hb* alleles the homeotic transformations are concealed by the gap phenotype, where the segments that are transformed in neomorphic mutants are deleted (Lehmann and Nusslein-Volhard, 1987). Thus, the *Dm'hb* gene acts as an essential morphogen in early embryogenesis to control proper metamerization via the regulation of segmentation genes and to assure the differentiation of these metameres into proper segment identity.

This essential role of *hb* in development substantiates its evolutionary conservation. The identification of a putative vertebrate homolog of *hb*, named Ikaros, suggests that the origin of the *hb* gene predates the split of the deuterostome and protostome phyla (Georgopoulos et al., 1992; Sun et al., 1996). Although the ancestral function seems to be distinct between vertebrate and protostomes, several features of the *hb* expression domains, like the maternal supply of transcripts and the expression in extra-embryonic cells and in the CNS, are shared by two of the metazoan superphyla, ecdysozoans and lophotrochozoans (Pinnell et al., 2006).

The role of *hb* in segmentation most likely evolved within arthropods (Pinnell et al., 2006). Among insects, *hb* plays an essential role in the segmentation process of all species where functional analyses are available (He et al., 2006; Liu and Kaufman, 2004a; Mito et al., 2005; Pultz et al., 2005; Tautz, 1987). In all the cases, *hb* was found to acts as an anterior patterning gene. As in *Drosophila*, lack of *hb* activity in other insects leads to a phenotype where only the anterior head and fewer abdominal segments are formed (Figure 2).

In *Nasonia* lose of *Nv'hb* function leads to deletion of the segments expressing *Nv'hb* in the wild type (Pultz et al., 2005). Although more segments are deleted in *Nasonia*, this phenotype is equivalent to the canonical gap phenotype described in *Drosophila*. In *Tribolium*, a previous study suggested that the *Tc'hb* phenotype is also caused in the same way as in *Drosophila*, i.e. by the deletion of posterior head and thoracic segments (Schroder, 2003). This finding would infer a conserved role of *hb* as a canonical gap gene among holometabolous insects, despite the distinct mode of embryogenesis between the short germ band of *Tribolium* and the long germ band of *Drosophila* and *Nasonia* (see above).
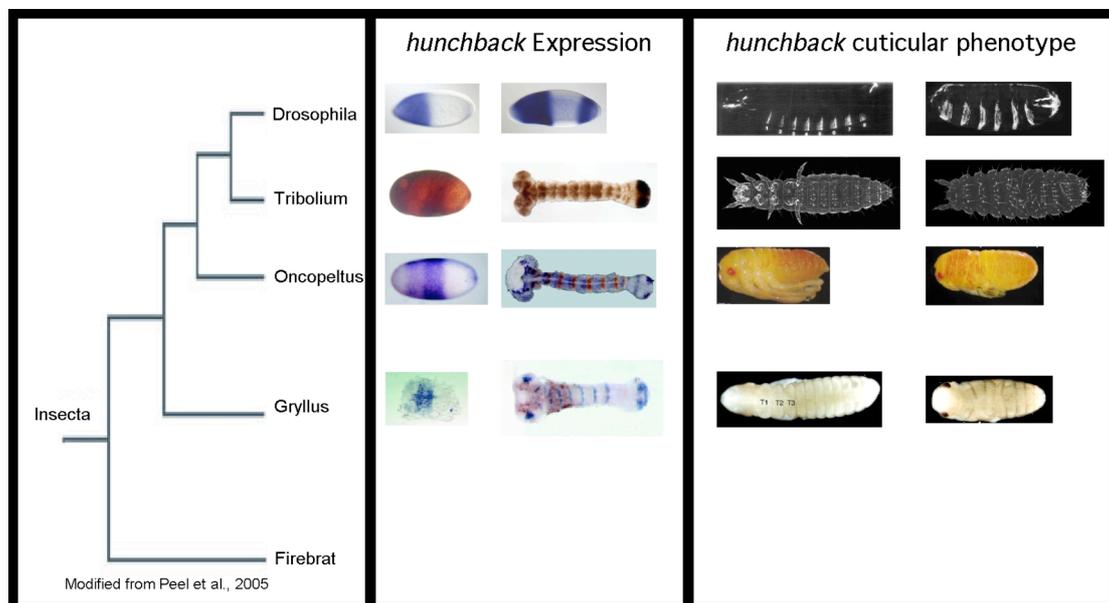
Figure 2. Phylogeny of insect orders discussed in the text showing distribution of the *hb* expression patterns and the cuticular phenotype after *hb* depletion in *Drosophila*, *Tribolium*, *Oncopeltus* and *Gryllus*. Phylogeny modified from Peel (2004). *Oncopeltus* pictures from (Liu and Kaufman, 2004a) and *Gryllus* pictures from (Mito et al., 2005).

Intriguingly, the loss of head and thoracic segments in the *hb* phenotype is caused by different means in hemimetabolous insects. In *Gryllus* and in *Oncopeltus*, the anterior expression of *hb* seems to be dispensable for the metamerization of the segments in which *hb* is expressed, with no segment deletions observed in head and anterior thoracic segments. On the other hand, the specification of segment identity seems to depend on the repressive function of *hb* on the expression of abdominal Hox genes. In these two short germ insects, however, it seems that the more evident role of *hb* is in the establishment of segment identity via the regulation of Hox genes. Nevertheless, *hb* also plays a role in the metamerization process of these insects, since the phenotype also displays segment deletions. Conversely to *Drosophila*, the segment deletions observed in *Gryllus* and *Oncopeltus* are of much less extent and lie in segments where *hb* is not expressed in the wild type.

Taken together, these findings would suggest (1) that the ancestral role of *hb* within insects was most likely the regulation of Hox genes with only a minor role in metamerization and (2) that most likely the canonical gap role of *hb* has evolved in the lineage leading to holometabolous insects.

Intriguingly, the holometabolous insect *Tribolium* shares the same short germ mode of embryogenesis as the hemimetabolous insects. Furthermore, the expression pattern of *Tc'hb* is also more comparable to other short germ insects then to *Drosophila*. Therefore, it would be hard to conceive that the role of *hb* was fundamentally changed between insects sharing the same embryogenesis mode (e.g. *Tribolium* and *Gryllus*) while conserved between species displaying distinct developmental mechanisms (*Tribolium* and *Drosophila*).

In this chapter, I describe the functional characterization of the segmentation gene *hb* in *Tribolium* through the use of morphological and molecular markers as well as the analysis of candidate target genes of *Tc'hb* known from *Drosophila* and propose a new interpretation of the *Tc'hb* phenotype in *Tribolium* and an evolutionary scenario for the role of *hb* in insects.

Results

Injection of double stranded RNA into *Tribolium* pupae (pRNAi) generates females lacking both maternal and zygotic *Tc'hb* function (Bucher et al., 2002). In order to characterize the role of *Tc'hb* in *Tribolium* segmentation, embryos depleted for *Tc'hb* (*Tc'hb*^pRNAi embryos) were generated.

Morphological characterization

The role of *hunchback* in segmentation

Loss of *Tc'hb* function does not affect the pre-gnathal segments labrum (lab), antenna (an) and mandible (md). All other segments bare no appendages and appear to have abdominal identity (Figure 3C). This phenotype was originally interpreted as a canonical gap phenotype in which the maxillary (mx), labial (lb) and thoracic (T#) segments are deleted (Schroder, 2003).

The intermediate *Tc'hb*^pRNAi phenotypes indeed show larvae with abdominal segments beyond the md, which could be interpreted as segments formed after the deletion of the gnathal and thoracic segments. However, the number of visible abdominal segments in these larvae is at least 10 (counting the fusion point as two segments), while the expected number of abdominal segments is eight (Figure 3C). In addition, the partial segment fusions in these embryos are observed around the 4th to 5th post-mandibular segment, hence outside of the *Tc'hb* expression domain (arrowhead in Figure 3C).

Weak *Tc'hb*^pRNAi phenotype larvae display the total number of segments as in the wild type, but all segments posterior to the md display abdominal identity (Figure 3B). In some larvae, an underdeveloped limb bud in the segment corresponding to the second (T2) and third (T3) thoracic segment in wild type larvae can be observed (arrow in Figure 3B). The detailed view in Figure 3 shows that the gnathal and thoracic segments are not deleted in *Tc'hb*^pRNAi larvae, but are instead transformed into abdominal identity (Figure 3D and 3E).
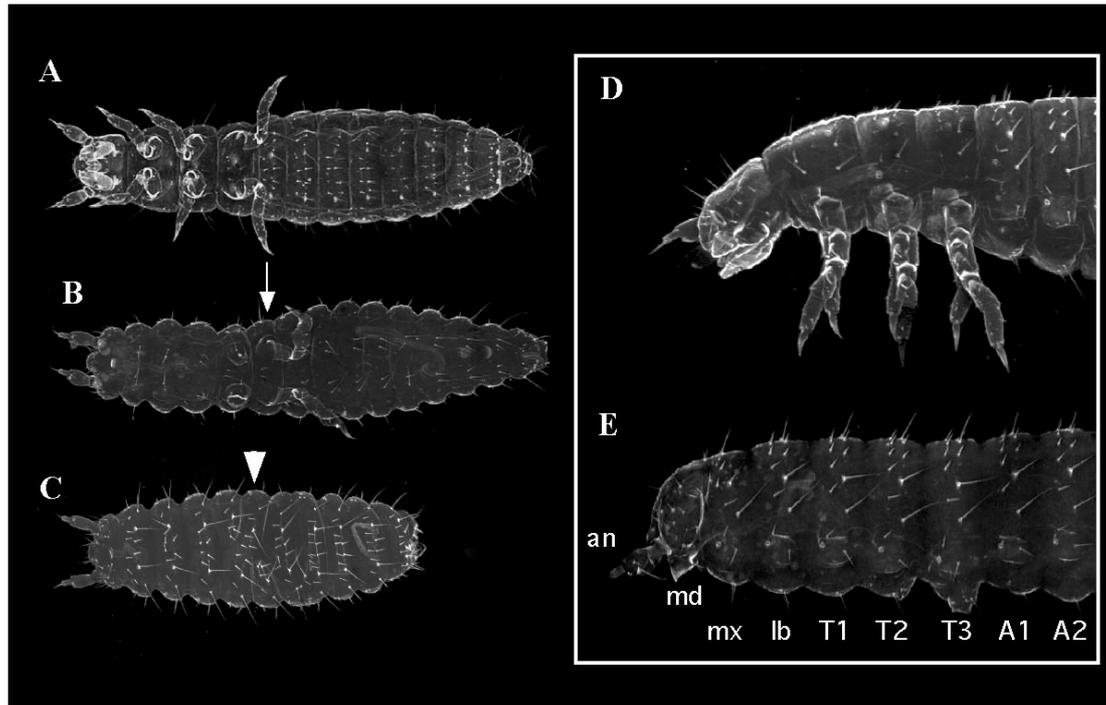
Figure 3. Cuticular preparation of (A, D) wild type larvae and depleted larvae showing (B, E) weak and (C) strong *Tc'hb*[pRNAi] phenotype. (B) All body segments are formed but gnathal and thoracic segments transformed to abdominal segments. (Arrow in B) Underdeveloped limbs in T3. (C) Larva displaying approximately 10 segments showing abdominal identity and (arrowhead) fusion of segments. (D-E) Detailed view of the homeotic transformations observed in *Tc'hb*[pRNAi] larva. (D) Wild type larvae with normal head and thoracic segments. (E) Weak *Tc'hb*[pRNAi] phenotype larva displaying the transformation of segments after the md segment. (E) Primorida of (an) antenna, (mx) maxilla, (lb) labial and the (T1) first, (T2) second and (T3) third thoracic as well as the (A1) first and (A2) second abdominal segments are depicted.

The strongest $Tc'hb^{pRNAi}$ phenotype (Figure 3C) was originally interpreted as a lack of anterior abdominal segments, in addition to the gnathal, thoracic deletions (Schroder, 2003). However, based on the homeotic transformation of the thoracic segments, as well as the fact that segment loss progresses from the point where the segmental fusions are seen in the weak phenotypes, the strongest $Tc'hb^{pRNAi}$ phenotype can be characterized as a progressive loss of posterior abdominal segments (further discussed in General Discussion).

These results indicate that the loss of *hb* function in *Tribolium* leads to (1) metamerization defects and (2) transformation of segment identities.

The role of *hunchback* in extraembryonic membranes

Maternal $Tc'hb$ ($Tc'hb^{mat}$) transcripts are loaded into the *Tribolium* oocyte , where they are ubiquitously distributed (Wolff et al., 1995) and translated after fertilization. As in *Drosophila* and in Grasshoppers, the $Tc'hb^{mat}$ seems to be translationally repressed from the posterior pole of the egg by a so far unknown mechanism. By the end of the blastoderm stage, $Tc'hb$ expression is restricted to an anterior domain where the cells are going to be specified to become the serosa. This domain is co-localized with the expression of *zerknüllt* (*Tc'zen1*), an essential factor for the specification of extraembryonic tissue (van der Zee, personal communication).

In order to investigate a possible early function of the $Tc'hb^{mat}$ and/or anterior cap expressions I analyzed $Tc'hb^{pRNAi}$ embryos using fluorescent DAPI (4',6-diamidino-2-phenylindole) staining. Here I demonstrate that $Tc'hb$ has an early role in the specification of the germ rudiment.

At the "differentiated blastoderm stage" (Roth, 2004), $Tc'hb^{pRNAi}$ embryos display no distinction between serosa and the germ rudiment nuclei (more detailed, size of nuclei etc.) (Figure 4B). This phenotype is nevertheless observed only in ca. 10% of the analyzed embryos.

With the onset of gastrulation, $Tc'hb$ seems to play an additional role during germband invagination. As in wild type, the primitive pit is formed at the posterior pole of $Tc'hb^{pRNAi}$ eggs, however the invagination appears to be abnormal in these embryos (Figure 4D). In addition, the position of the germband in relation to the egg seems to be affected. (Figure 4F, H). The embryo displays segmental groves characteristic of the abdomen of a fully elongated germband (arrows in Figure 4H),

but instead it occupies only the ventral-posterior portion of the egg (compare Figure 4H with G). This late effect is most probably caused by the arrest of segmentation in *Tc'hb*$^{\text{pRNAi}}$ embryos (see below).
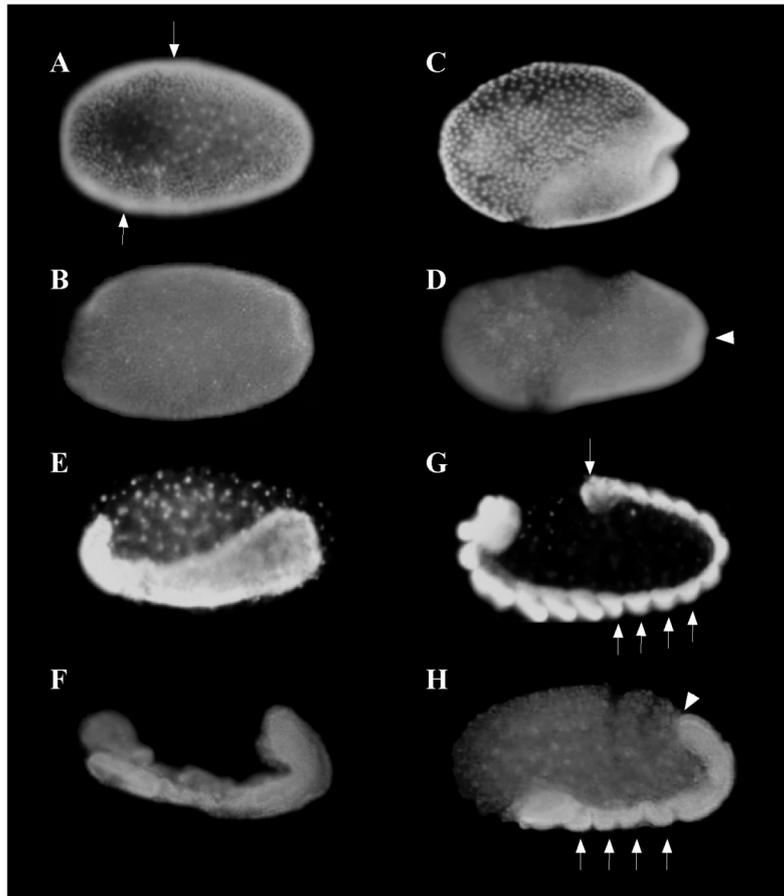
Figure 4. DAPI staining of *Tribolium* (A, B, E, F) wild type and (C, D, G, H) *Tc'hb*^pRNAi embryos. (A) Blastoderm stage of a wild type embryo showing the distinction between embryonic and serosal cells. (B) *Tc'hb*^pRNAi embryo at similar stage as A, showing no distinction between embryonic and serosal cells. (C) Wild type embryo at the stage of embryonic cells condensation and posterior pit formation. (D) *Tc'hb*^pRNAi embryo with distinction between embryonic and serosal cells to a lesser extent compared to C, but displaying failure of the germband invagination (arrowhead). (E) Wild type and (F) *Tc'hb*^pRNAi embryos during germ band elongation. (G) Fully extended germband of a wild type embryo. (H) *Tc'hb*^pRNAi embryo displaying segmental groves characteristic of the wild type abdominal segments (compare arrows between G and H). These embryos (H) represents the strong *Tc'hb*^pRNAi phenotype, where only four segments are formed afther the mandibule (see text). The wild type pictures are from Beermann (2006).

Maternally provided *hunchback* in *Tribolium*

In order to determine the role of *Tc'hb^mat* in *Tribolium* I performed an embryonic RNA interference (eRNAi) experiment. Since the eRNAi does not deplete the maternal transcripts, the observation of indistinguishable phenotype between eRNAi and pRNAi at the cuticular level would rule out a separate effect of the maternal product. On the other hand, any variation in the phenotype would suggest that *Tc'hb^mat* transcripts would contribute to the *Tc'hb*^RNAi phenotype.

Although no difference in the character of the *Tc'hb* phenotype was observed, a much higher occurrence of the weak phenotype showing more normally formed segments of abdominal identity was observed in the *Tc'hb*^eRNAi embryos. For example, the weakest class of *Tc'hb*^eRNAi phenotype displaying the total number of segments with underdeveloped limbs (Figure 3B) is only observed in eRNAi experiments. The weakest phenotype observed after pRNAi is depicted in figure 3C, displaying segment fusions and ten abdominal segments.

These observations have limitations because of the time that the RNAi takes to silence the target gene. A late RNAi effect would deplete the transcripts when its early function was already accomplished. However, all other genes analyzed with eRNAi showed identical or stronger phenotypes compared to the pRNAi experiment (bucher). Although this observation cannot be considered as conclusive the weaker effect observed when the maternal product is not depleted (eRNAi) could indicate that the maternal expression of *hb* plays a role in *Tribolium* as it does in *Drosophila*.

Molecular Characterization

In order to characterize the segmental function of *Tc'hb* at the molecular level *Tc'hb*^pRNAi embryos were analyzed for the expression of candidate target genes of *Tc'hb*. To assess the segmental register in these embryos, the *Tribolium* ortholog of the segment polarity gene gooseberry (*Tc'gsb*) was used as marker. Double staining with *Tc'en* shows that *Tc'gsb* is expressed at the posterior border of every segment, thus overlapping with *Tc'en* expression (Figure in supplementary data).

Figure 5. Expression of *Tc'gt* (blue) in (A, B) wild type and in (C, D) *Tc'hb*[pRNAi] embryos. The embryos are double stained for *Tc'gsb* (red). Although the anterior domain is not significantly affected, the posterior expression of *Tc'gt* in T3 and A2 (arrows in B) is absent in *Tc'hb*[pRNAi] embryos. (D) The segments where *Tc'gt* should have been expressed are fused in *Tc'hb*[pRNAi] embryos, visible by the fusion of the *Tc'gsb* stripes (arrowheads in D).
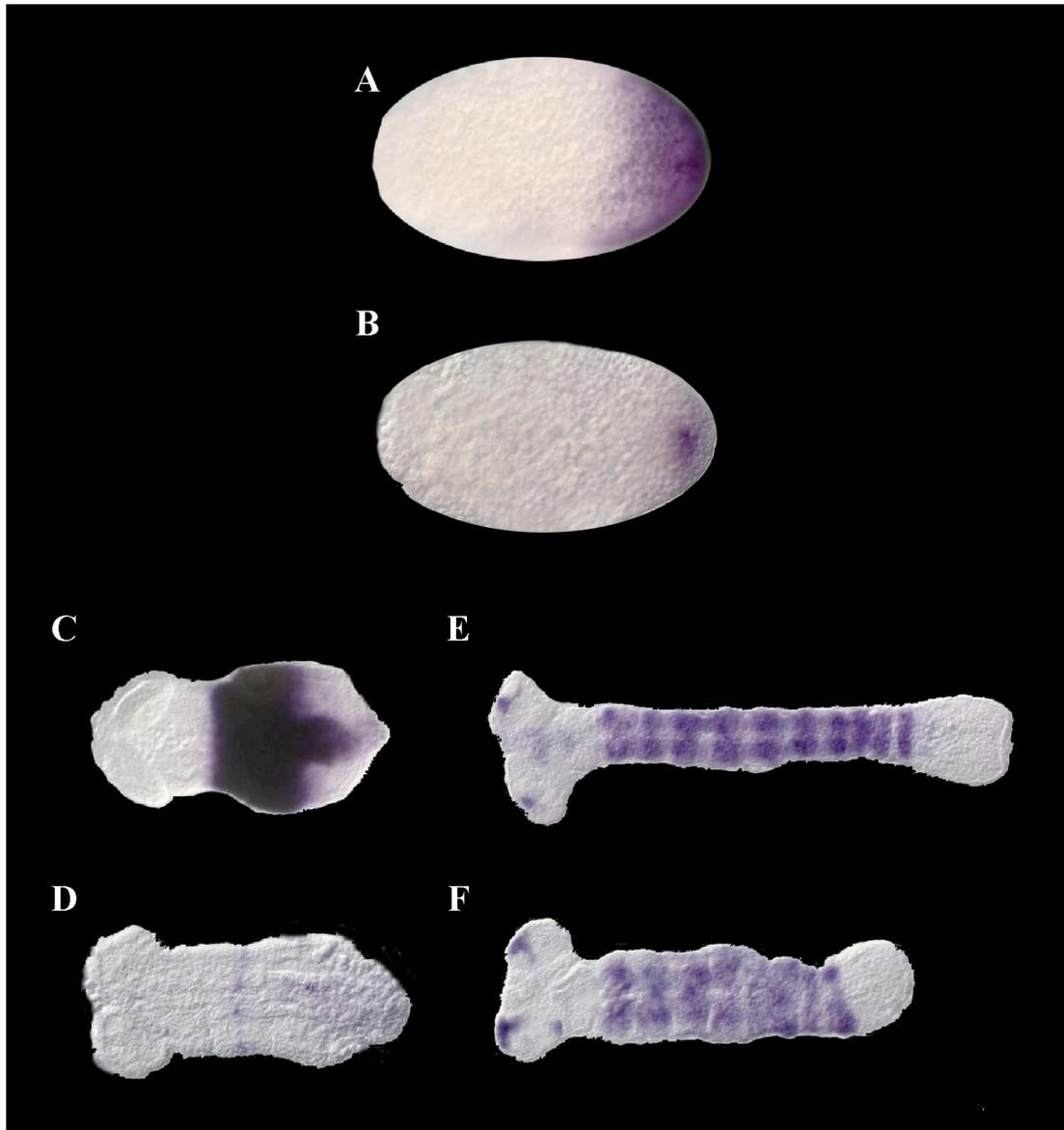
Figure 6. Expression of *Tc'Kr* in (A, C, E) wild type and in (B, D, F) *Tc'hb*[pRNAi] embryos. The central *Tc'Kr* expression is completely absent in *Tc'hb*[pRNAi] embryos (B, D). With further development the secondary expression of *Tc'Kr* (F) is seen in these embryos.

hunchback regulates the expression domains of gap genes

In *Drosophila*, the Dm'Hb gradient is required to regulate other gap genes, in particular *Dm'Kr* and *Dm'gt* (Hulskamp et al., 1994; Hülskamp et al., 1990; Struhl et al., 1992). In *Tribolium*, the location of the fused stripes in the *Tc'hb*[pRNAi] phenotype correspond to the segments where *Tc'Kr* and *Tc'gt* are expressed in wild type embryos. To understand the basis of the segment deletions observed in *Tc'hb*[pRNAi] embryos, these embryos were analyzed for the expression of *Tc'Kr* and *Tc'gt* as putative target genes.

Consistent with the interpretation of the cuticular phenotype, normal *Tc'gsb* stripes are formed in *Tc'hb*[pRNAi] embryos up to the 4th post-mandibular stripe, where a partial fusion with the following stripe occurs (Figure 5D). Adjacent to this, a segment showing normal width between *Tc'gsb* stripes is formed, followed by another pair of partially fused stripes (Figure 5D). In strong *Tc'hb*[pRNAi] phenotype, no further segmental stripes are visible posterior to this point.

Tc'gt is initially expressed in a broad domain during blastoderm stage, covering the future head and gnathal segments but excluding the lb (Bucher and Klingler 2004). The trunk expression appears during germband elongation (Figure 5A) and converges into two stripes in T3 and second abdominal (A2) segments respectively (Figure 5B - Bucher and Klingler 2004). In *Tc'hb*[pRNAi] embryos, the anterior *Tc'gt* domain is not visibly affected (Figure 5C, D), while the posterior *Tc'gt* stripes are not formed (Figure 5C, D). Later, it becomes apparent that the segments that should have expressed *Tc'gt* are fused, as evidenced by the *Tc'gsb* expression (Figure 5D). These experiments suggest that *Tc'hb* possibly acts as an activator of *Tc'gt*, in contrast to its role in *Drosophila*, where it acts as a repressor (Struhl et al., 1992).

Tc'Kr expression starts at the blastoderm stage with a broad domain at the posterior pole (Figure 6A) (Sommer and Tautz, 1993), which covers the three thoracic segments in the early germband (Cerny et al., 2005). In *hb*[pRNAi] embryos this domain is absent (Figure 6B, D), indicating that *Tc'hb* is required for its activation. There is a secondary segmental expression of *Tc'Kr*, which is generated during segment differentiation (compare Figure 6E and F) (Cerny et al., 2005). This expression is not affected in *Tc'hb*[pRNAi] embryos, although fewer segmental stripes are generated (compare Figure 6F), which is consistent with the loss of segments in such embryos.

Thus, Tc'Hb is required for the activation of the early *Tc'Kr* domain. The regulation of *Kr* by *hb* appears to be conserved between *Tribolium* and *Drosophila* (Hulskamp et al., 1994; Hülskamp et al., 1990; Struhl et al., 1992).

These results suggest that in *Tribolium*, the *Tc'hb* gene acts as a general activator of the *Tc'Kr* and *Tc'gt* trunk domains.

hunchback interacts with the pair rule circuit via *giant*

In *Drosophila*, the segmentation defects observed in gap gene mutants are a reflection of the misregulation of their targets: the gap genes themselves and the periodically expressed pair rule and segment polarity genes.

In *Dm'hb* mutant embryos, *Dm'Kr* expression is expanded anteriorly and the striped pattern of the pair rule genes is severely affected (Hulskamp et al., 1994; Hülskamp et al., 1990; Kraut and Levine, 1991a; Kraut and Levine, 1991b). This expansion leads to the formation of two enlarged metameres comprising the cells that would normally form around 4-6 metameres (White and Lehmann, 1986). These enlarged metameres undergo a resizing process, via cell death, acquiring normal width by the end of embryogenesis (See General Discussion).

In *Tribolium*, the orthologs of the pair rule genes have been shown to form a genetic circuit that plays an essential role in the metamerization process (Choe et al., 2006). Disruption of the orthologs of *Tc'run*, *Tc'eve* and *Tc'odd* leads to almost completely asegmental embryos (Choe et al., 2006).

To test whether the segmentation defects observed in the *Tc'hb*[pRNAi] phenotype could be due to misregulation of pair rule genes, the expression of *Tc'run* and *Tc'eve* was analyzed in *Tc'hb*[pRNAi] embryos.

The anterior stripes of *Tc'run* and *Tc'eve* in gnathal segments appear to be unaffected in *Tc'hb*[pRNAi] embryos (not shown). More posterior stripes, however, are not properly separated and both genes are expressed in a broad domain covering the growth zone (Figure 7B, G). In wild type, these stripes are within the region where segment deletions are observed in *Tc'hb*[pRNAi] embryos.

Since the effects observed in the expression pattern of the pair rule genes lie outside the *Tc'hb* expression domain, this effect could indicate a misregulation of *Tc'Kr* or *Tc'gt* in *Tc'hb*[pRNAi] embryos. Cerny and colleagues (2005) already showed

that *Tc'Kr* has no effect on *Tc'eve* stripes in the segments where *Tc'Kr* is expressed in the wild type; therefore the expression of the pair rule genes *Tc'run* and *Tc'eve* were analyzed in *Tc'gt*^pRNAi embryos. In accordance with the lack of *Tc'gt* in *Tc'hb*^pRNAi embryos, *Tc'gt*^pRNAi embryos display fusions of *Tc'run* (Figure 7C) and *Tc'eve* (Figure 7H) stripes in the trunk segments T3 and A2, where *Tc'gt* is expressed in wild type embryos (Figure 7E, J).

Depletion of *Tc'hb* leads to the loss of the *Tc'gt* trunk stripes, resulting in ectopic expression of the pair rule genes *Tc'eve* and *Tc'run* and subsequently to the metamerization defects observed in the trunk segments of *Tc'hb*^pRNAi and *Tc'gt*^pRNAi embryos (Figure 10).
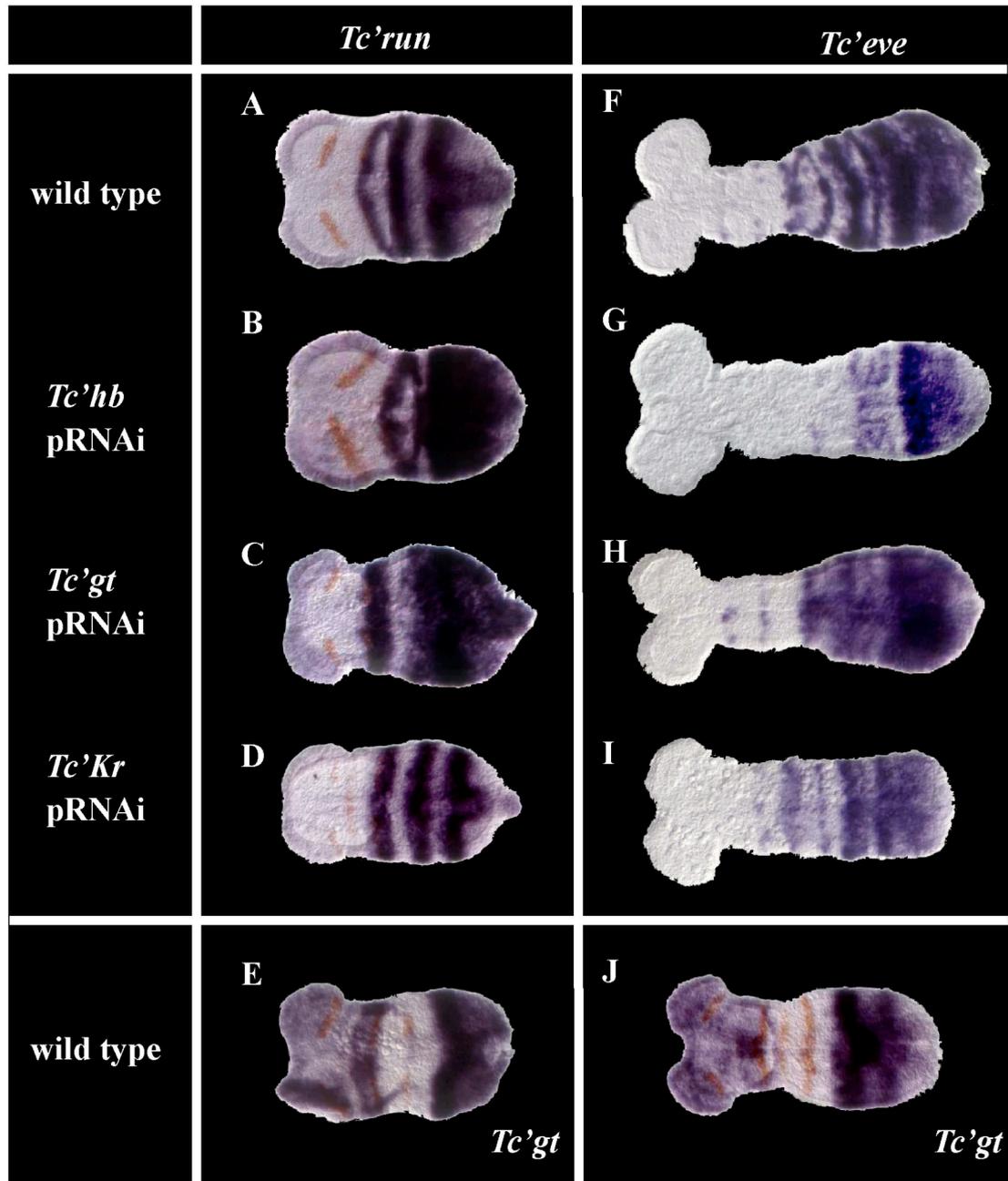
Figure 7. Expression of the pair rule genes (A-D) *Tc'run* and (F-I) *Tc'eve* in embryos depleted for the gap genes. (A, F,) Wild type embryos, (B, G) *Tc'hb*[pRNAi] embryos, (C, H) *Tc'gt*[pRNAi] embryos, (D, I) *Tc'Kr*[pRNAi] embryos are depicted. (E, J) Wild type *Tc'gt* expression for comparison with the gap gene depleted embryos. Effects observed on pair rule genes seems to lye in the segments were *Tc'gt* is expressed in the wild type.

hunchback regulates trunk Hox genes

In parallel to the role in metamerization via pair rule genes regulation, *Tc'hb* seems also to affect the expression of Hox genes in *Tribolium*.

Embryos depleted for *Tc'hb* develop into larvae displaying normal segment identity until the md. The subsequent segments however, appear to have abdominal identity.

To test whether these abdominal segments are the result of an expansion of abdominal Hox gene expression up to the md, I analyzed the expression of the gnathal hometic genes, *Tc'Dfd* and *Tc'Scr* as well as the trunk Hox genes *Tc'Antp*, *Tc'Ubx* and *Tc'AbdA* in *Tc'hb* knockdown embryos. In *Drosophila*, *Dm'hb* regulates *Dm'Antp*, *Dm'Ubx* and *Dm'AbdA* by repressing their expression in anterior segments (Casares and Sánchez-Herrero, 1995; Irish et al., 1989b).

In wild type *Tribolium* embryos, *Tc'Dfd* is expressed in the md and mx (Figure 8A) followed by the expression of *Tc'Scr* in the lb (Figure 8C). Although *Tc'Dfd* expression is not strongly affected in *Tc'hb*$^{\text{pRNAi}}$ embryos (Figure 8B), *Tc'Scr* is completely absent (Figure 8D).

Tc'Antp is expressed in all thoracic and abdominal segments in wild type embryos (Figure 8E). After the germ band completed elongation, *Tc'Antp* shows an increased level of expression in the thoracic region (arrows in Figure 8E). In *Tc'hb*$^{\text{pRNAi}}$ embryos, *Tc'Antp* expression is prematurely activated and covers all segments formed after the md (Figure 8F). Although ectopically expressed, the *Tc'Antp* domain does not show enhanced expression in specific segments as observed in thoracic regions in wild type embryos (compare Figure 8E and F). Additionally, the *Tc'Antp* expression domain is also expanded posteriorly covering the growth zone in *Tc'hb*$^{\text{pRNAi}}$ embryos (Figure 8F).

The domains of the abdominal Hox genes *Tc'Ubx* and *Tc'AbdA*, normally expressed in the abdomen (Figure 8G, I), are also expanded towards anterior in *Tc'hb*$^{\text{pRNAi}}$ embryos, expressed in all segments posterior to the md segment (Figure 8H, J).

The fact that the expansion of Hox genes in *Tc'hb*$^{\text{pRNAi}}$ embryos does not extend into the anterior head segments indicates that additional factors are responsible for repressing trunk Hox genes in segments anterior to the md. Although the

expansions correspond to different segments, similar effects on *Antp*, *Ubx* and *AbdA* expression domains are also observed in *Dm'hb* mutants in *Drosophila* (Casares and Sánchez-Herrero, 1995; Irish et al., 1989b).
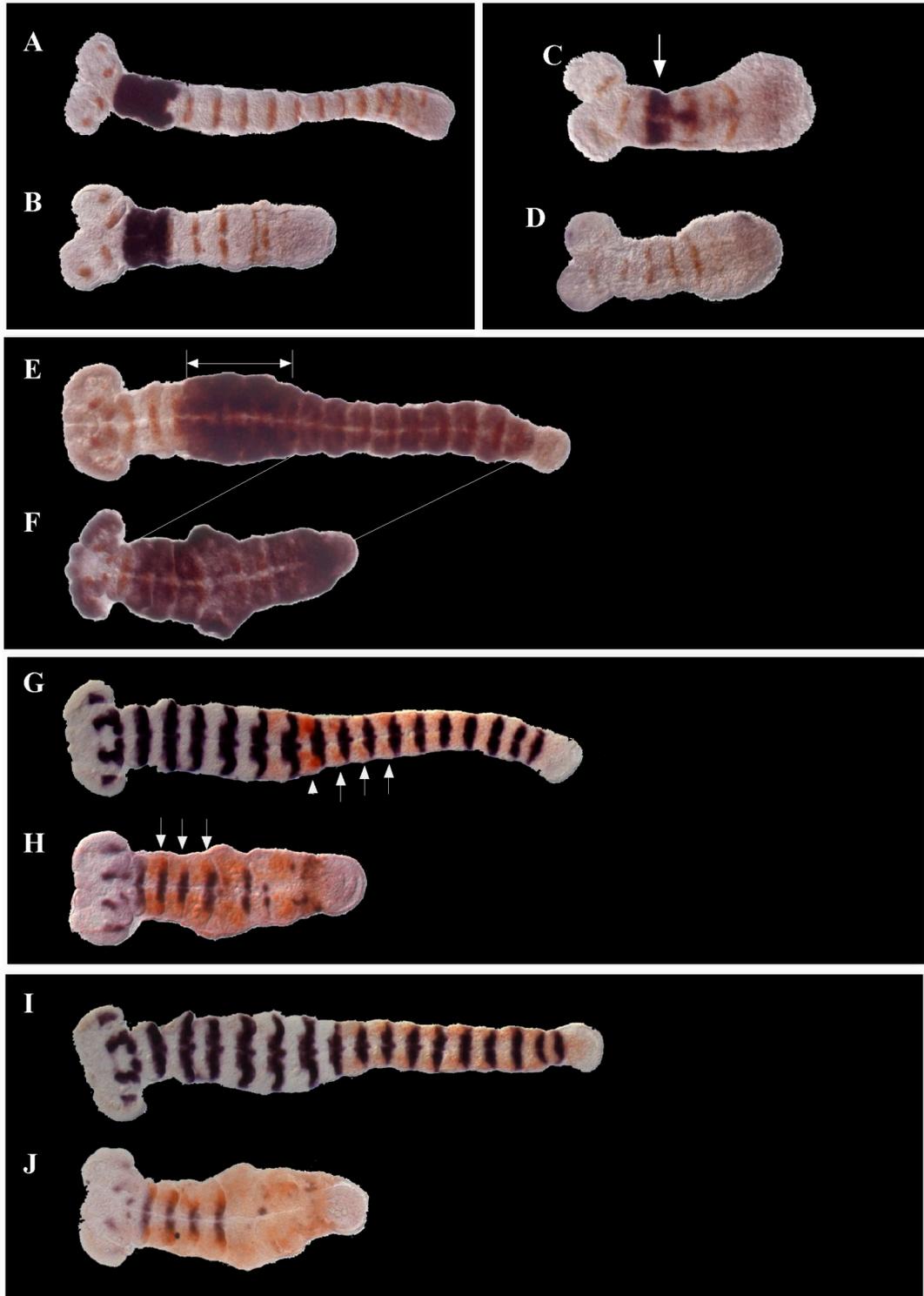
Figure 8. In situ hybridization of Hox genes in (A, C, E, G, I) wild type and in (B, D, F, H, J) *Tc'hb*^pRNAi embryos. *Tc'Dfd* expression domain seems to be smaller in *Tc'hb*^pRNAi embryos (B) compared to wild type (A). (D) *Tc'Scr* is not expressed in *Tc'hb*^pRNAi embryos. (F) *Tc'Antp* is shifted anteriorly in *Tc'hb*^pRNAi embryos but its strong expression observed in the wild type thoracic region (E) is not observed in *Tc'hb*^pRNAi embryos. *Tc'Ubx* and *Tc'AbdA* are shifted anteriorly in *Tc'hb*^pRNAi embryos.

Discussion

hunchback function in *Tribolium*

Molecular comparative analysis between long and short germ insects has revealed fundamental variations in the function of the *gap* genes. While their expression domains cover comparable segments in both long and short germ insects, the phenotypes observed after functional disruption differs significantly.

In this chapter I described the morphological and molecular characterization of the function of *Tc'hb* and provide a reinterpretation of the canonical gap function previously reported (Schroder, 2003). The results demonstrate that the *Tc'hb*^{pRNAi} phenotype can be interpreted as a deletion of segments formed outside of the *Tc'hb* expression domain followed by the homeotic transformation of the remaining post-mandibular segments.

In addition to the role in segmentation, the *Tc'hb* gene is also involved in early morphogenesis at the blastoderm and early gastrula stages in *Tribolium*.

Extra-embryonic membranes in *Tribolium*: assigning fate out of the embryo

Although *hb* orthologs have been analyzed in many insects and other arthropods, an early function of *hb* on the specification of extraembryonic membranes and early germ band morphogenesis has never been reported for any other organism. A possible role in differentiating extraembryonic membranes from the germ rudiment was already suggested for the *hb* ortholog in the Grasshopper (Patel et al., 2001).

The undifferentiated blastoderm formed in *Tc'hb*^{pRNAi} embryos (Figure 4A, B) indicates that *Tc'hb* is involved in the assignment of extra-embryonic fate to cells located within its anterior cap of expression. This effect is comparable to the loss of *Tc'zen1* function, where no serosal fate is established in the egg and all nuclei integrate into the germ rudiment (van der Zee et al., 2005). Interestingly, the commitment of more anterior cells to an embryonic fate in *Tc'zen1*^{pRNAi} embryos leads to the development of a head region containing an increased number of unpatterned cells. Although this intermediate stage (enlarged head) is somehow

rescued later in development (van der Zee et al., 2005), no embryos displaying enlarged head region are observed in *Tc'hb*$^{\text{pRNAi}}$ embryos.

Tc'hb expression has been shown to co-localize with *Tc'zen* in most of the insects analyzed so far (Patel, van der Zee, Lynch, personal communication). Our results represent the first proof that this co-localization might be functional in insects, corroborating with its strong conservation throughout the Insecta.

The low frequency observed for this phenotype might indicate that the role of *Tc'hb* in patterning extraembryonic tissue requires only very low levels of the Tc'Hb protein, perhaps still present in most of the *Tc'hb*$^{\text{pRNAi}}$ embryos.

The lack of evidence for this early function of *hb* in other insects (e.g. cricket) might be due to the limitation in accessing early steps of embryogenesis for molecular stainings (Taro Mito, personal communication).

An additional effect observed in DAPI stained *Tc'hb*$^{\text{pRNAi}}$ embryos is the failure of proper germband invagination. In wild type embryos, the formation of the germband is initiated with the onset of gastrulation, when the primitive pit is formed at the posterior pole of the egg (Figure 4C; Handel et al., 2001). Amniotic cells located at the posterior pole of the egg and at the anterior part of the head lobes fold towards the ventral side of the embryo forming the serosal window (ventral region of the embryo between both amniotic folds not covered by amniotic cells). When the serosal window closes, the elongating germ anlage is ventrally covered by a membrane composed of amniotic cells (Handel et al., 2001).

In *Tc'hb*$^{\text{pRNAi}}$ embryos, the posterior amniotic cells seem to be unable to fold ventrally over the germ rudiment. The germ anlage is therefore abnormally formed and much shorter as in the wild type. Figure 4H shows a *Tc'hb*$^{\text{pRNAi}}$ embryo displaying segmental groves characteristic of the wild type abdominal segments (compare arrows between G and H). Nevertheless, the early effect in *Tc'hb*$^{\text{pRNAi}}$ embryos at blastodermal stage (Figure 4B, D) is observed only at low frequencies. One explanation would be that this effect represents a stronger class of phenotype, where the loss of *Tc'hb* function would cause the embryos to fail further development.

The role of *hunchback* in *Tribolium*: patterning segment formation and differentiation

When compared between insects, the larval phenotype observed after disruption of the *hb* function is remarkably similar (Figure 2). In flies, beetles, wasps, crickets and bugs, strong *hb* phenotypes lead to the development of larvae displaying normal anterior head segments followed by a series of abdominal segments (Lehmann and Nusslein-Volhard, 1987; Liu and Kaufman, 2004a; Mito et al., 2005; Pultz et al., 2005; Schroder, 2003).

The mechanism underlying this phenotype has been exhaustively characterized in the long germ insect *Drosophila melanogaster*. In the fly, *Dm'hb* operates as a crucial anterior morphogen in the early embryo activating anterior gap and pair rule genes while repressing posterior ones (Hulskamp et al., 1994; Hülskamp et al., 1990; Struhl et al., 1992). *Dm'hb* mutant larvae display deletions of the segments were *Dm'hb* is expressed in the wild type (Figure 2).

The "head plus abdomen" phenotype obtained after *hb* depletion is caused by different means in basal insects. In contrast to the situation in *Drosophila*, the loss of *hb* function leads to segmentation defects in the posterior thoracic and abdominal segments, which do not express *hb* in wild type embryos. Segments lying anterior to this defective region are metamerized as in the wild type. Although normally formed, these segments lack the expression of *hb*, which leads to the ectopic expression of abdominal Hox genes, resulting in the assignment of abdominal identity to segments specified as head and thoracic in the wild type.

In *Tribolium*, *Tc'hb* was originally described as a canonical gap gene (Schroder, 2003). However, larvae cuticles displaying a phenotypic series of the *Tc'hb* phenotypes suggest a different interpretation.

These results show that *Tc'hb* plays a very similar role to its counterparts in other short germ insects. Therefore, the ancestral role of the *hb* gene might have consisted of major regulation of Hox genes and minor effects on pair rule genes (Figure 9). In the lineages leading to long germ insects, *hb* evolved a major role in metamerization by increasing its regulatory influence on the expression of pair rule genes.

The morphological characterization of the *Tc'hb*[pRNAi] phenotype raised a series of fundamental questions concerning the *Tc'hb* function in *Tribolium* and the

evolution of body patterning among insects and arthropods. Why are the segments expressing *Tc'hb* still formed after depletion of *Tc'hb*? Which factors are involved in the metamerization of the defective segments after *Tc'hb* knockdown? Is it an ancestral feature of gap genes to mainly affect Hox gene expression? To answer these questions I approached the molecular basis of the *Tc'hb*$^{\text{pRNAi}}$ phenotype by analyzing the genetic interactions between *Tc'hb* and some of the candidate target genes of *hb* described in *Drosophila*.
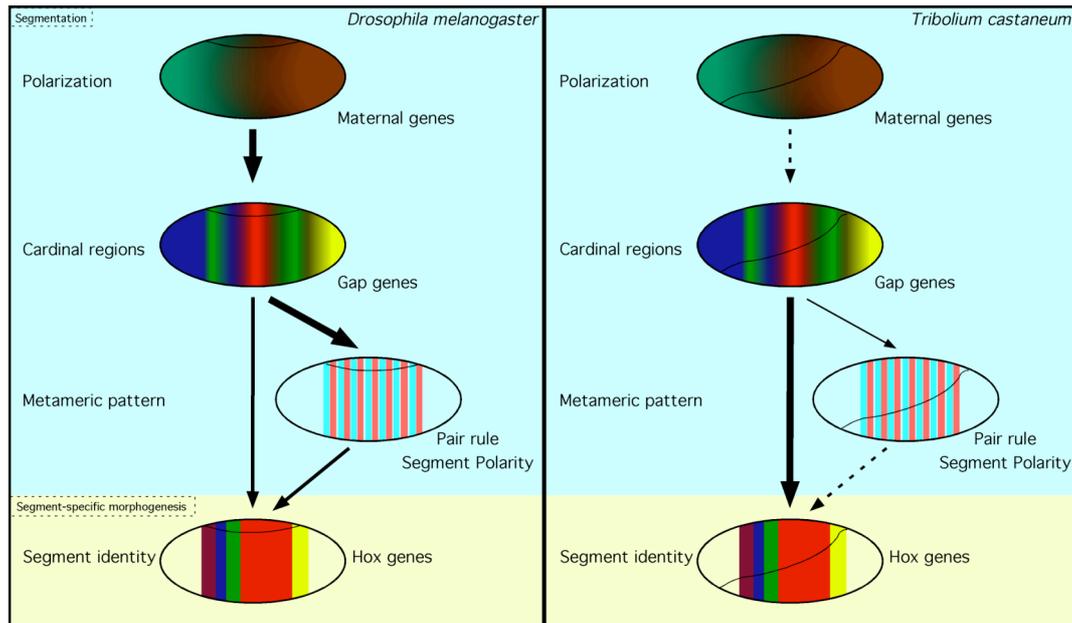
Figure 9. Schematic drawing illustrating the comparison of the segmentation cascade between *Drosophila* (left panel) and *Tribolium* (right panel). Arrows indicate regulation (directional) between classes of segmentation genes. Contransting with the situation in *Drosophila*, the *Tribolium* gap genes perform a more proeminent function in the regulation of Hox genes with minor control of the pair rule genes. The extent to which maternal determinants are controlling gap genes as well that pair rule genes are controlling Hox genes in *Tribolium* is still unknown (dashed arrows). Black line within the eggs depicts the division between extraembryonic and embryonic tissue (dorsal up and anterior to the left). For *Tribolium*, the expression domains of the segmentation and Hox genes do not correspond to the region depicted to be the embryo in the scheme.

The role of *hunchback* in *Tribolium*: interplay among gap genes

The early establishment of the expression domains of the *gap* genes is the first zygotic activity. Gap gene regulatory regions are able to interpret the relatively crude positional information from the maternally provided factors, thus creating cardinal regions along the anteroposterior axis of the embryo (Meinhardt, 1986). Together with Dm'Bcd, Dm'Hb acts as a morphogen along the embryonic axis regulating the expression of *gap* and pair rule genes involved in the formation of head, thoracic and abdominal segments (Simpson-Brose et al., 1994; Wimmer et al., 2000).

The molecular characterization of the *Tc'hb* phenotype in *Tribolium* revealed interesting similarities and variations compared to the regulatory model described for *Dm'hb*.

As in *Drosophila*, *Tc'hb* expression is essential for the activation of the *Tc'Kr* thoracic domain in *Tribolium* (Figure 6). However, the nature of the interaction between *Tc'hb* and *Tc'Kr* remains unknown. Although the maternal product of *Tc'hb* is ubiquitously distributed in the early blastoderm, the zygotic expression of *Tc'hb* and *Tc'Kr* does not seem to overlap in the embryo.

Tc'hb is also required for the activation of the *Tc'gt* expression in T3 and A2 (Figure 5). This interaction is also most likely indirect since *Tc'hb* is not expressed in thoracic and anterior abdominal segments until late in segmentation (Wolff et al., 1995). Furthermore, *Dm'hb* acts as a repressor of the posterior *Dm'gt* expression domain in *Drosophila* (Struhl et al., 1992).

Although not conclusive, the observation that depleting both, *Tc'hb*^{mat} and *Tc'hb*^{zyg} yielded stronger phenotypes compared to depletion of solely *Tc'hb*^{zyg} could suggest a role for the maternal transcript in activating early *Tc'Kr* and *Tc'gt* expression in *Tribolium*.

Taken together, this analysis suggests that *Tc'hb* is a general activator of the *Tc'Kr* and *Tc'gt* trunk domains in *Tribolium* (Figure 20). Interestingly, the deleted region showing fusions of *Tc'gsb* stripes in *Tc'hb*^{pRNAi} embryos correspond to the trunk segments that express *Tc'gt* in the wild type (T3 and A2).

Noteworthy, *Oncopeltus* and *Gryllus hb* knockdown embryos show fusion of the segmental markers *Tc'wg* and *Tc'en* at a very similar position as observed in

Tribolium (Liu and Kaufman, 2004a; Mito et al., 2005). However, no information is available on the function or expression of *gt* in this species.

Segmentation in *Tribolium*: gap gene regulation of the pair rule circuit

Recent analysis of the *Tribolium* pair rule gene orthologs revealed a self-maintaining regulatory circuit responsible for the metamerization of segments before and after the cellularization of the blastoderm. Disturbance of the expression of primary pair rule genes (*Tc'eve*, *Tc'run* and *Tc'odd*) results in asegmental embryos, while disruption of secondary pair rule genes (*Tc'prd* and *Tc'slp*) leads to the canonical pair rule phenotype known from Dm, in which segments are deleted in a double segmental periodicity (Choe and Brown, 2006; Choe et al., 2006).

The analysis of the *Tribolium* orthologs of the pair rule genes *Tc'eve* and *Tc'run* in *Tc'hb*[pRNAi] embryos revealed that indeed their periodic patterning is disrupted in the region where the segmentation defects are observed (Figure 7). Since these regions lie outside of the *Tc'hb* wild type expression domain I investigated whether the effect on pair rule gene expression would be mediated by the loss of *Tc'gt* expression in T3 and A2. Indeed, *Tc'gt* knockdown embryos show disruption of *Tc'eve* and *Tc'run* expression in the segments were *Tc'gt* is expressed in the wild type (Figure 7).

Taken together, these results indicate that the metamerization defects observed in *Tc'hb*[pRNAi] embryos are achieved by interfering with the first level of the proposed pair rule gene circuit (Choe et al., 2006), most likely via the misregulation of *Tc'gt* in these embryos (Figure 10). Additionally, it raises the question whether the similar segmentation defects observed in *hb* knockdown embryos in the basal insects *Oncopeltus* and *Gryllus* might also be mediated by *gt*.

Segmentation genes - wild type
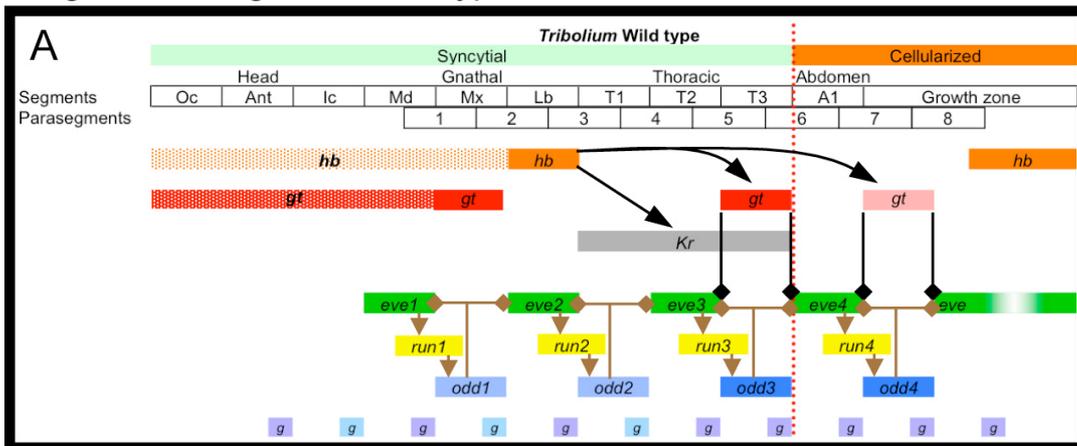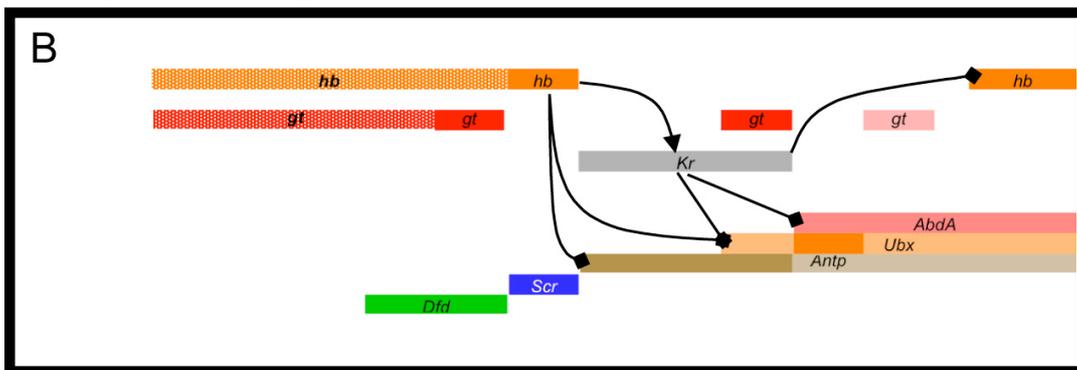
Hox genes - wild type

Segmentation genes - *Tc'hb* pRNAi
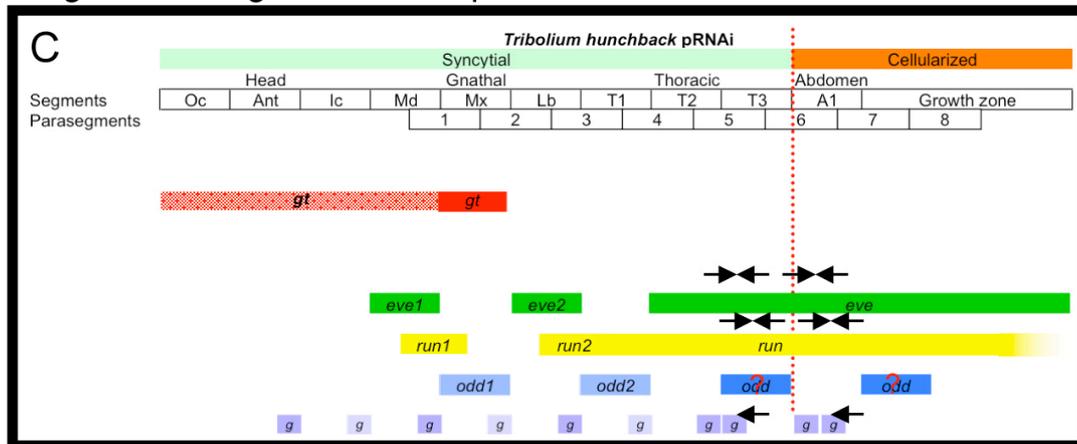
Hox genes - *Tc'hb* pRNAi
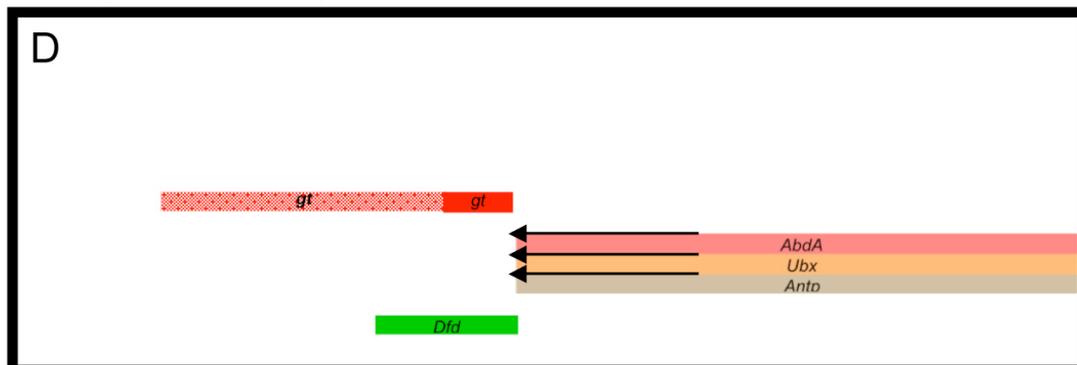
Figure 10. Schematic drawing of the segmentation cascade in *Tribolium*. (A) Wild type (B) *Tc'hb* pRNAi. (A) The wild type function of *Tc'hb* in segmentation is mediated via the activation of *Tc'gt*. (C) In *Tc'hb*^pRNAi embryos, the trunk *Tc'gt* stripes are missing, which leads to the fusion of *Tc'eve* and *Tc'run* stripes in this region. (B) The major role of *Tc'hb* is to regulate the expression domains of trunk Hox genes, partially mediated by the activation of *Tc'Kr*. (D) In *Tc'hb*^pRNAi embryos, the lack of *Tc'Kr* result in the anterior expansion of abdominal *Tc'Ubx* and *Tc'AbdA* that repress *Tc'Scr* and the thoracic *Tc'Antp* domain. The gap and Hox gene misexpression in *Tc'hb*^pRNAi embryos results in a phenotype where only anterior head is normally formed with posterior head and thoracic segments transformed into abdominal identity and most of the abdominal segments not formed.

Abdominal identity in *Tribolium*: prohibitory role of *hunchback*

The most evident characteristic of the *hb* phenotype in insects is the generation of mainly abdominal segments posterior to the pre-gnathal segments. Ironically, this obvious lack of post-gnathal and thoracic segments might be a source of ambiguous interpretation.

There are two distinct, and at a certain level independent, ways of interfering the generation of a specific segment. In the natural conditions, the insect body plan is morphologically subdivided into undifferentiated segmental units, also called metameric units. Depending on their position along the embryonic AP axis, each of the metameres will be assigned a specific identity, generating the distinct head, thoracic and abdominal segments (Akam, 1987). Therefore, it is possible, for example, to prevent the formation of thoracic segments by either (1) affecting the genes necessary for the metamerization of segments located at central positions in the embryo (e.g. *Dm'Kr*) or (2) affecting the genes required for assigning thoracic identity (e.g. *Dm'Antp*).

The distinction between the segmentation process and establishment of segment identity is therefore crucial for the proper interpretation of phenotypes obtained after depletion of gap gene transcripts, especially in short germ insects.

The specification of segment identity in the insect body plan depends on the expression of Hox genes (Carroll, 2002). Genes belonging to the Antennapedia complex (ANT-C) and the bithorax complex (BX-C) are known to be required for the proper identity of trunk segments (post-gnathal, thorax and abdomen) in insects. In *Drosophila, Dm'hb* is essential for the establishment of the expression domain of the trunk Hox genes *Dm'Antp, Dm'Ubx* and *Dm'AbdA* (Casares and Sánchez-Herrero, 1995; Irish et al., 1989b; White and Lehmann, 1986).

Staining *Tc'hb*[pRNAi] embryos for the expression of *Tc'Antp, Tc'Ubx* and *Tc'AbdA* revealed that the role of *hb* in repressing the trunk Hox genes is conserved between *Tribolium* and *Drosophila* (Figure 8). The ectopic expression of the abdominal genes *Tc'Ubx* and *Tc'AbdA* in *Tc'hb*[pRNAi] embryos (Figure 8H, J) leads to the homeotic transformation of gnathal and thoracic segments into abdominal identity. This transformation of thoracic to abdominal identity occurs despite the expression of *Tc'Antp* in these segments(Figure 8F). Notably, this co-localization of *Tc'Antp*,

Tc'Ubx and *Tc'AbdA* is also observed in the abdominal segments in wild type embryos, where *Tc'Antp* expression is weaker when compared to the thoracic domain (compare Figure 8E, G, I). The function of *Tc'Ubx* and *Tc'AbdA* were shown to be required for patterning abdominal segments in the *Tribolium* embryo (Bennet et al., 1999; Lewis et al., 2000). Additionally, the expansion of *Tc'Antp* domain in *Tc'hb*[pRNAi] embryos most likely leads to the repression of *Tc'Scr* (Figure 8D), since *Dm'Antp* is known to have an epistatic effect (posterior prevalence) on *Dm'Scr* in *Drosophila* (Carroll et al., 1988; Pelaz et al., 1993).

The primary wild type function of *Tc'hb* is therefore to provide the positional information in gnathal and thoracic segments. This positional information is essential for the metamerization process, through the regulation of the gap and pair rule genes and for the specification of segment identity, through the repression of the trunk Hox genes (Figure 10). When *Tc'hb* is depleted in *Tribolium*, thoracic and abdominal segments are fused while ghantal and thoracic segments develop as abdominal segments (Figure 10).

In support to these findings, in addition to the similar cuticular phenotype, *hb*[pRNAi] embryos in *Oncopeltus* and in *Gryllus* also display segment fusions and anterior shifts of trunk Hox genes strikingly similar to those observed in our analysis (Liu and Kaufman, 2004a; Mito et al., 2005). Taken together, these observations suggest that the role of *Tc'hb* described here most likely represents the ancestral state of the *hb* function shared by the last common ancestor of insects. Thus, the canonical gap function of *hb* described in *Drosophila* and *Nasonia* is so far restricted to holometabolous insects developing as long germ embryos, and might represents a derived state of the role of the *hb* gene among insects (see General Discussion).

Chapter II – mille-pattes: a novel segmentation gene in Tribolium

Introduction

The identification of the first orthologs of the *Drosophila* segmentation genes in other insects, showing high sequence conservation and similar expression patterns led to the assumption that the mechanism underlying the syncytial segmentation patterning in the fly could be functional in several other instances where segmentation happens under cellularized conditions. However, further embryological and molecular analysis in an increasing number of model systems has revealed significant differences in the mechanism of embryonic axis pattering among insects (Lynch and Desplan, 2004; Tautz and Sommer, 1995). The embryonic fate map during the blastoderm stage illustrates precisely such differences. According to the distinctions between the fate map of short and long germ insects, genes expressed in anterior and central regions of long germ insects are expressed in central and posterior regions of short germ insects, respectively. Genes expressed in the posterior half of the egg of long germ insects are therefore not present in the syncytial blastoderm of short germ insects (Tautz and Sommer, 1995). These genes are thus expressed only after the onset of gastrulation. Nevertheless, the expression domains of those genes are often found in homologous segments in insects displaying short or long germ mode. The head gap gene orthodenticle is zygotically expressed in the ocular segment in insects irrespective of their type of embryogenesis, whilst Kr, a gene expressed in the thoracic segments in *Drosophila*, is also expressed in those segments in every insect in which its expression has been analyzed (Cerny et al., 2005; Finkelstein and Perrimon, 1990; Gaul et al., 1987; Liu and Kaufman, 2004b; Lynch et al., 2006b; Mito et al., 2006; Preiss et al., 1985; Schroder, 2003; Sommer and Tautz, 1993).

Remarkably, genes showing both, an anterior and a posterior expression domain at the blastoderm stage of *Drosophila*, such as *gt* and *hb* also show an anterior and posterior expression domain in *Tribolium*, however the second domain is formed in a cellular environment (Bucher and Klingler, 2004; Wolff et al., 1995).

Furthermore, the expression of at least one gap gene is found in all segments formed at the blastoderm stage in both, short germ and long germ insects. Conversely,

some segments without any gap gene expression are seen in the abdominal segments of short germ insects, which are formed after cellularization.

These findings suggest that additional segmentation genes patterning trunk segments remain to be identified in insects developing as short germ. Despite genetic (Maderspacher et al., 1998; Sulston and Anderson, 1998; Sulston and Anderson, 1996) and EST (Savard, 2004) screens in short germ insects, over the past 15 years, molecular comparative embryology has been heavily based on the candidate gene approach with no additional discoveries of novel genes playing a role in segmentation.

To further understand the role of gap genes in *Tribolium*, as well as to identify new factors involved in segmentation, I decided to characterize the function of a novel gene, *mille-patte*, (*mlpt*) identified in a *Tribolium* EST expression screen and which is expressed in head and abdominal segments (Savard, 2004).

In this chapter I describe the morphological and molecular characterization of *Tc'mlpt*. First, I generated embryos carried out RNAi knockdown of this gene and analyzed the phenotype at the cuticular level. Next, I asked whether *Tc'mlpt* would interact with the orthologs of the *Tribolium* gap genes. Finally, I investigate whether the phenotype observed was achieved via the regulation of the *Tribolium* orthologs of the gap genes.

Results

Morphological characterization of *mille-pattes*

Cuticle phenotype

In order to assess the loss of function phenotype of mille-pattes (*Tc'mlpt*) I performed parental RNAi as described in Bucher et al. (2002). The phenotype was primarily characterized with cuticle preparations of 1st instar larvae (see material and methods). The pRNAi experiments yielded a consistent phenotype, with strength varying slightly depending on the age of the injected female on the egg lay (see Material and Methods for phenotypic series). The knockdown larvae display a loss of abdominal segments with additional generation of up to seven pairs of legs in the most severe cases, hence the name milles pattes (Figure 11B-E; Savard et al., 2006a). In *Tc'mlpt*^{pRNAi} larvae, the head, gnathal and the three thoracic segments are formed like in the wild type (Figure 11; detain in 11G). The posterior abdominal segments display thoracic identity and segment losses. Weak *Tc'mlpt*^{pRNAi} phenotypes display deletions of a few posterior segments, including the terminal structures pygodopes and urugomphi (Savard et al., 2006a), while stronger phenotypes display deletion of most of the abdominal segments therefore fewer leg baring segments (Figure 11B, D). The larval cuticles showed in Figure 11C and E represents an intermediate phenotype, where more abdominal segments are formed. In addition to the segment deletions and transformations, strong *Tc'mlpt*^{pRNAi} phenotype fail to develop the more distal segment of the antenna (arista) (Figure 11B, D; detail in 11G). Once again, the larval cuticles showed in Figure 11C and E display an intermediate state of the 'aristaless' phenotype, where the aristae are only reduced. Furthermore, all appendages formed in *Tc'mlpt* larva appear compacted (Figure 11).

The identity of the transformed thoracic segments can be addressed since wild type larvae have a pair of tracheal pits on the lateral sides of the second thoracic and all abdominal segments (arrows in Figure 11A). The additional thoracic segments in *Tc'mlpt* larvae show tracheal pits in alternating odd numbered segments (arrows in Figure 11B, C), suggesting a transformation of abdominal segments into a sequence

of T2 and T3. This effect is however variable with some larvae showing adjacent segments displaying or lacking tracheal pits (arrows in Figure 11D, E).

In view of the pRNAi phenotypes obtained for *Tribolium* orthologs of the gap genes and their expression pattern (this thesis; Bucher and Klingler, 2004; Cerny et al., 2005), the segment deletions and transformations observed in *Tc'mlpt*[pRNAi] larvae, together with the "gap" like expression pattern, suggest that *Tc'mlpt* plays a role in segmentation that is similar to the orthologs of the gap genes, however, unlike these other genes, *mlpt* does not encode a transcription factor.
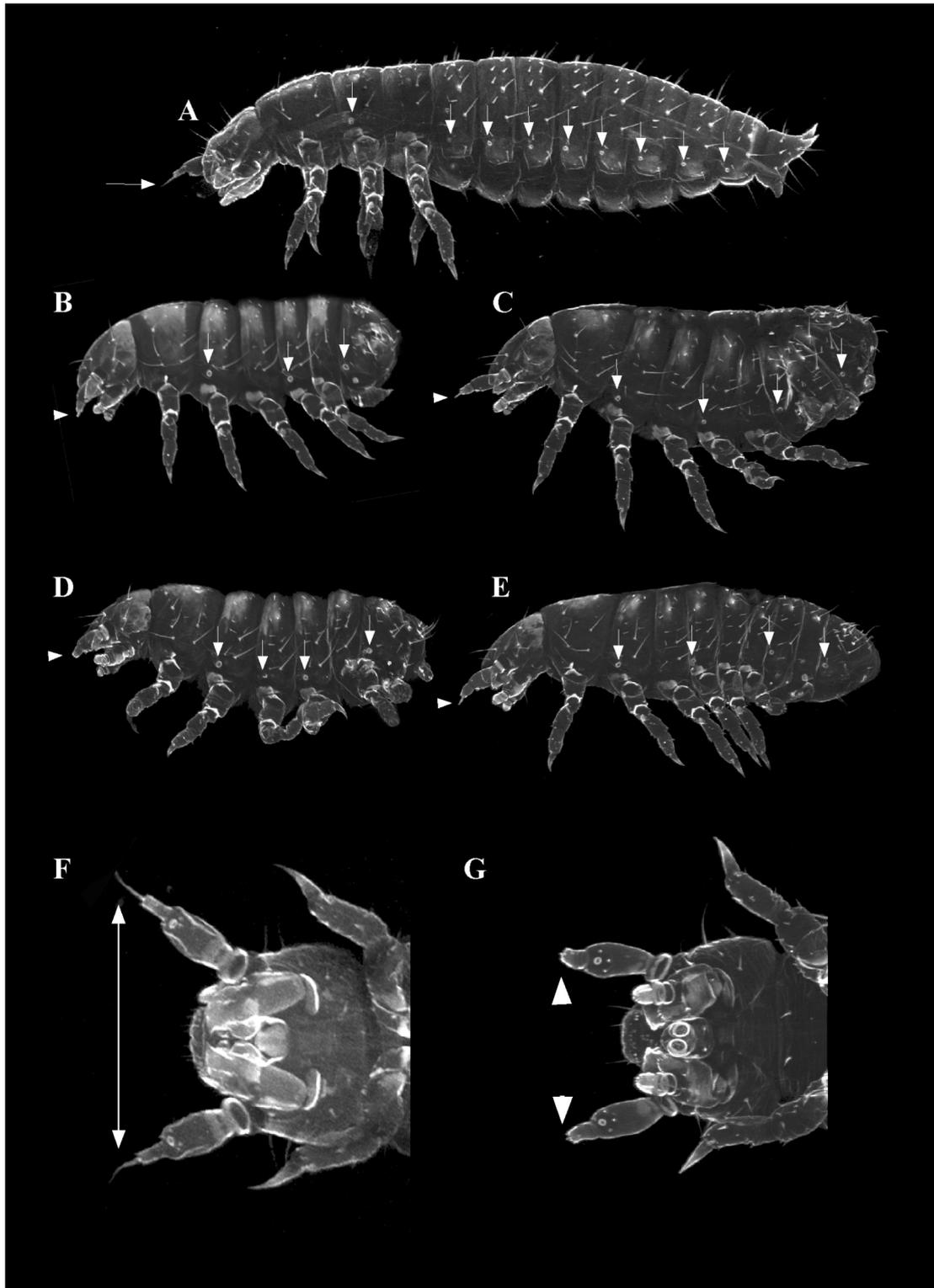
Figure 11. Cuticular preparations of (A,F) wild type and (B, C, D, E, G) *Tc'mlpt*$^{\text{pRNAi}}$ larvae. Wild type larva displaying head, thoracic and abdominal segments. (B-E) *Tc'mlpt*$^{\text{pRNAi}}$ larva displaying head segments and thoracic segments apparently normal. The abdominal segments are transformed into thoracic segments and posterior abdominal segments are deleted. The transformed thoracic segments can be distinguished by the presence of tracheal pits (arrows in B-E), characteristic of wild type T2 and abdominal segments (arrows in A). *Tc'mlpt*$^{\text{pRNAi}}$ larvae also fail to develop aristae. (F) Detailed view of a wild type head. Wild type aristae are depicted (arrows). (G) Detailed view of a strong *Tc'mlpt*$^{\text{pRNAi}}$ 'aristaless' phenotype (arrowhead). The 'aristaless' phenotype can also be observed in B-E, varing in respect to the strength of the RNAi effect.

Molecular Characterization

In order to verify whether *Tc'mlpt* function is achieved by interaction with other gap genes, *Tc'mlpt* expression was analyzed in embryos depleted for *Tc'hb*, *Tc'gt* and *Tc'Kr* as well as their own expression patterns in *Tc'mlpt*^pRNAi embryos. The phenotype was further characterized with respect to the expression of the trunk Hox genes *Tc'Antp*, *Tc'Ubx* and *Tc'AbdA*.

Gap genes regulate *mille-pattes* expression

Embryos depleted for *hb*, *gt* and *Kr* (*Tc'hb*^pRNAi, *Tc'gt*^pRNAi, and *Tc'Kr*^pRNAi embryos, respectively) were stained for *Tc'mlpt* (Figure 12). The ortholog of the segment polarity gene gooseberry (*Tc'gsb*) was used for double staining as a segmental reference. The early *Tc'mlpt* blastodermal expression domain in the anterior head and mandibular segments is not significantly altered in any of the three gap gene knockdown embryos. However, clear effects on the *Tc'mlpt* trunk expression domains become apparent during germband growth.

In *Tc'hb*^pRNAi embryos, the first *Tc'mlpt* trunk domain appears to form normally at first and exhibits the wild type anterior border (Figure 12A). However, this domain does not become properly established and eventually fades (Figure 12B, C). The second *Tc'mlpt* trunk domain in A7 is still visible in these embryos, indicating that its regulation is independent of the proper progression of trunk segmentation (Figure 12C).

The first *Tc'mlpt* trunk domain is initially only weakly visible in *Tc'Kr*^pRNAi embryos and appears to be shifted towards posterior (Figure 12D). At later stages, the domain is completely lost (Figure 12E). As in *Tc'hb*^pRNAi embryos, the second *Tc'mlpt* trunk domain is formed in *Tc'Kr*^pRNAi embryos (Figure 12F).

In *Tc'gt*^pRNAi embryos, the first *Tc'mlpt* trunk domain is expressed more strongly than in the wild type and its anterior border is shifted anteriorly by one segment, i.e., overlapping with the fourth *Tc'gsb* stripe instead of forming posterior to the fifth stripe (Figure 12G). The strong expression is retained during further development (Figure 12H, I). The second *Tc'mlpt* trunk domain also forms in these embryos (Figure 12I).
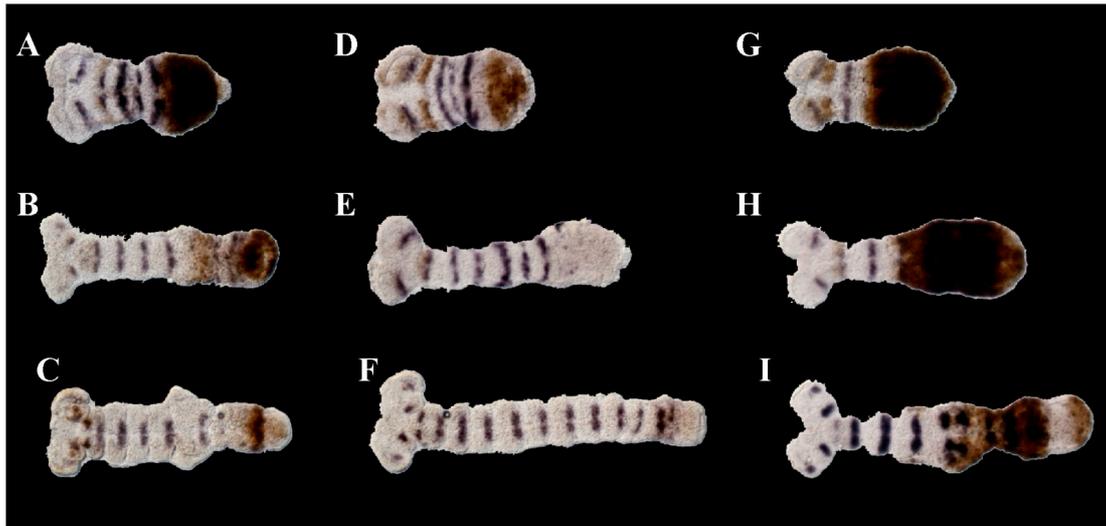
Figure 12. *Tc'mlpt* expression pattern in in embryos depleted for the gap genes. (A-C) In *Tc'hb*[pRNAi] embryos, *Tc'mlpt* trunk expression fails to be properly established while the posterior abdominal domain is formed. (D-E) In *Tc'Kr*[pRNAi] embryos, *Tc'mlpt* trunk expression is strongly reduced while the posterior domain seems to form in these embryos. (G-I) In *Tc'gt*[pRNAi] embryos, the *Tc'mlpt* trunk expression is expanded towards anterior and posterior.
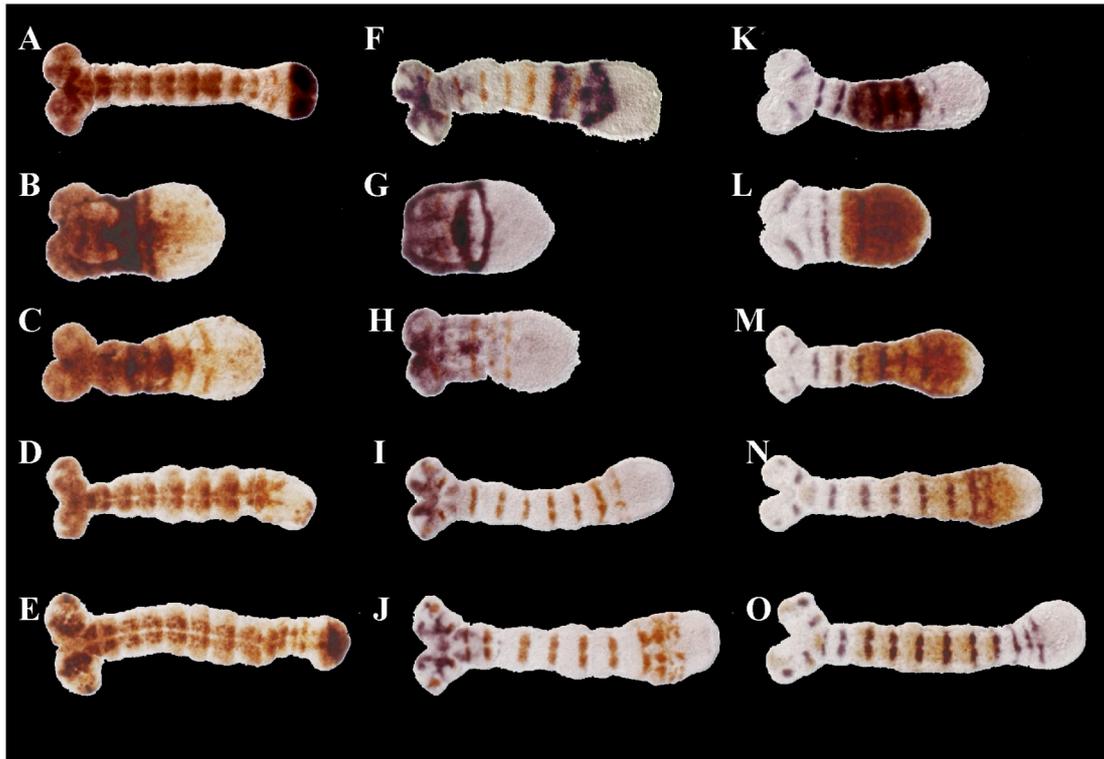
Figure 13. Expression patterns of gap genes in (A, F, K) wild type and (B-E, G-J, L-O) *Tc'mlpt*^{pRNAi} embryos. (A-E) *Tc'hb* expression is normally formed *Tc'mlpt*^{pRNAi} embryos except for the posterior abdominal domain, which appears later (E) compared to the wild type (A). (G-J) The anterior *Tc'gt* domain is properly established in *Tc'mlpt*^{pRNAi} embryos but the posterior T3 and A2 stripes are missing. (L-O) *Tc'Kr* expression domain is expanded towards posterior in *Tc'mlpt*^{pRNAi} embryos.

mille-pattes regulates other gap genes

To determine whether *Tc'mlpt* is acting at the same level of the segmentation gene hierarchy as the other gap genes, the expression of *Tc'hb*, *Tc'gt* and *Tc'Kr* was assessed in *Tc'mlpt*^pRNAi^ embryos (Figure 13).

The extra-embryonic expression (not shown) as well as the anterior *hb* domain in the gnathal segments is not significantly affected in *Tc'mlpt*^pRNAi^ embryos (Figure 13B). Wolff et al. (1995) have mapped the border of the anterior *hb* domain to the border between the gnathal and thoracic segments. Double staining with *Tc'gsb* shows that the *hb* border overlaps with the fifth stripe (Figure 13B), i.e. in approximately the same location. On the other hand, a clear effect is evident on the formation of the posterior *hb* domain. This should already be present in an embryo of the age shown in Figure 13C, however in *Tc'mlpt*^pRNAi^ embryos it is initially absent and eventually forms with some delay at a late stage (Figure 13E).

Tc'gt is expressed in the early blastoderm in the head segments up to the maxillal segment, refining later into a stripe in this segment (Bucher and Klingler, 2004). During germband extension, *Tc'gt* is expressed in two stripes in T3 and A2, respectively (Figure 13F; Bucher and Klingler, 2004). *Tc'mlpt* seems not to affect the blastodermal expression of *Tc'gt*, however the trunk domains are clearly disrupted (Figure 13G-J). Both *Tc'gt* stripes lie within the *Tc'mlpt* expression domain found in wild type embryos. In *Tc'mlpt*^pRNAi^ embryos the two *Tc'gt* stripes are completely absent (Figure 13G-J), suggesting that *Tc'mlpt* acts as an activator of this domain.

In line with the role of mille pattes as a gap gene in *Tribolium*, the *Tc'Kr* expression domain is also affected *Tc'mlpt* knockdown embryos. Cerny et al. (2005) mapped the wild type *Tc'Kr* domain to the three thoracic segments, which would correspond to the *Tc'gsb* stripes five to eight. The anterior border of the *Tc'Kr* domain in *Tc'mlpt*^pRNAi^ embryos starts with *Tc'gsb* stripe five (Figure 13L), but extends clearly beyond stripe eight (Figure 13L-N). Thus, *Tc'mlpt* appears to be required for setting the posterior border of *Tc'Kr* expression. At later stages, *Kr* shows a weak expression in most segments in wild type embryos (Cerny et al., 2005), which is also normally formed in *Tc'mlpt*^pRNAi^ embryos (Figure 13O).

mille-pattes regulates trunk Hox genes

In order to reveal the molecular scenario responsible for the changes of segment identity in the *Tc'mlpt* phenotype I decided to investigate the expression domains of Hox genes in *Tc'mlpt*[pRNAi] embryos. Since these changes involve thoracic and abdominal segments, only the expression of the trunk Hox genes *Tc'Antp*, *Tc'Ubx* and *Tc'AbdA*, were assessed in *Tc'mlpt*[pRNAi] embryos (Figure 14).

In *Tribolium*, *antennapedia* expression starts during germ band elongation posterior to the *Tc'gsb* labial stripe, i.e., in the first thoracic segment (not shown), This expression expands posteriorly, during germ band elongation, in each subsequent segment formed from the growth zone (Figure 14A). According to its role in the specification of thoracic identity in insects, *Tc'Antp* expression is expression is increased in the thoracic segments at later stages, just prior to the development of the limbs (arrows in Figure 14A). In *Tc'mlpt*[pRNAi] embryos, the initial expression of *Tc'Antp* is apparently unaffected, initially expressed in T1 like in the wild typem(Figure 14B). However, after germband extension, the level of *Tc'Antp* expression in the entire embryonic trunk is comparable to the stronger expression domain in the thoracic region in the wild type (compare Figure 14A and B).

The expression of the abdominal Hox genes *Tc'Ubx* and *Tc'AbdA*, normally covering all abdominal segments (Figure 14C, G), is drastically reduced and in some cases absent in *Tc'mlpt*[pRNAi] embryos (Figure 14D, H). Interestingly, although *Tc'Ubx* expression is observed in some *Tc'mlpt*[pRNAi] embryos at later stages, the increased level of *Tc'Ubx* expression observed in the A1 segment is found in the posterior most segments in these embryos.

Figure 14. Expression pattern of Hox genes (red) and *Tc'gsb* (blue) in (A, C, E ,G) wild type and (B, D, F, H) in *Tc'mlpt*^{pRNAi} embryos. Expression of *Tc'Antp* in (A) wild type and (B) *Tc'mlpt*^{pRNAi} embryos. Although the anterior *Tc'Antp* expression border is established as in wild type, the increase level of *Tc'Antp* expression, found in wild type thorax, is observed in all abdominal segments formed in *Tc'mlpt*^{pRNAi} embryos (compare arrow areas in A and B). Expression of *Tc'Ubx* in (C, E) wild type and (D, F) *Tc'mlpt*^{pRNAi} embryos. During germband elongation the *Tc'Ubx* expression is drastically reduced a shifted posteriorly (D). In later stages, the anterior *Tc'Ubx* expression border is observed in similar region as in wild type, however the increased expression observed in wild type A1 is found in the posterior most segments of *Tc'mlpt*^{pRNAi} embryos (compare arrows in E, F). Expression of *Tc'AbdA* in (G) wild type and (H) *Tc'mlpt*^{pRNAi} embryos. Expression of *Tc'Ubx* is drastically reduced and shifted towards posterior in *Tc'mlpt*^{pRNAi} embryos.

Discussion

Functional analyses of gap genes in *Tribolium*, *Gryllus* and in *Oncopeltus* have suggested that the "gap" function is a feature restricted to their orthologs in higher dipterans. The main ancestral role of the gap genes in insects, and perhaps also in arthropods, is to provide positional information along the embryonic AP axis for the establishment of the expression domains of the Hox genes (see above). The gap genes have nevertheless an important role in metamerization, since knocking down their function in *Tribolium*, *Gryllus* and *Oncopeltus* leads to segment deletions and a breakdown of segmentation. In the previous chapter, I demonstrated that at least in *Tribolium*, segmentation defects observed in *Tc'hb*[pRNAi], *Tc'Kr*[pRNAi] and *Tc'gt*[pRNAi] embryos can be explained by misexpression of the *Tc'gt* trunk domain in these embryos.

In this chapter, I describe the characterization of the novel segmentation gene *mille-pattes* in *Tribolium* (*Tc'mlpt*). *Tc'mlpt* is expressed in a contiguous abdominal domain. This region is primarily affected by loss of function of *Tc'mlpt*, where homeotic transformations and segment fusions are observed. As for the depletion of *Tc'hb*, *Tc'Kr* and *Tc'gt*, the segments lying beyond the *Tc'mlpt* expression domain are also affected after depletion of *Tc'mlpt*, either by transformation or by segmental loss. Furthermore, the function of *Tc'mlpt* not only involves the regulation of Hox genes, but is also most likely accomplished by cross-regulatory interactions with at least *Tc'hb*, *Tc'Kr* and *Tc'gt* in *Tribolium*. In this analysis, it was possible to observe an additional role of *Tc'mlpt* in specifying the most distal segment of the antenna (arista), structure that is missing in strong *Tc'mlpt* phenotypes.

Surprisingly, sequence analysis revealed that the *Tc'mlpt* gene represents a novel class of eukaryotes genes, coding for multiple conserved peptides (Savard et al., 2006a).

Cross-regulation between *mille-pattes* and gap genes: a new fellow

In wild type embryos, *Tc'mlpt* is co-expressed in the T2 with *Tc'Kr*, in T3 with *Tc'Kr* and *Tc'gt*, and in A2 only with *Tc'gt*. In the segments A1 and A3, *Tc'mlpt*

is the only gap gene playing a role in segmentation known to be expressed in this region (Savard et al., 2006a).

Consistent with the co-localization, *Tc'mlpt* regulates the trunk expression domains of both *Tc'Kr* and *Tc'gt*. While *Tc'mlpt* expression is essential for activating the T3 and A2 *Tc'gt* stripes, it is required to repress *Tc'Kr* expression in the abdominal segments, the latter being most likely indirect since both genes show overlapping expression in wild type.

Although no effect is observed on the blastodermal expression of *Tc'hb* in *Tc'mlpt*^{pRNAi} embryos, the temporal expression of the posterior *Tc'hb* domain seems to be affected in these embryos. This domain seems to form slightly later (about two segments posteriorly) in *Tc'mlpt*^{pRNAi} embryos when compared to the wild type. In the wild type, both posterior domains of *Tc'hb* and *Tc'mlpt* seem to co-localize in the A6 and A7 segments. However, it is difficult to determine their precise localization since both domains start to be expressed in the growth zone, region where no segmental marker is expressed. The lack of effect on the anterior expressions of *Tc'hb* and *Tc'gt* is expected since both domains are known to be strictly regulated by maternal factors in *Drosophila* and in *Nasonia* (Brent et al., 2007; Kraut and Levine, 1991b; Tautz, 1988).

Taken together, these results prove that *Tc'mlpt* acts at the same level as the gap genes in *Tribolium* and not only at a subsequent stage.

Tc'mlpt plays therefore a role in the segmentation process via cross-regulatory interactions with other gap genes and in the specification of proper identity for those segmental units formed within its expression domain.

Regulation of Hox genes by *mille-pattes*: assuring abdominal fate.

The novel segmentation gene *Tc'mlpt* indeed mimics all features of the orthologs of the gap genes in *Tribolium*. In addition to the cross-regulatory interactions with the gap genes, *Tc'mlpt* is essential for the proper expression patterns of the Hox genes.

The wild type expression domain with high levels of *Tc'Antp* in the thoracic region is expanded towards posterior segments in *Tc'mlpt*^{pRNAi} embryos (compare Figure 14A with B). This effect coincides with the expansion observed for *Tc'Kr* in

Tc'mlpt[pRNAi] embryos (Figure 13L-O) as well as the transformation of anterior abdominal segments towards thoracic identity in these embryos (Figure 11).

Consistent with the reduced number of abdominal segments observed in *Tc'mlpt*[pRNAi] embryos, the expression of the abdominal Hox genes *Tc'Ubx* and *Tc'Abd-A* are drastically reduced and even absent in some embryos (Figure 14D, H).

In summary, the essential role in the regulation of Hox genes further supports the nomination of *Tc'mlpt* as a novel gap gene in *Tribolium*.

mille-pattes: a polycistronic peptide coding RNAs (''ppcRNAs'')

By far the most unexpected feature of the *Tc'mlpt* gene is that, in contrast to all gap genes as well as most of the segmentation genes described so far, *Tc'mlpt* does not code for a transcription factor. Instead, it has a coding capacity for three small peptides repeated in tandem, containing a conserved LDPTGXY motif of 7 aa. Additionally, a fourth, larger arginine rich peptide is found downstream of the repeats (Savard et al., 2006a). While no sequence similarity at the nucleotide level is found in any other organism, the amino acid sequences of the peptides as well as their order along the transcript is conserved among several insects of different insect orders. Therefore, the evolutionary constraint acting only at the amino acid level over more than 260 million years (evolutionary distance between *Tribolium* and *Drosophila*) strongly suggests that *Tc'mlpt* peptides are being translated and play an essential role in those organisms.

The analysis of the ortholog of the *Tc'mlpt* gene in *Drosophila* (*Dm'mlpt/tal* from *tarsalless*) revealed that the gene has indeed an important function throughout development controlling embryonic development, such as tracheal development and denticle belts pattern, as well as in the formation of the tarsal segment of the adult leg (van der Zee, unpublished; Galindo et al., 2007) The analysis of rescue constructs containing only some of the peptides in *tal* mutants suggests that only one of the LDPTGXY motif-containing pepetides (type-A peptides) provides the *tal* function and that the arginine-rich peptide (type-B peptides) is indispensable for the fly development. The work further shows that the type-A pepetides are translated both in vivo and in vitro (Galindo et al., 2007).

It is interesting to observe that, although *Dm'mlpt/tal* is expressed in a pair rule stripes early in embryogenesis, no effect is observed in *Dm'mlpt/tal* mutants on

the expression of any of the tested patterning gene (van der Zee, Wurm and Aranda unpublished; Galindo et a., 2007). As discussed in Savard et al., (2006a), one possible reason that *mlpt* is not playing a role in *Drosophila* segmentation as it is in *Tribolium*, is the distinct mode of embryogenesis displayed by these two insects (see above). While the early steps of the *Drosophila* embryogenesis is regulated by diffusing gradients of large transcription factors in a syncytium, it is still unclear how these protein gradients would be functional in *Tribolium*, where most of the segments are patterned in a cellularized environment. The discovery of small peptides that are apparently able to cross cell membranes and transport cargo proteins several folds bigger then their own sizes (Lindgren et al., 2000; Rohrig et al., 2002) led to the speculation that in *Tribolium*, the function of the peptides encoded by *Tc'mlpt* would be to provide morphogentic gradients that, instead of diffusible as in Drosophila, would be carried through cell membranes. The fourth peptide (C-terminal) coded by *Tc'mlpt* shows a conserved core of four arginines very similar to a class of cell penetrating arginine-rich peptides (Savard et al., 2006a).

Interestingly, the most obvious feature of the *Tc'mlpt* function presented here is the regulation of gap and Hox genes in segments patterned after cellularization. The suggestion that the fourth arginine-rich peptide encoded by *Dm'mlpt/tal* is not functional in *Drosophila* (Galindo et al., 2007), might indeed indicate that in the lineage leading to in higher dipterans, the cell penetrating function of *mlpt* became dispensable for the patterning in syncytial blastoderm. Therefore one might expect that the reduced selective pressure on the sequence of this peptide would allow the accumulation of deleterious mutations during the hundreds of million years separating *Tribolium* from *Drosophila*. Nevertheless, the arginine-rich core of the fourth peptide is highly conserved between *Tc'mlpt* and *Dm'mlpt/tal* genes. Therefore further studies will be needed to test the function of this *mlpt* arginine-rich peptided in *Tribolium*.

Chapter III – Gap and pair rule gene interactions in Tribolium

Introduction

Although orthologs of segmentation genes have been identified throughout the Arthropoda, the function of this class of genes in other organisms seems to vary significantly from the one described in *Drosophila*. Since many of the regulatory factors are highly conserved proteins among organisms, it has been proposed that evolutionary changes occur primarily by substitutions in cis-regulatory sequences rather than in the proteins themselves (Averof et al., 1996).

The detailed characterization of the regulatory interactions among regulatory factors has been fundamental to the establishment of the *Drosophila* segmentation cascade as one of the best-understood developmental models. These analyses have revealed the maternal morphogen gradients regulating the dynamic expression of the gap genes (Gaul and Jäckle, 1989; Grossniklaus et al., 1994; Hülskamp et al., 1989; Lehmann and Nuesslain-Volhard, 1986; Lehmann and Nuesslain-Volhard, 1991; Tautz, 1988), the precise regulation of individual stripes of pair rule genes by specific combination of gap genes (Arnosti et al., 1996; Hader et al., 1998; Riddihough and Ish-Horowicz, 1991; Small et al., 1992; Stanojevic et al., 1991) as well as many other genetic interactions underlying the synchronous genetic patterning of the Drosophila embryo (reviewed in Ingham, 1988).

As a model, these genetic interactions have been thoroughly tested in many other organisms and have revealed a surprising plasticity of the developmental mechanism controlling segmentation among insects. One example comes from the analysis of orthologs of the genes regulated by *Dm'bcd* in organisms without a *bcd* ortholog. The anterior *hb* and the central *Kr* expressions described in *Drosophila* are formed in strikingly similar regions in the embryos from *Nasonia* and *Clogmia albipunctata*, a dipteran displaying features of short and long germ modes. Further surprising was the finding that the Dm'Bcd regulates the expression of the homologues of *caudal* and *hb* from *Tribolium*. While Dm'Bcd and Dm'Cad activate the transcription of *Tc'hb*, Dm'Bcd represses the translation of *Tc'cad* (Wolff et al., 1998).

This flexibility of the gene regulatory network controlling segmentation in insects is also evident when the regulation of the pair rule gene *eve* is compared between *Drosophila* and the mosquito *Anopheles gambiae* (Goltsev et al., 2004). Although the dynamic pattern of *eve* is mainly conserved, the expression patterns of the maternal factors and gap genes vary significantly between both species. In *Drosophila*, the posterior domain of *Dm'hb* sets the posterior border of the stripes 6 and 7 while in *Dm'gt* sets the posterior border of the stripe 5. In anopheles, the posterior expression of *Ag'hb* and *Ag'gt* are inverted compared to *Drosophila*. (posterior *Dm'gt* domain forms anteriorly to *Dm'hb* while the posterior *Ag'hb* forms anteriorly to *Ag'gt*). Therefore, it is likely that the posterior border of *Ag'eve* stripe 5 is regulated by the posterior *Ag'hb* expression while *Ag'gt*, probably in concert with *Ag'tll*, regulates the posterior border of the stripes 6 and 7 in *Anopheles* (Goltsev et al., 2004).

Taken together, these observations indicate that although the expression patterns of essential genes need to be conserved (phylotypic stage), the regulatory input responsible for establishing these patterns can be significantly modified over the time. Additionally, since several patterning genes involved in segmentation has been described to play a role in ancestral developmental mechanism such as neurogenesis, it is likely that this plasticity of regulatory interactions was essential for the recruitment of ancestral patterning genes into a role in segmentation.

In order to identify the regulatory interactions among the gap genes that are controlling segmentation in *Tribolium* I further characterized orthologs of the gap genes in *Tribolium* in their regulatory context.

Results

giant represses *Krüppel* but does not regulate *hunchback*.

Embryos depleted for *Tc'gt* show no significant change in the expression pattern of *Tc'hb* (Figure 15A-D). Although the posterior expression appears to be activated a bit prematurely, this impression might be caused by the deletion of segments observed in *Tc'gt*[pRNAi] embryos (arrows in Figure 15D). On the other hand, the lack of *Tc'gt* leads to an expansion of the thoracic *Tc'Kr* domain, shifting its anterior border to the mx (Figure 15F). The expression of *Tc'Kr* is also expanded posteriorly in these knockdown embryos (Figure 15H).

Krüppel represses *hunchback* and *giant* expression

In *Tc'Kr*[pRNAi] embryos, the blastodermal *Tc'hb* expression does not clear from the posterior pole of the egg. However, given the overlapping expression of maternal and zygotic transcripts at the blastoderm stage it is not possible to show this unequivocally. A major effect is observed with the formation and elongation of the germ band. In the early wild type germ-band, the anterior *Tc'hb* expression is converged into a stripe in the *lb* (Figure 16). Thoracic and abdominal segments do not express *Tc'hb* and the posterior domain arises only in A7, showing stronger intensity compared to the gnathal stripe (Figure 16). In *Tc'Kr*[pRNAi] embryos, *Tc'hb* is expressed throughout the extending germ band starting from the *lb* (Figure 16). This ectopic expression shows a strong intensity and is similar to the expression level of the posterior *Tc'hb* domain in the wild type (compare Figure 16) that suggests that the ectopic expression of *Tc'hb* in *Tc'Kr*[pRNAi] embryos is caused by a premature initiation of the posterior *Tc'hb* domain. This effect results in the overlapping expression of the posterior *Tc'hb* domain with the gnathal domain (Figure 16). Thus, *Tc'Kr* acts formally as a repressor on the posterior *Tc'hb* domain, a role that is not known from *Drosophila*.

Figure 15. (A-D) Expression patterns of *Tc'hb* (brown) and *Tc'gsb* (red) in (A, C) wild type and (B, D) in *Tc'gt*^pRNAi^ embryos. (E, H) Expression patterns of *Tc'Kr* (brown) and *Tc'gsb* (blue) in (E, G) wild type and (F, H) in *Tc'gt*^pRNAi^ embryos. Expression of *Tc'hb* is not significantly affected in *Tc'gt*^pRNAi^ embryos (B, D). Fusion of segments in *Tc'gt*^pRNAi^ embryos are depicted (arrowheads in D). Expression of *Tc'Kr* is expanded towards anterior and posterior in *Tc'gt*^pRNAi^ embryos (F, D).

Tc'Kr^pRNAi embryos were then analyzed for the expression of *gt*. Cerny et al. (2005) have already shown that the most posterior *gt* stripe is missing in *Tc'Kr*^pRNAi embryos. One can therefore assume that the absence of abdominal *Tc'gt* stripes in *Tc'hb*^pRNAi embryos (Figure 5, chapter I) is not a direct effect of *Tc'hb* function, but an indirect effect caused by the loss of *Tc'Kr* expression in these embryos. Cerny et al. (2005) also observed that two ectopic stripes of expression appear in the T1 and T3 in *Tc'Kr*^pRNAi embryos. Figure 16 shows a double staining with *Tc'gsb* at this stage. The positions of the two ectopic stripes correspond to the segments transformed into mx identity in *Tc'Kr*^pRNAi embryos (Cerny et al., 2005). It therefore seems likely that these stripes do not correspond to abdominal stripes, but to duplicated head stripes. Nevertheless, in *Tc'hb*^pRNAi embryos the lack *Tc'gt* expression in T3 and A2 segments leads to the deletion of these segments via the misregulation of the pair rule circuit (Chapter I). In *Tc'Kr*^pRNAi embryos, however, the ectopic expression of *Tc'gt* shifts the trunk stripes two segments towards anterior, now expressed in T1 and T3 (compare Figure 16). Therefore, while the T3 segment retains *Tc'gt* expression and is normally formed, the A2 segment lacks the expression of *Tc'gt* and is deleted in these embryos (Figure 16). Expression analysis of the pair rule genes in *Tc'Kr* mutants indeed shows normal patterning of *Tc'eve*, *Tc'run* and *Tc'h* only until their fifth stripe of expression (Cerny et al., 2005 and own observations).

Together with the *Tc'hb* regulatory interactions described in Chapter I, these results provide a genetic regulatory scenario that reveals a cross-regulatory network among gap genes in *Tribolium* (resumed in Figure 17). While *Tc'hb* expression activates the *Tc'*Kr and *Tc'gt* trunk domains, *Tc'gt* expression domains are required to repress *Tc'Kr* in the thoracic regions of the embryo. Furthermore, the central *Tc'Kr* domain acts as a repressor in the establishment of the *Tc'hb* and *Tc'gt* expression borders.
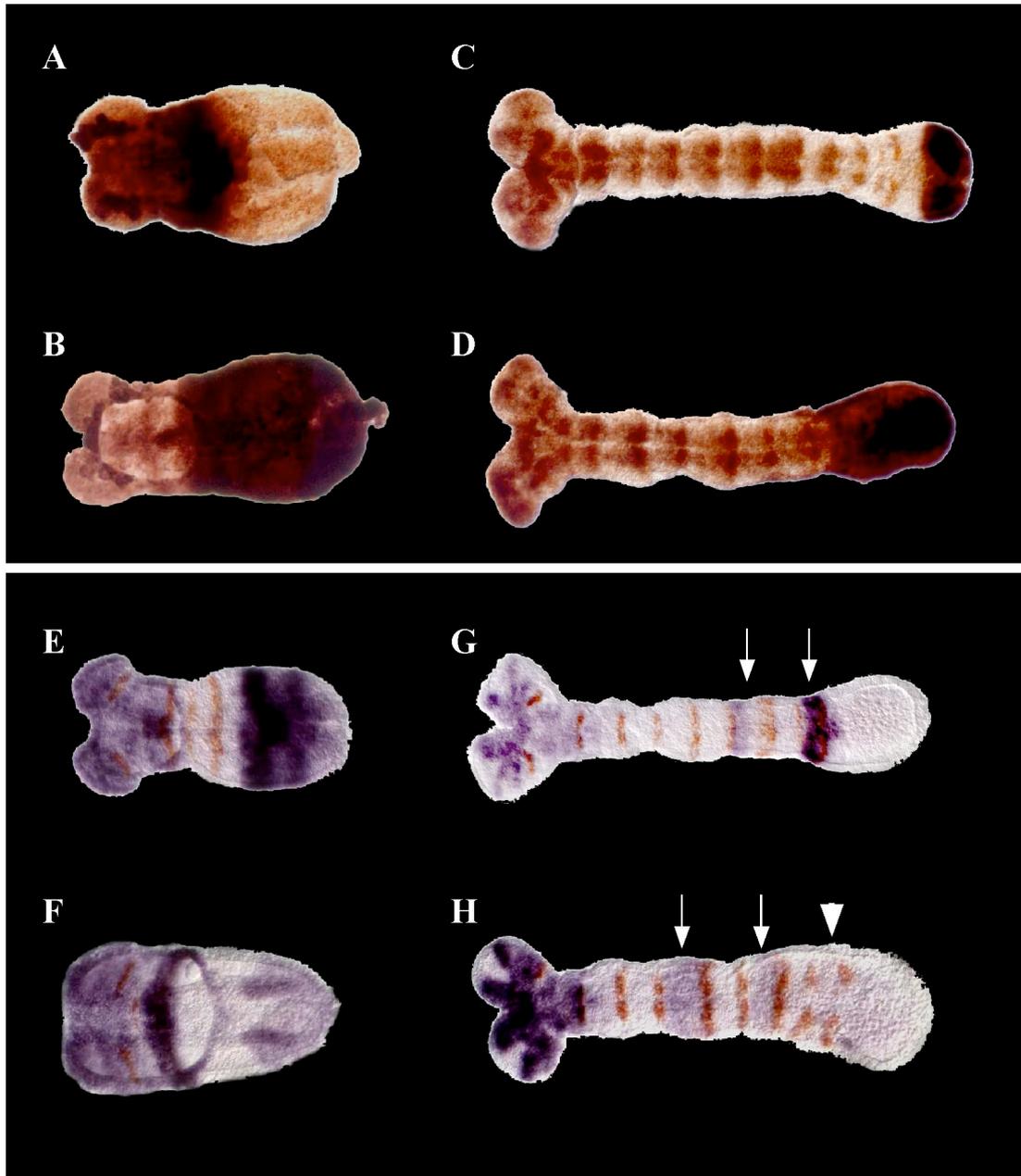
Figure 16. (A-D) Expression patterns of *Tc'hb* (brown) and *Tc'gsb* (red) in (A, C) wild type and (B, D) in *Tc'Kr*^{pRNAi} embryos. (E, H) Expression patterns of *Tc'gt* (blue) and *Tc'gsb* (red) in (E, G) wild type and (F, H) in *Tc'Kr*^{pRNAi} embryos. *Tc'hb* is expressed ubiquitously in a strong domain posterior to the mx segment in *Tc'Kr*^{pRNAi} embryos (B, D). The anterior *Tc'gt* expression is no strongly affected in *Tc'Kr*^{pRNAi} embryos however the trunk *Tc'gt* stripes are shifted anteriorly by two segments (F, H; compare arrows in G and H). Segment fusion observed in *Tc'Kr*^{pRNAi} embryos is depicted (arrowhead in H).

Segmentation genes - wild type
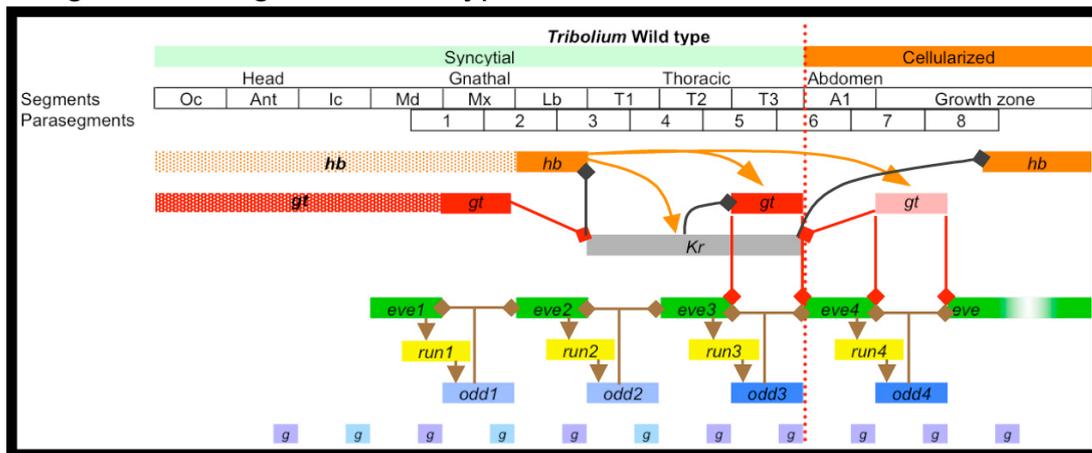
Figure 17. Schematic drawing of the cross-regulatory interactions among gap genes in Tribolium and their role in regulating the pair rule circuit. *Tc'hb* activates *Tc'Kr* central domain and *Tc'gt* expression in T3 and A2 (orange arrows). *Tc'Kr* represses *Tc'hb* in the thoracic and anterior abdominal segments and is important for the proper expression of the T3 and A2 *Tc'gt* stripes (gray arrows). *Tc'gt* sets the borders of *Tc'Kr* expression and is the regulatory link betheen the gap and the pair rule genes (red arrows). The primary pair rule genes of the Tribolium pair rule circuit (Choe et al., 2006) are depicted. Regulation among pair rule genes is depicted with brown arrows.

Hierarchical test: Gap gene expression independent of the pair rule genes

In the hierarchical segmentation cascade described in *Drosophila*, genes belonging to the downstream levels of the cascade do not regulate the expression of upstream ones (Ingham, 1988). For instance, a maternally provided gene can be required for the regulation of genes from any of the downstream levels of the cascade, however a segment polarity gene is never required for the regulation of pair rule genes and so on.

Intriguingly, in the short germ insect *Oncopeltus fasciatus* depletion of *Of'eve* function leads to severe effects on the expression of the gap genes *Of'hb* and *Of'Kr*, resulting in a severe head-only phenotype (Liu and Kaufman, 2005).

Given that both, *Tribolium* and *Oncopeltus* share the same mode of embryogenesis i.e., short germ band, it is tempting to assume that *Tc'eve* is also required for the early regulation of gap genes in the beetle.

I therefore analyzed the effects of pair rule genes on the regulation of gap genes in *Tribolium*. Embryos depleted for *Tc'eve* were generated and analyzed for the expression of different gap genes (Figure 18).

This analysis revealed that, although the morphology of embryos depleted for *Tc'eve* is strongly affected, the expression patterns of *Tc'hb* (Figure 18A-D), *Tc'gt* (Figure 18E, F) and *Tc'Kr* (Figure 18G, H) reveals that the expression of the gap genes seems to be properly established. The embryos depicted in Figure 18E and F are double stained for *Tc'gt* and *Tc'gsb*. The strength of the phenotype can be observed by the severe effect on the *Tc'gsb*, as it is expressed in broad domains in different regions of the asegmental embryo (Figure 18F). As in *Drosophila*, the expression of the gap genes in *Tribolium* seems to be independent of the expression of the pair rule genes.
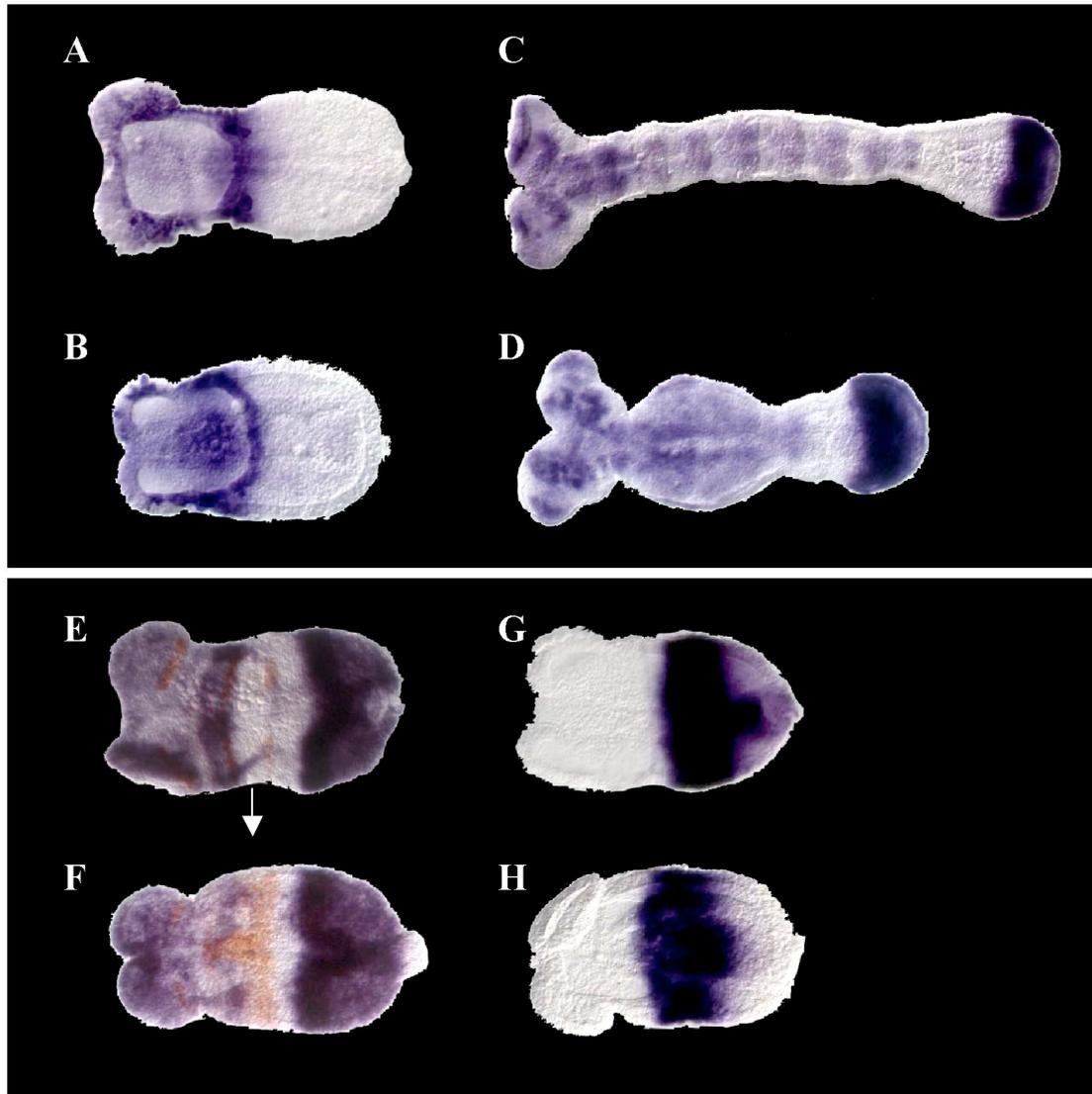
Figure 18. Hierarchical test of the segmentation cascade in Tribolium. Expression patterns of gap genes in *Tc'eve*[pRNAi] embryos. The expression of *Tc'hb* in *Tc'eve*[pRNAi] embryos (B, D) is formed similarly as in wild type embryos (A, C). The expression of *Tc'gt* (F) and *Tc'Kr* (H) are also not significantly affected in *Tc'eve*[pRNAi] embryos when compare to *Tc'gt* (E) and *Tc'Kr* (G) wild type expressions.

Pair rule gene circuit: a model without gaps?

In *Drosophila*, the hierarchy among the pair rule genes is defined by their regulatory input and their influence on each others expression. Primary pair rule genes are regulated exclusively by maternal or gap genes and regulate the expression of the secondary pair rule genes. The secondary are regulated by the primary pair rule genes and provide the link to the regulation of the segment polarity genes (Hartmann et al., 1994). The effects observed for the gap gene class of mutant phenotypes in *Drosophila* is therefore a result of the misregulation of the pair rule genes and consequently of the segment polarity genes.

In other to further characterize the segmentation cascade in *Tribolium* the cross-regulatory interactions between pair rule genes were assessed in *Tribolium*.

For this analysis, *Tc'eve* and *Tc'run* depleted embryos were generated via pRNAi and the expression of these genes were analyzed in each other's knockdown embryos. The morphological phenotypes observed for both genes were undistinguishable from the ones reported in Choe et al. (2006). However, the effects observed in the expression analysis of knockdown embryos are not fully equivalent with the previously reported ones.

In the model of Choe et al. (2006) model, the hierarchy among the pair rule genes was determined from the embryonic phenotype observed for each of the genes analyzed. *Tc'eve*, *Tc'run* and *Tc'odd*, which generate severely truncated embryos when knocked down, were classified as primary pair rule genes. The unaffected expression of these genes in knockdown embryos for other pair rule genes supports this classification. The other two pair rule genes analyzed, *Tc'prd* and *Tc'slp*, were classified as secondary pair rule genes, since their expressions depend on the regulation of the primary pair rule genes *Tc'eve*, *Tc'run* and *Tc'odd*. Accordingly, *Tc'prd* and *Tc'slp* do not regulate the expression of any of the primary pair rule genes. Interestingly, since *Tc'prd* and *Tc'slp* also do not regulate each other, a clear canonical pair rule phenotype (loss of alternating segments) is observed after pRNAi experiments for either *Tc'prd* or *Tc'slp* (Choe et al., 2006).

The regulatory interactions among the primary pair rule and on the secondary pair rule genes lead the authors to suggest a genetic circuit where *Tc'eve* is required for the activation of *Tc'run*, which, in turn, is required for the activation of *Tc'odd*.

Furthermore, the activation of *Tc'odd* by *Tc'run* is essential for the repression of *Tc'eve* in its interstripes regions (Figure 20). The authors also demonstrated that the effects on the regulation of the secondary pair rule by the primary pair rule genes are most likely mediated by *Tc'run*. Therefore in *Tribolium*, *Tc'eve* and *Tc'odd* would be required for the proper establishment of run expression in stripes, resulting in proper metamerization via the regulation of the secondary pair rule genes by *Tc'run*.

In the results presented here, the expression pattern of eve is clearly disrupted in *Tc'run*[pRNAi] embryos, at the blastoderm stage (not shown). However, *Tc'eve* expression refines into stripes during further development (Figure 19B, D, F), even in embryos displaying the most severe phenotype as reported by Choe et al. (2006) (compare Figure 19F with Figure 2M in Choe et al., 2006).

Furthermore, the expression of *Tc'run* was reported to be dependent on *Tc'eve* activation (Choe et al., 2006). Conversely, in embryos displaying the strongest *Tc'eve*[pRNAi] phenotype *Tc'run* is still expressed (Figure 19H, J, L). The ectopic expression of *Tc'gsb*, which is seen as a broad domain in these embryos, serves as a control for the penetrance of the phenotype (Figure 19H, J, L)

It is unlikely that the expression pattern presented here represent any contamination or unspecific detection methods during the in situ hybridization, since these effects would result in a reduced detection of expression.

It has already been observed that injections of dsRNA experiments for different target genes might lead to the contamination of the injection tool, thus resulting in a combined effect of both genes in the knockdown embryos (Bucher and Schroder personal communication and own observations). When the expected phenotypes are distinct, such an incident can be discriminated on the resulting phenotype. In this case, the phenotypes observed after knockdown of either *Tc'eve* or *Tc'run* can be morphologically almost indistinguishable (compare Figure 19D with J) even at the cuticular level (Figures 1b and c in Choe et al., 2006).

Therefore, a combined effect of these genes would also lead to highly similar embryo morphology. In case one of the genes is not completely dependent on the other gene (as we suggest for the effect of *Tc'eve* in *Tc'run*); a combined effect would abolish the remaining expression of the co-injected gene in these embryos without changing significantly the embryonic or cuticular phenotype.

Nevertheless, results presented here do not invalidate the model proposed by Choe et al. (2005), but our findings that stripes of expression are still seen for *Tc'run* and *Tc'eve* in each other's knockdown suggest that the factors involved in the reported analysis do not completely explain the proposed model. The effects observed on pair rule stripes in embryos depleted for gap genes might indeed indicate that the circuit need gap gene input, perhaps in the transition from syncytial to cellularized segmentation.
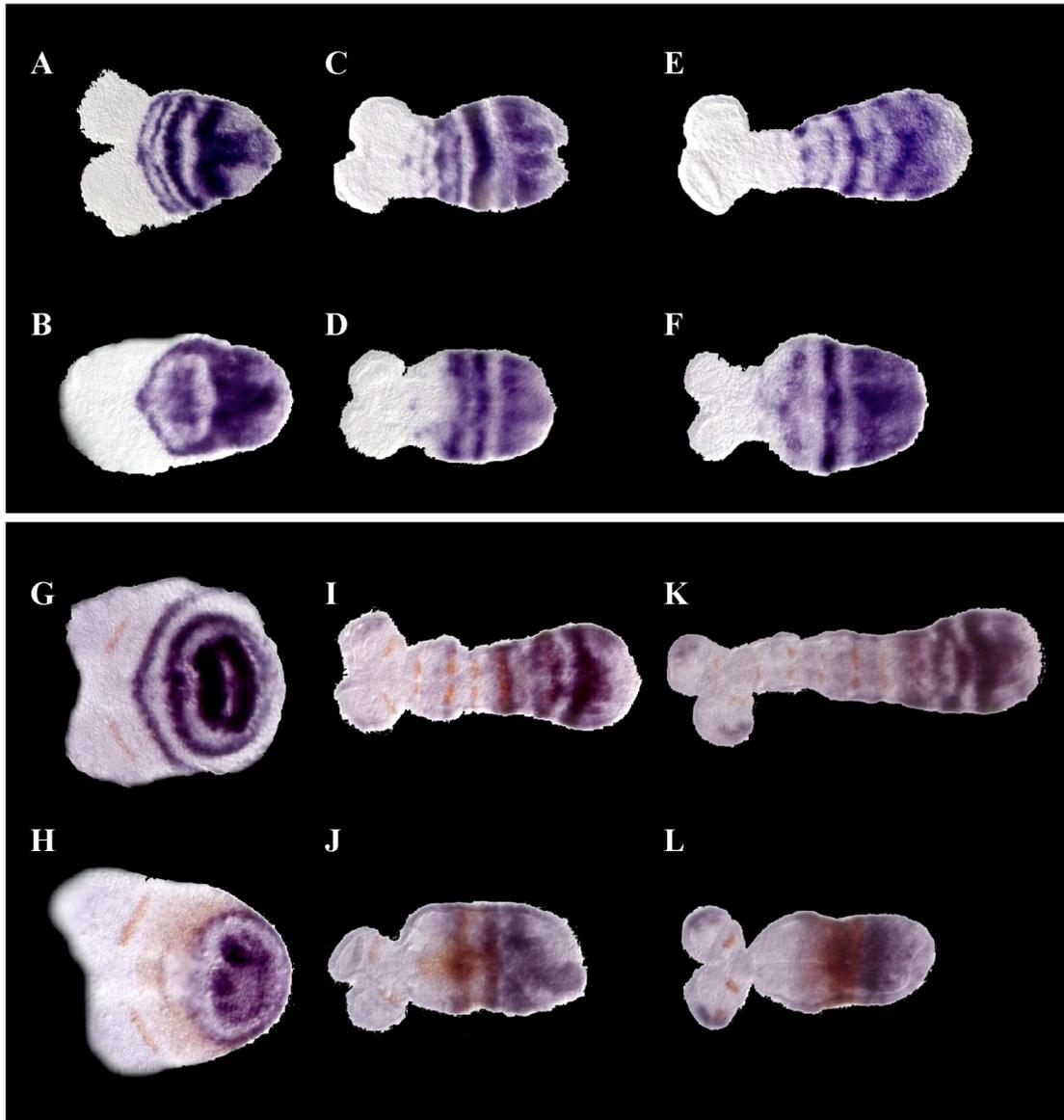
Figure 19. Analysis of the expression of *Tc'eve* in (A, C, E) wild type and (B, D, F) *Tc'run*^pRNAi embryos. Expression of *Tc'run* in (G, I, K) wild type and (H, J, L) *Tc'eve*^pRNAi embryos. Stripes of *Tc'eve* expression is observed in *Tc'run*^pRNAi embryos after blastoderm stage (D, F). Expression of *Tc'run* is still visible in *Tc'eve*^pRNAi embryos and expression stripes are visible in J. (G-L) Embryos double stained for *Tc'run* (blue) and *Tc'gsb* (red). (H, J, L) *Tc'eve*^pRNAi embryos displaying expression of *Tc'gsb* in a broad central domain, indicating strong effect in the segmentation process in these embryos.

Discussion

The analysis of gap gene orthologs in short germ insects has so far been mainly restricted to single gene functions, characterized by the use of molecular markers. One exception is the recent study on the orthologs of the pair rule genes in *Tribolium* that provided regulatory information leading to the suggestion of a pair rule gene circuit (Choe et al., 2006).

This thesis represents the first extensive analysis of gap gene orthologs in a short germ insect. The simultaneous analysis of an extensive number of segmentation genes provides a powerful data set on the regulatory interactions among segmentation genes, (1) contributing to a working model for the regulatory network of segmentation genes in *Tribolium* and (2) enabling consistent interpretation of loss of function phenotypes in *Tribolium*.

Cross-regulation among gap genes: same affairs, different intentions

Several interactions among gap genes observed in *Tribolium* seem to be conserved from the ones described in *Drosophila* and in *Nasonia*. *Tc'hb* acts as an activator of *Tc'Kr* expression (Figure 10), while *Tc'gt* act as repressor of *Tc'Kr* (Figure 20). Additionally, the expression of *Tc'Kr* in the trunk is required for the repression of *Tc'hb* in this region (Figure 21). Nevertheless, the results showed that *Tc'Kr* represses the posterior *Tc'hb* domain, a role that is not known from *Drosophila*. This negative effect of *Tc'Kr* on the posterior *Tc'hb* domain could be indirect, due to the distinct spatiotemporal expression of both genes. However, at the blastoderm stage, *Tc'Kr* is expressed in the posterior egg pole, in the cells that will give rise to the growth zone. It is possible, therefore, that in these cells the expression of *Tc'Kr* acts directly on the *Tc'hb* locus to silence the expression of *Tc'hb* until later stages of germ band elongation. In *Drosophila*, *Dm'hb* has been shown to act directly as a "long range" silencer of *Dm'Ubx* by recruiting PcG proteins that maintain, throughout development, the repression domain established by *Dm'hb* at the blastoderm stage (Kehle et al., 1998; Zhang and Bienz, 1992). In *Tribolium*, one can speculate that *Tc'Kr* might repress *Tc'hb* in a similar way. At later stage during germ band

elongation, additional factor(s) would suppress the silencing effect, activating *Tc'hb* expression in the posterior abdominal segments.

In summary, the interactions among the *Tc'hb, Tc'Kr* and *Tc'gt* observed in *Tribolium* are specified in different regions of the embryo compared to *Drosophila* and *Nasonia*. While in Drosophila the gap genes interactions are essential for the regulation of the pair rule genes, in *Tribolium* these interactions are important for the regulation of the Hox genes (Figure 23).

Interestingly, anterior patterning in *Drosophila* and *Nasonia* has been recently shown to be established by two independent mechanisms, despite the similar expression pattern of the genes involved in these mechanisms (Brent et al., 2007). In *Drosophila*, the anterior *Dm'hb* domain is formed by a combined input of Dm'Bcd and Dm'Hb (Simpson-Brose et al., 1994). The central *Dm'Kr* domain is in turn established by the activation by Dm'Bcd and Dm'Hb and responds to in a concentration-dependent maner to levels of Dm'Hb expression (Schulz and Tautz, 1994). In *Nasonia,* the anterior expression domain of *Nv'hb* is established by a combination of the instructive role of *Nv'otd1* in activating *Nv'hb* in the anterior domain and the permissive role of *Nv'gt* in repressing *Nv'Kr* that would otherwise repress *Nv'hb*. In spite of the distinct gene combinations use for anterior patterning in both organisms, both mechanisms involve the localization of maternally provided RNA. It was therefore proposed, based on the basal phylogenetic position of Nasonia (Savard et al., 2006c), that the mechanism involving the *bcd* gene in the fly took over the ancestral role of *gt* and *otd* as maternal determinants, thereby restricting *gt* and *otd* to zygoti gap genes in the fly (Brent et al., 2007).

These observations corroborate the assumption that the genetic toolkit controlling insect segmentation, and most of the developmental processes, would consist of essentially the same set of genes displaying precise and at the same time flexible interaction ability that can be recruited for different developmental tasks.

Based on these observations, an evolutionary scenario can be proposed for the role of *hb*. Among protostome (nematodes, annelids and arthopods), *hb* is expressed in extraembryonic epithelium and in the nervous system (Fay et al., 1999; Savage and Shankland, 1996; Werbrock et al., 2001). Within arthropods, this expression was co-opted into the anterior 'gap' expression. This anterior expression became then essential for patterning the AP embryonic axis in insects through the regulation of

other gap genes and Hox genes. In the lineage leading to the higher dipterans, the role of *hb* became essential for the regulation of the pair rule and segment polarity genes controlling the metamerization process.

Segmentation genes - wild type
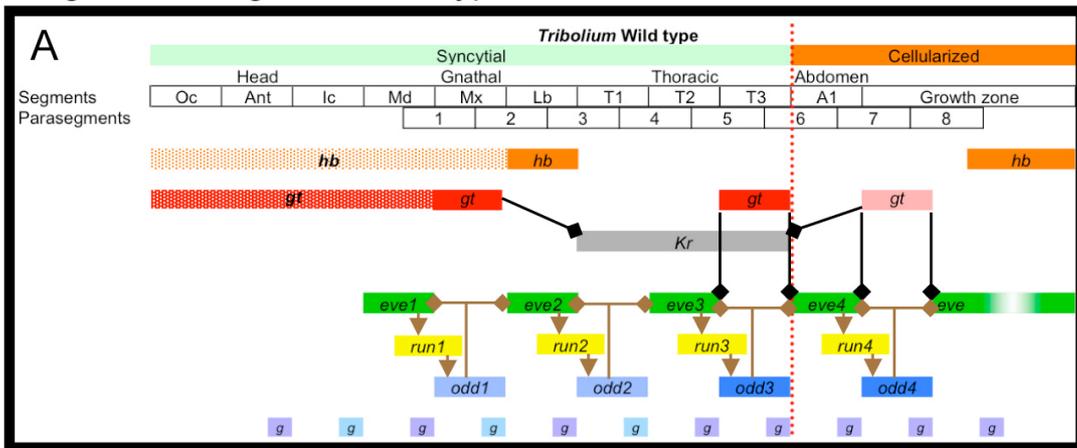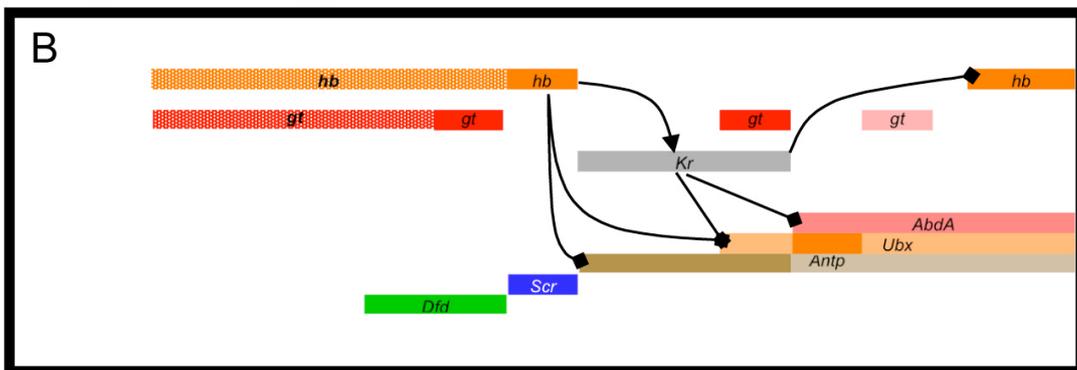
Hox genes - wild type

Segmentation genes - *Tc'gt* pRNAi
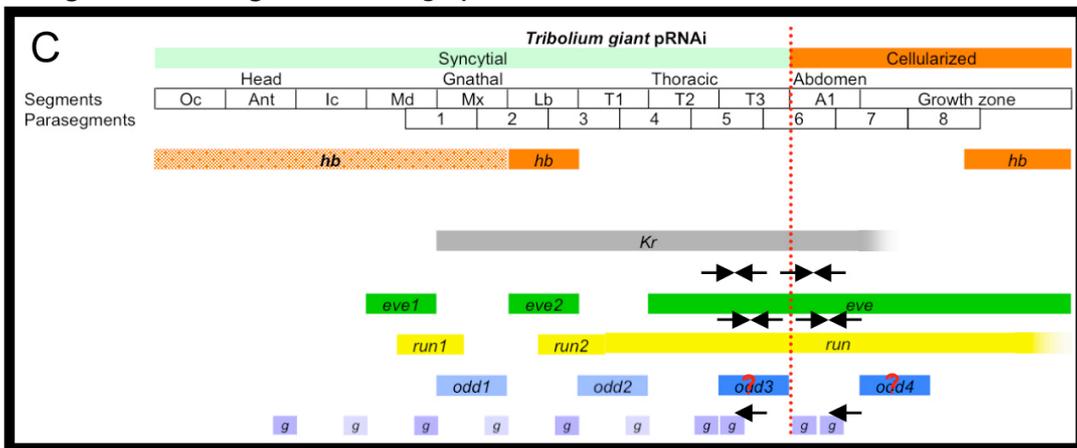
Hox genes- *Tc'gt* pRNAi
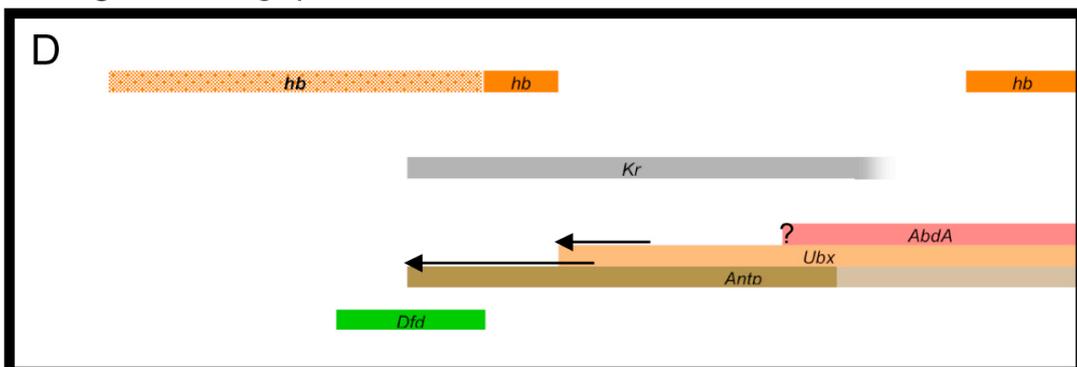
Figure 20. Schematic drawing of the segmentation cascade in *Tribolium*. (A) Wild type (B) *Tc'gt* pRNAi. (A) The wild type function of *Tc'gt* in the segmentation process is to regulate pair rule genes in thoracic and anterior abdominal segments. (C) In *Tc'gt*^pRNAi^ embryos, the expression of *Tc'eve* and *Tc'run* are affected in this region. (B) The role of *Tc'gt* in segment identity specification is mediated via *Tc'Kr*. (D) In *Tc'gt*^pRNAi^ embryos, *Tc'Kr* expression expands and represses the abdominal homeotic genes *Tc'Ubx* and *Tc'AbdA*. The *Tc'Kr* expansion leads to the expassion of the thoracic domain of *Tc'Antp*, resulting in homeotic transformation of gnathal and abdominal segments into thoracic. No data is available for the expression of *Tc'AbdA* in *Tc'gt*^pRNAi^ embryos.

Segmentation genes - wild type
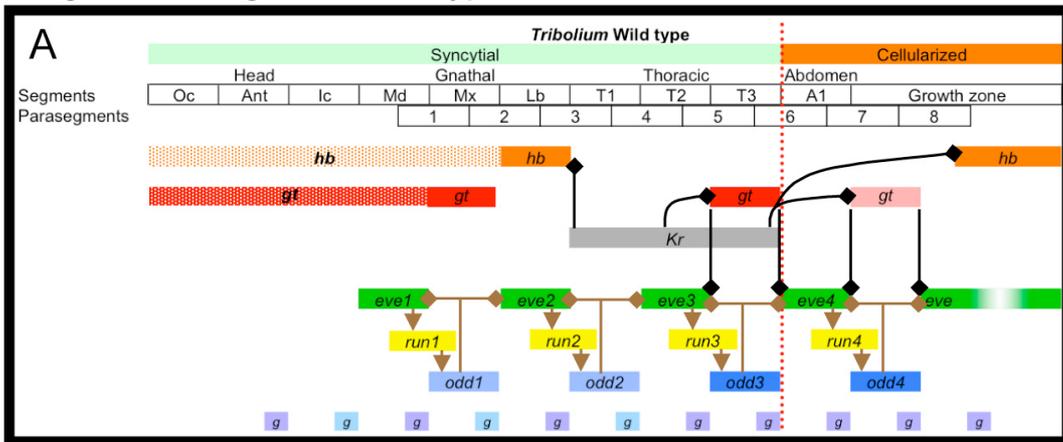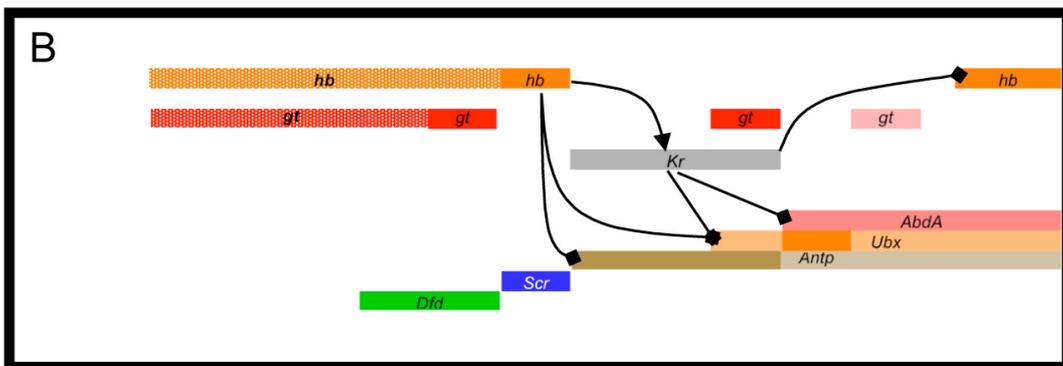
Hox genes - wild type

Segmentation genes - *Tc'Kr* pRNAi
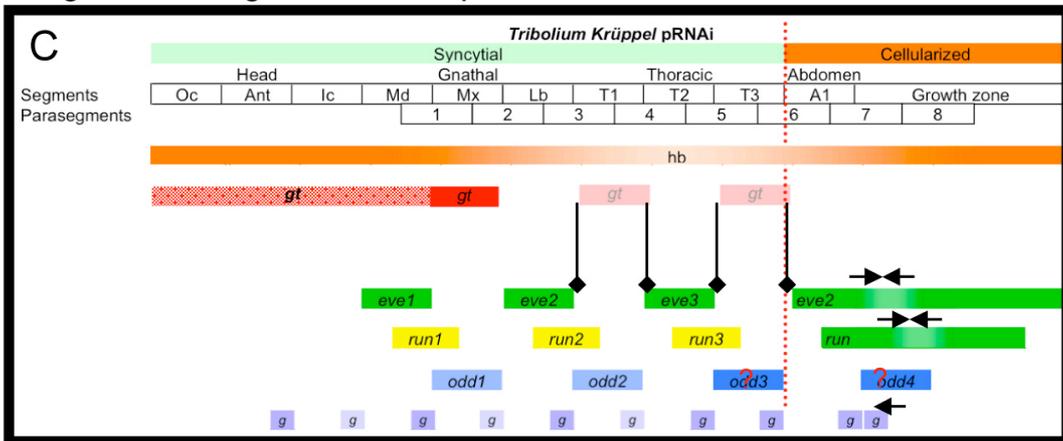
Hox genes - *Tc'Kr* pRNAi
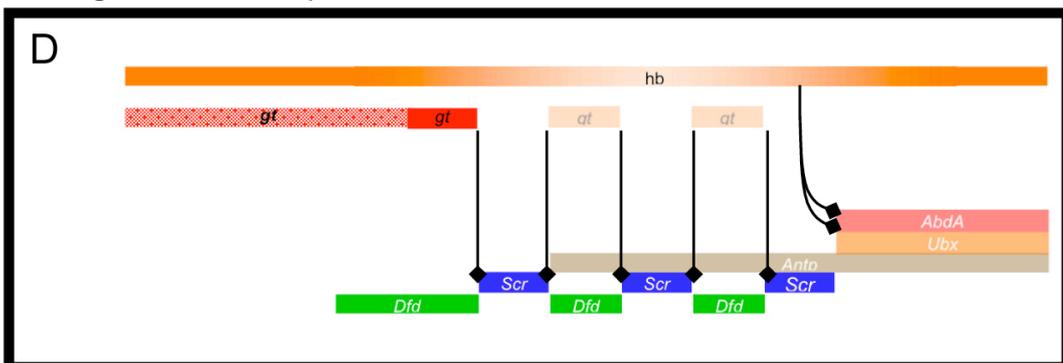
Figure 21. Schematic drawing of the segmentation cascade in *Tribolium*. (A) Wild type (B) *Tc'Kr* pRNAi. (A) The wild type function of *Tc'Kr* in the segmentation process is mediated via *Tc'gt*. (C) In *Tc'Kr*^pRNAi embryos, the lack of the A2 *Tc'gt* stripe leads to the misexpression of *Tc'eve* and *Tc'run* are affected in this region. (B) The role of *Tc'Kr* in segment identity specification is performed by the repression of *Tc'hb* expression in thoracic and anterior abdominal segments as well as the proper expression of *Tc'gt*. (D) In *Tc'Kr*^pRNAi embryos, *Tc'hb* ubiquitous expression represses the thoracic domain of *Tc'Antp* as well as the expression of *Tc'Ubx* and *Tc'AbdA*. The repression of posterior homeotic genes leads to the posterior expansion of anterior homeotic genes (elimination of the posterior prevalence). The ectopic expression of *Tc'hb* and *Tc'gt* in *Tc'Kr*^pRNAi embryos lead to the ectopic expression of *Tc'Dfd* and *Tc'Scr* in alternating trunk segments.

General Discussion

In the work of von Baer (1828), on the history of animal development, he postulated that the general features of a broad animal type appear earlier in the embryo than the special features (Translation in Gould, 1992). In other words, a feature conserved among all individuals of a group of animals tends to take place earlier in development when compared to more specialized ones. Therefore embryogenesis, the first steps of animal development, retains similar features among related organisms regarding the embryo formation. Specifically, embryogenesis in different species within a phylum pass through an early stage where all embryos are converged into a very similar morphology, the phylotypic stage. In insects, this stage corresponds to the fully extended and segmented germ band (Anderson, 1973). Accordingly, the genes expressed and operating at this stage show high sequence similarities and conserved expression patterns throughout Arthropoda.

The beetle *Tribolium castaneum* has become an attractive model organism for developmental biologists since it shares the short germ embryogenesis mode with most other insects. Although most of the regulatory genes described in *Drosophila* have been already identified in *Tribolium*, their functional characterization has usually been done as single gene analysis using molecular and morphological markers. One exception is the recent characterization of the pair rule gene orthologs (Choe et al., 2006). In this work, the simultaneous analysis of the orthologs of the *Drosophila* pair rule genes in *Tribolium* revealed the genetic interactions of the pair rule patterning in *Tribolium* and enabled the authors to propose a gene circuit model for the role of the pair rule genes in the segmentation of *Tribolium*.

Similarly, the concomitant analysis of segmentation genes described in this thesis revealed the genetic interactions underlying the regulatory network controlling trunk segmentation in *Tribolium*. Furthermore, it demonstrates that the expression domains of the gap genes, although conserved among insects, diverge significantly between short and long germ insects in respect to their role at distinct steps of the segmentation process.

The cardinal information of the gap genes

The gap gene definition was originally assigned to the segmentation genes that, when mutated in *Drosophila*, lead to "one continuous stretch of segments deleted" in the larval cuticle (Nüsslein-Volhard and Wieschaus, 1980). Although often exceeding their expression domains, the deletion of segments observed in gap gene mutants always coincides with the expression domains of the respective gene in the wild type.

In the late 80's, the identification of segmentation genes acting at the same level of the gap genes in the segmentation cascade and regulating pair rule gene expression without showing a gap phenotype at the cuticular level, was the fisrt evidence that the term "gap gene" could be misleading as a general concept (Gaul and Jäckle, 1990). From the 14 segmentation genes currently classified as having a gap gene function (The Interactive Fly), the majority of them (around 10) display no continuous stretch of segments deleted when mutated in *Drosophila*. Instead, these genes were classified as gap genes due to their role within the segmentation hierarchy in providing positional cues for the regulation of pair rule genes. Whether the genes classified as gap genes truly lead to a gap phenotype in the larva, seems to depend on the extent to which these genes are regulating the pair rule genes. In other words, mutations in segmentation genes that regulate adjacent pair rule gene stripes would result in the deletion of adjacent segments.

The expression pattern of the *Drosophila* gap genes as well as their level in the segmentation hierarchy seems to be conserved among insects. Although the cuticular phenotype observed after depletion of gap genes superficially display a continuous stretch of missing segments, this impression results from a combination of transformation as well as segment deletions whereby the missing segments often lie outside of the expression domain of the knockdown gene in the wild type (this thesis; Liu and Kaufman, 2004; Mito et al., 2006).

Compared to other insects, the results presented here further support the assumption that all the segmentation genes expressed in broad domains prior to, and regulating the expression of the pair rule genes (classified as gap genes), share a common feature; they provide refined positional information along the AP axis that serves as essential molecular cue for the expression of downstream genes. The lack of

this positional information results in pattern disturbance that might, or might not, lead to a loss of adjacent segments (classical gap phenotype). In most of the cases, disruption of gap gene function results in the loss of single or few segments and subsequent homeotic transformations.

This essential role of the gap genes in establishing positional information along the embryonic AP axis led Mainhardt (1986) to suggest the term 'cardinal' to define the function of this class of genes. The short-range gradient formed by gap gene proteins would form cardinal regions with adjacent gap genes overlapping their borders of expression. Therefore the distribution of the cardinal regions and their overlapping borders along the AP axis would provide the necessary positional cue for the regulation of the double segmental expression pattern of the pair rule genes (Meinhardt, 1986; Ingham et al., 1986).

This terminology was further supported by Akam (1987) who suggested the use of the term 'cardinal gene' to describe this class of segmentation genes.

This thesis provides further support for the use of the 'cardinal gene' definition to specify the class of segmentation genes that are maternally activated, display cross-regulatory interactions and regulate primary pair rule and hox gene expression.

The cardinal function of *hunchback* in the beetle *Tribolium castaneum*

The molecular and morphological characterizations of the *Tc'hb* gene presented here provide the base for a reinterpretation of the ancestral role of *hb* in holometabolous insects. The extensive regulation of hox genes and the probably indirect regulation of pair rule genes, reject the canonical gap function previously suggested for *Tc'hb*. The results present here corroborate with the universal role of the genes *hb*, *Kr* and *gt* as 'cardinal genes', providing compartments with positional information along the AP axis during early steps of embryogenesis in all insects.

A cardinal function of *hunchback* in *Drosophila*?

As proposed in this thesis, the main evolutionary difference between the roles of *hb* in short and long germ insects lies in the regulatory interactions between the gap

genes and their target genes (see above). While the lack of *Dm'hb* and *Dm'Kr* in *Drosophila* results in misregulation of the pair rule stripes within their expression domains, in short germ insects, depletion of these genes cause no effect on the pair rule pattern where *hb* or *Kr* are expressed in the wild type (Chapter I, Cerny et al., 2005).

In this section I would like to review the phenotype described for the *Dm'hb* gene based on the results presented by White & Lehmann (1986). In this work, the authors describe an interesting phenomenon during the development of the *Dm'hb* phenotype, which is observed only during embryogenesis. Immunohistochemical analysis of these embryos using an antibody against Dm'Ubx, allows visualization of the metameric pattern in the developing embryo as it is first established (Figure 22).

In class III (weak) *Dm'hb* mutants, the region where four metameres should have formed (corresponding to two thoracic and two abdominal segments), only two large metameres spanning this entire region are formed. Due to a resizing process via cell death, these two enlarged metameres approach wild type width later in development (White and Lehmann, 1986). Since *Dm'Ubx* is ubiquitously expressed in these embryos, the two enlarged metameres, although containing primordial cells of the thoracic segments, are specified as abdominal segments. The phenotype is therefore characterized as a loss of T2 and T3 (Lehmann & Nuesslein-Volhard, 1986).

Therefore, the canonical gap phenotype described for *Dm'hb* is not a result of the deletion of gnathal and thoracic segments. Instead, the cells forming each of these segments in the wild type are incorrectly patterned as part of two large metameres and the deletion occurs through loss of cells that are distributed throughout these enlarged metameres (Figure 22).

Intriguingly, the posterior border of the anterior *Dm'hb* expression domain is thought to lie around the (PS4), i.e. between the T1 and T2 segments. This posterior border slightly overlaps with the anterior border of the *Dm'Kr* domain. In wild type, *Dm'Kr* is expressed in the primordia of the three thoracic and the first five abdominal segments (Hoch et al., 1990). Notably, as in short germ insects, the fusion of thoracic and abdominal segment primordia seems to lay outside of the wild type *Dm'hb* expression domain. While in *Tribolium* the segmentation role of *Tc'hb* is most likely mediated via the regulation of *Tc'gt*, in *Drosophila*, this role could be indirectly mediated via the regulation of the central *Dm'Kr* domain. In *Dm'hb* mutant embryos,

the central expression of *Dm'Kr* is expanded anteriorly. In stronger phenotypes, the additional deletion of segments could therefore be caused by the increased expansion of *Dm'Kr* towards anterior. Noteworthy, it has been shown that the *Dm'eve* stripe 2 enhancer contains one Dm'Hb binding site that was experimentally proven to be necessary for the activation of this stripe.

One way to test the hypothesis presented here could be by introducing a copy of a *Dm'hb*-independent *Dm'Kr* gene in *Dm'hb* mutant embryos. If the gap phenotype observed in *Dm'hb* mutants is indirectly mediated via regulation of *Dm'Kr*, one would expect that the resulting phenotype from the proposed transgenic line would reflect a major homeotic transformations and minor segmentation defects.

Alternatively, embryos carrying the homeotic *Dm'hb* alleles could be analyzed for the expression of *Dm'Kr*. These alleles are mutated only in the conserved domains involved in the repression of trunk Hox genes (C and D boxes). The functional *Dm'hb* domains that have been shown to mediate *Dm'Kr* expression are unaltered in these mutant embryos (Hulskamp et al., 1994).

Similarly, the segmentation defect observed in posterior segments (PS13) in *Dm'hb* mutant embryos could be indirectly mediated by the posterior shift of the *Dm'gt* posterior domain in these embryos. This effect would be similar to the one reported for *Tc'hb* (Chapter I). If this is hypothesis is correct, than the main difference between the roles of *hb* in *Tribolium* and *Drosophila* would be the change in the regulation of pair rule genes by *Kr* in *Drosophila*.
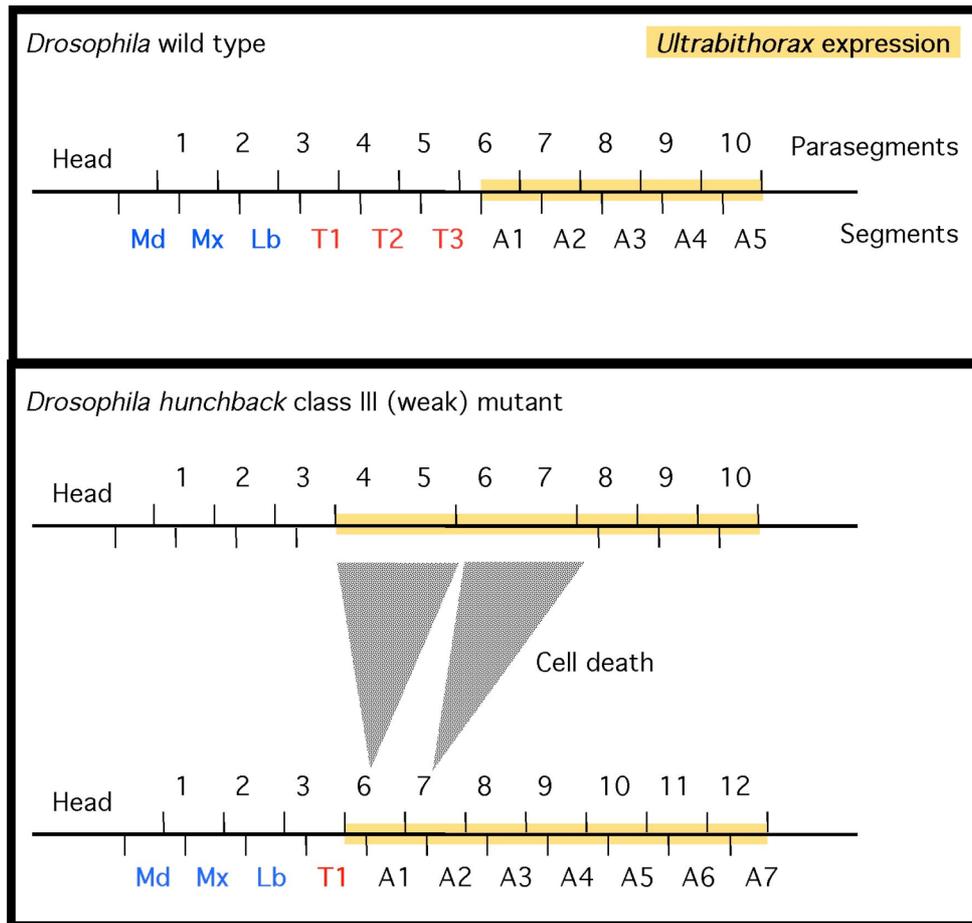
Figure 22. Schematic drawing of the development of a Dm'*hb* mutant phenotype. In *Dm'hb* mutants thoracic and anterior abdominal segments are formed as part of two enlarged metameres. By a segment resizig mechanism the cells that should formed the primordia of T2, T3, A1 and A2 in the wild type are formed as A1 and A2 in *Dm'hb* mutants, due to the segment fusions and the ectopic expression of *Dm'Ubx* in the *Dm'hb* mutants embryos.

A cardinal function of *hunchback* in *Nasonia*?

The proposal of a reinterpretation of the function of *Dm'hb* raised the question whether the *Nasonia hb* (*Nv'hb*) phenotype could help to clarify the role of *hb* in long germ insects. The recently proposed new phylogenetic position of *Nasonia* at the base of the holometabolous insects suggests that the long germ mode of embryogenesis found in *Nasonia* has evolved independently from the one found in *Drosophila*.

The molecular characterization of the phenotype of the *Nv'hb* mutant embryos was performed by the analysis of the patterns of Nv'Ubx and Nv'Abd-A. The phenotype displays loss of head and thoracic segments as well as the three most posterior segments (Pultz et al., 2005). Although more extensive than in *Drosophila*, the deletions observed in *Nv'hb* mutant embryos were interpreted as the deletions of the segments where *Nv'hb* is expressed (canonical gap).

As in *Drosophila* and in all insects analyzed, the lack of *Nv'hb* leads to the expansion of Nv'Ubx expression towards anterior segments. However, the formation of the metameric pattern during the development of the *Nv'hb* mutant embryos was not analyzed using molecular or morphological markers. It is therefore tempting to ask whether the deletions observed in the *Nv'hb* phenotype are indeed restricted to the segments were *Nv'hb* is expressed in the wild type. With the ubiquitous expression of Nv'Ubx in *Nv'hb* mutant embryos, any segment formed in these embryos, no matter if head or trunk, would display abdominal identity at later stages as observed in all other insects.

Based on the cardinal role of *Tc'hb* described here, it is nevertheless likely that the *hb* gene present in the last common ancestor of *Drosophila* and *Nasonia* was a cardinal gene involved in the regulation of Hox genes with a minor role in regulating pair rule genes.

The analysis of pair rule genes in *Nv'hb* mutant embryos could shed light on the role of *Nv'hb* in the segmentation process in this insect and further test the homology of the segmentation process between these two long germ insects.

The genes *hunchback* and *orthodenticle* substitute for *bicoid* in *Tribolium*

Anterior patterning in *Drosophila* is performed by a synergistic activity of the genes Dm'Bcd and Dm'Hb (Simpson-Brose et al., 1994). The activation of head and thoracic gap genes by Dm'Bcd is also dependent on the expression of Dm'Hb. Although *hb* orthologs have been identified in all arthropods analyzed so far, *bcd* orthologs are only present in higher dipterans (Brown et al., 2001). This evolutionary paradigm has long stimulated the identification of genes and gene regulatory interactions that might establish anterior patterning in the absence of a *bcd* ortholog (Lynch et al., 2006b; Schroder, 2003; Wolff et al., 1998).

The *hb* gene is a good candidate for this role due to its conservation among arthropods and the fact that several experiments in *Drosophila* have shown that *Dm'hb* can rescue gnathal and thoracic segments in *Dm'bcd* mutant embryos (Hülskamp et al., 1990; Schulz and Tautz, 1994; Struhl et al., 1992; Wimmer et al., 2000). The rescue is however never complete, supporting the synergistic role between *Dm'hb* and *Dm'bcd* genes (Wimmer et al., 2000). Another candidate to replace *bcd* is the *otd* gene. Also conserved throughout metazoa, *otd* encodes for a homeodomain protein that contains a lysine at position 50 (K_{50}), which is also present in the *bcd* gene and is required for its DNA and RNA biding specificity (Gao and Finkelstein, 1998).

In *Tribolium*, the genes *Tc'otd* and *Tc'hb* were suggested to act synergistically in the formation of almost all body segments (Schroder, 2003). While RNAi experiments for either *Tc'otd* or *Tc'hb* lead to the deletion of head and thoracic segments, double knockdown of *Tc'otd* and *Tc'hb* results in deletion of head, thoracic and almost all abdominal segments, resembling the strong *Dm'bcd* mutant phenotypes. Based on the results described here, I propose an additive instead of a synergistic effect in the phenotype observed in *Tc'otd* and *Tc'hb* double knockdown embryos.

Strong depletion of *Tc'otd* leads to the loss of all head segments and the first thoracic segment. All segments after T2 are normally formed in these larvae. Weaker *Tc'otd* phenotypes display the additional formation of more anterior segments (Schroder, 2003). In contrast to this, the strongest phenotype described here for *Tc'hb*, forms anterior segments normally up to the thorax with and no further segments

posterior to T3 and A2. Weaker *Tc'hb* phenotypes display the additional formation of more posterior segments (Figure 3B).

When both gene functions are disrupted, the segmentation of the head up to T1 is affected by the lack of *Tc'otd* activity while the segmentation of T3 and most of the abdominal segments is affected by the lack of *Tc'hb*, with only T2 and A1 segments being patterned independently. With the ubiquitous expression of *Tc'Ubx* and *Tc'AbdA* in these embryos, both remaining segments (T2 and A1) would be specified as abdominal segments. This hypothesis explains the germ band shown by Schroeder (2003), which displays a strong *Tc'hb+otd*[pRNAi] phenotype, with only two abdominal engrailed stripes formed, with the anlage for the hindgut and the malpighian tubules posterior to it. These structures are also observed in the strongest phenotypes for *Tc'hb* alone (not shown).

Therefore, I propose that *Tc'otd* and *Tc'hb* are responsible for patterning adjacent, but not overlapping regions along the *Tribolium* embryo. Noteworthy, the synergistic effect proposed for Dm'Hb and Dm'Bcd is still not fully demonstrated and further experiments involving promoter analysis with combinations of binding sites for Dm'Hb and Dm'Bcd would need to be performed (Tautz, personal communication).

Thoracic identity in *Tribolium*: differential levels of *Antennapedia* expression

The Hox gene *Dm'Antp* is required for the proper development of thoracic segments. *Dm'Antp* loss-of-function alleles result in homeotic transformations of thoracic segments into structures characteristic of more anterior segments (Abbott and Kaufman, 1986; Schneuwly et al., 1987; Struhl, 1981; Wakimoto and Kaufman, 1981). Ectopic expression of *Dm'Antp* in more anterior segments results in the transformation of the adult antennae by thoracic structures (e.g., Schneuwly et al., 1985). Outside *Drosophila*, the function of *Antp* orthologs is so far unclear. Nevertheless, *Antp* expression has been widely used together with other Hox genes in segment homology studies among arthropods (Averof and Akam, 1993; Averof and Akam, 1995; Hughes and Kaufman, 2002; Walldorf et al., 2001; Walldorf et al., 1989; Zhang et al., 2005).

Here I propose a model for the regulation and function of *Tc'Antp* in *Tribolium*, based on analyses of the *Tc'Antp* expression in several gene silencing experiments and the resulting phenotypes observed.

In wild type embryos, *Tc'Antp* is expressed in the entire thoracic and abdominal regions (Figure 8E or Figure 14A). After the germband extension is completed, the level of *Tc'Antp* expression increases in the entire thoracic region, preceding the development of the limbs (Figure 8E or Figure 14A). With the expansion of the *Tc'Antp* domain in *Tc'hb*^pRNAi^ embryos, expression of *Tc'Antp* is still observed in the entire abdominal trunk (Figure 8E), even though no segment with thoracic identity is formed in these embryos (Figure 3C). Notably, when compared to the different levels of *Tc'Antp* expression in the wild type, it is evident that the ectopic expression observed in *Tc'hb*^pRNAi^ embryos is comparable to the abdominal expression observed in wild type embryos (Figure 8E, F). Therefore one can conclude that the enhanced thoracic expression of *Tc'Antp* is absent in *Tc'hb*^pRNAi^ embryos.

The enhanced expression of *Tc'Antp* is shifted posteriorly in *Tc'mlpt*^pRNAi^ embryos (Figure 14A,B) is analogous to the effects of *Tc'Antp* in the *Tc'hb*^pRNAi^ phenotype. This posterior shift of *Tc'Antp* results in the transformation of anterior abdominal segments into segments of thoracic identity (Figure 11).

This observations lead to the hypothesis that the development of thoracic segments in *Tribolium* does not depend solely on the presence of *Tc'Antp* expression, but needs a certain threshold of *Tc'Antp* activity to be triggered. When *Tc'Antp* is expressed at low levels, thoracic development is suppressed (wild type abdomen and *Tc'hb*^pRNAi^ embryos; Figure 3). When *Tc'Antp* is expressed at high levels, thoracic development is triggered (wild type thorax and *Tc'mlpt*^pRNAi^ embryos; Figure 11).

Further support for this theory comes from the analysis of *Tc'gt*^pRNAi^ embryos. Embryos depleted of *Tc'gt* display homeotic transformation of gnathal and abdominal segments towards thoracic identity (Bucher and Klingler, 2004). Once again, the strong expression domain of *Tc'Antp* is expanded in gnathal and abdominal segments (Cerny et al., 2005).

To further strengthen this hypothesis, I suggest two possible alternatives for the regulation of *Tc'Antp*.

First, the expression of the gap gene *Tc'Kr* would be required to increase *Tc'Antp* expression in the thoracic region. However, although *Tc'Kr* expression starts

at the blastoderm stage, when the increased *Tc'Antp* expression in the thorax is observed, the thoracic expression of *Tc'Kr* is no longer detectable (compare Figures 6E and Figure 14A).

Therefore, the role of *Tc'Kr* on activating *Tc'Antp* could be indirect via repressing the repressors of *Tc'Antp* in the thoracic region.

Indeed, the gene *hb* acts as a repressor of *Antp* several insects (this thesis; Irish et al., 1989b; Mito et al., 2005). In *Tc'Kr*[pRNAi] embryos, the ubiquitous expression of *Tc'hb* would result in the repression of *Tc'Antp*.

A second source of repression of *Tc'Antp* would come from the posterior prevalence rule among Hox genes (see above). In *Tc'hb*[pRNAi] embryos, the expressions of *Tc'Ubx* and *Tc'AbdA* are shifted anteriorly (Figure 8H, J). This shift would lead to the repression of *Tc'Antp* in these embryos. Likewise, in *Tc'mlpt*[pRNAi] embryos, the expressions of *Tc'Ubx* and *Tc'AbdA* are drastically reduced or absent (Figure 14D, H), therefore allowing the expansion of the strong expression of *Tc'Antp* in abdominal segments (Figure 14B).

Therefore the wild type function of *Tc'Kr* would be to prevent the expression of *Tc'hb* and the Hox genes *Tc'Ubx* and *Tc'AbdA* in the thoracic region allowing the increased levels of *Tc'Antp* necessary for triggering thoracic morphogenesis in these segments (Figure 21).

Still, it is very likely that as in *Drosophila*, these repression domains established by *Tc'hb* and *Tc'Kr* are being maintained throughout development by the recruitment of additional factors as the PcG proteins (Lewis, 1978; Struhl 1981; Struhl & Akam 85; Breen & Ducan, 86; Juergens, 85; Soto, 95).

Hox genes domains in *Tribolium*: between legs and mouth, a gap choice

In *Tribolium*, *Tc'Kr* mutant larva display normal head and gnathal segments (Cerny et al., 2005). The following four to six segments, however, are transformed into a series of alternating mx and lb. At the molecular level, this phenotype is generated by the misexpression of the gnathal Hox genes *Tc'Dfd* and *Tc'Scr*. In addition to the wild type expression of *Tc'Dfd* and *Tc'Scr* in the mx and lb, respectively, both genes are ectopically expressed in a double segmental periodicity in the thorax and abdomen of *Tc'Kr* mutant embryos. The effect on *Tc'Dfd* and *Tc'Scr*

expressions observed in *Tc'Kr* mutants was suggested to involve an underlying pair rule mechanism generating the double segmental expression (Cerny et al., 2005).

Remarkably, the analysis of the cross-regulatory interactions among gap genes and their influence on Hox gene expression provide an attractive explanation for the effects observed in the *Tc'Kr* mutant phenotype.

In wild type *Tribolium* embryos, the gnathal and trunk Hox genes are expressed as the following: *Tc'Dfd* expression in the md and mx, *Tc'Scr* expression in the *lb*, high levels of *Tc'Antp* expression in the next three segments and the remaining segments express lower levels of *Tc'Antp* expression, together with *Tc'Ubx* and *Tc'AbdA* expressions (Figure 21).

In *Tribolium*, it has been shown that the gap genes act as the additional repressors on the expression of Hox genes (this thesis; Cerny et al., 2005). By modifying the configuration of the gap gene expression in the *Tribolium* embryo via pRNAi it is possible to shift back and forth the expression pattern of the several Hox genes.

The expression patterns of the gap genes in *Tc'Kr*^pRNAi embryos (Figure 21) suggest a possible repressive scenario involving cross-regulation among gap genes combined with the posterior prevalence rule among Hox genes.

In *Tc'Kr*^pRNAi embryos, Hox genes specifying gnathal structures are expressed in more posterior segments (Figure 21). Due to posterior prevalence, the posterior expansion of gnathal Hox genes requires the suppression of more posterior Hox genes in these segments. This effect is achieved by the ubiquitous expression of *Tc'hb* in these embryos (Figure 16B, D), resulting in the repression of the trunk Hox genes *Tc'Antp*, *Tc'Ubx* and *Tc'AbdA* (Figure 21). But how would the double-segmental periodicity of *Tc'Dfd* and *Tc'Scr* be established in these embryos?

To approach this question, I observed the expression of the gap genes in the segments expressing *Tc'Dfd* and *Tc'Scr* in the wild type, the mx and lb, respectively. *Tc'gt* expression in the head region shows a stripe of increased expression in the *mx* already at the blastoderm stage (Figure 21). In the *lb*, only *Tc'hb* is expressed (Figure 21). Considering a posterior prevalence of *Tc'Scr* on the expression of *Tc'Dfd*, one could hypothesize that *Tc'gt* could act as a repressor of *Tc'Scr* in the *mx,* thus allowing *Tc'Dfd* to be expressed.

Based on these observations and the results presented here, the wild type expression domains of the Hox genes in *Tribolium* would be established as the following (Figure 23): In the mx, *Tc'gt* expression represses *Tc'Scr*, establishing the *Tc'Dfd* domain; in the lb, *Tc'hb* expression represses *Tc'Antp*, establishing the *Tc'Scr* domain; and finally, in the thoracic segments, *Tc'Kr* expression represses *Tc'Ubx* and *Tc'AbdA*, establishing the *Tc'Antp* domain.

Therefore in *Tc'Kr*[pRNAi] embryos, the ubiquitous expression of *Tc'hb* represses *Tc'Antp*, *Tc'Ubx* and *Tc'AbdA* from the thoracic and anterior abdominal segments. Consequently, the more anterior Hox gene, *Tc'Scr*, is expressed in these segments. However, *Tc'Kr*[pRNAi] embryos show ectopic expression of *Tc'gt* in T1 and T3, where *Tc'gt* expression would repress *Tc'Scr*. In the segments where *Tc'Scr* is repressed, the anterior Hox gene *Tc'Dfd* is then expressed (Figure 23).

These observations suggest that a repressive scenario involving the gap genes *Tc'hb*, *Tc'gt* and *Tc'Kr* establishes the domains of the Hox genes along the AP axis in *Tribolium*. The model does not explain, however, the differential expression of the Hox genes within segments, like for example the stronger expression of *Tc'Ubx* in A1 (arrowhead in Figure 8G) and some interactions assumed here, like the repression of *Tc'Scr* by *Tc'gt*, are speculative.

Hox genes - wild type
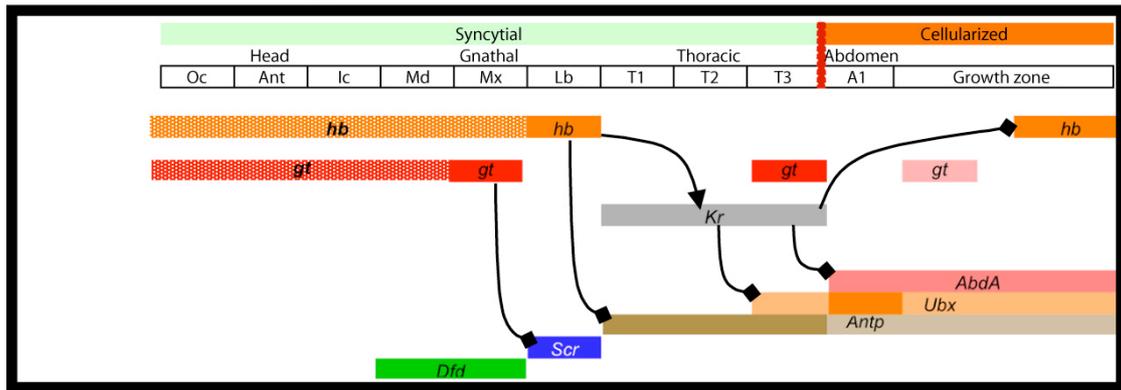
Figure 23. Schematic drawing of the establishment of Hox genes expression in *Tribolium* by gap gene regulation. The expression domain of *Tc'Kr* sets the anterior border of *Tc'AbdA* and *Tc'Ubx*, leading to the strong expression of *Tc'Antp* in the segments formed within the *Tc'Kr* domain. The expression of *Tc'gt*, and perhaps the high levels of *Tc'hb*, sets the anterior border of *Tc'Kr* and therefore of *Tc'Antp*. The next anterior segment, where only *Tc'hb* is expressed (from the one analyzed here), the *Tc'Scr* expression domain is formed with the posterior border formed by the posterior prevalence of *Tc'Antp* while the anterior border is formed by repression of *Tc'gt*. The anterior *Tc'Scr* border sets then the posterior border of the *Tc'Dfd* expression domain that expands until the mandible.

Conclusions

The best way of noticing that something is important for you is when you, in need, cannot find it. The analysis of gene function has mainly been performed by "preventing" an organism to use a certain gene. This can be achieved by techniques such as mutagenesis and RNA interference. Another way to understand the organization of a certain genetic process is to change the spatiotemporal expression of genes by expressing them ectopically. The morphological consequence of the gene loss, the lack-of-function phenotype, or the gene disturbance, gain-of-function phenotype, will reveal to which developmental process their function is important.

With the development of molecular probes, these phenotypes could be analyzed for the effects that the missing gene causes on the spatiotemporal expression of other genes. Since then, molecular characterizations of gene function have uncovered the tip of an endless iceberg of gene regulatory interactions involved in all biological processes.

This thesis represents the first characterization of the gene regulatory network among the gap genes known from *Drosophila* in the beetle *Tribolium castaneum*. The analysis involved the orthologs of the gap genes; *hb, gt* and *Kr*, pair rule genes; *eve* and *run* and Hox genes; *Dfd, Scr, Antp, Ubx* and *AbdA*. The results demonstrate that in *Tribolium*, these genes are involved in the patterning of similar embryonic regions as their homologues among insects. However, significant changes are observed in the way these genes are interacting within the network and important implications can be derived from this.

One evolutionary change that can illustrate the plasticity of the segmentation system is the role of *hb* in the metamerization process in *Tribolium* and *Drosophila*. In the beetle, this role is most likely mediated by the regulation of *Tc'gt*, with no direct effects of *Tc'hb* on pair rule genes. I therefore hypothesize that the canonical gap function described for *Dm'hb* could be instead mediated by the regulation of *Dm'Kr* and *Dm'gt*, with no (or little) direct effect of *Dm'hb* on the regulation of pair rule genes. Therefore, Hunchback in both species would form cardinal regions along the AP axis, essential for the proper expression of other gap genes.

But why then does depletion of *hb* in *Tribolium* and *Drosophila* lead to the deletion of different segment primordia? Considering that *hb* is expressed in similar

embryonic regions and interacts with similar gap genes in both species, the answer to this question might lie in the role of the target genes of *hb*. In Tribolium, *Tc'Kr* has no effect on the pair rule genes expressed within the expression domain of *Tc'Kr* in the wild type (Cerny et al., 2005). Additionally, I proposed here that the effects on pair rule genes observed in *Tc'hb, Tc'gt* and *Tc'Kr* depleted embryos, can be explained by the lack of the posterior *Tc'gt* expression in these embryos. In *Drosophila*, depletion of *Dm'Kr* leads to severe defects on the expression pattern of pair rule genes (Carroll and Scott, 1986). Additionally, *Dm'gt* mutants also display misregulation of pair rule genes. In both cases, the effects on the pair rule genes, leading to segment deletions, are observed for the pair rule stripes expressed within the respective expression domains of *Dm'Kr* and *Dm'gt*. Therefore the distinct regions of the embryo affected by depletion of *hb* in *Tribolium* and in *Drosophila* could be explained by the role of *Dm'Kr* in the regulation of pair rule genes.

As previously suggested, the transition from short to long germ mode most likely involved changes in the regulatory regions of pair rule genes, allowing that successive increase of gap gene input, like in case of *Dm'Kr*, in the regulation of the pair rule stripes (Peel, 2004).

But which genes are regulating the pair rule stripes in short germ insects? A recent analysis of the orthologs of the pair rule genes in *Tribolium* revealed that this class of genes is forming a gene regulatory circuit controlling segmentation. The model presents several variations from the model described in *Drosophila* (Jaynes and Fujioka, 2004). For example, essential primary and secondary pair rule genes from *Drosophila*, like *h* and *ftz*, are not involved in the *Tribolium* pair rule gene circuit. Nevertheless, the regulatory variations between both gene networks converge into the expression of *Tc'en* and *Tc'wg* in the same compartments within the parasegmental units in both species, anterior and posterior, respectively.

It is still not clear whether the pair rule gene circuit observed in *Tribolium* is conserved in hemimetabolous insects. Nevertheless, *even-skipped*, a primary pair rule gene in both *Tribolium* and *Drosophila*, is not expressed in a pair rule pattern in *Schistocerca* and *Oncopeltus* (Liu and Kaufman, 2005; Patel et al., 1992).

Among non-insect arthropods, there is no evidence that such a pair rule system would pattern the early embryo. Instead, most of the orthologs of the pair rule genes

analyzed in chelicerates and myriapods are expressed in segmental stripes (reviewed in Damen, 2007).

An alternative system for the metameric pattern formation in non-insect arthropods comes from the analysis of orthologous genes of the Notch-Delta signaling pathway in the spider *Cupiennius salei*. Similarly to its role in the vertebrate segmentation clock, in this spider, the expression pattern of the ortholog of *hairy* (*Cs'h*) is severely affected in embryos depleted for the spider orthologs of *Notch* and *Delta* (Stollewerk et al., 2003). This finding suggests that the striped expression of *Cs'h* is established in the spider in the same way as it is in vertebrates.

Taken together, these finding suggest that the mechanism controlling the formation of segments exhibit high plasticity allowing adaptive changes during evolution. On the other hand, the selective constraint on the expression of the segment polarity genes and Hox genes assures that all the essential segment units and identities are properly formed in arthropods as distinctive as mites and lobsters.

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Marques-Souza H, Aranda M, Tautz D. "A conserved role for *hunchback* in trunk organisation of insects". **Submitted**.

Marques-Souza H, Aranda M, Wurm M, Tautz D. "Segmentation genes controlling homeotic gene expression in *Tribolium*". **In preparation**

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Erklärung

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

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Erklärung