Comparative studies on the role of Egfr, Wingless and Decapentaplegic signalling in leg development in the red flour beetle *Tribolium castaneum*

Dissertation submitted in partial fulfilment of the requirements for the degree of
"Doctor rerum naturalium" (Dr. rer. nat.)
of the Georg-August-University Göttingen

submitted by
Daniela Großmann

Hamburg-Bergedorf
Germany

Göttingen
2011

Referee: Jun. Prof. Dr. Gregor Bucher

Second Referee: Nikola-Michael Prpic-Schäper

Date of the oral examination: 25th of January 2011

Declaration

Herewith I ensure, that the thesis “Comparative studies on the role of Egfr, Wingless and Decapentaplegic signalling in leg in the red flour beetle *Tribolium castaneum*” has been written independently and with no other sources and aids than quoted. I also declare that I have not previously applied for a doctoral degree at another university.

_____ Göttingen, December 20th, 2011

Daniela Großmann

• Table of contents

1 Summary

Leg development and its underlying genetic and molececular mechanisms are best understood in the prime insect model *Drosophila melanogaster* and this knowledge can serve as a first guide to study leg development in other arthropod species. The pattering of the proximo-distal axis in the leg is guided by a hierarchic gene cascade and the morphogens Wingless (Wg) and Decapentaplegic (Dpp) are the key factors which control the expression of proximo-distal patterning genes at the next level of the cascade. Beside its function in distal development, *wg* is also crucial for the allocation of the the limb primordia and specification of ventral cell fate in the leg.

The development of the leg in *Drosophila melanogaster* presents a highly derived mode of arthropod leg development and differs from other insects and arthropod groups, where the limbs are direct outgrowths of the embryonic body wall. Recent work provides evidence against a conservation of the Wg/Dpp leg pattering system. Although the expression pattern of *wg* is highly conserved in arthropods, functional studies in the insects *Oncopeltus* and *Gryllus* could not support a role of *wg* in leg development of these species. At least, in the coleopteran *Tribolium castaneum* a role of *wg* in leg allocation could be proven. Also the role of *dpp* in leg development has been questioned. The expression of *dpp* is not conserved between *Drosophila* and other arthropod species and indicates a non-conserved role of *dpp* in limb development of arthropods.

In the first part of this thesis, I present a functional analysis of *wg* and *dpp* in leg development in the red flour beetle *Tribolium castaneum.* This analysis has been performed via staggered stage-specific embryonic RNAi and different gene expression studies in RNAi embryos. This analysis revealed that the function of *wg* is similar between *Drosophila* and *Tribolium*. *Wg* is essential in leg allocation and distal leg development during early development as well as during late development in establishment of ventral cell fate in the legs. In addition, I could demonstrate, that *dpp* is indeed required for distal leg development in *Tribolium* but lacks a function in establishment dorsal cell fate in the legs.

Despite of these results in *Tribolium* there is increasing evidence that in other arthropod groups the Wg/Dpp system is not that crucial in leg axis pattering as in *Drosophila.* Instead EGFR signalling, which in *Drosophila* is needed in late

development for pattering the tarsal region, might have a more decisive role in proximo-distal leg formation in other arthropod species. Therefore, in the second part of my thesis, I present a functional analysis of the *Egf* receptor, its ligand *spitz* and the transcription factor *pointed* in leg development in *Tribolium castaneum*. This functional analysis revealed, that Egfr signalling fulfills a more complex role in pattering and formation of the legs in *Tribolium* as it is known in *Drosophila*. I could demonstrate that the requirement of Egfr signalling is not restricted to the distal part of the leg but also includes pattering and formation of the medial parts of the leg. Interestingly, my results strongly suggest that Egfr signalling in the medial leg is not mediated via *pointed* as in the distal portion of the leg but a different, not yet identified transcription factor

In summary, in this thesis I could show that the beetle *Tribolium castaneum* likely represents an intermediate evolutionary state as far as the proximal-distal pattering mechanisms in the legs are concerned. Although the Wg/Dpp system is already the predominant system in leg development in *Tribolium*, Egfr signalling has an important and more complex role than in *Drosophila*.

1 Zusammenfassung

Bisher ist die Beinentwicklung und die zugrunde liegenden genetischen und molekular-biologischen Mechanismen maßgeblich in dem Modellorganismus *Drosophila melanogaster* untersucht worden. Die entsprechenden Kenntnisse können als erster Anhaltspunkt zur Untersuchung der Beinentwicklung in anderen Arthropodenarten dienen. Die Musterbildung der proximalen-distalen Achse in den Beinen wird in *Drosophila* über eine hierarchische Genkaskade gesteuert, an deren Spitze die Morphogene Wingless and Decapentaplegic stehen, die die Expression der proximalen-distalen Gene kontrollieren. Neben dieser distalen Funktion ist *wg* zudem entscheidend an der Allocation der Beinscheiben und der Spezifizierung des ventralen Zellschicksals in den Beinen beteiligt.

Die Beinentwicklung in *Drosophila melanogaster* stellt allerdings eine sehr abweichende Form der Arthropoden-Beinentwicklung dar und unterscheidet sich von der Entwicklung in anderen Insekten- bzw. Arthropodenarten, in denen sich die Gliedmaßen als direkte Auswüchse aus der embryonalen Körperwand entwickeln.

Vorliegende vergleichende Studien sprechen gegen eine Konservierung des Wg/Dpp-Systems bei der Beinmusterbildung. Obwohl die Expression von *wg* in Arthropoden hochkonserviert ist, konnten funktionale Studien in den Insekten *Oncopeltus* und *Gryllus* eine Rolle von *wg* in der Beinentwicklung nicht unterstützen. In dem Käfer *Tribolium castaneum* konnte zumindest eine Rolle von *wg* bei der Allocation der Beinscheiben nachgewiesen werden. Ebenso wird eine Funktion des Gens *dpp* in der Beinentwicklung weiterer Arthropodenarten in Frage gestellt. Die Expression von *dpp* ist nicht konserviert und deutet demnach eher auf eine nicht konservierte Rolle von *dpp* in der Gliedmaßenentwicklung von Arthropoden hin.

Im ersten Teil der vorliegenden Arbeit wurde eine funktionale Analyse von *wg* und *dpp* in der Beinentwicklung des Reismehlkäfers *Tribolium castaneum* durchgeführt. Mittels zeitversetzter RNAi in Embryonen sowie der Analyse verschiedener Expressionsdaten in RNAi-Embryonen konnte gezeigt werden, dass die Funktion von *wg* in *Tribolium* der in *Drosophila* stark ähnelt. Ebenso wie in *Drosophila* ist wg in *Tribolium* zum einen in der frühen Phase der Beinentwicklung notwendig für die Entstehung der Beinentstehungsorte sowie für die distale Musterbildung, zum anderen wird *wg* in der späteren Phase der Beinentwicklung für die Spezifizierung

der Ventralseite des Beines benötigt. Darüber hinaus konnte ich zeigen, dass neben wg auch *dpp* eine Funktion in der proximalen-distalen Achsenbildung in *Tribolium* hat, allerdings keine Funktion in Bezug auf die Spezifizierung der Dorsalseite des Beines ausübt wie es in *Drosophila* der Fall ist.

Trotz dieser Ergebnisse in *Tribolium* gibt es zunehmend Hinweise darauf, dass in anderen Arthropodengruppen das Wg/Dpp Systems nicht so entscheidend für die Musterbildung der proximalen-distalen Achsenbildung entlang der Beine ist wie in *Drosophila*. Stattdessen hat möglicherweise der Egfr Signalweg, der in *Drosophila* in der späten Beinentwicklung für die Musterbildung der tarsalen Region entscheidend ist, eine weitreichendere Funktion in der proximo-distalen Achsenbildung anderer Arthropdenarten. Im zweiten Teil der vorliegenden Arbeit habe ich eine entsprechende funktionale Studie des *Egf* Rezeptors, dessen Liganden s*pitz* und des Transkriptionsfaktors *pointed* in der Beinentwicklung von *Tribolium castaneum* durchgeführt. Anhand dieser Analyse konnte gezeigt werden, dass der Egfr-Signalweg eine komplexere Funktion in der Musterbildung der Beine in *Tribolium* ausübt als in *Drosophila*. Der Egfr Signalweg ist nicht nur involviert in die Musterbildung des distalen Bereiches des Beines, sondern darüber hinaus auch für die Musterbildung des medialen Beines notwendig. Die vorliegenden Ergebnisse deuten zudem stark darauf hin, dass der Egfr Signalweg im medialen Bereich des Beines nicht über den Transkriptionsfaktor *pointed* wirkt, sondern einen anderen, bisher nicht identifizierten Transkriptionsfaktors nutzt.

Zusammenfassend konnte anhand der vorliegenden Studie gezeigt werden, dass der Käfer *Tribolium castaneum* bezüglich der Beinentwicklungsmechanismen eine evolutionäre Übergangsposition einnimmt, in dem das Wg/Dpp System bereits die entscheidende Rolle in der proximalen-distalen Achenbildung in den Beinen ausübt, allerdings der Egfr Signalweg noch eine komplexere Rolle in der Beinentwicklung einnimmt als später in der Fruchtfliege *Drosophila melanogaster.*

2 Introduction

2.1 The arthropod phylum: the most dominating group of living animals on the planet

Arthropods are the most successful and diverse animal group on the planet and have reached an unparalleled evolutionary and ecological success. They have adapted to almost every ecological niche on earth and are the most dominating group of marine, freshwater, land and air ecosystems (Rupert et al. 2004). In terms of the number of species and the number of individual animals, arthropods are the dominating metazoan group on the planet. Estimating the total number of living species is extremely difficult. It is estimated that more than 1.000.000 arthropod species have been described so far and more than 5.000.000 arthropod species exist on earth (Chapman 2009). The arthropod phylum includes four main classes: Crustaceans (e.g. crabs), Hexapoda (e.g. beetles, bees, ants), Myriapoda (mainly centipedes and millipedes) and Chelicerata (e.g. spiders, scorpions, mites) (for a deeper insight in the phylogenetic relationships of arthropods see the recent study by Regier et al. 2010). The remarkable diversity and great evolutionary success of arthropods have been due mainly to two characteristics of arthropods: their segmented body plan and particularly the diversity of their articulated appendages (Davis and Patel 1999; Angelini and Kaufman 2005b; Prpic and Damen 2008). The appendages of arthropods are unparalleled in their immense number of novel forms and have been adapted to a large number of functions. Depending on the life style of a species different appendage types have evolved, e.g. for walking, swimming, prey-capture, biting, egg-laying etc. (Prpic and Damen, 2008, see Fig. 1). This diversity makes arthropods a prime model for studying the ground-principles of adaptive evolution as well as the underlying genetic mechanisms and evolutionary changes in these mechanisms in order to produce morphological innovations (Prpic and Damen 2008).

The appendages of insects can be classified into two groups: ventral appendages (antennae, mouthparts, legs and genitalia) and dorsal appendages (wings and halteres) (reviewed by Kojima 2004 and Angelini and Kaufman 2005b). Ventral appendages are a common feature in all arthropods and despite of their great variety

in form and function it is thought that all these appendages diverge from a shared single ancestral appendage, the so called "ground-state" appendage.

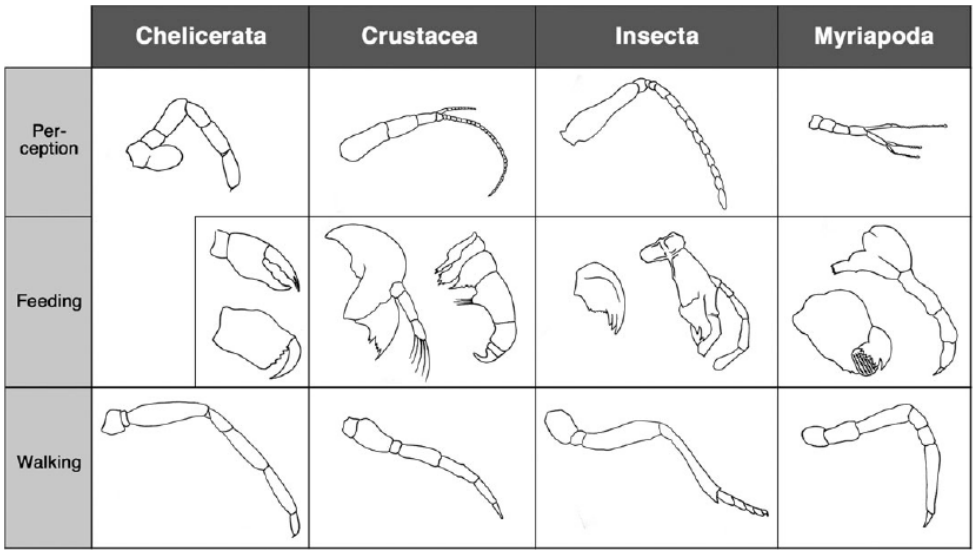


Fig 1. Schematic overview of a small portion of the diversity of arthropod appendages
The diagram shows simplified and generalised appendages for sensory perception (top row), feeding (gnathal) appendages (centre row) and walking appendages (lower row). Top row from left to right: spider pedipalp, amphipod first antenna, bee antenna, pauropod antenna. Centre row from left to right: scorpion chelicera and spider chelicera, syncarid mandible, first maxilla of Remipedia, ectognathan mandible, ectognathan maxilla, millipede mandible, chilopod second maxilla. Bottom row from left to right: spider prosomal walking leg, amphipod pereiopod, dipteran thoracic leg, millipede trunk leg. Modified after Prpic and Damen (2008).

According to Snodgrass the ground-state appendage consisted of two podomeres, the proximal coxopodite and the distal telopodite (Snodgrass 1935). Casares and Mann support the idea of an ancestral unbranched appendage consisting of a proximal and distal part. By removing the activity of Hox genes in *Drosophila melanogaster* they produced an appendage with a large proximal segment and a largely normal tarsus (Casares and Mann 2001). However, in recent years, it has been questioned, if the common ancestor was really that simple. Data from Trilobita, one of the earliest arthropod fossils, and extant arthropod species indicate that the ground-state was much more complex and the appendages already have branched limbs and is made up of three structures: a protopodite, a telopodite and an exopodite (reviewed by Boxshall 2004; Giorgianni and Patel 2005 and Prpic and Damen 2008). It is clear that

these data give rise to controversy and that more data from fossil groups as well as extant arthropod species are needed to answer the question about the common ancestral appendage.

As mentioned above, one major question of evolutionary developmental biology is to learn more about the changes in genetic mechanisms leading to new and modified appendages which have the ability to adapt to new functions. Legs are considered to be the least divergent appendage within the arthropod group and in the last decades researchers focused on genetic and developmental studies in the prime model *Drosophila melanogaster* to get more insight in the underlying molecular and genetic mechanisms of leg development.

2.2 Leg development in *Drosophila melanogaster*

The fruit fly *Drosophila melanogaster* (Order: Diptera) is a holometabolous insect and due to its phylogenetic position within insects and its rapid development it is suggested to present a highly derived state of development. As a holometabolous insect, *Drosophila* undergoes complete metamorphosis, which means, that almost all adult tissues are derived from groups of cells that are set aside from the larval tissue during embryogenesis. These imaginal discs grow within the larva but do not contribute to the larval body plan (Cohen 1993; reviewed by Kojima 2004 and Angelini and Kaufman 2005b). Limb development from imaginal discs is unique to the Holometabola, but even in this group appendage development from imaginal discs is not universal. In Coleoptera and Lepidoptera, for instance, only some adult appendages develop from imaginal discs (Svacha 1992). Indeed, in many other arthropod groups limb development proceeds directly from three dimensional embryonic limb buds instead from a two-dimensional field of cells as in *Drosophila* (reviewed by Angelini and Kaufman 2005b).

So far, leg development is best understood in the prime insect model *Drosophila melanogaster* and although it represents a highly derived process, in the last couple of years several comparative studies in other insects and various arthropod species have shown that many genes, which are involved in *Drosophila* leg development, have also a function in leg development of other arthropods species (e.g. Panganiban

et al. 1994; Niwa et al. 1997; Abzhanov and Kaufman 2000; Jokusch et al. 2000; Inoue et al. 2002; Prpic et al. 2003; Prpic and Tautz 2003; see section 2.4). Therefore, a complete understanding of the developmental processes and the underlying molecular and genetic mechanisms in *Drosophila* helps to get more insight in leg development in other arthropod groups and a better understanding of the conservation of involved genes.

Drosophila leg development can be separated into three different stages (Fig. 2):

1. Allocation of the limb primordium during embryogenesis (Fig.2 A-C).
2. Subdivision of the leg disc via Wg-and Dpp signalling along the PD-axis (Fig. 2 D-F).
3. Metamorphosis of the adult leg during pupal stages (Fig. 2 G).

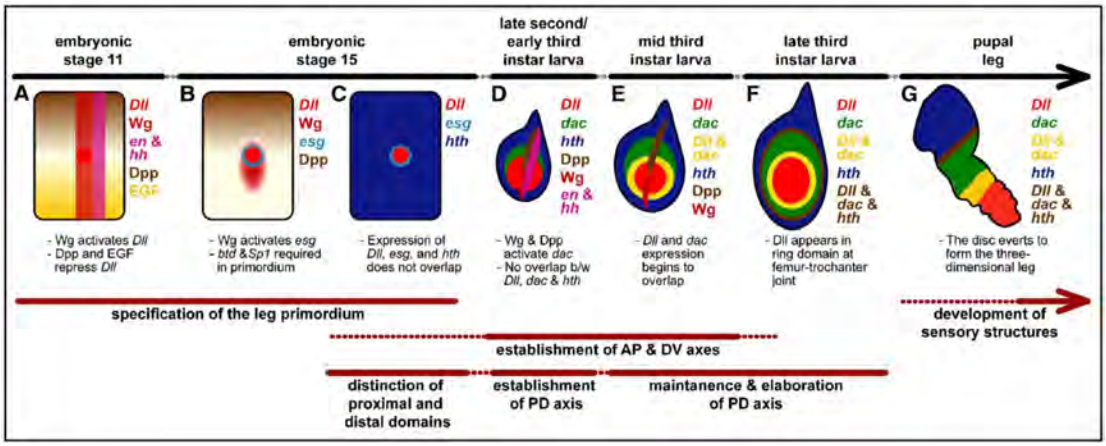


Fig. 2 Development of the *Drosophila* leg.
The diagram summarizes the important processes of the development of the *Drosophila* leg. (A-C) Allocation of the limb primordium. At embryonic stage 11 *Dll* is activated by Wg and it is repressed dorsally by Dpp and ventrally by EGFR. At embryonic stage 15 Wg is required for proximal identity and the activation of *esg*. (D-F) Subdivision of the leg disc by the morphogens Wg and Dpp via the activation and repression of specific target genes (*Dll*, *dac*, *hth*), which give rise to circular, overlapping expression domains along the PD-axis. (G) During pupal stage the leg disc telescopes outward and forms a three-dimensional leg.
Early developmental processes are depicted in the context of a thoracic body segment (A-C), later processes are depicted in the context of the imaginal disc (D-G). Modified after Angelini and Kaufman (2005b).

2.2.1 Allocation of the limb primordium during embryogenesis

The process of leg development starts during embryonic stage 11, when a small

number of cells in each thoracic segment are specified to become the leg imaginal disc (Cohen 1993). At embryonic stage 11 the leg and wing primordia are still combined in a so-called thoracic limb primordium (Cohen 1993). *Distal-less* (*Dll*), which encodes a helix-loop-helix homeodomain transcription factor, is one of the first genes that is expressed in the limb primordial cells (Cohen et al. 1989; Cohen 1993).

Wg, Dpp and Egfr signalling play an essential role during this early stage of leg development and are necessary for proper allocation of the imaginal disc (Cohen et al. 1993; Goto and Hayashi 1997). *Wg*, a Wnt family member, is required for the specification of the entire thoracic limb primordium, where it activates the initial expression of *Dll* via an early acting enhancer called *Dll*304 (Vachon 1992; Kubota et al. 2003; McKay et al. 2009; Fig. 2 A).

The expression of *Dll* is restricted to the ventro-lateral side of each thoracic segment. On the dorsal side, *Dll* is inhibited by *decapentaplegic* (*dpp*), a member of the TGF-ß family, and on the ventral side *Dll* is repressed in response of receiving negative input from Epidermal growth factor receptor signalling (Golembo et al. 1996; Goto and Hayashi 1997; Kubota et al. 2000, Fig. 2 A).

During this early stage of development the cells of the thoracic limb primordium are still multipotent and have the ability to contribute to the wing, haltere, leg or the Keilin's organ (McKay et al. 2009, Fig. 3). At stage 14 the cells of the second and third thoracic segment, that give rise to the wing and haltere, downregulate Wg signalling, stop expressing *Dll* and separate from the leg primordium to develop to the wing and haltere imaginal discs (Cohen et al. 1993; McKay et al. 2009).

The cells of the leg primodium further subdivide into proximal and distal cells and the *Dll*-expressing cells will only contribute to the distal part of the leg (telopodite) (McKay et al. 2009). During this later stage *Dll* expression is not longer controlled by the cis-regulatory element *Dll*304, but the LT-element (leg trigger element) and the maintenance-element (Estella and Mann 2008; Estella et al. 2008; McKay et al. 2009, Fig.3). Both elements are only active in response to high levels of Wg-and Dpp-signalling (McKay et al. 2009).

Dll expression is limited to the head and the thoracic segments. Appendage formation is repressed in abdominal segments by the activity of abdominal Hox-genes, which directly repress the activity of *Dll*304 in the abdomen (Vachon et al. 1992; Gebelein

et al. 2002; reviewed by Hughes and Kaufmann 2002, Fig. 3).

In addition to *Dll*, the two Zn-finger transcription factors *Sp1* and *buttonhead* (*btd*) are also activated by Wg signalling during limb development (Wimmer et al. 1993; Wimmer et al. 1996; Estella et al. 2003). Both genes are expressed in the thoracic limb primordia and play a crucial but non-redundant role in ventral appendage specification (Estella et al. 2003; McKay et al. 2009; Estella and Mann 2010). The complete knockdown of *Sp1* and *btd* results in the loss of all leg structures and can lead to a complete transformation of leg to wing (ventral to dorsal fate change) (McKay et al. 2009; Estella and Mann 2010). Rescue-experiments showed that only *SP1* but not *btd* plays an essential role in early leg development (Estella and Mann 2010).

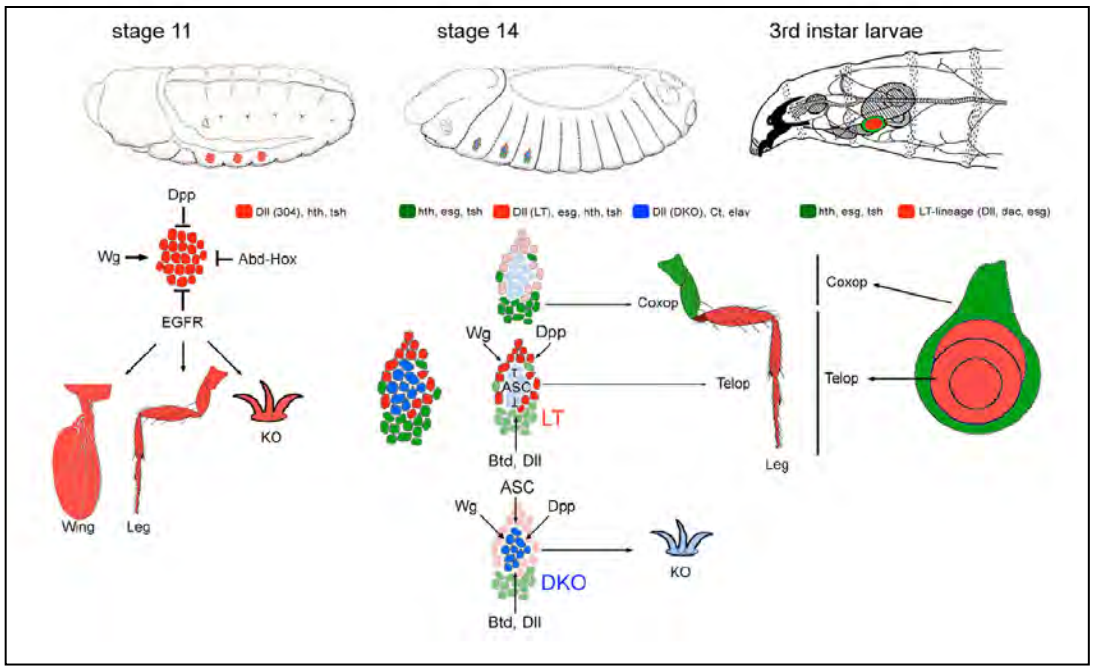


Fig. 3 Development of the thoracic limb primordia
The diagram summarizes the temporal control of the *Dll* expression in the limb primordia by three cis-regulatory elements. During stage 11 the enhancer element *Dll*304 is active; the cells are still multipotent and give rise to any part of the dorsal or ventral appendages or the Keilin's Organ (KO). From stage 14 on, the control over *Dll* expression shifted to the LT enhancer-element and the Keilin's Organ enhancer-element (DKO). The LT-enhancer-element is active in the progenitors of the telopodite (red); DKO is only active in the progenitors of the Keilin's Organ (blue). Cells that express *tsh* as well as *hth* but not *Dll* give rise to the coxopodite (green). Modified after McKay et al. (2009).

Dll as well as *Sp1* and *btd* are initially activated by Wg signalling in the limb primordium, but *Sp1* and *btd* are not involved in the activation of the early *Dll*304 enhancer. However, Estella and Mann could demonstrate that *SP1* functions upstream of *Dll* and is required for the specification of the limb primordia (Estella and Mann 2010). Although *SP1* is not involved in early activation of *Dll*, during later stages it is in addition to Wg- and Dpp- signalling required for the activation of the late *Dll* enhancer (LT-element). Unlike *Dll*, *Sp1* plays next to its function in telepodite formation also a role in specifying more proximal leg segments and repression of dorsal fate (Estella et al. 2010).
Last, Estella and Mann could show that during late development *Sp1* and *btd* act together with *Dll* to promote the growth of the entire leg (Estella and Mann 2010).

2.2.2 Subdivision of the leg disc via Wg- and Dpp signalling along the proximo-distal axis

After establishment of the leg disc a hierarchic gene cascade guides proximo-distal axis formation (Rauskolb and Irvine 1999). At the top level of this cascade Wg and Dpp are acting as long-range morphogens. Both genes are expressed along the anterior-posterior-(AP)-compartment boundary. Hedgehog is expressed in the posterior compartment and with help of a short-range signal it activates the expression of *wg* and *dpp* in the anterior compartment. Both genes are repressed in the posterior compartment by the Engrailed protein (Basler and Struhl 1994; Diaz-Benjumea et. al. 1994; Sanicola et al. 1995; Jiang and Struhl 1996) Once activated, the expression domains of *wg* and *dpp* are stabilized by mutually antagonistic repression. Therefore, *wg* is expressed in a ventral sector and *dpp* in a dorsal sector (Brook and Cohen 1996; Jiang and Struhl 1996; Penton and Hoffmann 1996; Johnston and Schubiger 1996; Theisen et al. 1996).
In contrast to early limb development, where Dpp represses the activity of Wg, in the leg disc the combined activity of both genes is required for further subdivision and specification of the leg disc via activation and repression of specific target genes (PD domain genes) (e.g. *Dll, dac, hth*). The PD domain genes give rise to circular, partially overlapping expression domains along the PD axis (Diaz-Benjumea et al. 1994; Abu-Shaar and Mann 1998; Lecuit and Cohen 1997; reviewed by Kojima

2004; Fig.2 D-F). When the leg disc is established, *Dll* is expressed in a central domain that corresponds to the presumptive tarsal segments and distal tibia (Cohen 1997). *Dll* is not longer activated via the early *Dll*304-element, but the late acting enhancer, the LT-element. For its activation, the LT-element requires high input of Wg-signalling as well as Dpp-signalling (Lecuit and Cohen 1997; Abu-Shaar and Mann 1998; Estella et al. 2008; McKay et al. 2009). Later, *Dll* expression is maintained in these cells and their progeny by the maintenance (M) -enhancer ("trigger-maintenance-model") (Estella et al. 2008; McKay et al. 2009).

Dac, which encodes a transcription factor, is expressed in an intermediate domain between *Dll* and *hth* and is required for proper development of the presumptive femur and proximal tibia (Lecuit and Cohen 1997; Mardon et al. 1994; Abu-Shaar and Mann, 1998). In contrast to *Dll*, *dac* is induced in cells, which receive lower activity of Wg and Dpp signalling (Lecuit and Cohen 1997).

Different other genes e.g. the homebox gene *homothorax (hth*), *extradenticle* (*exd*) and *teashirt* (*tsh*) are expressed in the leg disc, but their expression is limited to the peripheral domain that corresponds to the future body wall, the coxa and trochanter (Wu and Cohen 1999). Hth is required for the activity of Exd, since it binds to Exd and regulates its translocation into the nucleus (Rieckhof et al. 1997; Rauskolb et al. 1995). Activity of *hth* and *tsh* in the distal domain is repressed by combined Wg and Dpp-signalling (Abu-Shaar and Mann 1998; Wu and Cohen 1999; Wu and Cohen 2000).

Hth, exd and *tsh* are all required for normal development of the proximal leg segments (Wu and Cohen 1999; Wu and Cohen 2000). Besides, *hth* works together with *Dll* and *dac* to form a functional boundary between proximal and distal leg domains (Wu and Cohen 1999).

Finally, during pupal development the "inward leg" telescopes outwards and forms a normal three-dimensional adult insect leg (Fristrom and Fristrom 1993, Angelini and Kaufman 2005b, see Fig. 2 G).

2.2.3 Critical remarks on the "gradient model"

This mechanism of leg disc patterning thus mainly relies on the formation of Wg and

Dpp protein gradients ("gradient model"). Recently, Giorgianni and Mann (2011) have argued that the gradients are only required during a short period of time and other mechanisms are equally important for leg disc patterning. By analysing the *dac* regulatory element (RE) in detail they could show that Wg and Dpp are not the primary activators of *dac*, since eliminating the Pan- and Mad- binding sites has only a minimal effect on the enhancer activity. Instead they could show, that *Dll*, which is activated by high levels of Wg and Dpp signalling, is an essential and direct activator of *dac*. In addition, they argue, that Wg and Dpp signalling is not sufficient for repressing *dac* in the distal-most domain. Instead, Giovanni and Mann suggest that high levels of Wg and Dpp signalling activate the Epidermal Growth Factor Receptor (EGFR) pathway and one of its target genes, *Bar,* functions also as a repressor of *dac* in distal cells.

This study shows that there are indeed still open questions concerning the function of Wg, Dpp, *Dll* and Egfr in establishing the proximo-distal axis in leg development.

2.3 Specification of dorsal and ventral leg cell fates by Wg and Dpp signalling

Next to its function in pattering the AP-axis, Wg and Dpp are also required for specifying ventral and dorsal fate, respectively (Struhl and Basler 1993; Wilder and Perrimon 1995; Jiang and Struhl 1996; Brook and Cohen, 1996; Penton and Hoffmann, 1996). In contrast to the establishment of the AP axis, where Dpp and Wg act combinatorially, organizing the DV-axis depends on mutual repression of Wg and Dpp (Brook and Cohen 1996; Theisen et al. 1996; Jiang and Struhl 1996; Penton and Hoffmann 1996). Mutations in *wg* lead to the deletion of all ventral leg structures and to symmetric duplications of dorsal structures (Cousco et al. 1993; Held et al. 1994). Mutations in *dpp* have the opposite effect and are caused by ectopic expression of *wg* in the dorsal domain (Brook and Cohen 1996; Brook 2010). In addition to the antagonistic relationship between *wg* and *dpp*, different target genes are required for specifying dorsal and ventral fate in the leg (Estella and Mann 2008; Brook 2010).

Dpp represses *wg* in the dorsal part of the leg disc and activates the dorsal specific T-box gene *optomotor-blind* (*omb*) (Brook and Cohen 1996). Interestingly, Dpp signalling controls the DV axis formation in a different way than the AP axis

formation. Concerning the AP axis, Dpp regulates the expression of *Dll* and *dac* via repression of *brinke*r (*brk*), a transcriptional repressor of Dpp target genes. In contrast, Dpp does not use this *brk*-dependant mechanism to control the activation of *omb* and repression of *wg* to establish dorsal fate. So far, it is still unclear, how Dpp regulates the activities of *omb* and *wg* (Estella and Mann 2008).
In the ventral sector *wg* establishes ventral fate by repressing the activity of *dpp* and activation of *midline* (*mid*) and *H15* (Wilder and Perrimon 1995; Brook and Cohen 1996; Svendsen et. al 2009; Brook 2010). It could be shown that this ventral function of *wg* is local and does not require Wg protein to form a morphogen gradient. Instead *wg* acts through inactivation of the *shaggy/zeste-white 3 kinase* (*sgg/zw3)*, a negative regulator of the Wnt pathway, to activate *H15* and to specify ventral cell fate in the leg (Diaz-Benjumea and Cohen 1994; Wilder and Perrimon 1995).

2.4 Leg development in other arthropods: conserved and derived aspects

The development of the leg in *Drosophila melanogaster* from a flat, almost "two-dimensional" leg disc presents a highly derived mode of arthropod leg development and differs in other insects and arthropod groups, where the limbs are direct outgrowths of the embryonic body wall (see section 2.2., reviewed by Kojima 2004 and Angelini and Kaufman 2005b). In *Drosophila*, the PD axis pattering is guided by a hierarchic gene cascade. Wg and Dpp are the key factors, acting as long-range morphogens and control the expression of the proximo-distal pattering genes at the next level of the cascade.

Given the derived mode of limb development in *Drosophila* a major and central question is to what extent this cascade and the function of the involved genes is conserved in species with a different mode of appendage development. Since *wg*, *dpp* and the PD domain genes *Dll, dac* and *hth* are the main factors during leg development in *Drosophila*, their expression and function have been studied in a number of insect species as well as in other arthropod groups (Panganiban et al. 1994; Niwa et al. 1997; Abzhanov and Kaufmann 2000; Jokusch et al. 2000; Inoue et

al. 2002; Prpic et al. 2003; Prpic and Tautz, 2003).
One of the best studied PD domain genes is *Dll* that has been studied in insects (e.g. *Tribolium castaneum*, *Oncopeltus fasciatus*, *Schistocerca gregaria*, *Gryllus bimaculatus*) as well as in various arthropod species such as *Glomeris marginata* and *Cupiennius salei*. It could be shown that the expression pattern of *Dll* is widely conserved and that it is expressed normally in the distal part of appendages (Abzhanov and Kaufman 2000; Jockusch et al. 2000; Beermann et al. 2001; Schoppmeier and Damen 2001; Inoue et al. 2002; Prpic and Tautz 2003).
In addition, comparative studies confirmed that also the *dac* orthologs show conserved expression patterns, for instance in *Tribolium* (Prpic et al. 2001), *Oncopeltus* (Angelini and Kaufman 2004) and *Gryllus* (Inoue et al. 2002).
Moreover, comparative functional studies could confirm that beside the expression patterns also the function of *Dll* and *dac* is more or less conserved between *Drosophila* and other arthropods (Beermann et al. 2001; Schoppmeier and Damen 2001; Angelini and Kaufman 2004). The relatively high degree of conservation in expression and developmental function of the PD domain genes leads to the question, if the regulation of these genes by a Wg and Dpp morphogen gradient as in *Drosophila* is also evolutionary conserved.

In the leg disc of *Drosophila wg* is expressed in a ventral domain along the AP boundary. Several studies in various arthropod groups such as myriapods (Hughes and Kaufmann 2002; Prpic 2004), chelicerates (Damen 2002, Prpic et al. 2003), crustaceans (Nulsen and Nagy 1999, Prpic 2008) and in different insect species including *Tribolium, Oncopeltus* and *Gryllus* (Nagy and Caroll 1994; Niwa et al. 2000; Angelini and Kaufman 2005a) could confirm that the expression pattern of *wg* is highly conserved in arthropods. All data have shown that *wg* orthologs are expressed along the ventral side of the embryonic limb buds and resembles the *wg* expression known from the leg disc in *Drosophila*.
Surprisingly, studies concerning the expression of *dpp* orthologs could not confirm a conserved expression pattern, neither in insect species nor in other arthropod species. In all arthropods studied so far, the expression of *dpp* differs from the expression pattern found in *Drosophila*, but is more or less consistent among the diverse species examined. In contrast to *Drosophila*, in most species *dpp* is not expressed in a dorsal

sector, instead it is expressed throughout the limb buds and later on in the distal tip or rings proximal of the distal tip (Sanchez-Salazar et al. 1996; Jokusch et al. 2000, Niwa et al. 2000, Prpic et al. 2003; reviewed by Angelini and Kaufman 2005b).

Although the conserved function and expression of the PD domain genes initially indicated a conservation of the whole hierarchic gene cascade with Wg and Dpp morphogens at the top level, the divergence in *dpp* expression is striking and questions this hypothesis and speaks more for a different role and function of *dpp* in the specification of the limb PD axis as it is known in *Drosophila*.
Interestingly, although the differences in *dpp* expression between *Drosophila* and other arthropod species indicate a non-conserved role of *dpp* in limb development, Prpic et al. proposed a hypothetical model (termed "topology model" by Angelini and Kaufmann 2005b), which indeed support the idea that the function of *dpp* and subsequently the Wg/Dpp leg pattering system is conserved in more basal arthropods and different insect species (e.g. *Cupiennius salei, Tribolium, Gryllus*) (Prpic et al. 2003). Actually, due to the different morphology of precursor leg structures in *Drosophila* and more basal arthropods (two-dimensional leg disc versus three-dimensional limb bud) they suggest that the expression of at least one morphogen, Dpp or Wg respectively, has to be restricted to the distal tip of the limb bud. Indeed, only such a pattern would allow the Dpp/Wg system to activate specific target genes in the same ring-shaped manner in the distal region as known in *Drosophila*.

Angelini and Kaufman tested the hypothesis of Prpic et al. and performed a functional analysis of *wg* and *dpp* in the hemimetabolous milkweed bug *Oncopeltus fasciatus*. They could show, that the knockdown of *wg* or *pangolin* (*pan*), the transducer of canonical Wnt-signalling, via maternal and zygotic RNA-interference produced defects in eye development as well as in dorsal segmentation. Surprisingly, depletion of *wg* or *pan* does not generate any defect in the appendages. The role and function of *Of'dpp* in leg development could not be analysed, since *dpp*RNA-interference lead to early and severe developmental defects and the embryos fail to initiate later processes like appendage development (Angelini and Kaufman 2005a).
Knockdown of *wg* and *armadillo* in another hemimetabolous and more basal insect, the two-spotted cricket *Gryllus bimaculatus*, could show that *wg* is required for

normal body segmentation, but does not seem to have a role in leg development (Miyawaki et al. 2004). Consequently, this data provides evidence against the hypothesis of a conserved Wg/Dpp-system and Angelini and Kaufman suggested that the role of *wg* and *dpp* in limb development evolved only later in the holometabolous insects (Fig. 4) (Angelini and Kaufman 2005b).

In contrast, Ober and Jokusch could produce different results in the holometabolous insect *Tribolium castaneum*. *Tribolium* shows direct limb development as well as *O. fasciatus* and *G. bimaculatus*, but is more closely related to *Drosophila* than the hemimetabolous insects. Downregulation of *wg* via pupal RNAi results in loss of the thoracic limbs or at least in malformed thoracic appendages (Ober and Jokusch 2006). This RNAi data indicate that *wg* is indeed necessary for the initiation of limb outgrowths (appendage allocation) in *Tribolium*. Due to the complete loss of the limbs, a later role of *wg* in distal and ventral limb development could not be further analysed and remains unclear. In addition, Ober and Jokusch analysed the function of *dpp* in *Tribolium*, but could not find evidence for a conserved role of *dpp* in early limb development. *dpp*RNAi embryos did not show any defects and appendages underwent normal outgrowth (Ober and Jokusch 2006).

Interestingly, the results concerning the role of *dpp* in *Tribolium* could not be confirmed by van der Zee et al. The downregulation of the activity of *Tc'dpp* via maternal RNAi caused severe defects. The embryos showed enlarged headlobes and were completely ventralized, further development failed and any analysis of the role of *Tc'dpp* in limb development was not possible (van der Zee et al. 2006)

Counter to the results of Ober and Jokusch, Pechmann (2011) could show that *dpp* has indeed an essential and central role and function in *Tribolium* leg development. In the background of a *Tribolium castaneum omb* mutant it could be shown that the expression pattern of *Tc'dpp* as well as the expression pattern of *Tc'wg* has changed and both genes were mis-expressed along the dorsal side of the embryonic limb bud. The following mis-expression of different genes (e.g. *Dll, dac, SP8*) seems a direct response to the altered *dpp* expression along the dorsal side. These results indicate an essential role of *dpp* in the leg development of *Tribolium* and support the idea of a conserved Wg/Dpp-system in leg development as proposed by Prpic et al. (Pechmann 2011).

In summary, all these data give rise to controversy and so far the conservation of the

Wg/Dpp system is highly debated and no final answer can be given.

Besides, it has to be asked, if the Wg/Dpp leg patterning system is not evolutionary conserved in arthropods and represents a more derived system in the holometabolous insects as proposed by Angelini and Kaufman, which ancestral system controls the proximal-distal leg pattering in the majority of other arthropod species? One possible ancestral system might be the EGFR signalling pathway.

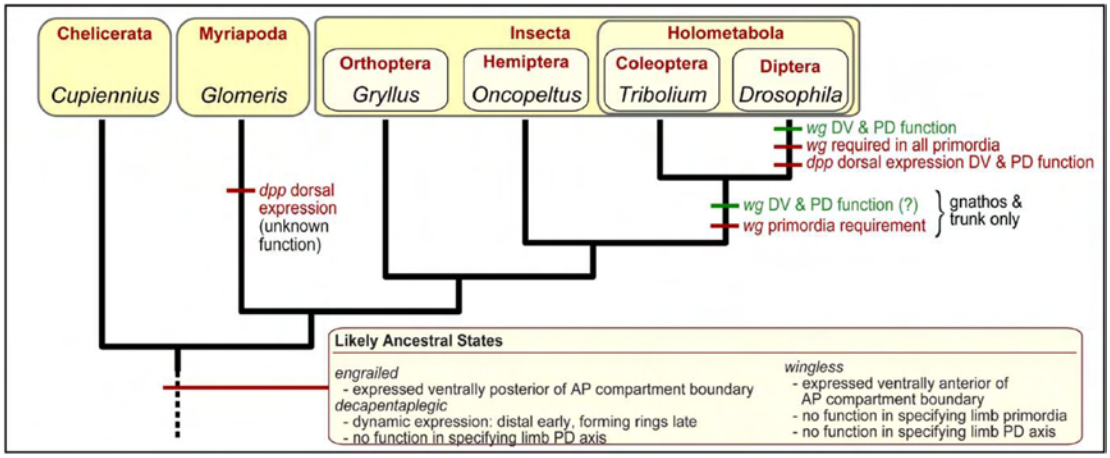


Fig. 3 Evolutionary changes in expression and function of *wg* and *dpp* in arthropod limb development. Angelini and Kaufman suggest that neither *wg* nor *dpp* have an ancestral function in leg development (see "Likely Ancestral States"). The role of *wg* and *dpp* in appendage development evolved later in the holometabolous insects. Since it is not clear when *wg* and *dpp* exactly acquired its function in limb development, possible upper and lower limits of this event are indicated on the tree in green. Modified after Angelini and Kaufman 2005b.

2.5 Egfr signalling: a possible, ancestral system in leg patterning

2.5.1 The Egfr pathway in *Drosophila melanogaster*

The epidermal growth factor receptor (Egfr) belongs to a large family of cell surface receptors with protein tyrosine kinase activity (reviewed by Schweitzer and Shilo 1997 and Perrimon and Perkins 1997). In contrast to vertebrates, where four EGF receptors are involved in signalling (EGFR, ErbB2, ErbB3, ErbB4), in *Drosophila melanogaster* only one receptor of the pathway is known (DER/EGFR) (Harris et al. 2003; Shilo 2005).

In *Drosophila*, the Egfr pathway is involved in many aspects of development e.g. cell

survival, proliferation and differentiation in embryos, imaginal discs and oogenesis (Shilo and Raz 1991; Shilo 2002; reviewed by Schweitzer and Shilo 1997 and Shilo 2005). One known way of intracellular signal transduction from the activated receptor is via the highly conserved canonical Ras/Raf/MEK/MAPK pathway (Perrimon 1994; Perrimon and Perkins 1997; Schweitzer et al. 1995b; Shilo 2005). Gene expression by Egfr is mainly induced through the *Pointed ETS* (*Pnt*) transcriptional activator (Gabay et al. 1996; O'Neill et al. 1994). The gene pnt exists in *Drosophila* in two isoforms, Pnt-P1 and Pnt-P2. While Pnt-P1 acts as a constitutive transcriptional activator and does not need modulation for its activity by Egfr signalling, does Pnt-P2 requires phosphorylation by MAP kinase to become an active transcriptional activator (Klämpt et al. 1993; Klaes et al. 1994; Gabay et al. 1996). Since the receptor itself and its downstream signalling components are broadly expressed during development and Egfr signalling is involved in so many different processes, regulatory mechanisms are essential for controlling precisely the activity of Egfr in different tissues (Zak et al. 1990; Freeman 1998). A key way of regulation is through a network of different ligands, which controls the activity of the receptor in a tissue-specific manner. In *Drosophila*, at least four ligands are known: Spitz, Gurken, Vein and Keren (Rutledge et al. 1992; Neumann-Silberberg and Schüpbach 1993; Schnepp et al. 1996; Golembo et al. 1999; Reich et al. 2002).

The primary Egfr ligand in *Drosophila* is Spitz, which is used repeatedly during all stages and activates the receptor in most tissues. The gene is ubiquitously expressed in the embryo and encodes an EGF-like transmembrane protein (Rutledge et al. 1992). *Spitz* is produced as an inactive transmembrane precursor (m*Spi*), which needed to be processed into its active secreted form by the two proteins Rhomboid (Rho) and Star (S) (Bier et al. 1990; Kolodkin et al. 1994; Golembo et al. 1996). In contrast, the ligand Vein posses a weaker activation capacity, but in some tissues Vein functions as the main ligand (e.g. leg pattering, see section 2.5.2) (Schnepp et al. 1998; Golembo et al. 1999). The activity of the ligand Gurken is restricted to the follicle cells of the ovary (Neumann-Silberberg et al. 1993). The last activating ligand of Egfr is the recently found ligand Keren. Keren is structurally related to Spitz and regulated in a similar manner. So far, little is known about its function, but it seems to complement the activity of Spitz in certain tissues (Reich et al. 2002;

Brown et al. 2007).

Besides Spitz, Keren, Vein and Gurken, which regulates Egfr positively, also different negative regulators of the Egfr pathway are known in *Drosophila*, including Argos, Kekkon and Sprouty (Schweitzer et al. 1995, Ghiglione et al. 1999; Reich et al. 1999; Ghiglione et al. 2003; Casci et al. 1999).

2.5.2 Role of Egfr signalling in *Drosophila melanogaster* leg pattering

The establishment of the proximo-distal axis is guided by the morphogens Wg and Dpp. The Wg and Dpp proteins spread throughout the disc and activate the genes of the next level, the leg gap genes (e.g. *Dll*, *dac*, *hth*). Of course, the small number of leg gap genes is not sufficient to determine all ten leg segments. The five domains which have been created by the leg gap genes do not correspond to a single segment of the future leg and have to be further subdivided (reviewed by Kojima 2004).

This process has been well studied in the *Dll* domain, which is further subdivided into five tarsal segments and the pretarsus (reviewed by Kojima 2004). Interestingly, it could be shown that the pattering of the tarsal segments and the pretarsus is in later stages independent of Wg- and Dpp- signalling and is instead controlled by a different signalling pathway. The expression of genes subdividing these regions (e.g. *aristaless*, *Bar*, *bab* and *rn*, the so called "tarsal gap genes") is mainly controlled by the EGFR signalling pathway (Galindo et al, 2002; Campell 2002). In the leg disc, Vein is the only known active Egfr ligand. *Vein* is initially activated by Wg and Dpp signalling as well as *Dll* and is expressed in the very center of the *Dll*-expressing domain (Schnepp et al. 1998; Galindo et al. 2002). Secretion of the Vein protein leads to EGFR-mediated activation and repression of the tarsal gap genes *rn*, *Bab, aristaless*, *Bar* and *lim1*, which further define the tarsal and pretarsal region (Galindo et al. 2002; reviewed in Kojima 2004).

Taken together, Egfr signalling is a second system in addition to Wg/Dpp signalling which is required for proximo-distal axis formation in *Drosophila*, but its function is restricted to the patterning of the most distal end of the legs.

Moreover, Prpic could show, that Egfr signalling is not just in the insect *Drosophila melanogaster* involved in leg pattering, but also in another basal arthropod species, the spider *Cupiennius salei*. Interestingly, it could be shown that Egfr signalling has a

more complex role than in *Drosophila*, where its activity is restricted to the tarsal region. Instead, in *Cupiennius salei* the activity of Egfr signalling is crucial for the complete leg growth (Prpic 2004).
Together with the assumption that the tarsal region represents an ancestral ground-state limb structure (Casares and Mann 2001, see section 2.1), these data indicate that the Egfr pathway might be a possible ancestral system which controlled proximo-distal axis pattering before the Wg/Dpp system evolved its role in leg formation.

2.6 Aim of this thesis

Leg development is best studied in the prime insect model organism *Drosophila melanogaster*. Leg pattering is guided by a hierarchic gene cascade and the morhogens Wingless and Decapentaplegic are the key factors and control the expression of proximo-distal pattering genes. Unfortunately, due to its phylogenetic position *Drosophila* presents a highly derived mode of arthropod leg development and it is questionable if the Wg/Dpp leg pattering system is evolutionary conserved throughout arthropods. In most other arthropod species legs develop directly from three-dimensional limb buds. The underlying mechanisms of this mode of appendage development are less well studied and the data available to date are controversial. Recent studies concerning the function of *wg* and *dpp* in *Oncopeltus fasciatus*, *Gryllus bimaculatus* and *Tribolium castaneum* yield controversial results and it is still highly debated if the Wg/Dpp leg pattering system represents the conserved system in arthropod leg development.
In the first part of this thesis, I further analysed the role and function of *wg* and *dpp* in leg development in the red flour beetle *Tribolium castaneum*. *Tribolium* is a holometabolous insect and belongs to the order of the Coleoptera and is therefore a representative of the most species-rich metazoan taxon (Hunt et al. 2006). In contrast to *Drosophila*, leg development proceeds directly from three-dimensional limb buds and *Tribolium* therefore presents a suitable model for analysing the Wg/Dpp system in an organism with a more basal mode of limb development as known in *Drosophila*. Besides, the genome is sequenced and a robust reverse genetic method to knock down gene function (RNA-interference, RNAi) is established (Bucher et al

2002; Tribolium Genome Sequencing Consortium 2008). Double-stranded RNA can be injected in embryonic, larval, pupal and adult stages and the RNAi effect is transmitted systemically to the offspring. These benefits make *Tribolium* an ideal model organism for this study. Previous data concerning the role of *wg* and *dpp* in *Tribolium* leg development are controversial but indicated that at least *wg* is required for appendage allocation. To circumvent early and severe developmental defects and to get further insight in the temporal requirement of *wg* and *dpp* I performed staggered stage-specific RNA-interference. In addition, different gene expression studies in *wg* and *dpp*RNAi embryos were performed to get insight of the mechanistic cause of the leg phenotypes and a better understanding of the later role of *wg* and *dpp* in *Tribolium* leg development.

As already mentioned, recent works provide evidence against a conserved role of the Wg/Dpp leg pattering system. It has been suggested that the Egfr signalling pathway represents a possible ancestral system. To gain insight of a possible ancestral function of the Egfr pathway, I isolated cDNA fragments of the genes encoding the *Tribolium* Egf receptor, its ligand Spitz and its target gene *pointed*. Beside gene expression studies I performed functional analyses using embryonic, pupal and larval RNA-interference.
Overall, both projects are supposed to give more insight in the role, conservation and diversity of the Wg, Dpp and Egfr pathways in arthropod leg development.

3 Results

Each chapter within the results section starts with a one-page description of:

- The aim of the particular manuscript in the context of the thesis as a whole.
- The status of the manuscript.
- The authors contribution to the practical work.

3.1 Separable functions of *wingless* in distal and ventral pattering in the *Tribolium* leg

The purpose of this work was to study the role of *wingless* in leg development in *Tribolium castaneum*. Therefore, we performed stage-specific staggered embryonic RNAi in wildtype and transgenic EGFP expressing enhancer trap lines. For the analysis of *wg*RNAi larvae and to identify specific leg parts, we used a specific map of cuticle markers. Furthermore, to demonstrate a role of *wingless* in distal and ventral pattering, we used molecular markers for the analysis of ventral and distal tissue in the developing *Tribolium* leg.

Daniela Großmann, Johannes Scholten, Nikola-Michael Prpic

Status: published in "Development, Genes and Evolution"
(2009) 219: 469-479

Authors contribution to the practical work:
Daniela Großmann performed all RNAi experiments and did all in situ hybridizations.
Johannes Scholten established the map of cuticle markers.

Dev Genes Evol (2009) 219:469–479
DOI 10.1007/s00427-009-0310-z

ORIGINAL ARTICLE

Separable functions of *wingless* in distal and ventral patterning of the *Tribolium* leg

Daniela Grossmann · Johannes Scholten · Nikola-Michael Prpic

Received: 14 October 2009 / Accepted: 23 November 2009 / Published online: 19 December 2009

Abstract The gene *wingless* (*wg*) in *Drosophila* is an important factor in leg development. During embryonic development *wg* is involved in the allocation of the limb primordia. During imaginal disk development *wg* is involved in distal development and it has a separate role in ventral development. The expression pattern of *wg* is highly conserved in all arthropods (comprising data from insects, myriapods, crustaceans, and chelicerates), suggesting that its function in leg development is also conserved. However, recent work in other insects (e.g. the milkweed bug *Oncopeltus fasciatus*) argued against a role of *wg* in leg development. We have studied the role of *wg* in leg development of the flour beetle *Tribolium castaneum*. Using stage-specific staggered embryonic RNAi in wild-type and transgenic EGFP expressing enhancer trap lines we are able to demonstrate separable functions of *Tribolium wg* in distal and in ventral leg development. The distal role affects all podomeres distal to the coxa, whereas the ventral role is restricted to cells along the ventral midline of the legs. In addition, severe leg defects after injection into early embryonic stages are evidence that *wg* is also involved in proximal development and limb allocation in *Tribolium*. Our data suggest that the roles of *wg* in leg development are highly conserved in the holometabolous insects. Further studies will reveal the degree of conservation in other arthropod groups.

Keywords Wingless · Leg development · Arthropods · Appendage evolution · *Tribolium castaneum*

Communicated by S. Roth

D. Grossmann · N.-M. Prpic (✉)
Johann-Friedrich-Blumenbach-Institut für Zoologie und Anthropologie, Georg-August-Universität Göttingen,
Abteilung Entwicklungsbiologie, GZMB Ernst-Caspari-Haus,
Justus-von-Liebig-Weg 11,
37077 Göttingen, Germany
e-mail: nprpic@uni-goettingen.de

J. Scholten
Department of Biology II,
Ludwig-Maximilians-Universität München,
Großhaderner Straße 2,
82152 Planegg-Martinsried, Germany

Present Address:
J. Scholten
Julius-Schaxel-Institut für Experimentelle Theorie,
Seelheimer Weg 5,
35043 Marburg, Germany

Introduction

The gene *wingless* (*wg*) is mainly known for its role in segment polarity specification during body segmentation in *Drosophila* (reviewed in Sanson 2001). However, *wg* is also an important factor in *Drosophila* leg development (e.g., Cohen et al. 1993; Struhl and Basler 1993; Campbell et al. 1993). The leg primordium in *Drosophila* is already specified in the embryo. At embryonic stage 11 the primordium of the leg is still combined with the wing primordium in the so-called thoracic limb primordium (Cohen et al. 1993). Wg is required for the specification of the entire thoracic limb primordium where it activates the *Distal-less* (*Dll*) gene via the early *Dll* enhancer 304 (Cohen et al. 1993; Kubota et al. 2003; McKay et al. 2009). This early function of Wg is repressed on the dorsal side by Decapentaplegic (Dpp) signaling and on the ventral side by epidermal growth factor receptor signaling (Goto and Hayashi 1997; Kubota et al. 2000), thus restricting the thoracic limb

primordium to the ventral–lateral side. At embryonic stage 14 the cells of the wing primordium downregulate Wg signaling and separate from the leg primordium (Cohen et al. 1993). The cells of the leg primordium subdivide further into proximal and distal cells. Wg is also involved in this process by activating the late enhancer LT ("leg trigger") of *Dll* in the distal cells, whereas the proximal cells do not require *wg* function at this stage (McKay et al. 2009; Estella and Mann 2008; Estella et al. 2008). This function of *wg* is separate from the early *wg* function as evidenced by the fact that Wg cooperates with Dpp rather than being repressed by it (Held et al. 1994; Diaz-Benjumea et al. 1994; Penton and Hoffmann 1996; Jiang and Struhl 1996; Theisen et al. 1996; Lecuit and Cohen 1997; Theisen et al. 2007). Finally, *wg* is then required for the specification of ventral fate in the legs (Struhl and Basler 1993; Theisen et al. 1996; Brook and Cohen 1996; Johnston and Schubiger 1996). This function, however, appears to be mechanistically different from the previous function where Wg protein forms a morphogen gradient. The ventral function of *wg* is local and does not require Wg protein to form a morphogen gradient (Diaz-Benjumea and Cohen 1994; Wilder and Perrimon 1995; Theodosiou et al. 1998). Instead, Wg might interact with other local factors to specify ventral fate through its target gene *H15* (Wilder and Perrimon 1995). In summary, *wg* has three temporally separate and mechanistically different functions during the development of the *Drosophila* leg. The earliest function specifies and allocates the entire leg primordium including the proximal cells. By contrast, the later function in organizing pattern formation along the proximal–distal axis concerns only distal cells and the proximal cells do not require *wg* at this stage. The ventral role, finally, is temporally and functionally separate from the proximal–distal role.

The *wg* gene was the first segment polarity gene that has been shown to have a conserved expression pattern in long-germ and short-germ insects (Nagy and Carroll 1994). In the meantime, *wg* has been isolated and studied in a number of other arthropods including different insect species (e.g. Jockusch et al. 2000; Niwa et al. 2000; Dearden and Akam 2001; Miyawaki et al. 2004), as well as myriapods (Hughes and Kaufman 2002; Prpic 2004; Janssen et al. 2004), crustaceans (Duman-Scheel et al. 2002; Nulsen and Nagy 1999; Prpic 2008), chelicerates (Damen 2002; Prpic et al. 2003), and an onychophoran (Eriksson et al. 2009). These data show that the expression pattern of *wg* in all arthropods is highly conserved suggesting conserved functions. Surprisingly, however, functional tests have so far provided no support for highly conserved functions. Especially the three separate roles of *wg* in leg development (allocation, distal, and ventral development) seem not to be conserved. In the milkweed bug *Oncopeltus fasciatus* and the cricket *Gryllus bimaculatus wg* is necessary for normal body segmentation, but does not seem to have a role in leg development (Angelini and Kaufman 2005a; Miyawaki et al. 2004). These two species belong to more basal hemimetabolous insect groups and Angelini and Kaufman (Angelini and Kaufman 2005b) have therefore suggested that the role of *wg* in appendage development evolved only later in the holometabolous insects. Previous data from *Tribolium castaneum* indicated that in this holometabolous insect *wg* is indeed required for appendage allocation, because legs were absent after *wg* RNAi, but a role of *wg* in distal and ventral development remained unclear (Ober and Jockusch 2006). We have therefore studied the temporal requirement of *wg* in *Tribolium* leg development using staggered stage-specific RNAi. Our results show that in *Tribolium wg* has three consecutive roles in allocation, distal, and ventral development, like in *Drosophila*. Our data suggest that the origin of these functions in insect evolution must predate the split between Diptera and Coleoptera.

Materials and methods

Parental and embryonic staggered RNAi

Parental RNAi was performed as described previously (Bucher et al. 2002). The pupae were injected with wg dsRNA with a concentration of 3,000 ng/µl and incubated at 32°C until eclosion. Eclosed females were mated with wild-type males and the eggs of two consecutive egg lays (24 h each) were collected. For embryonic RNAi, eggs were collected directly after egg deposition and incubated at 25°C until injection (4, 8, 12, and 18 h after egg laying). Injections were performed with beveled borosilicate needles using a micromanipulator and a FemtoJet injection controller (Eppendorf). Injected embryos were incubated at 25°C until hatching or (for in situ expression analysis) until germ band retraction. Removal of the *wg* mRNA below the level of detection was confirmed in each case by whole mount in situ hybridization using a *wg* probe.

Embryo fixation and in situ hybridization

Embryos for in situ hybridization were dechorionized with a 50% solution of DanKlorix (Colgate-Palmolive) in water and fixed with 4% formaldehyde in a mixture of PEMS (0.1 M Pipes, 2 mM $MgSO_4$, 1 mM EDTA; pH=6.9) and heptane. Vitelline membranes were removed by methanol shock and subsequent shearing through a syringe needle (19 G gauge). Whole-mount in situ hybridization detection of expression of enhanced green fluorescent protein (EGFP) mRNA was performed as described previously (Prpic et al. 2001). After in situ hybridization embryos were fixed with

4% formaldehyde in phosphate-buffered saline with 0.02% Tween-20, pH=7.4 and embedded in 80% glycerol for microscopy.

Microscopy and imaging

Analysis of the cuticle markers (leg bristles) was performed with a laser scanning microscope (Zeiss LSM 510). Cuticles and embryos from the in situ hybridizations were observed with differential interference contrast microscopy (Zeiss Axioplan-2). Images were captured with an Intas digital camera and were subjected to adjustment of brightness, contrast, and color values using Adobe Photoshop image processing software (Version 7.0 for Apple Macintosh).

Results

A map of surface cuticle markers for the legs of *T. castaneum*

In order to identify leg parts in the appendages of *Tribolium* with morphological markers we obtained a comprehensive map of cuticle markers comprising sensorial bristles and campaniform sensillae (Fig. 1a, b). Each leg segment (podomere) has a characteristic set of cuticle markers. However, not all markers are present in all individuals or on all legs and sometimes the number of certain markers may differ between individuals.

The coxa has 10 cuticle markers, two of which are especially suitable, because they are present in all individuals (denoted by a red dot in Fig. 2). These two markers are bristles, cx-1 and cx-2, that are located close to each other on the anterior dorsal side of the coxa. Two groups of smaller bristles, cx-a and cx-p, are present in most individuals and mark the anterior and posterior part of the coxa, respectively. However, the number of bristles in each group is variable (see Fig. 2). The remaining bristles occur singly (except cx-bp which may be present as a pair) and are present in most individuals. The tiny ventral bristle cx-v, however, is more frequently missing on thoracic leg 1 than on legs 2 and 3 (boxed in Fig. 2). The trochanter has eight cuticle markers most of which are present in all individuals. The best trochanter marker is a group of campaniform sensillae, tr-cs, that may comprise two or three members (denoted by a red dot in Fig. 2). The dorsolateral bristle tr-l is less suited, because it is more frequently missing on thoracic legs 1 and 2 than on leg 3 (boxed in Fig. 2). The femur has 10 cuticle markers of which the long ventral bristle fe-v1 is the most conspicuous one and is present in all individuals. The

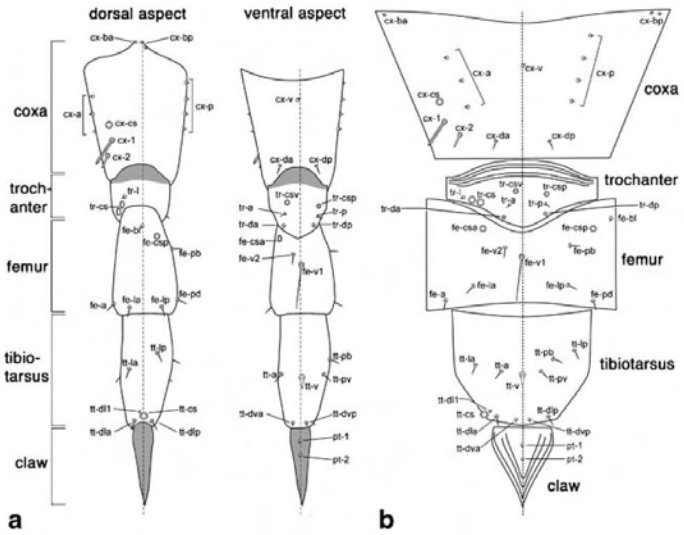


Fig. 1 A map of cuticular markers on the thoracic appendages of *T castaneum*. **a** Schematic drawing of a leg in dorsal (*left*) and ventral (*right*) aspect. The podomeres are indicated on the left side. **b** Schematic map of a leg opened along the dorsal side. The ventral midline of the leg is indicated by the *dotted line*. The cuticular markers are named according to their position on a specific podomere. The *letters before the dash* stand for the podomere, the *letters after the dash* denote the position on that podomere. For example, fe-pd indicates that this marker is on the femur, and there it is located posterior and distal. *Podomere abbreviations*: *cx* coxa, *tr* trochanter, *fe* femur, *tt* tibiotarsus, *pt* pretarsus (claw). *Position abbreviations*: *v* ventral, *l* dorsal, *a* anterior, *p* posterior, *b* proximal, *d* distal. *Other abbreviations*: *cs* campaniform sensilla

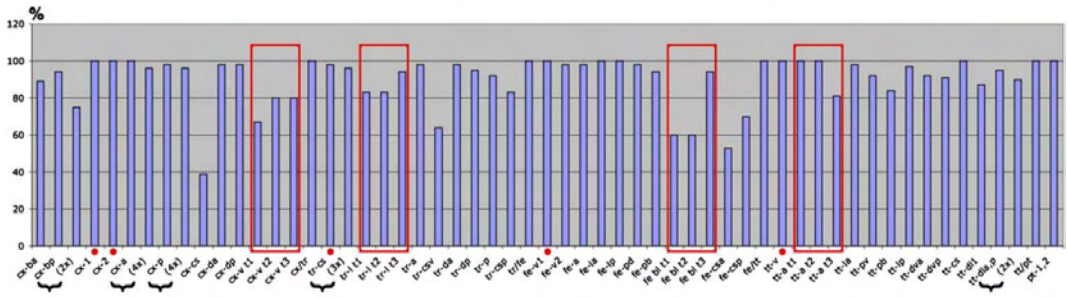


Fig. 2 Frequency of occurrence of cuticular leg markers on all three thoracic legs. The numbers were compiled from the study of 12 larvae. The cuticular markers are given on the *x*-axis (named according to the map in Fig. 1), and their frequency is given on the *y*-axis. Some markers may also be duplicated or quadruplicated; these cases are denoted by the *bracket*. A *red box* includes markers that have very different frequencies of occurrence on the different thoracic legs. The *red dot* denotes the most reliable and easy to discern markers for each podomere. Abbreviations: see Fig. 1

remaining bristles are present in most individuals; the two smaller bristles fe-la and fe-lp can serve as markers for the anterior and posterior femur as they are present in all individuals (see Fig. 2). The campaniform sensillae fe-csa and fe-csp are less suited as markers, because they are lacking in a large portion of individuals. The tibiotarsus has 11 cuticle markers of which the thorn-like bristle tt-v and the dorsal campaniform sensilla tt-cs are good markers, because they are present in all individuals. The remaining bristles are present in the majority of individuals. The two dorsal bristles tt-dla and tt-dlp are conspicuous, but one or both may be missing making them less suitable as markers. The claw finally has mostly two tiny bristles (pt-1, pt-2); at least one of them is always present.

The present map of cuticular markers identifies several markers—distributed over most podomeres—that do not display individual variation. These markers are useful morphological landmarks for phenotypic analysis of leg phenotypes in the larva. The remaining markers are present in the majority of wild-type specimens and may be used for phenotypic analysis if the low amount of individual variation is taken into account. Only few markers, especially campaniform sensillae, display higher rates of individual variation and should not be used for phenotypic analysis.

Parental RNAi with *Tc-wg*

Parental RNAi (pRNAi) in *Tribolium* has been shown to lead to significant down-regulation of a gene in the progeny of beetles injected at the pupal stage (Bucher et al. 2002). We have performed pRNAi experiments with *wg* in order to study the role of *wg* in development. The eclosion rate and the survival of eclosed imagines after *wg* dsRNA injection (injected pupae: $n=118$) was similar to the GFP dsRNA control injection (injected pupae: $n=55$), but slightly lower than in the uninjected control pupae (pupae: $n=42$; Fig. 3a). This indicates that *wg* pRNAi has no effect on the eclosure rate or the subsequent survival of the imagines, but the injection per se leads to a slightly reduced rate of eclosure and survival. A significant effect was seen in the productivity of the females after injection. The uninjected control females layed approximately 13 eggs per female during the first egg-laying period after eclosure and this rate increased to approximately 17 eggs per female during the second egg-laying period (Fig. 3b, left). In dsGFP-injected females the productivity was lower, probably owing to the injection procedure (Fig. 3b, center). However, productivity was even lower in females injected with *wg* dsRNA (Fig. 3b, right). Thus, this effect cannot be attributed to injection stress alone, and must have been caused by the *wg* dsRNA.

The layed eggs were incubated further and the rate of hatching was recorded. In the uninjected controls around half of the eggs were empty shells (hatching rate first egg lay, 61%; second egg lay, 42%; Fig. 3c). The percentage of empty egg shells increased in the GFP dsRNA-injected animals, and finally increased to over 95% in *wg* dsRNA injected animals (Fig. 3c). All eggs that reached hatching in both controls and also in the experiment gave rise to wild-type larvae. These data indicate that pRNAi with *wg* is leading to reduced female productivity (probably by interfering with gonad development or oogenesis), is embryonically lethal and the few obtained wild-type larvae in the *wg* dsRNA-injected animals likely are escapers.

Embryonic RNAi effects after staggered injections

Since the pRNAi experiments lead to either empty egg shells or wild-type cuticles, we turned to staggered embryonic RNAi (eRNAi). eRNAi circumvents the problem of effects already during gonad formation or oogenesis, and by staggering the injections the effects that lead to early embryonic lethality can also be excluded.

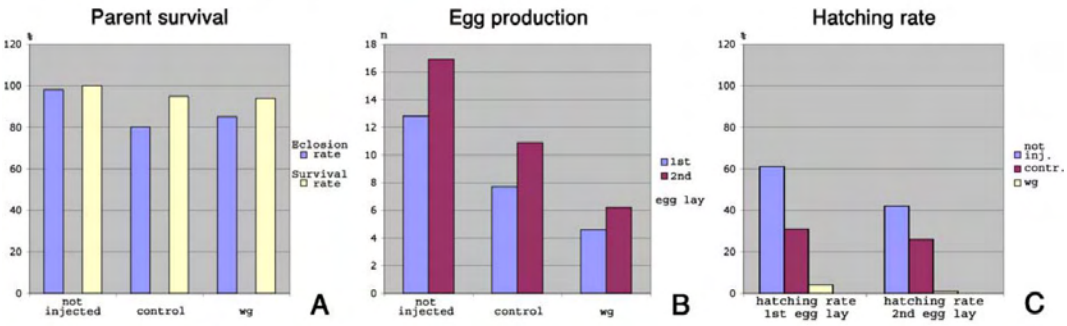


Fig. 3 Results of the parental RNAi experiments. **a** Eclosion rates and survival rates of the eclosed beetles (in percent). These are similar for not injected, control injected and *wg* injected animals. **b** Female productivity measured after the first and second egg lay. The mean number of the eggs per female is given on the *y*-axis. **c** Hatching rate (in percent) of the first and second egg lay and the percentage of wild-type cuticles after hatching. The hatching rate decreases dramatically after wg dsRNA injection, but all hatched larvae from uninjected, control injected and wg dsRNA injected mothers are wildtype. *inj.* injected

We performed the earliest injections 4 h after egg laying. At this time point the embryo is still at a very early stage of development and the cleavage nuclei are in the process of approaching the egg periphery. Not unexpected therefore, these injections led to 90% empty egg shells (Fig. 4b) indicating early embryonic lethality similar to the results of the pRNAi. However, 10% of the larvae developed a cuticle. Three percent of these displayed only irregular cuticular structures (Fig. 5a) that were also present in the control injections (see Fig. 4a), 1% were wildtype, and 6% of the larvae had legs with severely disturbed proximal–distal axis formation (Fig. 5b, c). These results indicate that eRNAi can produce weaker phenotypes than pRNAi and that these weaker phenotypes include severe defects in morphology (including leg formation).

We have therefore performed staggered injections 8, 12, and 18 h after egg laying (these values are approximate values; actual injections may deviate from these values by ±1.5 h). At 8 h of development the embryo has reached the blastoderm stage, and we anticipated to circumvent in this way to interfere with very early *wg* functions before blastoderm formation. At 12 h of development serosal closure takes place, the thoracic segments have already formed, but the leg primordia are not yet specified. We anticipated that in this way we could avoid to interfere with thoracic segment formation, but affect leg bud formation from the start. At 18 h of development germ band elongation is complete and all segments are formed, and the leg buds are already present. In this way we expected to be able to specifically interfere with late processes of leg development, without affecting segmentation or limb primordium specification. Injections later than 18 h after egg laying were not possible, because the vitelline membrane becomes too rigid and cannot be penetrated with the beveled borosilicate needles used in our experiments without squashing the embryo. The results of the 8-h injections were very similar to the 4-h injections. There was no significant decrease of empty egg shells or increase of proximal–distal leg axis phenotypes (Figs. 4b and 5d–f). The 12-h injections lead to a strong decrease of the amount of empty egg shells, and the occurrence of proximo-distal leg phenotypes increased simultaneously to almost 30%. This proximo-distal leg

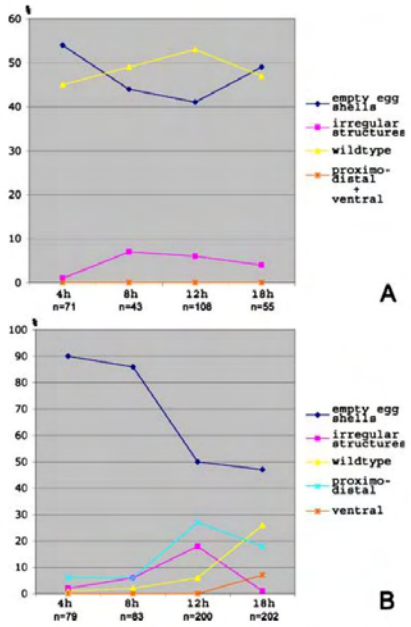


Fig. 4 Results of the staggered embryonic injections. **a** Control injections (injection buffer). **b** *wg* dsRNA injections. The time after egg laying is given on the *x*-axis. For details please refer to the text

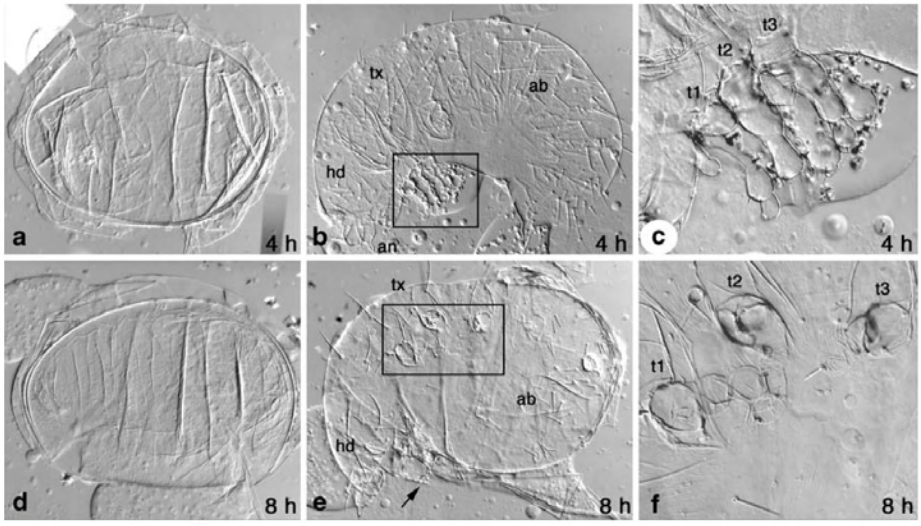


Fig. 5 Cuticle preparations of larvae hatched from eggs injected after 4 (**a**–**c**) and 8 (**d**–**f**) hours after egg laying. **a** and **d** are examples for eggs containing irregular cuticular structures. **b** and **e** show severely malformed larval cuticles with defective head, thorax, and abdomen. All appendages are affected; the thoracic legs are shown enlarged in panels (**c**) and (**f**). **c** is the enlargement of the area *boxed* in (**b**) and **f** is the enlargement of the area *boxed* in (**e**). The *arrow* in (**e**) points to the remnants of a maxilla. *an* antenna, *hd* head, *tx* thorax, *ab* abdomen, *t1* prothoracic leg, *t2* mesothoracic leg, *t3* metathoracic leg

phenotypes included specimens with malformed legs that nevertheless were composed of some leg segments (Fig. 6d, f), and specimens with more severely malformed legs (Fig. 6e, g). In these legs it was not possible to confidently establish the identity of the remaining leg portions because the morphology was too severely disturbed and most cuticle markers were absent. However, two additional phenotypes, that we term "candy cane" and "nonpareille" phenotypes, could be analyzed in more detail. In the "nonpareille" phenotype (named for Nonpareils pearls) all remaining leg segments appear rounded (Fig. 6b) and in severe cases they are lined up like pearls on a chain (Fig. 6c). In these phenotypes several cuticular markers were present (Fig. 6b, c) that indicate that the legs comprise a coxa with largely wild-type morphology, a long femur with ectopic constrictions, and a shortened tibiotarsus, and are lacking the claw. Thus, this phenotype reveals severe problems with proximal–distal axis formation distal to the coxa and distal-most structures are even lacking. The severe form of the "nonpareille" phenotype apparently is the "candy cane" phenotype where the podomeres do not form a chain anymore, but are fused into a long cane with a curiously bent distal end (Fig. 6a). Many of these specimens were simultaneously lacking the flagellum on the antennae (not shown).

In the 18-h injections the amount of empty egg shells dropped to control levels (Fig. 4a, b) and the amount of wild-type cuticles increased to almost 30%, indicating that at these late stages many *wg*-dependent processes are already complete and cannot be disturbed by the injection of *wg* dsRNA anymore. The overall amount of leg phenotypes remained constant, but the distal phenotype decreased and a novel, weaker phenotype appeared that does not lack distal leg segments. Instead, the claw is abnormally shaped: thin and pin-shaped and without the normal ventral bend (Fig. 7f). This phenotype also lacks the typical marker bristles that grow on, or very close to the ventral midline of the legs: on the femur (fe-v1) and tibiotarsus (tt-v, tt-pv). The lack of the ventral bend of the claw together with the lack of the ventral marker bristles indicates that this phenotype is caused by the loss of ventral tissue in the legs and we have therefore termed this phenotype "ventral-less".

The distal phenotypes of the 18-h injections were similar to the "nonpareille" phenotypes of the 12-h injections, but were weaker in most cases. The weakest cases had all typical podomeres including the claw, but the femur was elongated and thinner than in the wildtype and the tibiotarsus showed ectopic constrictions (Fig. 7d). The intermediate phenotype had normal coxa and trochanter, but a shortened tibiotarsus, no claw and a single ectopic constriction in the femur (Fig. 7b), and the most severe phenotypes were "nonpareille" phenotypes with pearl-shaped tibiotarsus and rounded ectopic subdivisions of the femur, and the trochanter seems to be fused entirely to the coxa (Fig. 7c).

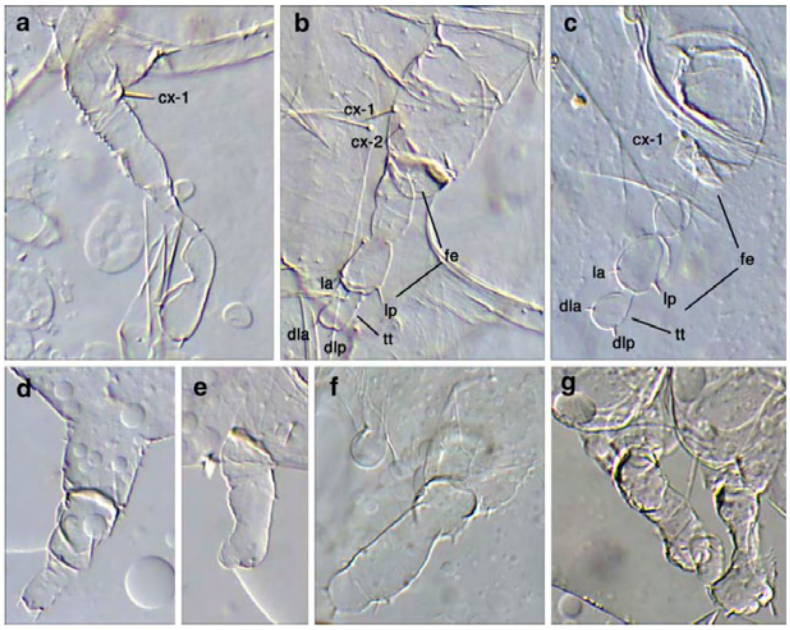


Fig. 6 Thoracic appendages of larvae hatched from eggs injected after 12 h after egg laying. **a** Typical "candy cane" leg phenotype. **b**, **c** "Nonpareille" leg phenotypes. Note the additional constrictions in the femur and the absence of the pretarsal claw. **d**–**g** More severe truncations of the legs. In some cases only stumps of the legs remain (**e**, **g**), in other cases some leg segments remain, but cannot be identified because of the lack of cuticle markers (**d**, **f**). For the labeling of the cuticle markers please refer to Fig. 1. *fe* femur, *tt* tibiotarsus

We have then used molecular markers for the ventral and distal tissues in the developing *Tribolium* leg. The GEKU insertional mutagenesis screen has produced about 50 enhancer trap lines with EGFP expression in the legs (Trauner et al. 2009). We have used two of these lines to perform staggered RNAi with *wg*. The line Goe-04609 expresses EGFP mRNA along the ventral side of the legs, and in addition in a segmentally repeated pattern and in the developing heart (Fig. 8a). In embryos injected 18 h after egg laying the expression is largely identical to the expression in the wildtype, but the ventral expression in the legs is lacking (Fig. 8b). This demonstrates that ventral leg tissue is missing. In the embryos injected earlier, the pattern of Goe-04609 is disturbed more severely: the segmental pattern is fused along the anterior–posterior axis and the expression in the heart is missing (Fig. 8c). The line Goe-12407 expresses EGFP mRNA in a ring of cells near the tips of the legs, and in addition in a punctate pattern in the central nervous system (Fig. 8d). In embryos injected 18 h after egg laying the expression is very similar to the expression in the wildtype, but the distal ring in the legs is reduced to a spot of expression on the dorsal side of the legs (Fig. 8e). This indicates that ventral leg tissue is missing. In the embryos injected earlier, the pattern of Goe-12407 is weak (Fig. 8f). However, the expression in the antennae is much stronger, indicating that the enhancer trapped in Goe-12407 is normally repressed in the antenna by *wg*.

Discussion

Evidence for a role of *wg* in leg allocation in *Tribolium*

Our experiments with pupal injections were not able to reveal a function of *wg* in leg development. Despite good eclosion and survival rates, the females that eclosed from pupae injected with *wg* dsRNA had a very low productivity and from the few eggs almost no larvae hatched; those larvae that did hatch were wildtype. The low productivity suggests that *wg* might have a role already during oogenesis. The low hatching rate indicates that *wg* has functions early in development that lead to early embryonic lethality and the few wild-type larvae must be escapers that by chance did not receive *wg* dsRNA.

Early injections into eggs lead to similar effects. Most of the eggs of the 4- and 8-h injections were empty or contained irregular cuticular structures similar to those observed by Bolognesi et al. (2008). The few larvae that developed showed severe phenotypes indicating problems with several developmental processes including segmentation. The legs of these larvae were severely malformed. The defects we have observed are very similar to those reported by Ober and Jockusch (2006). These authors also found segmentation defects and severely truncated or missing legs after *wg* RNAi. The specimens figured in their Fig. 2d–g are very similar to our results after early injections after 4 and 8 h. The specimen figured in their Fig. 2i shows

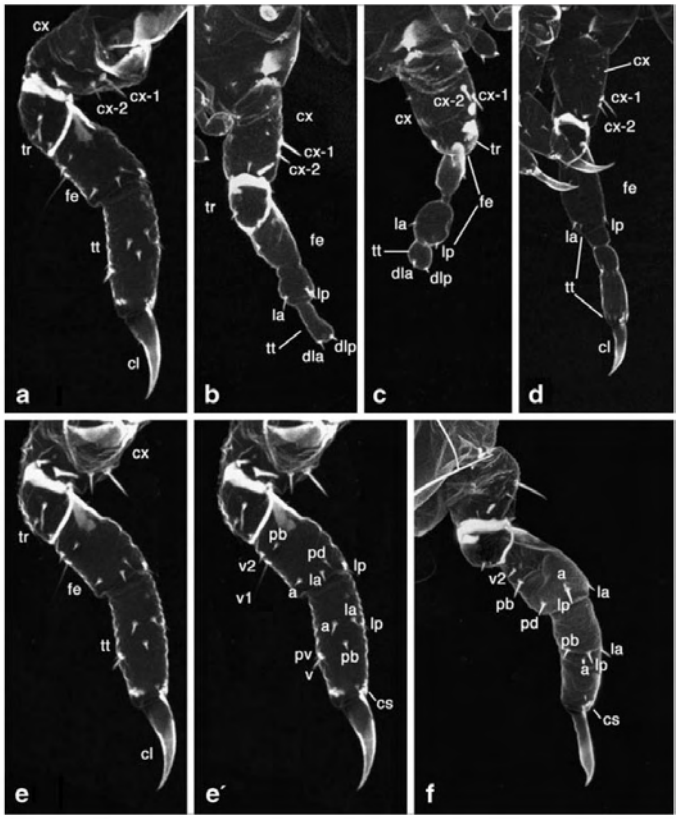


Fig. 7 Thoracic appendages of larvae hatched from eggs injected after 18 h after egg laying. **a** Wild-type larval leg for comparison. **b**–**d** Different "nonpareille" phenotypes. Note the additional constrictions in the femur and the absence of the pretarsal claw in the stronger "nonpareille" phenotype (**b**, **c**) and the additional constrictions in the tibiotarsus and the presence of the pretarsal claw in the weaker "nonpareille" phenotype (**d**). Also note the presence of a largely normal coxa in these phenotypes. (**e**, *E´*) Wild-type larval leg for comparison and labeled for the podomeres (**e**) and the cuticle markers of the femur and tibiotarsus (*E´*). **f** A "ventral-less" phenotype labeled for the cuticle markers of the femur and the tibiotarsus. Note the absence of fe-v1, tt-pv, and tt-v. Also note the lacking ventral bend of the pretarsal claw. For the labeling of the cuticle markers please refer to Fig. 1. *cx* coxa, *tr* trochanter, *fe* femur, *tt* tibiotarsus, *cl* pretarsal claw

shortened and severely malformed appendages, which is also very similar to what we have found in the early injections. Ober and Jockusch (2006) have interpreted these phenotypes as support for a role of *wg* in appendage allocation. However, because in all of these specimens the segments are also malformed, it is unclear whether the leg defects are true leg developmental defects or secondary defects because of the defective segment formation.

We avoided interfering with the early functions of *wg* in body segmentation by applying later injections after 12 and 18 h. The most severe leg phenotypes in these injections are seen in the 12-h injections: the legs are malformed and all parts of the leg are affected including the coxa. This is very similar to the early function of *wg* in leg allocation in *Drosophila* when Wg is required for activating the 304 enhancer of *Dll* in all cells of the leg primordium including proximal (coxal) cells (Cohen et al. 1993; McKay et al. 2009). We thus interpret the severe malformations in 12-h injections as evidence for a role of *Tribolium wg* in the specification and allocation of the entire leg primordium.

Separate functions of *wg* in distal and ventral development

In the "candy cane" and "nonpareille" phenotypes in the 12- and 18-h injections the coxa is not affected indicating that at this stage of development *wg* is not required for the development of the proximal leg parts anymore. The distal leg parts, however, are malformed and the distal-most portion, the claw, is missing in most specimens. This distal function is very similar to the distal function in *Drosophila* leg imaginal precursors where *wg* is required for the development of all leg portions distal to the coxa by activating the LT enhancer of *Dll* (Cohen et al. 1993; Estella et al. 2008; McKay et al. 2009). The extra constrictions in the femur or the tibiotarsus in the strong and weak "nonpareille" phenotypes, respectively, are unexpected and their origin is unclear. The normal number of constrictions (future joints) in *Drosophila* is specified by the action of the Notch pathway (de Celis et al. 1998; Rauskolb and Irvine 1999; Bishop et al. 1999) which is regulated by the leg gap genes and these are regulated by Wg signaling in conjunction with Dpp signaling (Lecuit and Cohen 1997; Estella and Mann 2008). It is

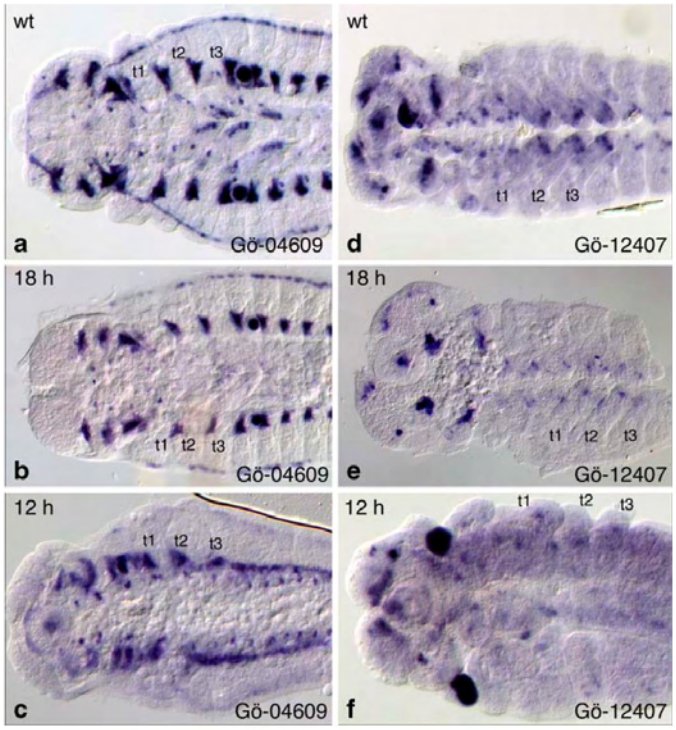


Fig. 8 Expression of molecular markers for ventral and distal leg fate is altered in *wg* RNAi embryos. **a**–**c** Expression of the ventral leg marker enhancer trap Goe-04609 in wild-type embryos (**a**) and embryos injected after 18 (**b**) and 12 h (**c**) after egg laying. **d**–**f** Expression of the distal leg marker enhancer trap Goe-12407 in wild-type embryos (**d**) and embryos injected after 18 (**e**) and 12 h (**f**) after egg laying. *t1* prothoracic leg, *t2* mesothoracic leg, *t3* metathoracic leg. Anterior is to the *left* in all panels

currently unknown whether a similar cascade exists in *Tribolium*, but this is very likely because the role of the Notch pathway in leg segmentation is also conserved in the spider *Cupiennius salei*, a basally branching arthropod (Prpic and Damen 2009). Thus, it is possible that interference with the distal function of *wg* disturbs the normal segmentation of the *Tribolium* legs leading to ectopic constrictions in the distal segments.

The "ventral-less" phenotype appeared only in the 18-h injections. In these legs all segments are normal except that the ventral-most tissue is lacking. This ventral function appears to be separate from the distal function since the "ventral-less" phenotype does not show any distal defects. Again this is very similar to the ventral function of *wg* in *Drosophila* where Wg does not act as a concentration-dependent morphogen, but rather acts locally on the ventral side probably in conjunction with other ventrally expressed factors (Diaz-Benjumea and Cohen 1994; Wilder and Perrimon 1995; Theodosiou et al. 1998).

Conclusions

In summary, our results indicate three separate functions of *wg* in leg development of *Tribolium*, and these functions also are temporally separated. The early function of *wg* is the specification of the entire leg primordium. This is supported by the strong leg defects after the 12-h injections that affect all leg parts including the coxa. Later injections cannot interfere with normal coxa development (the "candy cane" and "nonpareille" phenotypes in which distal leg portions are affected, but the coxa is normal) suggesting that at this stage *wg* has a role in the formation of the entire distal leg portion, but not in the proximal cells anymore. Future studies have to reveal whether this distal function is performed by *wg* as a morphogen in conjunction with *dpp* as the second morphogen as it is the case in *Drosophila* (e.g. Lecuit and Cohen 1997). Finally, the late function of *wg* is the specification of ventral leg cells. This function is independent of the distal function, because in the "ventral-less" phenotype the formation of the proximal–distal axis is not affected. The fact that the effect is restricted to ventral leg cells is evidence that it does not involve a Wg gradient.

Our results indicate that the separate functions of *wg* in leg allocation, distal, and ventral leg development must have evolved before the split between Diptera and Coleoptera. Without further information from more basal insects or non-insect arthropods this suggests that the leg functions of *wg* have evolved in the last common ancestor

of the holometabolous insects and thus earlier than suggested by Angelini and Kaufman (2005b). In order to exactly pinpoint the origin of the involvement of *wg* in arthropod leg development, the function of *wg* has to be studied in additional holo- and hemimetabolous insect species and also in other arthropod groups where functional data are currently lacking.

Acknowledgements We thank Gregor Bucher, Ernst A. Wimmer, and Johannes Schinko for providing us with the enhancer trap lines Goe-04609 and Goe-12407 from the GEKU insertional mutagenesis screen. We especially thank Martin Klingler for his support and advice. We also thank the members of our group for stimulating discussions and Marco Winkler for technical assistance. This work has been supported by the Deutsche Forschungsgemeinschaft (grant number PR 1109/1-1).

References

Angelini DR, Kaufman TC (2005a) Functional analyses in the milkweed bug *Oncopeltus fasciatus* (Hemiptera) support a role for Wnt signaling in body segmentation but not appendage development. Dev Biol 283:409–423

Angelini DR, Kaufman TC (2005b) Insect appendages and comparative ontogenetics. Dev Biol 286:57–77

Bishop SA, Klein T, Martinez Arias A, Couso JP (1999) Composite signalling from *Serrate* and *Delta* establishes leg segments in *Drosophila* through *Notch*. Development 126:2993–3003

Bolognesi R, Farzana L, Fischer TD, Brown SJ (2008) Multiple Wnt are required for segmentation in the short-germ embryo of *Tribolium castaneum*. Curr Biol 18:1624–1629

Brook WJ, Cohen SM (1996) Antagonistic interactions between Wingless and Decapentaplegic responsible for dorsal-ventral pattern in the *Drosophila* leg. Science 273:1373–1377

Bucher G, Scholten J, Klingler M (2002) Parental RNAi in *Tribolium* (Coleoptera). Curr Biol 12:R85–R86

Campbell G, Weaver T, Tomlinson A (1993) Axis specification in the developing *Drosophila* appendages: the role of *wingless*, *decapentaplegic*, and the homeobox gene *aristaless*. Cell 74:1113–1123

Cohen B, Simcox AA, Cohen SM (1993) Allocation of the thoracic imaginal primordia in the *Drosophila* embryo. Development 117:597–608

Damen WGM (2002) Parasegmental organization of the spider embryo implies that the parasegment is an evolutionary conserved entity in arthropod embryogenesis. Development 129:1239–1250

de Celis JF, Tyler DM, de Celis J, Bray SJ (1998) Notch signalling mediates segmentation of the *Drosophila* leg. Development 125:4617–4626

Dearden PK, Akam M (2001) Early patterning in the grasshopper, *Schistocerca gregaria*: *wingless*, *decapentaplegic* and *caudal* expression. Development 128:3435–3444

Diaz-Benjumea FJ, Cohen SM (1994) *wingless* acts through the *shaggy/zeste-white 3* kinase to direct dorsal-ventral axis formation in the *Drosophila* leg. Development 120:1661–1670

Diaz-Benjumea FJ, Cohen B, Cohen SM (1994) Cell interactions between compartments establishes the proximal-distal axis of *Drosophila* legs. Nature 371:175–179

Duman-Scheel M, Pirkl N, Patel NH (2002) Analysis of the expression pattern of *Mysidium columbiae wingless* provides evidence for conserved mesodermal and retinal patterning processes among insects and crustaceans. Dev Genes Evol 212:114–123

Eriksson J, Tait NN, Budd GE, Akam M (2009) The involvement of engrailed and wingless during segmentation in the onychophoran Euperipatoides kanangrensis (Peripatopsidae: Onychophora) (Reid, 1996). Dev Genes Evol 219:249–264

Estella C, Mann RS (2008) Logic of Wg and Dpp induction of distal and medial fates in the *Drosophila* leg. Development 135:627–636

Estella C, McKay DJ, Mann RS (2008) Molecular integration of Wingless, Decapentaplegic, and autoregulatory inputs into *Distalless* during *Drosophila* leg development. Dev Cell 14:86–96

Goto S, Hayashi S (1997) Specification of the embryonic limb primordium by graded activity of Decapentaplegic. Development 124:125–132

Held LI, Heup MA, Sappington M, Peters SD (1994) Interactions of *decapentaplegic*, *wingless*, and *Distal-less* in the *Drosophila* leg. Roux Arch Dev Biol 203:310–319

Hughes CL, Kaufman TC (2002) Exploring myriapod segmentation: the expression patterns of *even-skipped*, *engrailed*, and *wingless* in a centipede. Dev Biol 247:47–61

Janssen R, Prpic NM, Damen WGM (2004) Gene expression suggests decoupled dorsal and ventral segmentation in the millipede *Glomeris marginata* (Myriapoda: Diplopoda). Dev Biol 268:89–104

Jiang J, Struhl G (1996) Complementary and mutually exclusive activities of Decapentaplegic and Wingless organize axial patterning during *Drosophila* leg development. Cell 86:401–409

Jockusch EL, Nulsen C, Newfeld SJ, Nagy LM (2000) Leg development in flies versus grasshoppers: differences in *dpp* expression do not lead to differences in the expression of downstream components of the leg patterning pathway. Development 127:1617–1626

Johnston LA, Schubiger G (1996) Ectopic expression of *wingless* in imaginal discs interferes with *decapentaplegic* expression and alters cell determination. Development 122:3519–3529

Kubota K, Goto S, Eto K, Hayashi S (2000) EGF receptor attenuates Dpp signaling and helps to distinguish the wing and leg cell fates in *Drosophila*. Development 127:3769–3776

Kubota K, Goto S, Hayashi S (2003) The role of Wg signaling in the patterning of embryonic leg primordium in *Drosophila*. Dev Biol 257:117–126

Lecuit T, Cohen SM (1997) Proximal-distal axis formation in the *Drosophila* leg. Nature 388:139–145

McKay DJ, Estella C, Mann RS (2009) The origins of the *Drosophila* leg revealed by the cis-regulatory architecture of the *Distalless* gene. Development 136:61–71

Miyawaki K, Mito T, Sarashina I, Zhang H, Shinmyo Y, Ohuchi H, Noji S (2004) Involvement of Wingless/Armadillo signaling in the posterior sequential segmentation in the cricket, *Gryllus bimaculatus* (Orthoptera), as revealed by RNAi analysis. Mech Dev 121:119–130

Nagy LM, Carroll SB (1994) Conservation of *wingless* patterning functions in the short-germ embryos of *Tribolium castaneum*. Nature 367:460–463

Niwa N, Inoue Y, Nozawa A, Saito M, Misumi Y, Ohuchi H, Yoshioka H, Noji S (2000) Correlation of diversity of leg morphology in *Gryllus bimaculatus* (cricket) with divergence in *dpp* expression pattern during leg development. Development 127:4373–4381

Nulsen C, Nagy LM (1999) The role of *wingless* in the development of multibranched crustacean limbs. Dev Genes Evol 209:340–348

Ober KA, Jockusch EL (2006) The roles of *wingless* and *decapentaplegic* in axis and appendage development in the red flour beetle, *Tribolium castaneum*. Dev Biol 294:391–405

Penton A, Hoffmann FM (1996) *Decapentaplegic* restricts the domain of *wingless* during *Drosophila* limb patterning. Nature 382:162–165

Prpic NM (2004) Homologs of *wingless* and *decapentaplegic* display a complex and dynamic expression profile during appendage development in the millipede *Glomeris marginata* (Myriapoda: Diplopoda). Front Zool 1:6. doi:10.1186/1742-9994-1-6

Prpic NM (2008) Parasegmental appendage allocation in annelids and arthropods and the homology of parapodia and arthropodia. Front Zool 5:17. doi:10.1186/1742-9994-5-17

Prpic NM, Damen WGM (2009) *Notch*-mediated segmentation of the appendages is a molecular phylotypic trait of the arthropods. Dev Biol 326:262–271

Prpic NM, Wigand B, Damen WGM, Klingler M (2001) Expression of *dachshund* in wild-type and *Distal-less* mutant *Tribolium* corroborates serial homologies in insect appendages. Dev Genes Evol 211:467–477

Prpic NM, Janssen R, Wigand B, Klingler M, Damen WGM (2003) Gene expression in spider appendages reveals reversal of *exd/hth* spatial specificity, altered leg gap gene dynamics, and suggests divergent distal morphogen signaling. Dev Biol 264:119–140

Rauskolb C, Irvine KD (1999) *Notch*-mediated segmentation and growth control of the *Drosophila* leg. Dev Biol 210:339–350

Sanson B (2001) Generating patterns from field of cells. Examples from *Drosophila* segmentation. EMBO rep 2:1083–1088

Struhl G, Basler K (1993) Organizing activity of Wingless protein in *Drosophila*. Cell 72:527–540

Theisen H, Haerry TE, O'Connor MB, Marsh JL (1996) Developmental territories created by mutual antagonism between Wingless and Decapentaplegic. Development 122:3939–3948

Theisen H, Syed A, Nguyen BT, Lukacsovich T, Purcell J, Srivastava GP, Iron D, Gaudenz K, Nie Q, Wan FYM, Waterman ML, Marsh JL (2007) Wingless directly represses DPP morphogen expression via an Armadillo/TCF/Brinker complex. PLoS ONE 2:e142

Theodosiou NA, Zhang S, Wang WY, Xu T (1998) *slimb* coordinates *wg* and *dpp* expression in the dorsal-ventral and anterior-posterior axes during limb development. Development 125:3411–3416

Trauner J, Schinko J, Lorenzen MD, Shippy TD, Wimmer EA, Beeman RW, Klingler M, Bucher G, Brown SJ (2009) Large-scale insertional mutagenesis of a coleopteran stored grain pest, the red flour beetle *Tribolium castaneum*, identifies embryonic lethal mutations and enhancer traps. BMC Biol 7:73

Wilder EL, Perrimon N (1995) Dual functions of *wingless* in the *Drosophila* leg imaginal disc. Development 121:477–488

3.2 Egfr signalling is mediated by spatially distinct mediators in proximo-distal leg pattering in *Tribolium castaneum*

The purpose of this work was to study the role of the *Epidermal growth factor receptor*, the ligand *spitz* and the transcription factor *pointed* in formation and pattering of the legs in *Tribolium castaneum*. Therefore, we isolated homologs of *Egfr*, *spitz* and *pointed* and analysed the expression pattern in wildtype embryos. Furthermore, we performed stage-specific staggered embryonic RNAi with all three genes, followed by embryonic and larval phenotype analysis. In addition, we analysed the effect of RNAi on the pattern formation in developing legs using different marker genes.

Daniela Großmann, Nikola-Michael Prpic

Status: manuscript in preparation

Authors contribution to the work:
Daniela Großmann performed all experiments.

Egfr signalling is mediated by spatially distinct mediators in proximodistal leg patterning in *Tribolium castaneum*

Daniela Grossmann and Nikola-Michael Prpic (*)

Georg-August-Universität Göttingen, Johann-Friedrich-Blumenbach-Institut für Zoologie und Anthropologie, Abteilung für Entwicklungsbiologie, GZMB Ernst-Caspari-Haus, Justus-von-Liebig-Weg 11, 37077 Göttingen, GERMANY

(*) author for correspondence

Tel.: ++49 551 39 101 24

Fax: ++49 551 39 5416

E-mail: nprpic@uni-goettingen.de

Number of words: 5930 (in total)

Number of figures: 6

Supporting information: 3 supplemental figures and legends

Abstract

The evolution of a mechanism to generate a proximal-distal axis perpendicular to the anterior-posterior body axis was a key event in arthropod evolution and facilitated the formation of appendages, which formed the basis for the diversification and evolutionary success of the phylum. The study of proximodistal leg patterning in extant arthropods can provide insight into the origin and evolution of the proximal-distal axis and its patterning mechanisms. In the fly *Drosophila melanogaster*, proximal-distal patterning is mainly organized by Wg/Dpp signalling. Egfr signalling is also involved in proximal-distal axis patterning, but is restricted to late stage and distal leg parts only. There is increasing evidence that this predominance of Wg/Dpp signalling in proximodistal axis patterning is not typical for the arthropods, and Egfr signalling could be more important in more basal insects and other arthropods. Here we study the role of *Epidermal growth factor receptor* (*Egfr*), *spitz* (*spi*), and *pointed* (*pnt*) in leg development in the beetle *Tribolium castaneum*. We show that Egfr signalling has a more complex role in *T. castaneum* than in *D. melanogaster* and is not only required in the distal leg, but is also involved in pattern formation in the medial leg. *Egfr* and *spi* are required for the regulation of *clawless* (*cll*), *Distal-less* (*Dll*) and *dachshund* (*dac*), and after RNAi lead to thickened and fused leg segments. Intriguingly, Egfr signalling in the medial leg appears functionally separate from its distal role, since it is not mediated by the transcription factor Pnt. This suggests that Egfr signalling has a dual role with separate mediators in proximodistal axis patterning. While the distal role is evolutionarily conserved, Wg/Dpp signalling apparently evolved as a parallel or redundant system driving the reduction/loss of Egfr signalling in the medial leg region.

Introduction

The arthropods comprise four major groups (so-called "classes"): Chelicerata (e.g. spiders, mites), Myriapoda (e.g. centipedes, millipedes), Crustacea (e.g. shrimps, lobsters), and Insecta (=Hexapoda; e.g. flies, beetles, butterflies). In all four classes together there are about 1 million described species and thus the arthropoda comprise about 50% of all known species of organisms on the planet (Purvis and Hector, 2000). This intriguing diversity and evolutionary success of the arthropods is to a large part based on the functional and morphological diversity of their segmental appendages. Thus, the innovation of appendages was a key event in arthropod evolution. To evolve from an appendage-less ancestor to the first appendage bearing arthropod a genetic system was needed that is capable of generating a novel axis on the body that is perpendicular to the anterior-posterior axis, the so-called proximal-distal axis. The study of mechanisms of proximodistal appendage patterning in extant species of arthropods can provide insights into the origin and evolution of this axis.

Drosophila melanogaster is a well-established genetical model system and many of its developmental processes have been studied in great detail. However, due to the rapid development of *D. melanogaster* many features of its development are derived. Leg development is an example for such a derived mode of development. Most arthropods develop their appendages as gradual outgrowths during embryonic stages. *D. melanogaster*, however, develops its legs via leg imaginal discs. The location of these discs is specified during embryogenesis (e.g. Cohen et al., 1991), but the physical formation of the discs takes place only later during larval development. In addition the legs are formed as flat sheets of tissue inside of the larva and thus without an obvious morphological proximal-distal axis. Nevertheless, studies of leg disc development have shown that the proximal-distal axis is laid down during the larval stages. The patterning of the proximodistal axis is mainly organized by a system using Wingless (Wg) and Decapentaplegic (Dpp) signalling (Struhl and Basler, 1993; Campbell et al., 1993; Diaz-Benjumea et al., 1994; Jiang and Struhl, 1996; Penton and Hoffmann, 1996; Theisen et al., 1996). While Wg ligands are produced on the ventral side, the dorsal side of the disc produces the Dpp ligand. These two ligands emanate from their area of origin and thus cover the entire leg disc

with gradients of Wg and Dpp concentration. By "measuring" the concentration of Wg and Dpp ligands, each cell in the disc can determine its distance from either source of the morphogens and thus its exact location within the disc. This positional information is then used to activate the appropriate proximal-distal patterning genes in the leg disc (Lecuit and Cohen, 1997; Estella et al., 2008; Estella and Mann, 2008; McKay et al., 2009; but see also Giorgianni and Mann, 2011). Thus, Wg/Dpp signalling is the main system of proximodistal leg axis patterning in *D. melanogaster*. There is a second system, that uses Egfr signalling, but this system is only used at later stages of leg disc development, when most of the proximodistal axis has already been patterned by Wg/Dpp signalling and thus is only required for the patterning of the distal end of the legs (Campbell, 2002; Galindo et al., 2002; Galindo et al., 2005).

Given this predominance of Wg/Dpp signalling in proximal-distal leg axis patterning in *D. melanogaster* it was generally assumed that it should also be the ancestral mechanism of proximal-distal leg axis development in arthropods in general, and thus should be evolutionarily conserved. Species-specific gene expression differences are believed to reflect differences in leg architecture rather than differences in Wg/Dpp function (Prpic et al., 2003). Surprisingly, however, there is accumulating evidence that Wg/Dpp signalling does not play a major role in proximodistal leg development in other arthropod species (Miyawaki et al., 2004; Angelini and Kaufman, 2005a; Ober and Jockusch, 2006). Thus, it has been suggested that Wg/Dpp signalling evolved its role in proximal-distal leg patterning only late in insect evolution in the lineage of the holometabolous insects (Angelini and Kaufman, 2005b; Shah et al., 2011). This leaves the question how proximal-distal leg axis patterning is organized in the majority of the other arthropod species. One possibility is that the Egfr signalling pathway, that only plays a minor role in *D. melanogaster*, has a more important role in these species.

Egfr signalling in *D. melanogaster* uses mainly three different ligands of the Egf receptor: Spitz (Spi), Vein (Vn), and Gurken (Grk). While Vein and Gurken serve as tissue specific ligands, Spitz is ubiquitously expressed and is an Egfr ligand in a large number of developmental processes (reviewed in Perrimon and Perkins, 1997). After the activation of the Egf receptor by the binding of any of the ligands a cascade of

kinases relays the signal to the nucleus. Most outcomes of Egfr signalling are mediated in the nucleus by the transcription factor Pointed (Pnt) (Rutledge et al., 1992; O´Neill et al., 1994). Pnt in *D. melanogaster* exists in two isoforms, called Pnt-P1 and Pnt-P2. Pnt-P2 protein is activated by phosphorylation through Egfr signalling; Pnt-P1 activity is independent from Egfr signalling, but its transcription depends on active Pnt-P2 (O´Neill et al., 1994; Gabay et al., 1996; Brunner et al., 1994). Thus, the complete activity of Pnt depends directly or indirectly on Egfr signalling.

We study leg development in the red flour beetle *Tribolium castaneum*. The beetles (Coleoptera) belong to the holometabolous insects, but are more basally branching than the flies (Diptera). Thus, from their phylogenetic position they might rely more on Egfr signalling during proximo-distal leg axis patterning than flies. It has been shown previously that *wg* and Wg signalling have a role in proximal-distal leg patterning in *T. castaneum* (Ober and Jockusch, 2006; Grossmann et al., 2009; Beermann et al., 2011; Bao et al., 2011). The role of *dpp* in this process is still unclear, because of its early function in dorsoventral patterning (Ober and Jockusch, 2006; Van der Zee et al., 2006; Nunes da Fonseca et al., 2008, 2010). We have here studied the role of *spi*, *Egfr* and *pnt* in the formation and patterning of the legs in *T. castaneum*. We show that RNAi with *Egfr*, *spi*, and *pnt* has effects on the patterning in the distal leg. RNAi with *Egfr* and *spi* has an additional effect in the medial leg. We conclude that in *T. castaneum* Egfr signalling is an additional patterning system that is required in parallel with Wg/Dpp signalling for the patterning of the proximal-distal leg axis.

Materials and methods

Embryonic RNAi

For embryonic RNAi, eggs were collected directly after egg deposition and incubated at 25°C until injection (approximately 12 h after egg laying). Injections were performed with beveled borosilicate needles using a micromanipulator and a FemtoJet injection controller (Eppendorf). The concentration of the dsRNA was 1,800 ng/µl. Injected embryos were incubated at 25°C until hatching or (for in situ

expression analysis) until germ band retraction. Removal of the mRNA below the level of detection was confirmed in each case by whole mount in
situ hybridization using the probes also used for the analysis of the wildtype expression pattern. To exclude off-target effects, separate non-overlapping fragments of *Egfr* and *pnt* were used for dsRNA synthesis and these dsRNA preparations were injected in parallel with the normal RNAi experiments (i.e. with the full gene fragment). In each case the normal RNAi and the off-target RNAi led to identical results. For *spi*, this off-target control was not possible, because the sequence was too short to design two non-overlapping fragments. In order to assess the probability of off-target effects with *spi* RNAi, we have performed a BLAST based search for possible unspecific targets in the *T. castaneum* genome as described previously (Posnien et al., 2011). This analysis showed that all possible 21mers of the *spi* sequence used for RNAi have no identical or highly similar matches anywhere in the *T. castaneum* genome sequence (Tribolium Genome Sequencing Consortium, 2008) except for the *spi* gene. Thus, off-target effects are unlikely.

Embryo fixation and in situ hybridization

Embryos for in situ hybridization were dechorionized with a 50% solution of DanKlorix (Colgate-Palmolive) in water and fixed with 4% formaldehyde in a mixture of PEMS (0.1 M Pipes, 2 mM MgSO4, 1 mM EDTA; pH=6.9) and heptane. Vitelline membranes were removed by methanol shock and subsequent shearing through a syringe needle (19 G gauge). Whole-mount in situ hybridization detection of mRNA expression was performed as described previously (Prpic et al. 2001). After in situ hybridization embryos were fixed with 4% formaldehyde in phosphate-buffered saline with 0.02% Tween-20, pH=7.4 and embedded in 80% glycerol for microscopy.

Microscopy and imaging

Larval cuticles and embryos from the in situ hybridizations were observed with differential interference contrast microscopy (Zeiss Axioplan-2). Images were captured with an Intas digital camera and were subjected to adjustment of brightness, contrast, and color values using Adobe Photoshop image processing software (Version CS5 for Apple Macintosh).

Results

Embryonic expression of *Egfr*, *spi*, and *pnt*

Expression of *Egfr* during the early stages of gastrulation is ubiquitous, but higher levels are detected at the posterior end of the germ band (Fig. 1A). During germ band elongation the low-level ubiquitous expression decreases, but high levels of expression persist in the growth zone (Fig. 1B) and later additional expression domains appear in the appendages (including the labrum) and segmental spots in the abdomen (Fig. 1C). The legs initially express *Egfr* ubiquitously and at a high level (Fig. 1D), but in the legs of embryos during germ band retraction *Egfr* expression resolves into a number of segmental rings (Fig. 1E).

Gastrulation stage embryos express *spi* at the anterior pole and extending from there are two stripes along the main body axis (Fig. 1F). Later, the anterior expression resolves into two expression spots in the brain and the longitudinal lines fuse into a single stripe along the ventral midline in the gnathal region, but remain separate in the growth zone, where *spi* expression forms a loop in the center of the growth zone (Fig. 1G). During germ band extension the expression along the ventral midline and the spots in the brain remain the most prominent expression domains, but small dots of expression appear in the tip of all appendage buds (Fig. 1H). This expression in a small group of cells in the tip of the legs persists during germ band extension (Fig. 1I) and germ band retraction (Fig. 1J). This suggests that a source at the leg tip that emanates the Spi ligand is active throughout leg development.

During gastrulation *pnt* is mainly expressed at the posterior end of the germ band (Fig. 1K), and later a strong domain at the presumptive site of the stomodeum is also visible (Fig. 1L). In elongated germ band embryos additional expression of *pnt* appears along the ventral midline, the brain and ocular region and the peripheral nervous system (Fig. 1M). In the legs, expression is restricted to small groups and rings of cells in the distal leg portion, and proximally there is also expression within the legs, probably correlated with leg innervation (Fig. 1N, O).

RNAi with *Egfr*, *spi*, and *pnt* leads to similar phenotypes

Parental RNAi experiments (data not shown) showed that injected females had severely reduced egg productivity, thus indicating that the three genes are involved in

oogenesis and/or gonad formation and function. Therefore, their role in leg development could not be studied with parental RNAi and we used embryonic RNAi instead to avoid interfering with oogenesis or other early developmental processes. All embryos were injected approximately 12 hours after egg laying (see also Materials and Methods) and thus at a time point shortly before the limb buds become specified. The embryonic RNAi phenotype was similar for all three genes and examples of embryos resulting from the injections are shown in Fig. 4. The head and most of the thorax show only mild defects; however, all appendages of these two tagmata are malformed and shortened. The embryos show problems with the development of dorsal tissue. The dorsal (= lateral) tissue along both sides of the embryos converges at the level of the second or third thoracic segment, thus leading to the development of a rim of dorsal tissue at the end of the thorax that crosses the ventral midline (e.g. arrow in Fig. 4C). This appears to cause problems with abdominal dorsoventral polarity, because the abdomen develops from this point as an invaginated tube of malformed segments. This "outside-in" abdomen is very thin and therefore easily breaks off during preparation. It is therefore unclear whether a normal number of abdominal segments develops in every case; however, in some preparations the malformed tube-shaped abdomen is very long (e.g. Fig. 4M), suggesting that an almost complete number of abdominal segments can be formed in some cases. Most of these embryos died before they were able to produce a cuticle, as indicated by the high number of egg shells in the cuticle preparations after RNAi (Supplementary Figures 1-3). Those embryos that survived to the larval stage (i.e. cuticle formation) mostly did not manage to hatch from the egg and had to be dissected from the egg by hand. Examples of these larvae are shown in Figs. 2 and 3. A head capsule is present in all of the larvae (Fig. 2A-F), but the thorax is often severely malformed (Fig. 2C). In all cases the abdomen is present as a long tube of cuticle with all bristles growing on the inside and directed towards each other. Thus, this "outside-in" cuticle tube is consistent with the "outside-in" abdomen seen in the embryos. The inverted abdomen is common to the RNAi phenotypes of all three genes (Fig. 2A-F). In contrast, gene specific differences are seen with respect to the formation of the legs. Those larvae with a severely malformed thorax do not show significant leg development (e.g. Fig. 2C), but in the remaining larvae leg morphology could be studied. In *Egfr* RNAi larvae the legs are shorter and visibly

thicker than the wildtype legs and the claw is missing (Fig. 3A). In addition, the segments distal to the trochanter are often fused together (e.g. legs on t1 and t2 in Fig 3A). A similar phenotype is observed in the legs of *spi* RNAi larvae, but the fusion and shortening of the segments distal to the trochanter is even stronger. All segments form a single lozenge-shaped segment without a claw (Fig. 3B). By contrast, the legs of *pnt* RNAi larvae are all virtually identical to wildtype legs, except that they are lacking the claw (Fig. 3C). In summary, the leg phenotypes of all three genes had in common that the proximal podomeres coxa and trochanter were never affected, but the distal claw was missing. Fusions of the medial leg segments (femur and tibiotarsus) were only observed after *Egfr* RNAi and *spi* RNAi, but not after *pnt* RNAi.

Changed proximal-distal pattern formation after RNAi with *Egfr*, *spi*, and *pnt*

The cuticle phenotypes already indicated a role of *Egfr*, *spi*, and *pnt* in leg formation. We have therefore examined the effect of the RNAi on pattern formation in the developing legs. The gene *clawless* (*cll*) is strongly expressed in the claw, and thus in the distalmost segment of the legs (Fig. 4A, B). In the legs of *Egfr* RNAi embryos and *pnt* RNAi embryos *cll* expression is completely abolished (Fig. 4C, D, G, H). In the legs of *spi* RNAi embryos *cll* expression is still detected, but with strongly reduced levels (Fig. 4E, F). The gene *Distal-less* (*Dll*) is expressed in the medial and distal portion of all legs (Beermann et al., 2001) (Fig. 4I, J). Expression of *Dll* is strongly reduced in the legs of *Egfr* RNAi embryos and *spi* RNAi embryos, but the overall expression pattern is not changed (Fig. 4K-N). In *pnt* RNAi embryos the expression pattern of *Dll* is virtually identical to the wildtype expression pattern (Fig. 4O, P). The gene *dachshund* (*dac*) is expressed in a ring in the medial portion of the legs (Prpic et al., 2001) (Fig. 5A). In *Egfr* RNAi embryos and *spi* RNAi embryos the size of the dac expression domain is strongly expanded and can include the distal end of the malformed appendage (Fig. 5B, C). In *pnt* RNAi embryos, however, the expression pattern of *dac* is unchanged (Fig. 5D). The gene *Sp8* is expressed in the distal and medial portions of the legs, and thus similar to the *Dll* gene (Schaeper et al., 2010) (Fig. 5E). However, there are two rings with increased expression levels, one ring at the proximal end of the expression domain, and the second ring near the

tip. In the RNAi embryos the expression pattern is more or less unchanged (Fig. 5F-H), but especially in *Egfr* RNAi the expression levels appear to be slightly reduced (Fig. 5F).

Discussion

Egfr signalling in embryonic leg patterning in *Tribolium castaneum*

As summarized in the introduction, the Spi protein is one of three cardinal ligands of the Egf receptor in *Drosophila melanogaster*, and Pnt is the main transcription factor that mediates most outcomes of signal transduction through the Egfr pathway. The similar effect on abdominal polarity observed after RNAi with *Egfr*, *spi*, and *pnt* in *Tribolium castaneum* suggests, that these factors form a similar pathway in *T. castaneum* as well (Fig. 6). This notion is also supported by the conserved action of Rhomboid and Star in *T. castaneum*, which take part in the proper activation of the Spi ligand in *D. melanogaster* as well as in *T. castaneum* (Rousso et al., 2010), and by the conserved role of Egfr signalling in dorsoventral patterning (Lynch et al., 2010).

However, the effect on leg formation and leg patterning shows some significant differences between the three studied genes. The expression of *cll* is abolished or reduced after RNAi with all three genes. This suggests that they are functionally related in a hierarchical chain that is required for the activation of *cll* in the distal leg. Interference with any segment of the chain thus leads to the downregulation of *cll* expression (Fig. 6). By contrast, the regulation of *Dll* and *dac* does not require *pnt*, but still is affected by RNAi with *Egfr* and *spi*. This suggests that in these cases the outcome of Egfr signalling is mediated by another as yet unidentified transcription factor ("X" in Fig. 6). We propose that this alternative mediator is required for *Dll* activation, but normally represses *dac* expression, because *dac* expression is strongly upregulated after *Egfr* RNAi and *spi* RNAi. However, the unidentified Egfr signalling mediator is unlikely to be the only activator of *Dll*, because *Dll* expression is reduced but not fully abolished after *Egfr* RNAi and *spi* RNAi. The relationship between Egfr signalling and *Sp8* is unclear from the present data ("?" in Fig. 6). At least there is no strong effect on *Sp8* expression after interference with Egfr signalling, which might indicate that the patterning function of *Sp8* is largely

independent from Egfr signalling.

These conclusions from the study of embryonic leg patterning are consistent with the leg cuticle phenotype. Because most of the severely affected embryos die before reaching the larval stage and thus do not form a cuticle, the phenotypes observed in the cuticle preparations likely do not display the full or strongest leg phenotypes. However, their morphology is fully compatible with the observed gene patterning defects. The legs in *pnt* RNAi larvae only show defects in the distal leg, consistent with the lack of *cll* expression, but normal patterning of the medial leg by *Dll* and *dac*. By contrast, the legs in *Egfr* RNAi and *spi* RNAi larvae (in addition to the distal defects) show fusions and malformations in the medial leg, consistent with the changed patterns of *Dll* and *dac*. In summary, our data suggest that the embryonic leg is divided into three portions that differ in their use of Egfr signalling for their formation and patterning. The proximal leg, comprising the coxa and trochanter, appears to be independent from Egfr signalling, because no defects were observed after RNAi. The distal leg, comprising the claw (pretarsus), requires canonical Egfr signalling mediated by its main transcription factor *pnt*. The medial leg, however, involves Egfr signalling, but uses a different transcription factor that still remains to be identified.

Evolutionary implications: exchange of Egfr and Wnt signalling in medial leg patterning?

The patterning of the proximal-distal leg axis in *D. melanogaster* is largely organized by Wg and Dpp signalling. Egfr signalling has a role in proximodistal patterning as well, but only late in development and only in the distal leg. However, there is increasing evidence that this predominance of Wg and Dpp signalling is not typical for leg development in the arthropods (see Introduction). Thus, without the predominance of Wg/Dpp in the control of proximodistal leg axis patterning, Egfr signalling could be more important in more basal insects and other arthropods. Indeed, our results in *T. castaneum* show that in contrast to *D. melanogaster* Egfr signalling is not only required in the distal leg, but is involved in pattern formation in the medial leg as well. Intriguingly, this role of Egfr signalling in the medial leg appears to be functionally separate from the role in the distal leg, because it is not mediated by the transcription factor Pnt. This suggests that the two roles of Egfr

signalling in proximodistal axis patterning have evolved separately and thus show different degrees of evolutionary conservation. We suggest an evolutionary scenario in which Egfr signalling initially had a dual role in proximodistal leg axis patterning: a role in medial leg patterning mediated by an as yet unidentified factor and a role in distal leg patterning mediated by Pnt. The distal role is highly conserved in the arthropods. However, in the medial leg Wg/Dpp signalling evolved as a parallel or redundant system and thus facilitated the subsequent reduction of the role of Egfr signalling in this leg region. According to this scenario, *T. castaneum* preserves an intermediate evolutionary state, while *D. melanogaster* shows a full reduction of Egfr signalling in the medial leg.

Acknowledgements

We thank Marco Winkler and Maja Gere for technical assistance, Beate Preitz for assistance with microscopy, Nico Posnien for help with bioinformatic off-target analysis of *spitz*. We thank Reinhard Schröder and Martin Klingler for critical discussions about leg formation in *Tribolium castaneum*. This work has been funded by the Deutsche Forschungsgemeinschaft and the Georg-August-University, Göttingen.

References

Angelini DR, Kaufman TC (2005a) Functional analyses in the milkweed bug *Oncopeltus fasciatus* (Hemiptera) support a role for Wnt signalling in body segmentation but not appendage development. Dev Biol 283, 409–423.

Angelini DR, Kaufman TC (2005b) Insect appendages and comparative ontogenetics. Dev Biol 286, 57–77.

Bao R, Fischer T, Bolognesi R, Brown SJ, Friedrich M (2011). Parallel duplication and partial subfunctionalization of beta-Catenin/Armadillo during insect evolution. Mol Biol Evol *in press*

Beermann A, Jay DG, Beeman RW, Hülskamp M, Tautz D, Jürgens G (2001). The *Short antennae* gene of *Tribolium* is required for limb development and encodes the orthologue of the *Drosophila Distal-less* protein. Development. 2001, 287-297.

Beermann A, Prühs R, Lutz R, Schröder R (2011). A context-dependent combination of Wnt receptors controls axis elongation and leg development in a short germ insect. Development 138, 2793-2805.

Brunner D, Dücker K, Oellers N, Hafen E, Scholz H, Klämbt C (1994). The ETS domain protein pointed-P2 is a target of MAP kinase in the sevenless signal transduction pathway. Nature 370, 386-389.

Campbell G (2002). Distalization of the *Drosophila* leg by graded EGF-receptor activity. Nature 418, 781-785.

Campbell G, Weaver T, Tomlinson A (1993). Axis specification in the developing *Drosophila* appendages: the role of *wingless*, *decapentaplegic*, and the homeobox gene *aristaless*. Cell 74, 1113–1123

Cohen B, Wimmer EA, Cohen SM (1991). Early development of leg and wing primordia in the *Drosophila* embryo. Mech Dev 33, 229-240.

Diaz-Benjumea FJ, Cohen B, Cohen SM (1994). Cell interaction between compartments establishes the proximal-distal axis of *Drosophila* legs. Nature 372, 175-179.

Estella C, McKay DJ, Mann RS (2008). Molecular integration of wingless, decapentaplegic, and autoregulatory inputs into *Distalless* during *Drosophila* leg development. Dev Cell. 14, 86-96.

Estella C, Mann RS (2008). Logic of Wg and Dpp induction of distal and medial fates in the *Drosophila* leg. Development 135, 627-636.

Gabay L, Scholz H, Golembo M, Klaes A, Shilo BZ, Klämbt C (1996). EGF receptor signalling induces pointed P1 transcription and inactivates Yan protein in the *Drosophila* embryonic ventral ectoderm. Development 122, 3355-3362.

Galindo MI, Bishop SA, Greig S, Couso JP (2002). Leg patterning driven by proxima-distal interactions and EGFR signalling. Science 297, 256-259.

Galindo MI, Bishop SA, Couso JP (2005). Dynamic EGFR-Ras signalling in Drosophila leg development. Dev Dyn 233, 1496-1508.

Giorgianni MW, Mann RS (2011). Establishment of medial fates along the proximodistal axis of the *Drosophila* leg through direct activation of dachshund by *Distalless*. Dev Cell 20, 455-468.

Grossmann D, Scholten J, Prpic NM (2009). Separable functions of *wingless* in distal and ventral patterning of the *Tribolium* leg. Dev Genes Evol 219, 469-479.

Jiang J, Struhl G (1996). Complementary and mutually exclusive activities of *decapentaplegic* and *wingless* organize axial patterning during *Drosophila* leg development. Cell 86, 401-409.

Lecuit T, Cohen SM (1997). Proximal-distal axis formation in the *Drosophila* leg.

Nature 388, 139-145.

Lynch JA, Peel AD, Drechsler A, Averof M, Roth S (2010). EGF signalling and the origin of axial polarity among the insects. Curr Biol 20, 1042-1047.

McKay DJ, Estella C, Mann RS (2009). The origins of the *Drosophila* leg revealed by the cis-regulatory architecture of the *Distalless* gene. Development 136, 61-71.

Miyawaki K, Mito T, Sarashina I, Zhang H, Shinmyo Y, Ohuchi H, Noji S (2004). Involvement of Wingless/Armadillo signalling in the posterior sequential segmentation in the cricket, *Gryllus bimaculatus* (Orthoptera), as revealed by RNAi analysis. Mech Dev 121, 119-130.

Nunes da Fonseca R, van der Zee M, Roth S (2010). Evolution of extracellular Dpp modulators in insects: The roles of *tolloid* and *twisted-gastrulation* in dorsoventral patterning of the *Tribolium* embryo. Dev Biol 345, 80-93.

Nunes da Fonseca R, von Levetzow C, Kalscheuer P, Basal A, van der Zee M, Roth S (2008). Self-regulatory circuits in dorsoventral axis formation of the short-germ beetle Tribolium castaneum. Dev Cell 14, 605-615.

Ober KA, Jockusch EL (2006). The roles of *wingless* and *decapentaplegic* in axis and appendage development in the red flour beetle, *Tribolium castaneum*. Dev Biol 294, 391-405.

O'Neill EM, Rebay I, Tjian R, Rubin GM (1994). The activities of two Ets-related transcription factors required for *Drosophila* eye development are modulated by the Ras/MAPK pathway. Cell 78, 137-147.

Penton A, Hoffmann FM (1996). Decapentaplegic restricts the domain of wingless during *Drosophila* limb patterning. Nature 382, 162-164.

Perrimon N, Perkins LA (1997). There must be 50 ways to rule the signal: the case of the Drosophila EGF receptor. Cell 89, 13-16.

Posnien N, Koniszewski N, Bucher G (2011). Insect *Tc-six4* marks a unit with similarity to vertebrate placodes. Dev Biol 350, 208-216.

Prpic NM, Wigand B, Damen WGM, Klingler M (2001). Expression of *dachshund* in wild-type and *Distal-less* mutant *Tribolium* corroborates serial homologies in insect appendages. Dev Genes Evol 211, 467-477.

Prpic NM, Janssen R, Wigand B, Klingler M, Damen WGM (2003). Gene expression in spider appendages reveals reversal of exd/hth spatial specificity, altered leg gap gene dynamics, and suggests divergent distal morphogen signalling. Dev Biol 264, 119-140.

Purvis A, Hector A (2000). Getting the measure of biodiversity. Nature 405, 212-219.

Rousso T, Lynch J, Yogev S, Roth S, Schejter ED, Shilo BZ (2010). Generation of distinct signalling modes via diversification of the Egfr ligand-processing cassette. Development 137, 3427-2437.

Rutledge BJ, Zhang K, Bier E, Jan YN, Perrimon N (1992). The *Drosophila spitz* gene encodes a putative EGF-like growth factor involved in dorsal-ventral axis formation and neurogenesis. Genes Dev 6, 1503-1517.

Schaeper ND, Prpic NM, Wimmer EA (2010). A clustered set of three Sp-family genes is ancestral in the Metazoa: evidence from sequence analysis, protein domain structure, developmental expression patterns and chromosomal location. BMC Evol Biol 10:88

Shah MV, Namigai EKO, Suzuki Y (2011). The role of canonical Wnt signalling in leg regeneration and metamorphosis in the red flour beetle *Tribolium castaneum*. Mech Dev *in press*.

Struhl G, Basler K (1993). Organizing activity of Wingless protein in *Drosophila*. Cell 72, 527–540

Theisen H, Haerry TE, O'Connor MB, Marsh JL (1996). Developmental territories created by mutual antagonism between Wingless and Decapentaplegic. Development 122, 3939-3948.

Tribolium Genome Sequencing Consortium (2008). The genome of the model beetle and pest *Tribolium castaneum*. Nature 452, 949-955.

Van der Zee M, Stockhammer O, von Levetzow C, Nunes da Fonseca R, Roth S (2006). Sog/Chordin is required for ventral-to-dorsal Dpp/BMP transport and head formation in a short germ insect. Proc Natl Acad Sci U S A 103, 16307-16312.

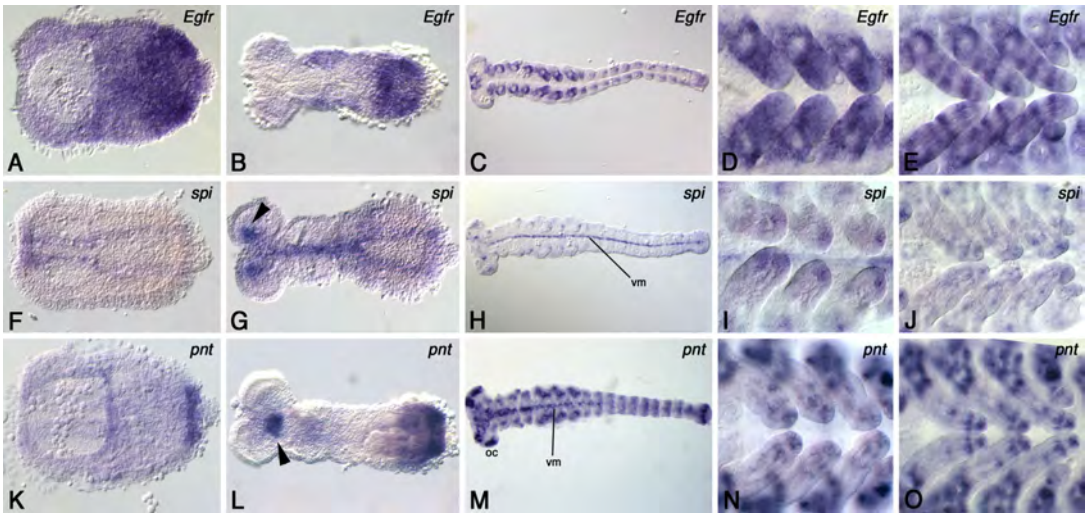


Figure 1. Expression of *Egfr* (A-E), *spi* (F-J) and *pnt* (K-O) during the embryonic development of *T. castaneum*. Expression patterns are shown in embryos during gastrulation (A, F, K), start of germ band elongation (B, G, L) and the end of germ band elongation (C, H, M). The arrowhead in G points to expression in the central brain. The arrowhead in L points to expression in the stomodeum. The six panels on the right show a magnified view of the thorax with the three developing legs of embryos towards the end of germ band elongation (D, I, N) and towards the end of germ band retraction (E, J, O). Anterior is to the left in all panels. Abbreviations: oc, ocular region; vm, ventral midline.

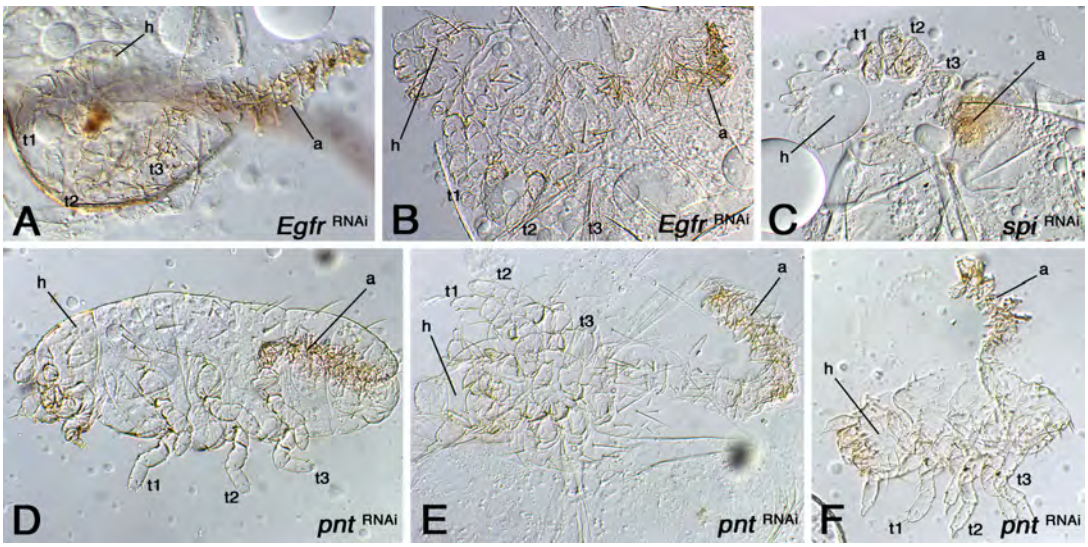


Figure 2. Larval cuticle phenotype of RNAi with *Egfr* (A, B), *spi* (C), and *pnt* (D-F). Note the "outside-in" abdomen in all cases. Anterior is to the left in all panels. Abbreviations: h, head capsule; t, larval thoracic segment; a, abdomen.

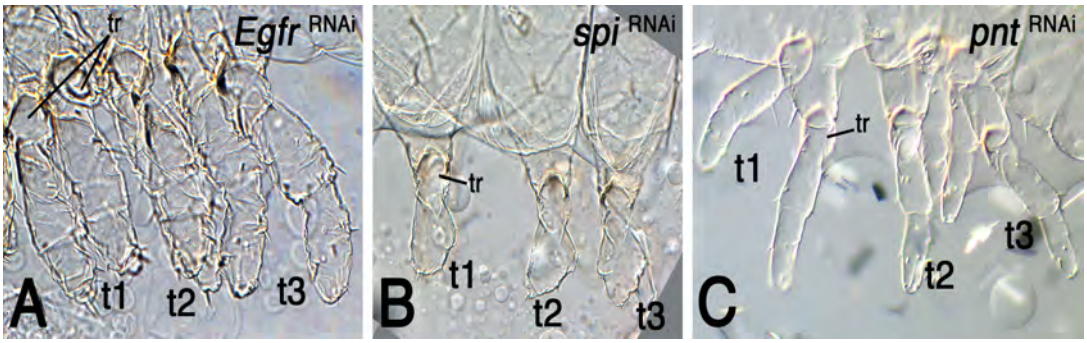


Figure 3. Larval leg phenotypes after *Egfr* (A), *spi* (B), and *pnt* (RNAi). In *Egfr* and *spi* RNAi animals the legs are broadened and show fusions of all segments distal to the trochanter and a missing distal claw, whereas in *pnt* RNAi animals the legs are normal except for the missing distal claw. The trochanter (tr) is indicated in all panels for easier comparison between the panels. Anterior is to the left in all panels. Abbreviations: tr, trochanter; t, larval thoracic leg.

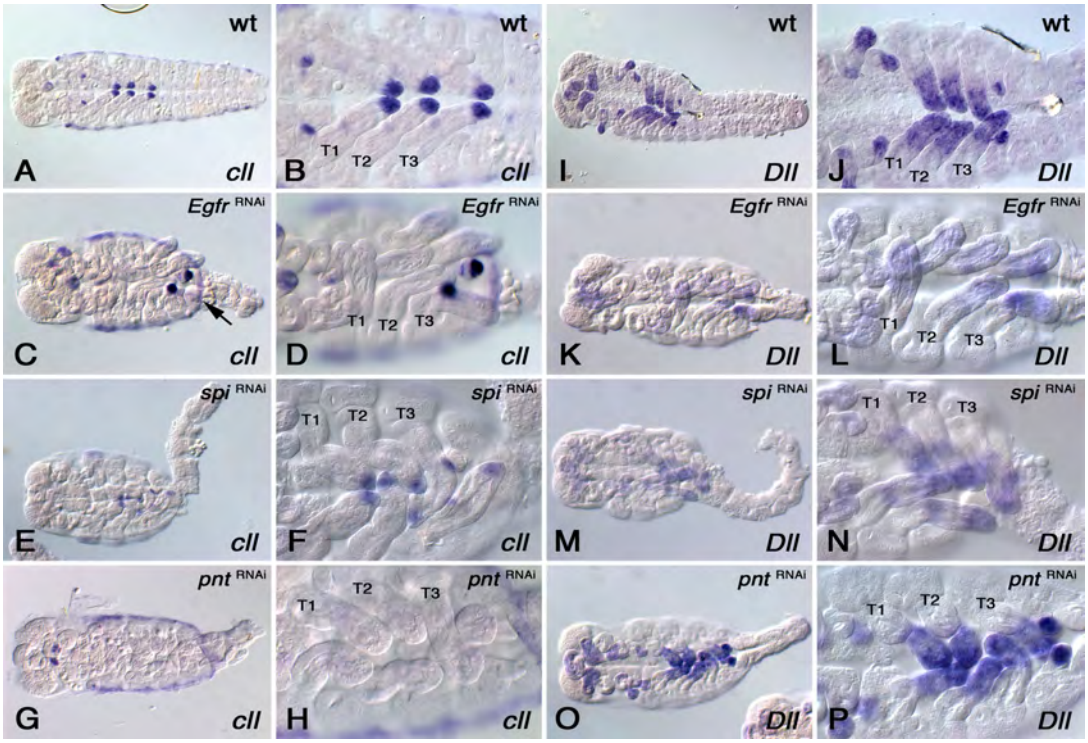


Figure 4. Expression of *cll* (A-H) and *Dll* (I-P) in *Egfr* (C, D, K, L), *spi* (E, F, M, N), and *pnt* (G, H, O, P) RNAi and wildtype (A, B, I, J) embryos. The embryonic phenotype is similar in all RNAi embryos: the dorsal thoracic tissue is fused at the end of the thorax. This is best seen in *cll* stained embryos, because *cll* is expressed in the presumptive dorsal tissue (i.e. along the lateral rim of the germ band). Staining of *cll* fuses at the end of the thorax (see arrow in C). The abdomen thus grows from there with inverted polarity as an invaginated ("outside in") tube that easily breaks off during preparation (see e.g. C, G). The *cll* gene is expressed in the tip of the legs

(A, B). In the RNAi embryos *cll* expression is strongly reduced or lacking. The *Dll* gene is expressed in the medial and distal portion of the legs (I, J). Its expression is reduced in *Egfr* (K, L) and *spi* (M, N) RNAi embryos, but not in *pnt* RNAi (O, P) embryos. Abbreviation: T, embryonic thoracic segment. Note: the blackish blue staining seen in the pleuropodia on abdominal segment 1 in some of the embryos (e.g. C, O) is a well-known artefact in whole mount in situ hybridization in *T. castaneum*.

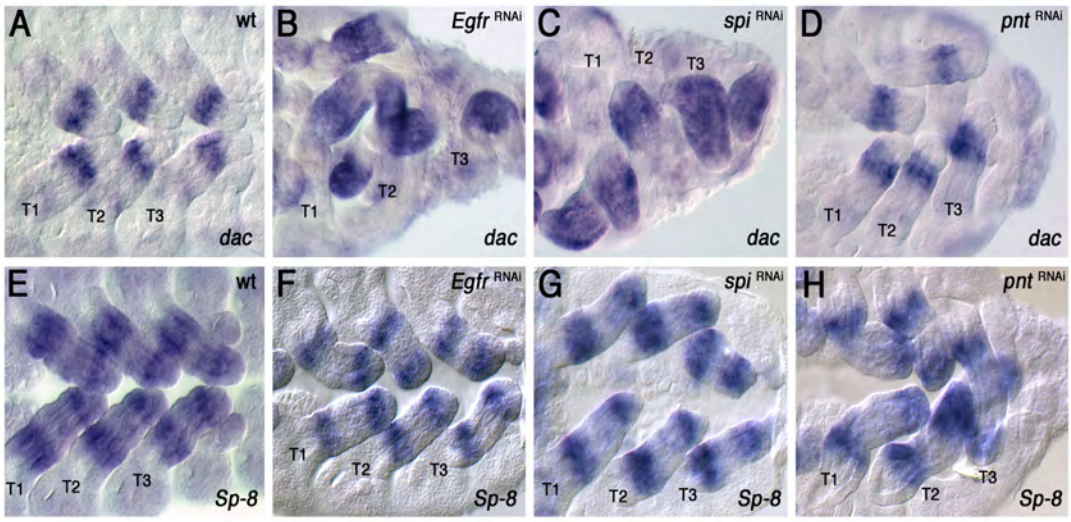


Figure 5. Expression of *dac* (A-D) and *Sp8* (E-H) in *Egfr* (B, F), *spi* (C, G), and *pnt* (D, H) RNAi and wildtype (A, E) embryos. The *dac* gene is expressed in a medial ring (E). Expression levels remain normal after *pnt* RNAi (D), but are upregulated after *Egfr* RNAi (B) and *spi* RNAi (C). The *Sp8* gene is expressed in the medial and distal portions of the legs with two rings of higher level expression (E). In the RNAi embryos *Sp8* expression is largely normal, but appears to be somewhat weaker, especially in *Egfr* RNAi embryos (F). Abbreviation: T, embryonic thoracic segment.

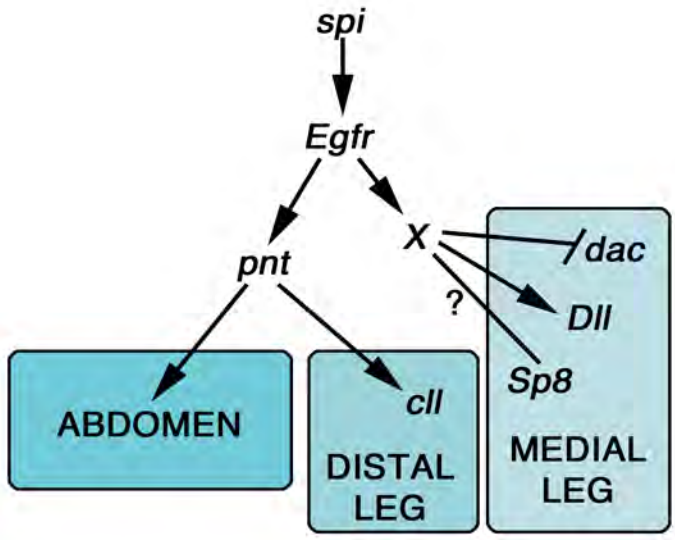
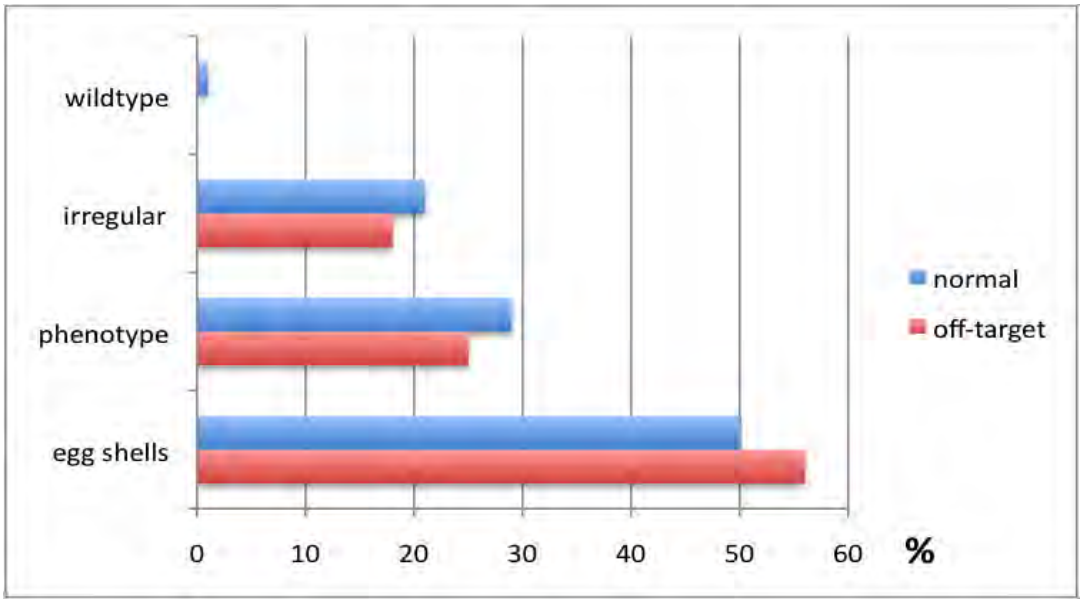
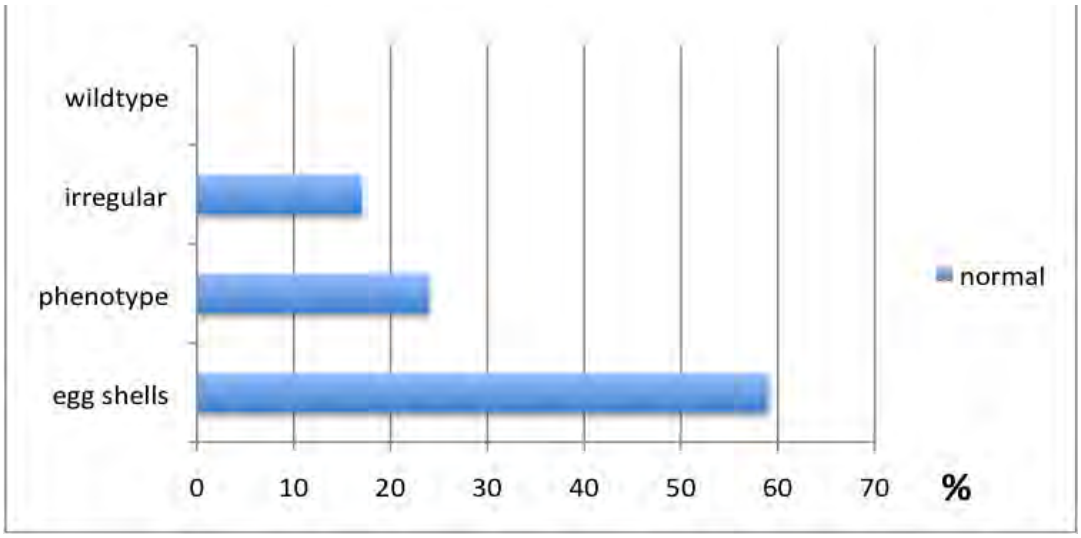


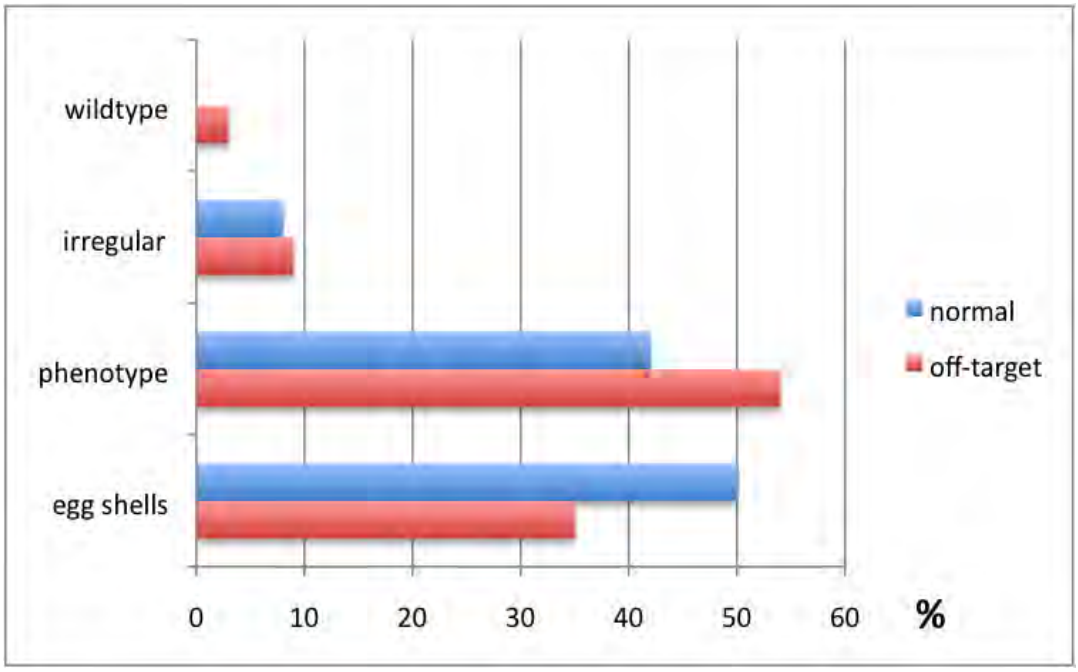
Figure 6. Proposed interactions of Egfr signalling pathway components and patterning genes in leg development and abdomen formation in *T. castaneum*. The available data suggest that *pnt* mediates the outcome of Egfr signalling in the abdomen and the distal leg, but another unidentified factor ("X") is the mediator of the Egfr pathway in the medial leg. The relationship between Egfr signalling and *Sp8* expression is unclear from the available data (denoted by "?").



Supplementary Figure 1. Results of the RNAi with *Egfr*. Numbers of animals scored: Normal RNAi: n=154; off-target RNAi: n=283. "Irregular" denotes the presence of some cuticular structures within the egg shell, whereas "egg shells" indicates that no traces of cuticle were found in the eggs (i.e. these animals have died before cuticle production).



Supplementary Figure 2. Results of the RNAi with *spi*. Numbers of animals scored: Normal RNAi: n=168. "Irregular" denotes the presence of some cuticular structures within the egg shell, whereas "egg shells" indicates that no traces of cuticle were found in the eggs (i.e. these animals have died before cuticle production).



Supplementary Figure 3. Results of the RNAi with *pnt*. Numbers of animals scored: Normal RNAi: n=98; off-target RNAi: n=277. "Irregular" denotes the presence of some cuticular structures within the egg shell, whereas "egg shells" indicates that no traces of cuticle were found in the eggs (i.e. these animals have died before cuticle production).

3.3 *Decapentaplegic (dpp)* is involved in embryonic proximo-distal leg patterning in *Tribolium castaneum*

The purpose of this work was to study the role of the gene *decapentaplegic* (*dpp*) in leg development in the red flour beetle *Tribolium castaneum*. Therefore, we performed stage-specific staggered embryonic RNAi followed by embryonic and larval phenotype analysis. To learn more about the function of *Tc'dpp* in formation and pattering of the leg, we additionally analysed the expression pattern of dorsal and medial patterning genes in *Tc'dppRNAi* embryos.

Daniela Großmann, Nikola-Michael Prpic

Status: work in progess

Authors contribution to the work:
Daniela Großmann performed all experiments.

Decapentaplegic (*dpp*) is involved in embryonic proximodistal leg patterning in *Tribolium castaneum*

Daniela Grossmann and Nikola-Michael Prpic (*)

Georg-August-Universität Göttingen, Johann-Friedrich-Blumenbach-Institut für Zoologie und Anthropologie, Abteilung für Entwicklungsbiologie, GZMB Ernst-Caspari-Haus, Justus-von-Liebig-Weg 11, 37077 Göttingen, GERMANY

(*) author for correspondence
Tel.: ++49 551 39 101 24
Fax: ++49 551 39 5416
E-mail: nprpic@uni-goettingen.de

Number of words: 2790 (in total)

Number of figures: 2

Abstract

The *decapentaplegic* (*dpp*) gene is an important factor in proximal-distal leg patterning in *Drosophila melanogaster*, where it functions together with the gene *wingless* (*wg*) to regulate the expression of leg patterning genes like *Distal-less* (*Dll*) and *dachshund* (*dac*). It is currently debated whether this mode of proximodistal leg patterning is evolutionarily conserved in other insects. While there is a hypothetical model (topology model) that argues for a conservation of the *wg/dpp* mode in all arthropod legs, experimental evidence for this model is lacking so far. Here we study the role of *dpp* in the legs of the red flour beetle *Tribolium castaneum*. We show that *dpp* has a role in leg development and *dpp* RNAi leads to characteristic morphological defects in the legs distal to the trochanter. In addition, we show that *dpp* is required for the regulation of *optomotor-blind* (*omb*) and *dachshund* (*dac*) in the developing legs of *T. castaneum* embryos. These results are fully compatible with the topology model and suggest that a *wg/dpp*-based mechanism similar to the known mechanism in *D. melanogaster* is operating during leg development in *T. castaneum*.

Introduction

The development of the leg imaginal discs of the fly *Drosophila melanogaster* has served as a paradigm for the mechanism of proximal-distal axis formation and patterning in the arthropods. In *D. melanogaster* the proximodistal leg axis is mainly organized by a gradient system of the morphogens Wingless (Wg) and Decapentaplegic (Dpp) (e.g. Campbell et al. 1993; Diaz-Benjumea et al., 1994; Jiang and Struhl 1996; Lecuit and Cohen 1997) and it has been thought that this mechanism is generally involved in leg patterning in other arthropods as well. Recent results from different arthropod species, however, have lent little support to this notion. Although the expression pattern of *wg* is widely conserved in the arthropods, its actual function in proximodistal leg axis patterning is debated (reviewed in Angelini and Kaufman, 2005b). Recent results suggest that the conserved expression reflects a conserved role in regeneration, rather than a role in proximodistal axis patterning, which has only evolved later within the holometabolous insects (Shah et

al. 2011). The role of the *dpp* gene is even less clear. The dorsal expression known from *D. melanogaster* is not found in other species; rather *dpp* is expressed early in a distal domain and later resolves in a divergent pattern of spots and rings (Sanchez-Salazar et al. 1996; Niwa et al. 2000) that appear to correlate with special features of the leg (e.g. *dpp* expression rings are especially strong in the thickened jumping legs of crickets and grasshoppers (Niwa et al. 2000; Jockusch et al. 2000)). Thus, in the case of *dpp* not even the expression pattern provides evidence for a conserved role in proximodistal leg axis patterning. Nevertheless, a hypothetical model (the "topology model") has been proposed that argues that despite the differences in *wg*/*dpp* expression the role of these genes in proximodistal leg axis patterning is conserved in all arthropods (Prpic et al. 2003). So far this model has received little support from studies dealing with *wg* and *dpp* function in arthropod leg formation (reviewed in Angelini and Kaufmann 2005b; Shah et al. 2011). Especially, the role of *dpp* in leg development has been questioned. As mentioned above, its divergent expression does not suggest a conserved function in proximodistal axis formation and its function proved to be difficult to study, because of the earlier roles of *dpp* in development. If *dpp* function is impaired early in embryogenesis of the beetle *Tribolium castaneum* the embryos fail to develop a proper dorsal-ventral body axis and only an enlaged head lobe and a tube shaped and thin trunk is formed (Van der Zee et al. 2006). The embryos do not develop any further and thus a role in leg development cannot be studied directly. Similarly, the embryos of the heteropteran *Oncopeltus fasciatus* die during early developmental stages after *dpp* RNAi (Angelini and Kaufman 2005a). Ober and Jockusch (2006) have studied weakly affected specimens of *T. castaneum* after *dpp* RNAi and were not able to detect leg defects. These authors therefore concluded that *dpp* does not have a role in leg development in *T. castaneum*.

We study leg development in the beetle *T. castaneum* and we have used staggered stage-specific embryonic RNAi before to investigate the role of *wg* in leg development (Grossmann et al. 2009). This method facilitates to administer the RNAi effect later in development and thus to study late developmental processes (e.g. leg development) without affecting early embryogenesis. We have also used this method to study the function of *dpp* in leg development in *T. castaneum* and we show here that *dpp* is involved in the development of the proximal-distal leg axis in this species and has an influence on the expression of dorsal as well as medial patterning

genes.

Materials and Methods

Embryonic RNAi

For embryonic RNAi, eggs were collected directly after egg deposition and incubated at 25°C until injection. The embryos were injected at different time points: approximately 4, 8, 12, and 18 h after egg laying. These time points correlate with different developmental stages and thus allow for normal embryogenesis before injection. The time points correlate with: early embryogenesis before the cleavage nuclei have reached the periphery (4h), blastoderm stage (8h), serosal closure, leg buds have not yet formed (12h), germ band elongation complete, small leg buds are visible (18h). Injections were performed with beveled borosilicate needles using a micromanipulator and a FemtoJet injection controller (Eppendorf). The concentration of the dsRNA was 1,800 ng/µl. Injected embryos were incubated at 25°C until hatching or (for in situ expression analysis) until germ band retraction. Removal of the mRNA below the level of detection was confirmed by whole mount in situ hybridization using the probe also used for the analysis of the wildtype expression pattern. To exclude off-target effects, separate non-overlapping fragments of *dpp* were used for dsRNA synthesis and these dsRNA preparations were injected in parallel with the normal 18h RNAi experiments (i.e. with the full gene fragment). Normal RNAi and the off-target RNAi led to identical leg phenotypes.

Embryo fixation and in situ hybridization

Embryos for in situ hybridization were dechorionized with a 50% solution of DanKlorix (Colgate-Palmolive) in water and fixed with 4% formaldehyde in a mixture of PEMS (0.1 M Pipes, 2 mM MgSO4, 1 mM EDTA; pH=6.9) and heptane. Vitelline membranes were removed by methanol shock and subsequent shearing through a syringe needle (19 G gauge). Whole-mount in situ hybridization detection of mRNA expression was performed as described previously (Prpic et al., 2001). After in situ hybridization embryos were fixed with 4% formaldehyde in phosphate-buffered saline with 0.02% Tween-20, pH=7.4 and embedded in 80% glycerol for microscopy.

Microscopy and imaging

Larval cuticles and embryos from the in situ hybridizations were observed with differential interference contrast microscopy (Zeiss Axioplan-2). Images were captured with an Intas digital camera and were subjected to adjustment of brightness, contrast, and color values using Adobe Photoshop image processing software (Version CS5 for Apple Macintosh).

Results and Discussion

Larval leg phenotype of *dpp* RNAi in *Tribolium castaneum*

The injections at 4h, 8h, and 12h resulted in mainly egg shells with no traces of larval cuticle and a very few wildtype larvae (Fig. 1). These results suggested that at these injection time points the effect of RNAi with *dpp* function was leading to embryonic lethality before they could form a larval cuticle and the few wildtype larvae did not receive *dpp* dsRNA ("escapers"). Indeed, inspection of embryos of these experiments revealed that they showed the previously described severe phenotype with the enlarged head lobe and thin trunk (Van der Zee et al., 2006; data not shown) and thus died before cuticle formation. However, the injections at 18h were sufficient to apply the RNAi effect after the development of dorsoventral polarity and metamerization of the body and specifically interfered with *dpp* expression in the developing legs. Therefore, these injections resulted in larvae that showed no or only mild malformations of the body, but had severe leg defects (Fig. 2A). These leg defects showed a very characteristic phenotype. The coxa and trochanter were morphologically normal, but the femur and the tibiotarsus were fused together into one thickened and shortened leg segment. The pretarsal claw was morphologically normal, but was attached to the femoro-tibiotarsal fusion podomere in an unusual angle at approximately 90°. This unusual phenotype suggested to us that *dpp* has a role in the proper development of the medial and distal leg fates. The abnormal orientation of the claw also suggests problems with distal polarity.

Expression of leg patterning genes in *dpp* RNAi embryos

We have therefore investigated the expression of previously known leg patterning

genes in *dpp* RNAi embryos injected at 18 h after egg laying. The gene *optomotor-blind* (*omb*) is normally expressed along the dorsal side of the legs (Fig. 2B). In *dpp* RNAi embryos the expression of *omb* is still strong in the antennae and the eye region, but is significantly reduced in the gnathal appendages and legs (Fig. 2C). By contrast, the *wg* gene is expressed along the ventral side of the legs in the wildtype and remains restricted to the ventral side in *dpp* RNAi embryos (Fig. 2E). The gene *dachshund* (*dac*) is normally expressed in a medial ring in the legs. In *dpp* RNAi embryos the expression of *dac* is completely abolished in all appendages except for the mandibles (Fig. 2D).

A role for *dpp* in embryonic leg patterning in *T. castaneum*

Previous results were inconclusive as to a role of *dpp* in leg development in *T. castaneum* (Ober and Jockusch 2006). Our results show that interference with *dpp* function during stages when the legs are developing lead to very specific leg phenotypes in the larva. This suggests that *dpp* has a specific role during leg formation in *T. castaneum*. The study of leg patterning gene expression in *dpp* RNAi embryos provides further insight into the mechanisms of *dpp* action during leg development. The complete loss of *dac* expression in the legs indicates that *dpp* is required for the regulation of proximal-distal patterning genes and thus is involved in the development of the proximodistal axis. This is consistent with the known role of *dpp* in *D. melanogaster*, where *dpp* functions together with *wg* to organize proximal-distal patterning. In *D. melanogaster dpp* has a second function in dorso-ventral leg patterning (Brook and Cohen 1996). The down-regulation of the dorsal leg expression of *omb* in *dpp* RNAi embryos of *T. castaneum* might be an indication that *dpp* has a similar role in *T. castaneum* as well. However, we did not observe a change of expression of the ventral factor *wg*, a fact that argues against a ventralization of the legs after *dpp* RNAi. In summary, *dpp* in *T. castaneum* has an important role in proximal-distal leg patterning affecting all leg parts distal to the trochanter, but a possible role in dorsal-ventral leg axis formation is unclear based on the available data.

It is interesting to note that the role of *dpp* in the head appendages appears to differ (at least in part) from the role in the thoracic legs. First, expression of *omb* in the antennae does not seem to require *dpp* (see Fig. 2B). Second, the expression of *dac*

in the mandible is independent from *dpp* (see Fig. 2D). This indicates that at least the antenna and the mandible employ alternative proximal-distal patterning mechanisms that do not require *dpp*. It has been suggested previously that alternative, *dpp*-independent proximal-distal patterning mechanisms exist in *T. castaneum* (Ober and Jockusch 2006) and probably other insects (Angelini and Kaufman 2005a, 2005b; Myiawaki et al. 2004). However, these mechanisms were also thought to pattern the thoracic legs, which is in conflict with the topology-model (Prpic et al., 2003) that posits that the legs are patterned by a *wg/dpp* mechanism in all arthropods. Our present results suggest that there are indeed alternative proximal-distal patterning mechanisms operating in some of the head appendages of *T. castaneum*, but our results are also fully compatible with the topology model as far as the thoracic legs of *T. castaneum* are concerned.

Acknowledgements

We thank Marco Winkler and Maja Gere for technical assistance, Beate Preitz for assistance with microscopy. This work has been funded by the Deutsche Forschungsgemeinschaft and the Georg-August-University, Göttingen.

References

Angelini DR, Kaufman TC (2005a) Functional analyses in the milkweed bug *Oncopeltus fasciatus* (Hemiptera) support a role for Wnt signalling in body segmentation but not appendage development. Dev Biol 283, 409–423.

Angelini DR, Kaufman TC (2005b) Insect appendages and comparative ontogenetics. Dev Biol 286, 57–77.

Brook WJ, Cohen SM (1996). Antagonistic interactions between Wingless and Decapentaplegic responsible for dorsal-ventral pattern in the *Drosophila* leg. Science 273, 1373-1377.

Campbell G, Weaver T, Tomlinson A (1993). Axis specification in the developing *Drosophila* appendages: the role of *wingless*, *decapentaplegic*, and the homeobox gene *aristaless*. Cell 74, 1113–1123

Diaz-Benjumea FJ, Cohen B, Cohen SM (1994). Cell interaction between compartments establishes the proximal-distal axis of *Drosophila* legs. Nature 372, 175-179.

Grossmann D, Scholten J, Prpic NM (2009). Separable functions of *wingless* in distal and ventral patterning of the *Tribolium* leg. Dev Genes Evol 219, 469-479.

Jiang J, Struhl G (1996). Complementary and mutually exclusive activities of *decapentaplegic* and *wingless* organize axial patterning during *Drosophila* leg development. Cell 86, 401-409.

Jockusch EL, Nulsen C, Newfeld SJ, Nagy LM (2000). Leg development in flies versus grasshoppers: differences in *dpp* expression do not lead to differences in the expression of downstream components of the leg patterning pathway. Development 127, 1617-1626.

Lecuit T, Cohen SM (1997). Proximal-distal axis formation in the *Drosophila* leg. Nature 388, 139-145.

Miyawaki K, Mito T, Sarashina I, Zhang H, Shinmyo Y, Ohuchi H, Noji S (2004). Involvement of Wingless/Armadillo signalling in the posterior sequential segmentation in the cricket, *Gryllus bimaculatus* (Orthoptera), as revealed by RNAi analysis. Mech Dev 121, 119-130.

Niwa N, Inoue Y, Nozawa A, Saito M, Misumi Y, Ohuchi H, Yoshioka H, Noji S (2000). Correlation of diversity of leg morphology in *Gryllus bimaculatus* (cricket) with divergence in *dpp* expression pattern during leg development. Development 127, 4373-4381.

Ober KA, Jockusch EL (2006). The roles of *wingless* and *decapentaplegic* in axis and appendage development in the red flour beetle, *Tribolium castaneum*. Dev Biol 294, 391-405.

Prpic NM, Wigand B, Damen WGM, Klingler M (2001). Expression of *dachshund* in wild-type and *Distal-less* mutant *Tribolium* corroborates serial homologies in insect appendages. Dev Genes Evol 211, 467-477.

Prpic NM, Janssen R, Wigand B, Klingler M, Damen WGM (2003). Gene expression in spider appendages reveals reversal of exd/hth spatial specificity, altered leg gap gene dynamics, and suggests divergent distal morphogen signalling. Dev Biol 264, 119-140.

Sanchez-Salazar J, Pletcher MT, Bennett RL, Brown SJ, Dandamudi TJ, Denell RE, Doctor JS (1996). The *Tribolium decapentaplegic* gene is similar in sequence, structure, and expression to the *Drosophila dpp* gene. Dev Genes Evol 206, 237-246.

Shah MV, Namigai EKO, Suzuki Y (2011). The role of canonical Wnt signalling in leg regeneration and metamorphosis in the red flour beetle *Tribolium castaneum*.

Mech Dev *in press*.

Van der Zee M, Stockhammer O, von Levetzow C, Nunes da Fonseca R, Roth S (2006). Sog/Chordin is required for ventral-to-dorsal Dpp/BMP transport and head formation in a short germ insect. Proc Natl Acad Sci U S A 103, 16307-16312.

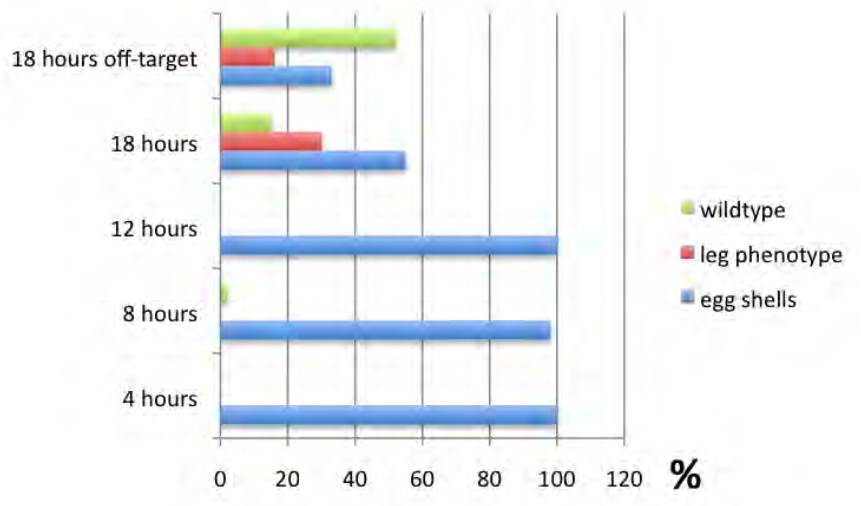


Figure 1. Results of the RNAi with *dpp*. The early injections (4h, 8h, 12h after egg laying) predominantly led to egg shells without formation of visible larval cuticle inside. The 18 h injections in addition resulted in a very characteristic larval leg phenotype (see Fig. 2A). This leg phenotype was also seen in the off-target controls using non-overlapping sub-fragments of the full-length fragment used in the normal RNAi experiments. Numbers of animals scored: n=230 (4h), n=114 (8h), n=113 (12h), n=142 (18h), n=74 (18h off-target).

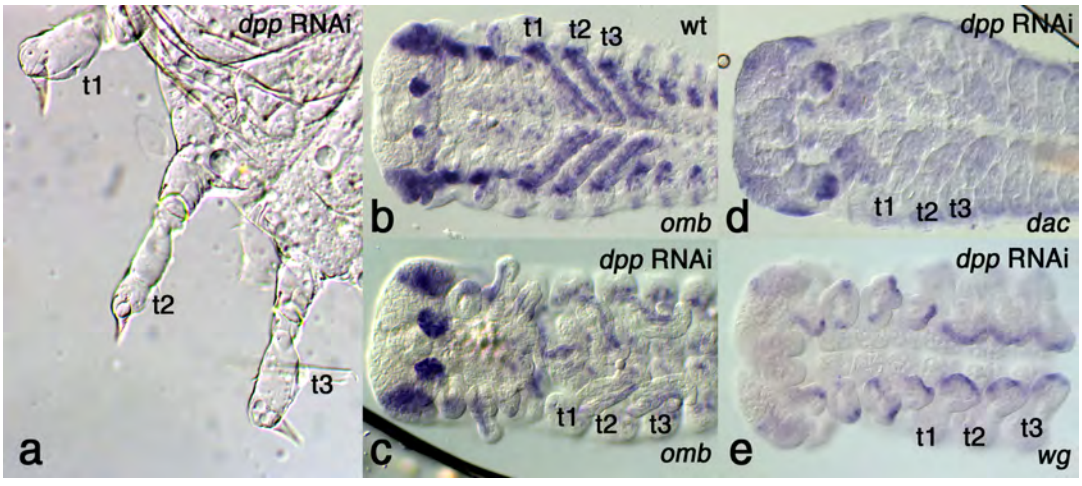


Figure 2. A role of dpp in proximodistal leg axis patterning in Tribolium castaneum. (A) Characteristic larval leg phenotype after dpp RNAi at 18 h after egg laying. (B, C) Expression of omb in wildtype (B) and dpp RNAi embryos (C). The expression of omb in the gnathal appendages and legs is reduced in dpp RNAi embryos. (D) The

expression of dac in dpp RNAi is fully absent in all appendages except for the antennae. (E) The expression of wg remains restricted to the ventral side of the appendages in dpp RNAi embryos. Anterior is to the left in all panels. Abbreviations: t, thoracic leg.

4 Discussion

4.1 The role of *wg* in leg development in *Tribolium castaneum*

The gene *wingless* is an important and crucial factor in leg development of the fruit fly *Drosophila melanogaster*. Wg signalling is at least involved in three different processes. First, during embryonic development *wg* is required for the allocation of the limb primordia, second, during imaginal disc development Wg signalling is involved in distal development and third it specifies ventral cell fate in the leg cells. The expression pattern of *wg* is highly conserved in all arthropods investigated to date and suggests that its function in leg development is also conserved. However, recent works in hemimetabolous insects (*Oncopeltus fasciatus*, *Gryllus bimaculatus*) argue against a conserved role of *wg* in leg development.

In our study, we tested the role of *wg* in leg development of the red flour beetle *Tribolium castaneum* (Order Coleoptera). *Tribolium* is a holometabolous insect and is therefore more closely related to *Drosophila* than *Oncopeltus* and *Gryllus*, but contrary to *Drosophila* the appendages develop directly from embryonic limb buds. We used parental and embryonic *wg*RNAi to find out if *wg* has a function in *Tribolium* leg development at all and if so, in which stages its function is conserved.

4.1.1 Wg signalling is essential for the specification of the leg primordia in *Tribolium castaneum*

Depletion of *wg* through pupal RNAi could not reveal a function of *wg* in leg development. Although the injected pupae eclosed and survived after *wg* dsRNA injection, the *wg*RNAi females showed a very low egg productivity. It is known that *wg* is involved in several crucial developmental processes, including segmentation and oogenesis. The low productivity of injected females indicates that *wg* has an early role during oogenesis and the knockdown prevents normal egg development. In addition, from the few layed eggs, almost no larvae hatched and those that hatched and could be investigated were classified as wildtype. These results point to a role of *wg* in early development that leads to severe defects and embryonic lethality.

To circumvent the problems of early embryonic lethality, we consequently performed

staggered stage-specific embryonic RNAi (eRNAi). Early injections in 4h and 8h old embryos still interfered with early functions of *wg* and produced only empty eggs or eggs with irregular structures. The few larvae that developed showed severe phenotypes including severely malformed legs (Fig. 5 B, C). Previous work by Ober and Jokusch has already indicated a role of *wg* in appendage allocation in *Tribolium* (Ober and Jokusch 2006). Knockdown of *wg* caused defects that affected a wide range of morphological structures, including segments, limbs and spiracles. In most cases the legs were completely missing. If present the legs were severely truncated and thinner (Fig. 2I, Ober and Jokusch 2006). These defects are very similar to our results we obtained after early injections in 4h and 8h old embryos. As mentioned, Ober and Jokusch suggested an early role of *wg* in leg allocation similar to its function in *Drosophila*. However, it has to be noted, that all *Tc'wg*RNAi embryos with leg defects shown in Fig. 2 C-G also show severe segmentation defects. Therefore, it can not be excluded that the leg defects are just secondary defects due to segmentation defects, instead of real leg developmental defects. The authors argue, that the observed leg phenotypes cannot be a downstream effect of altered segmentation because there are specimens with malformed legs but prober number of segments (Fig. 2 C, I). However, these phenotypes that the authors refer to only occur in 10%. (n=10) and therefore might partially represent naturally occurring mutants unrelated to the RNAi experiment. Furthermore, the embryo depicted in Fig 2 C never occurred in our samples after we performed *wg*RNAi; instead it represents a typical phenotype that we observed after *dpp*RNAi (see Fig. 2E in section 3.3) and thus it is at least a possibility that a confusion of the phenotypes occurred in this case. Another possible explanation for the occurrence of a mild leg phenotype but no segmental defects is that leg development might be much more sensitive even to a slight reduction of *wg* transcripts whereas the establishment of the body segments might be more robust. In our experiment, we therefore avoided interfering with the early segmentation function of *wg* by injecting *wg* dsRNA in 12h or 18h old embryos and consequently could separate the leg phenotype from the segmentation defects. The most severe leg phenotypes were obtained after injecting in 12h old embryos. This proximo-distal leg phenotype does not show just defects in the distal part of the leg, but also in the coxa, the most proximal part of the leg (Fig 6 E, G). This function of *wg* is very similar to its early function in *Drosophila*. In *Drosophila*, *wg* is

required during early stages for the activation of the *Dll*304-enhancer element in all cells of the leg primordium, which includes the distal as well as the proximal cells, which will later give rise to the coxa.

Taken together, the severe malformations in 12 hour injections, which affect the complete leg including the coxa, give strong evidence for a role of Wg signalling in specification the leg primordia. Although *Tribolium* and *Drosophila* show different modes of limb development, we conclude, that the earliest function of *wg* in leg allocation is similar between both insects. In *Drosophila*, Wg performed this distal function as a long-range morphogen and acts cooperatively with Dpp (Diaz-Benjumea et al. 1994; Abu-Shaar and Mann 1998; Lecuit and Cohen, see section 2.2.2). In *Tribolium*, it is so far not known how *wg* establish distal cell fate in the leg and future studies have to reveal weather *wg* also act as a morphgen and together with *dpp* as in *Drosophila*.

4.1.2 Separate function of *wg* in distal and ventral leg development

Beside its function in early *Drosophila* leg development, Wg signalling is also required for distalisation of the leg during imaginal disc development. After the cells of the thoracic limb primordium separate and the leg and wing primordia are established, the cells of the leg primordium further subdivide in proximal and distal cells. Wg signalling is not any longer required for the development of the proximal part of the leg but for the establishment of all leg portions distal to the coxa (Cohen et al. 1993; Estella et al. 2008; McKay et al. 2009). Our “candy cane” and “nonpareille” phenotypes, which we obtained after 12 hour and 18 hour injections, indicate a very similar distal role for wg in *Tribolium*. Both phenotypes reveal severe problems with proximal-distal axis formation distal to the coxa and distal-most structures are mostly lacking. In both cases the coxa is not affected and indicates that *wg* is not longer required for the development of proximal parts of the leg. Ober and Jokusch already suggested a later role of *wg* in distal appendage development, but due to the complete loss of limbs in their *wg* RNAi phenotypes, they were not able to prove this hypothesis (Ober and Jokusch 2006). But very recently Beermann et al. agreed with our results. Depletion of the Wnt receptor gene *Tc’frizzled-1* (*Tc’fz-1*) through

parental RNAi caused a leg phenotype that strongly resembles our *wg* RNAi phenotype (Fig. 6 D, E). In addition, their expression analysis with the distal marker genes *Tc' Lim1* and *Tc' dachsous* revealed the absence of the anlagen for the tibiotarsus and the pretarsal claw (Beermann et al. 2011). Due to this distal phenotype and the fact that the proximal part of the leg is intact, the authors suggest a functional role of *Tc'fz-1* in distal leg formation and propose *Tc'wg* as best candidate ligand for *Tc'Fz-1* in appendage formation (Fig. 6 A-E; Beermann et al. 2011).
Interestingly, our 18 hour injections revealed a third function of *wg* in *Tribolium* leg development. The overall amount of leg phenotypes after injecting in 18 hour old embryos remained more or less constant, but the distal phenotype decreased and the weaker "ventral-less"-phenotype could be observed. In contrast to the "nonpareille" and the "candy cane" phenotype, the "ventral-less"-phenotype does not show any distal defects, just the claw is abnormally shaped. The knockdown of *wg* in embryos of the enhancer trap lines Goe-04609 and Goe-12407 and the following expression analysis demonstrates that ventral leg tissue is missing and strongly indicates a function of *wg* in ventral patterning. This ventral function is also known in *Drosophila*, where *wg* does act locally together with ventrally expressed factors as *midline* and *H15* to establish ventral leg cell fate ((Diaz-Benjumea and Cohen 1994; Wilder and Perrimon 1995 and see section 2.3). The "ventral-less"-phenotype in *Tribolium* is restricted to cells along the ventral midline of the legs and we therefore strongly suggest that *wg* does not act as a morphogen instead locally as it is the case in *Drosophila*.

Taken together, our data strongly indicate three separate functions of *wg* in leg development and these functions are temporally separated. Beerman at al. disagree with our suggestion of a distinct time-dependent function of *Tc'wg*. They rather argue for a concentration-dependent regulation of the proximo-distal and dorso-ventral leg pattering (Beermann et al. 2011). However, the phenotypic series that Beermann et al. obtained after parental RNAi is due to the fact that the efficiency of RNAi decreases over time in the offspring of injected females and consequently the strength of caused phenotypes is also decreasing. In addition, they just obtained the distal phenotype and not as in our case the additional ventral phenotype. They indeed performed an expression analysis with *Tc'fz-1*RNAi embryos using *Tc'wg* as ventral

marker and suggest that their results indicate a loss of ventral tissue but in our opinion their interpretation of the results is not correct. They concluded that the *wg* expression is abolished in the ventral leg except for a ventral stripe in the coxa. A comparison with the *wg* expression in a wildtype leg shows that *wg* is never expressed in the whole length of the leg due to the repression of *wg* by *dpp* in the distal part of the leg. Taking into account that the legs of *Tc'fz-1*RNAi embryos are shortened, the *wg* expression shown in their Fig. 6 G-J does not differ from that in a wildtype embryo and is consequently not proof for a ventral phenotype in their *Tc'fz-1*RNAi embryos. In contrast, our observed ventral phenotype is not a result of a decreasing RNAi-effect, since we injected at all different time points the same amount and same concentration of *wg* dsRNA. In addition, the complete lack of the ventral expression marker in the legs of the analysed enhancer trap lines after depletion of *Tc'wg* further confirms our results and supports a role of *Tc'wg* in establishment of ventral cell fate in the leg. Therefore, we further strongly argue for a distinct and time-dependant role of *wg* in early and late leg development in *Tribolium*.

4.2 Dpp signalling is required for proximo-distal leg pattering in *Tribolium*, but lacks a function in dorsal leg pattering

The expression pattern of *wg* is highly conserved in all arthropods investigated to date and our data indicate a conserved role of *wg* at least in the holometabolous beetle *Tribolium*. However, several studies in insects and other arthropod groups revealed that the expression of *dpp* orthologs does not resemble that of the *Drosophila* leg disc. These unexpected differences in *dpp* expression between *Drosophila* and other arthropod species indicate a non-conserved role of *dpp.* Previous functional studies in *Tribolium* are not consistent. Ober and Jokusch for instance demonstrated that the downregulation of *dpp* in *Tribolium* is leading to subtle phenotypes but does not generate defects in limb development. Actually, the development of all appendages is morphologically normal (Fig. 5) and they suggest that *dpp* does not posses a role in appendage development in *Tribolium* (Ober and Jokusch 2006). Van der Zee et al. performed also parental RNAi with *dpp* in *Tribolium* and their experiment yield severely affected embryos. The embryos were

completely ventralized, further development was interrupted and consequently all appendages fail to develop (van der Zee et al. 2006).

In our experiment, we performed staggered stage-specific RNAi to circumvent these early and severe defects. Early injections in 2, 4, 8 and even 12 hour old embryos generated just empty eggs or severely disrupted and not analysable embryos as already described by van der Zee et al. (2006). These results indicate that *dpp* is still involved in a variety of different developmental processes and do not allow a statement about a potential function of *dpp* in leg development. Strikingly, the injections in 18 hour old embryos generated more or less normal embryos without any severe defects except for a distal leg phenotype. The claw is misshaped and attached to the tibiotarsus in an unusual angle. Furthermore, the analysis of specific cuticle markers revealed that the femur and the tibiotarsus are partially fused and strongly shortened. We interpret these severe malformations as strong evidence for a role of *Tc'dpp* in distal leg development in *Tribolium*. These malformations could be the result of the general miss-pattering of the distal leg. The missing leg expression pattern of the medial-distal marker gene *Tc' dac* supports this hypothesis. To better understand how this phenotype is caused expression analysis with more different marker genes has to be performed. Interestingly, Pechmann (2011) could already demonstrate in two different *Tc'omb* mutant lines that *Tc'dpp* fulfils an essential role in leg development of *Tribolium* and suggest that *Tc'dpp* is in contrast to *Tc'wg* the instructive morphogen and regulates several leg gap genes, including *Distal-less*, *dachshund* and *cll*. Pechmann could also demonstrate that *Tc'dpp* expression in a mutant *Tc'omb* line is not restricted to a small stripe distal to the limb tip, but is miss-expressed along the dorsal side. Pechmann suggests that *Tc'omb* acts as a repressor of *Tc'dpp* along the dorsal side (Pechmann 2011). The expression data in *Tc'dpp*RNAi-embryos revealed that the expression of the dorsal marker *omb* in the legs is strongly reduced, indicating that *Tc'dpp* activates *Tc'omb* as it is known in *Drosophila*. Together with the results of Pechmann we assume, that *Tc' omb* is indeed a repressor of *Tc'dpp* and that *Tc'dpp* activates its own repressor which restricts its expression domain to a small stripe distal to the limb bud.

In *Drosophila,* next to its function in proximo-distal leg pattering, *dpp* is also required for the establishment of dorsal cell fate in the leg and controls the activation of *omb* and the repression of *wg*. Mutations in *Dm'dpp* lead to the deletion of all

dorsal leg structures and to symmetric duplications of ventral structures (Brock and Cohen 1996; Brock 2010). Therefore, one might expect that the knockdown of *dpp* in *Tribolium* is leading to a ventralization of the leg. Surprisingly, the analysis of the *wg* expression pattern in *dpp*-RNAi-embryos revealed, that *wg* is expressed normally along the ventral side as in wildtype embryos and no additional expression domain could be detected which might indicate a ventralization of the legs in Tc'*dpp*-RNAi-embryos. This data together with the fact that in *Tribolium dpp* is not expressed along the dorsal side of the limb bud give rise to the assumption, that *dpp* in *Tribolium* is not involved in establishment of dorsal cell fate in the leg (Sanchez-Salazar et al. 1996).

Taken together, in contrast to Ober and Jokusch, we were able to generate via staggered stage-specific embryonic *Tc'dpp*RNAi a distal leg phenotype and we therefore strongly suggest that *dpp* indeed has a role in leg development in *Tribolium*. Furthermore, we assume that *dpp* in contrast to its function in *Drosophila* is not involved in the establishment of dorsal cell fate in the leg in *Tribolium* and that these function is only achieved in the lineage leading to *Drosophila* (see section 4.3).

4.3 The Wg/Dpp-leg pattering system: conserved and derived aspects in *Tribolium castaneum*

In *Drosophila melanogaster*, establishment of the appendage primordia and the limb proximo-distal axis requires Wg and Dpp signalling activity (Cohen et al. 1993). The PD axis pattering is guided by a hierarchic gene cascade and Wg and Dpp are the key factors and act as long-range morphogens. Both genes are expressed along the anterior side of the AP-compartment boundary of the leg disc. The ventral expression domain of *wg* and the dorsal domain of *dpp* are stabilized by mutually antagonistic repression. Combined activity of both genes is required for further subdivision and specification of the leg disc via activation and repression of specific target genes as *Dll*, *dac* and *hth* (Diaz-Benjumea et al. 1994; Abu-Shaar and Mann 1998; Lecuit and Cohen 1997, see section). Several studies revealed a relative high degree of conservation in expression and function at the middle level of the cascade.

Studies in various arthropod groups could show that the expression of *wg* is highly

conserved in arthropods, but a conserved expression pattern of *dpp* along the dorsal side of the limb bud could not be confirmed. Instead, *dpp* is broadly expressed in the early limb bud and later in a distal region close the distal tip. In addition, given the derived nature of limb development from two-dimensional imaginal discs in *Drosophila*, it was questioned, if the role and function of Wg and Dpp-signalling in leg development is evolutionary conserved in more basal insects and other arthropod groups.

Subsequently performed functional studies provide no clear answer and give rise to controversy concerning an evolutionary conserved Wg-Dpp leg pattering system outside *Drosophila*. Recent studies in *Oncopeltus fasciatus* and *Gryllus bimaculatus* were not able to confirm a role of *wg* in leg development of these hemimetabolous insects (Miyawaki et al. 2004; Angelini and Kaufman 2005). However, data from *Tribolium castaneum* indicated a role of *wg* in leg allocation (Ober and Jokusch 2006). The role of *dpp* in leg development of other arthropod species is hardly investigated and available data from *Tribolium* do not support an essential and conserved function of *dpp* in leg development (Ober and Jokusch 2006; Van der Zee). We performed functional analyses of *wg* and *dpp* in *Tribolium* and our results indeed indicate a role of both genes in leg development. Our RNAi data reveal that *wg* is not just involved in leg allocation but also in distal and ventral leg pattering. In addition, with staggered stage-specific embryonic RNAi we were able to knockdown the function of *wg* at specific time points and consequently we could reveal that all three functions of *wg* are temporally separated. Strikingly and in contrast to previous data we were able to demonstrate that next to *wg* also *dpp* is indeed involved in distal leg development in *Tribolium*. The knockdown of *dpp* caused severe defects in distal parts of the leg including the femur, tibiotarsus and the distal claw. In addition, we could demonstrate that the target gene *dac* is either directly or indirectly regulated by *dpp*.

The topology model by Prpic et al. (2003) suggests that despite the non-conserved expression pattern of *dpp*, the Wg-Dpp system is conserved in leg pattering of many arthropod species. Our findings support this hypothesis. Besides, Pechmann (2011) could demonstrate an essential and instructive role of *dpp* in leg development of *Tribolium*. Together with our results we strongly argue for a conserved Wg-Dpp system in proximo-distal axis formation in *Tribolium castaneum*.

4.4 Evolution of wg and dpp functions in insect leg development

The comparative analyses of *wg* (or downstream factors of the WNT-signalling pathway) and *dpp* function during leg development to date are mainly focused on insects. So far their function has been investigated in four distantly related insect orders: Orthoptera (*Gryllus bimaculatus*), Hemiptera (*Oncopeltus fasciatus*), Coleoptera (*Tribolium castaneum*) and Diptera (*Drosophila melanogaster*).

Functional data from *Gryllus*, *Oncopeltus* and *Tribolium* suggest that the Wg/Dpp system known in *Drosophila* was ancestrally not involved in specification and development of the limb primordia and proximo-distal axis (Miyawaki et al. 2004; Angelini and Kaufman 2005b; Ober and Jokusch 2006). Angelini and Kaufman have suggested that the genetic network with Wg and Dpp as upstream activators of proximo-distal pattering gens has evolved only later in the holometabolous insects, after the divergence of Diptera and Coleoptera (Angelini and Kaufman 2005b).

However, our data strongly suggest that the separate function of *wg* in leg allocation, distal and ventral leg development and the function of *dpp* in distal leg development of *Tribolium* must have evolved before the divergence of Diptera and Coleoptera and therefore supports the hypothesis of Prpic et al. of a conserved Wg/Dpp system in proximo-distal leg pattering (Prpic et al. 2003). However, *dpp* seems to have actually acquired its function in dorsal leg pattering only after the split between Coleoptera and Diptera since we were not able to observe a loss of dorsal cell fate or a ventralization effect after depletion of *dpp* function in *Tribolium*.

Although the data in *Oncopeltus* and *Gryllus* do not point to a conserved Wg/Dpp system we do not exclude the possibility of a conserved function of both genes in hemimetabolous insects. *wg* RNAi in *Oncopeltus* indeed does not generate a leg phenotype, but critical to note is, that no expression analysis of *wg* in the *Of'wg*RNAi embryos has be performed and therefore, it can not be excluded that still enough *Of'wg* transcript was present and leg development could proceed properly despite a reduced concentration of *Of'wg* transcripts. Also the RNAi data of *Gryllus* are difficult to interpret. Miyawaki et al. were not able to knock down the function of *wg* in *Gryllus bimaculata*. The expression data analysis revealed only a slight

reduction of *wg* expression and consequently no morphological changes in the embryo were observed (Miyawaki et al. 2004). Instead of a direct knockdown of *wg*, the authors depleted the function of *Gb'armadillo* (*Gb'arm*), a segment-polarity gene, which is involved in the canonical Wnt-pathway. Nevertheless, Miyawaki et al. suggest that *wg* is not involved in leg development just because of an indirect knockdown of the Wg signalling pathway via *Gb'arm*. However, only one armadillo homologue was isolated and tested. In vertebrates, *C. elegans* and *Tribolium castaneum* for instance there are two or more homologues of armadillo known (; Kolligs et al. 2000; Natarajan et al. 2001; Georg Oberhofer, personal communication). It might be, that also a second or more homologues of *arm* exists in *Gryllus bimacultaus* and that one of these is crucial for leg development instead of the one tested by Miawaki et al. It is thus important to establish the exact number of *arm* homolgues in the genome of *Gryllus* and to perform additional functional experiments. At last, although all available data so far indicate that *wg* regulates leg development in insects via the canonical Wnt-pathway and therefore via *arm*, it should be also considered that leg development in *Gryllus* might be regulated by a non-canonical pathway.

Strikingly, studies on leg regeneration in *Gryllus bimaculatus* demonstrate that the initiation of the proximo-distal axis in leg regeneration occurs at a site where *wg*-expressing cells abut *dpp*-expressing cells (Mito et al. 2002). Although these results are related to leg regeneration and not embryonic leg development these data might point to a potential function of *wg* and *dpp* also in embryonic leg development in *Gryllus*.

In conclusion, due to the small and doubtful dataset in hemimetabolous insects, we do not exclude a conserved function of the Wg/Dpp leg pattering system in more basal insect groups as well as other arthropod groups. Finally, it remains unclear to what extent the function of *wg* and *dpp* in arthropod appendage development is conserved and much more arthropod species (e.g. *Cupiennius salei*, *Achaearanea tepidariorum*, *Glomeris marginata*) and outgroup species (e.g. Onychophora) have to be involved in comparative studies to better define to what extant the Wg/Dpp leg pattering system is conserved.

4.5 Requirement of Egfr signalling in leg development in *Tribolium castaneum*

Studies on leg development in the prime insect model *Drosophila melanogaster* revealed over the last decades that Wg/Dpp signalling is the main system of proximo-distal leg axis pattering. Although it was generally assumed that the Wg/Dpp leg pattering system also represents the ancestral mechanism of proximo-distal axis formation in basal arthropods or arthropods in general, there is increasing evidence that this system in other arthropod groups is not that crucial in leg axis pattering as in *Drosophila* (Miaywaki et al. 2004; Angelini and Kaufman 2005a, Angelini and Kaufman 2005b). Actually, Angelini and Kaufman suggested that Wg and Dpp evolved their specific role in proximo-distal leg axis patterning only late in the lineage of the holometabolous insects (Angelini and Kaufman 2005b, see Fig. 4 in the introduction). Consequently, this raises the question which system controls proximo-distal axis pattering in the majority of the other arthropod species. It could be shown that EGFR signalling is in addition to the Wg/Dpp system involved in proximo-distal leg axis formation. In *Drosophila* Egfr signalling is required only late in development and its function is restricted to the tarsal region, but it is possible that the EGFR signalling pathway plays a more decisive role in proximo-distal leg formation in other arthropod species. Interestingly, Prpic (2004) could already show that the function of Egfr signalling in *Cupiennius salei* is not limited to the most distal region but crucial for the complete leg growth.

In our previous study, we already could demonstrate that *wg* and *dpp* have a role in proximo-distal leg formation in *Tribolium*. Due to its more basal phylogenetic position within the holometabolous insects, it might be possible that Egfr signalling in addition to Wg and Dpp has a more complex role in proximo-distal axis formation than in *Drosophila*. To address this issue we studied the role of the EGF receptor, its ligand Spitz and its target gene *pnt* in formation and pattering of the legs in *Tribolium castaneum*.

4.5.1 Interference with *Egfr*, s*pi* and *pnt* causes an abdominal "outside-in" phenotype

We were able to isolate the *Egf* receptor, its ligand s*pitz* and and the transcription factor *pointed* from a cDNA pool containing embryos of all developmental stages of *Tribolium*. We identified only a single *spi* homolog, which is highly similar to *Keren* and *Spitz* in *Drosophila melaogaster*. The function of all three genes could not be studied with parental RNAi. Injected females had severely reduced egg productivity which indicates that all three genes are involved in early developmental processes as oogenesis and/ or gonad development. To circumvent these early processes and to gain insight in their function in leg development in *Tribolium* we used instead staggered stage-specific embryonic RNAi. Early injections of *Egfr ds*RNA, *pnt* dsRNA and *spi* dsRNA before 12h after egg-laying interfered still with early functions of all three genes and caused mainly empty eggs or eggs with irregular structures. All embryos that were injected 12h after egg-laying and therefore at a time point when the limb buds become specified showed specific leg and abdominal phenotypes. Interestingly, the abdominal "outside-in" phenotype caused by RNAi is similar for all three genes. The dorsal tissue of all embryos converges at the level of the second and third thoracic segment, which leads to the development of a rim of dorsal tissue at the end of the third thoracic segment that crosses the ventral midline. This seems to cause problems with abdominal dorsoventral polarity and leads to a malformed tube-shaped abdomen, which we define as the "outside-in" abdominal phenotype. Since the knockdown of all three genes was leading to a similar effect on abdominal polarity in *Tribolium*, we suggest that *Egfr*, s*pi* and *pnt* form a similar signalling pathway as it is known in *Drosophila melanogaster*. So far, there are only a few studies concerning the function of *Egfr*, *spi* and *pnt* in *Tribolium* or other arthropod species, but a recent study by Rousso et al. (2008) could already demonstrate conserved aspects of the EGFR pathway in *Tribolium castaneum* and therefore support our hypothesis. In *Drosophila*, the ligand Spitz must be processed into an active secreted form by Rhomboid and Star (Bier et al. 1990; Kolodkin et al. 1994; Golembo et al. 1996). Rousso et al. (2008) could show that also in *Tribolium* the ligand Spitz has to be activated by the two proteins Rhomboid and Star.

4.5.2 Two roles of Egfr signalling in proximo-distal leg development in *Tribolium*

The expression pattern analysis of all three genes already indicated a role of *Egfr*, *spi* and *pnt* in leg development of *Tribolium*. Indeed, our RNAi data show that knockdown of *Egfr*, *spi* as well as *pnt* caused defects in *Tribolium* leg development. In contrast to the similar "outside-in" phenotype in the abdomen, gene specific differences could be observed with respect to the formation of the legs. *Egfr*RNAi and *spi*RNAi cuticles showed a similar leg phenotype. In both phenotypes we could observe that the femur and the tibiotarsus are fused and shortened. In addition, the distal claw was missing in both cases. In contrast, *pnt*RNAi caused a much minor leg phenotype. The legs were all virtually identical to wildtype legs, except that they were lacking the distal claw. Interestingly, the leg phenotype of all three genes had in common that the coxa and trochanter were never affected and the distal claw is missing, but the fusion of the femur and tibiotarsus could only be observed in the *Egfr*RNAi and *spi*RNAi larvae. Since RNAi does not cause any defects in the coxa and trochanter, we assume that Egfr signalling is not involved in forming and pattering of the proximal parts of the leg.

The analysis of specific marker genes in all RNAi embryos could reveal defects in the general pattering of the leg. Interestingly, the changed expression pattern of different marker genes was not identical after RNAi with *Egfr*, *spi* and *pnt*. Our results show differences between the three genes with respect to the regulation of specific target genes. The expression of *clawless* (*cll*) is abolished or at least strongly reduced after RNAi with all three genes, consistent with the RNAi cuticle leg phenotypes. This suggests that *Egfr*, *spi* and *pnt* are functionally related in a hierarchical chain that is required for the correct activation of *cll* in the distal leg and any interference with one part of the chain leads to the downregulation of *cll* expression. Strikingly, the regulation of *Dll* and *dac* does not require the activity of *pnt*, but seems to be directly or indirectly dependent on the activity of *Egfr* and *spi*. In *pnt*RNAi embryos the expression of *Dll* and *dac* is unchanged. This is consistent with the leg cuticle phenotype, where the medial parts such as the femur and the tibiatarsus are not affected after RNAi. The observed differences of the expression pattern of *Dll* and *dac* in *pnt*RNAi and *Egfr* as well as *spi*RNAi embryos suggests that in theses cases the outcome of Egfr signalling is not mediated by *pnt*, but another

and not yet identified transcription factor. We propose therefore, that this unknown factor is required for the activation of *Dll* and in parallel is required for the repression of *dac*. However, the expression of *Dll* is in *Egfr*RNAi embryos as well as *spi*RNAi embryos not completely abolished, which indicates that the unidentified transcription factor is not the only activator of *Dll* expression in *Tribolium*. We therefore suggest that next to the unidentified factor at least a second activator is required for the correct *Dll* expression in *Tribolium*. We also analysed the expression pattern of *Sp8* after RNAi with all three genes. The gene *Sp8* is normally expressed in the distal and medial portions of the leg and it could be shown that *Sp8* is crucial for allometric growth of the limbs in *Tribolium castaneum* (Beermann et al. 2004). Since we did not observe any strong effect on the expression pattern of *Sp8* after interference with *Egfr*, *spi* or *pnt*, we assume that the pattering function of Sp8 is independent from EGFR signalling.

Taken together, our findings from the RNAi and expression pattern analysis strongly suggest that the embryonic leg is divided into three parts. All parts differ significantly in their use of EGFR signalling for their specific formation and pattering. The proximal part seems to be independent from EGFR signalling. The medial part uses EGFR signalling for formation and pattering, but signalling is not mediated by *pnt* as in the distal part of the leg, and a different, unidentified transcription factor is required instead. These findings lead to the hypothesis that originally EGFR signalling fulfilled two different roles in proximo-distal leg formation and that these functions in medial and distal leg formation have evolved separately. The distal role of EGFR signalling is conserved in arthropods at least in holometabolous insects (Galindo et al, 2002; Campell 2002). In contrast to its distal role, we propose that the function of EGFR signalling in formation of the medial part of the leg has been substituted in the lineage leading to *Drosophila* by the Wg/Dpp leg pattering system. According to this hypothesis, *Tribolium castaneum* represents an intermediate evolutionary state. In the spider *C. salei Egfr* is expressed in each segment and signalling is required for the complete leg growth (Prpic 2004), in *Tribolium* EGFR signalling is still required for the correct formation and pattering of the medial and distal parts of the leg but not the proximal part (this thesis) and in *Drosophila* the requirement of EGFR signalling is only needed in the distal part of the leg (Galindo et al, 2002; Campell 2002).

4.6 Concluding remarks: evolutionary aspects on the requirement of the Wg/Dpp system and Egfr signalling in arthropod leg development

Our knowledge of leg development in arthropods is primarily based on studies performed in the prime model *Drosophila melanogaster*. In the last decades, genetic and molecular studies have given a deeper insight into the underlying mechanisms of leg development. In *Drosophila*, the establishment of the proximo-distal axis is largely guided by Wg and Dpp signalling. Besides the Wg/Dpp system, Egfr signalling is also involved in pattering the proximo-distal axis, but it is only required late in development and its function is restricted to the distal part of the leg. However, for a better understanding of evolutionary aspects of leg development in arthropods several comparative studies in other arthropod species including spiders, crickets and bugs have been performed. Given the derived nature of limb development in *Drosophila* and the fact that in most other arthropod species the legs are direct outgrowths of the embryonic body wall, it might not be surprising that available data so far are controversial and the conservation and predominance of the Wg/Dpp in leg development is questioned.

In our study, we investigated the function of Wg- and Dpp- signalling as well as the role of EGFR signalling in the coleopteran *Tribolium castaneum*. Our comparative approach revealed that in *Tribolium* Wg and Dpp signalling are indeed important signalling pathways in leg development. As in *Drosophila*, Wg signalling is required for three different processes. During early development Wg signalling is needed for the allocation of the leg and later its function is essential for distal and ventral leg development. Moreover, RNAi data revealed that Dpp signalling is required for proximo-distal leg pattering. Knockdown of *dpp* caused defects in all segments distal to the trochanter. In contrast to *Drosophila*, we could not find evidence for a role of *dpp* in dorsal pattering. This function seems to be acquired only later in the holometabolous insects.

Interestingly, we could find differences with respect to the requirement of EGFR signalling in *Tribolium* leg development. Our results indicate that EGFR signalling has a more complex role in *Tribolium* than in *Drosophila*. The requirement of EGFR signalling is not restricted to the distal part of the leg but it is also involved in pattern

formation in the medial leg. In addition, this role of EGFR signalling in the medial part of the leg appears functionally separate from its role in the distal leg, since EGFR signalling is not mediated by *pnt* but an different, unidentified transcription factor. However, this function in the medial leg seems to be substituted by the Wg/Dpp system on the lineage leading to *Drosophila melanogaster*.

In summary, the beetle *Tribolium castaneum* likely represents an intermediate evolutionary state, where the Wg/Dpp system is already the predominant system in leg development, but EGFR signalling is more important and has a more complex role than in *Drosophila*. Although there are controversial data concerning the function of *wg* and *dpp* in species like *Oncopeltus* and *Gryllus*, we do not exclude a conserved function of the Wg/Dpp leg pattering system in more basal insect groups as well as other arthropod species. Since our results indicate that EGFR signalling has more complex role in leg development in *Tribolium* it is now imporatnt to study the role of EGFR, Wg, and Dpp signalling in more arthropod species (e.g. *Cupiennius salei*, *Achaearanea tepidariorum*, *Glomeris marginata*) in order to reveal to what extant their functions in leg development are conserved in other species.

5 References

Abu-Shaar, M. and R.S. Mann (1998). “Generation of multiple antagonistic domains along the proximodistal axis during Drosophila leg development.” Development 125: 3821-30.

Abzhanov, A. and T. C. Kaufman (2000). “Homologs of Drosophila appendage genes in the pattering of arthropod limbs.“ Dev Biol 227: 673-89.

Angelini, D.R. and T.C. Kaufman (2004): „Functional analyses in the hemipteran Oncopeltus fasciatus reveal conserved and derived aspects of appendage pattering in insects.” Dev Biol 271: 306-21.

Angelini, D.R. and T.C. Kaufman (2005a). „Functional analyses in the milkweed bug Oncopeltus fasciatus (Hemiptera) support a role for Wnt signalling in body segmentation but not appendage development.” Dev Biol 283: 409-23.

Angelini, D. R. and T. C. Kaufman (2005b). „Insect appendages and comparative ontogenetics.“ Dev Biol 286: 57-77.

Basler, K. and G. Struhl (1994). “Compartment boundaries and the control of Drosophila limb pattern by hedgehog protein.” Nature 368: 208-14.

Beermann, A. M. Aranda and R. Schroeder (2004). “The Sp8 zinc-finger transcription factor is involved in allometric growth of the limbs in the beetle Tribolium castaneum.” Development 131: 733-742.

Beermann, A., D. G. Jay, R. W. Beeman, M. Hülskamp, D. Tautz and G. Jürgens (2001). “The Short antennae gene of Tribolium is required for limb development and encodes the orthologue of the Drosophila Distal-less protein.” Development 128: 287-297.

Beermann, A., R. Prühs, R. Lutz and R. Schröder (2011). „A context-dependent

combination of Wnt receptors controls axis elongation and leg development in a short germ insect.“ Development 138: 2793-2805.

Bier, E., L. Y. Jan and Y. N. Jan (1990). “rhomboid, a gene required for dorsoventral axis establishment and peripheral nervous system development in Drosophila melanogaster.” Genes Dev 4: 190-203.

Brook, W. J. and S. M. Cohen (1996). “ Antagonistic interactions between wingless and decapentaplegic responsible for dorsal-ventral pattern in the Drosophila leg.” Science 273: 1373-7.

Brook, W. J. (2010). “T-box genes organize the dorsal ventral leg axis in Drosophila melanogaster.” Landes Bioscience Fly 4: 159-162.

Brown, K. E., M. Kerr and M. Freeman (2007). “The EGFR ligands Spitz and Keren act cooperatively in the Drosophila eye.” Dev Biol 307: 105-113.

Bucher, G., J. Scholten and M. Klingler (2002). “Parental RNAi in Tribolium (Coleoptera).” Curr Biol 12: R85-6.

Campell, G. (2002). “Distalisation of the Drosophila leg by graded EGF-receptor activity.” Nature 418: 781-5.

Casares, F. and R. S. Mann (2001). “The ground state of the ventral appendage in Drosophila.” Science 293: 1477-80.

Casci, T., J. Vinos and M. Freemann (1999). “ Sprouty, an intracellular inhibitor of RAS signalling.” Cell 96: 655-665.

Chapman, A. D. (2009). “Numbers of living species in Australia and the world. 2nd ed. Australian Biological Resources Study, Canberra. http://www.environment.gov.au/biodiversity/abrs/publications/other/species-numbers/2009/pubs/nlsaw-2nd-complete.pdf”.

Cohen, B., A. A. Simox and S. M. Cohen (1993). “Allocation of the thoracic imaginal primordial in the Drosophila embryo.” Development 117: 597-608.

Cohen, S. M., G. Bronner, F. Kuttner, G. Jurgens and H. Jackle (1989). „Distal-less encodes a homeodomain protein required for limb development in Drosophila.” Nature 338: 432-4.

Cousco, J. P., M. Bate and A. Martinez-Arias (1993). “A wingless-dependant polar coordinate system in Drosophila imaginal discs.” Science 259: 484-489.

Damen, W. G. M. (2002). “Parasegmental organization of the spider embryo implies that the parasegment is an evolutionary conserved entity in arthropod embryogenesis.” Development 129: 1239-1250.

Davis, G. K. and N. H. Patel (1999). “The origin and evolution of segmentation.” Trends Cell Biol 9: M68-72.

Diaz-Benjumea, F. J., B. Cohen and S. M. (1994). “Cell interaction between compartments establishes the proximal-distal axis of Drosophila legs.” Nature 372: 175-9.

Estella, C., G. Rieckhof, M. Calleja and Gines Morata (2003). “The role of buttonhead and SP1 in the development of the ventral imaginal discs of Drosophila.” Development 130: 5929-5941.

Estella, C. and R. S. Mann (2008). “Logic of Wg and Dpp induction of distal and medial fates in the Drosophila leg.” Development 135: 627-36.

Estella, C. and R. S. Mann (2010). “Non-redundant selector and growth-promoting functions of two sister genes, buttonhead and SP1, in Drosophila leg development.” PLoS Genet 6: e1001001.

Estella, C., D. J. McKay and R. S. (2008). “Molecular integration of wingless, decapentaplegic and autoregulatory inputs into Distalless during Drosophila leg development.” Dev Cell 14: 86-96.

Estella, C., G. Rieckhof, M. Calleja and G. Morata (2003). “The role of buttonhead and SP1 in the development of the ventral imaginal discs of Drosophila.” Development 130: 5929-41.

Fristrom, D. and J. W. Fristrom (1993). “The metamorphic development of adult epidermis.” In M. Bate and A. Martinez Arias (eds.) The Development of Drosophila melanogaster, Vol II, Cold Spring Habor, New York: Cold Spring Harbor Laboratory Press, pp. 843-897.

Gabay, L., H. Scholz, M. Golembo, A. Klaes, B. Z. Shilo and C. Klambt (1992). “EGF receptor signalling induces pointed P1 transcription and inactivates Yan protein in the Drosophila embryonic ventral ectoderm.” Development 122: 3355-3362.

Galindo, M. I., S. A. Bishop, S. Greig and J. P. Couso (2002). “Leg patterning driven by proximal-distal interactions and EGFR sigaling.” Science 297: 256-9.

Gebelein, B., J. Culi, H. D. Ryoo, W. Zhang and R. S. Mann (2002). „Specificity of Distalless repression and limb primordia development by abdominal Hox proteins.“ Dev Cell 3: 487-98.

Ghiglione, C., K. L. Carraway, 3rd, L. T. Amundadrottir, R. E. Boswell; N. Perrimon and J. B. Duffy (1999). “The transmembrane molecule kekkon 1 acts in a feedback loop to negatively regulate the activity of the Drosophila EGF receptor during oogenesis.” Cell 96: 847-856.

Ghiglione, C., L. T. Amundadrottir, L. Andresdotti, D. Bilder, J. A. Diamonti, S. Noselli, S., N. Perrimon and K. L. Carraway (2003). “Mechanism of inhibition of the Drosophila and mammalian EGF receptors by the transmembrane protein Kekkon 1.”

Development 130: 4483-4493.

Giorgianni, M. and N. H. Patel (2005). “Conquering land, air and water: the evolution and development of arthropod appendages. “Briggs D.E.G (ed.) Evolving Form and Function: Fossils and Development, New Haven: Peabody Museum of Natural History, Yale University: 159-188.

Giorganni, M. W. and R. S. Mann (2011). “Establishment of Medial Fates along the Proximodistal Axis of the Drosophila Leg through Direct Activation of dachshund by Distalles.” Dev Cell 20 (4): 455-68.

Golembo, M., T. Yarnitzky, T. Volk and B. Z. Shilo (1999). ”Vein expression induced by EGF receptor pathway to provide a positive feedback loop in pattering the Drosophila embryonic ventral ectoderm.” Genes Dev 13: 158-162.

Golembo, M., E. Raz, B. Z. Shilo (1996). ”The Drosophila embryonic midline is the site of Spitz processing, and induces activation of the EGF recptor in the ventral ectoderm.“ Development 122: 3363-70.

Goto, S. and S. Hayashi (1997). “Specification of the embryonic limb primordium by graded activity of Decapentaplegic.” development 124 (1): 125-32.

Harris, R. C., E. Chung and R. J. Coffey (2003). “EGF receptor ligands.” Experimental Cell Research 284: 2-13.

Held, L. I., M. A. Heup, J. M. Sappington and S. D. Peters (1994). “ Interactions of of decapentaplegic, wingless and Distal-less in the Drosophila leg.” Roux’s Arch Dev Biol 203: 310-319.

Hughes, C. L. and T. C. Kaufman (2002). “Hox genes and the evolution of the arthropod body plan.” Evol Dev 4 (6): 459-99.

Hunt T, J. Bergsten, Z. Levkanicova, A. Papadopoulou, O. S. John, R. Wild, P. M.

Hammond, D. Ahrens, M. Balke, M. S. Caterino (2007). "A comprehensive phylogeny of beetles reveals the evolutionary origins of a superradiation." Science 318: 1913-1916

Inoue, Y., T. Mito, K. Miyawaki, K. Matsushima, Y. Shinmyo, T. A. Heanue, G. Mardon, H. Ohuchi and S. Noji (2002). "correlation of expression patterns of homothorax, dachshund, and Distal-less with the proximo-distal segmentation of the cricket leg bud." mech Dev 113: 141-148.

Jiang, J. and G. Struhl (1996)."Complementary and mutually exclusive activities of decapentaplegic and wingless organize axial patterning during Drosophila leg development." Cell 86 (3): 401-9.

Jokusch, E. L., C. Nulsen, S. J. Newfeld and L. M. Nagy (2000). "Leg development in flies versus grasshoppers: differences in dpp expression do not lead to differences in the expression of downstream components of the leg patterning pathway." Development 127 (8): 1617-26.

Johnston, L. A. and G. Schubinger (1996). "Ectopic expression of wingless in imaginal discs interferes with decapentaplegic expression and alters cell determination." Development 122: 3519-3529.

Kojima, T. (2004). "The mechanism of Drosophila leg development along the proximodistal axis." Dev Growth Differ 46 (2): 115-29.

Kojima, T., T. Tsuji and K. Saigo (2005). "A concerted action of paired-type homeobox gene, aristaless, and a homolog of Hox11/tlx homeobox gene, clawless, is essential for the distal tip of the Drosophila leg." Dev Biol 279: 434-445.

Kolligs F. T., B. Kolligs, K. M. Hajra, G. Hu, M. Tani, K. R. Cho and E. R. Fearon (2000). "γ-Catenin is regulated by the APC tumor suppressor and its oncogenic activity is distinct from that of β-catenin". Genes Dev 14: 1319–1331.

Kolodkin, A. E., A. T. Pickup, D. M. Lim, C. S. Goodmann and U. Banerjee (1994). "Characterization of Star and its interactiosn with sevenless and EGF receptor during photoreceptor cell development in Drosophila." Development 120: 1731-1745.

Kubota, K.,S. Goto and S. Hayashi (2003). "The role of Wg signalling in the pattering of embryonic leg primordium in Drosophila. Dev Biol 257: 117-126.

Kubota, K., T. Tsuji and K. Saigo (2005). "EGF attenuates Dpp signalling and helps to distinguish the wing and leg cell fates in Drosophila." Development 127 (17): 3769-76.

Lecuit, T., and S. M. cohen (1997). "Proximal-distal axis formation in the Drosophila leg." Nature 388 (6638): 139-45.

Mardon, G., N. M. Solomon and G. M. Rubin (1994). "dachshund encodes a nuclear protein required for normal eye and leg development in Drosophila." Development 120 (12): 3473-86.

McKay, D. J., C. Estella and R. S. Mann (2009). "The origins of the Drosophila leg revealed by the cis-regulatory architecture of the Distalless gene." Development 136 (1): 61-71.

Miyawaki K., T. Mito, I. Sarashina, H. Zhang, Y. Shinmyo, H. Ohuchi and S. Noji (2004). "Involvment of Wingless/Armadillo signalling in the posterior sequential segmentation in the cricket Gryllus bimaculatus (Orthoptera), as revealed by RNAi analysis." Mech Dev 121: 119-130.

Mito, T., Y. Inoue, S. Kimura, K. Miyawaki, N. Niwa, Y. Shinmmyo, H. Ohuchi and S. Noji (2002). "Involvment of hedgehog, wingless and dpp in the initiation of the proximodistal axis formation during regeneration of insect legs, a verification of the modified boundary model." Mechanisms of Development 114: 27-35.

Nagy L. M. and S. B. Caroll (1994). "Conservation of wingless pattering functions in the short-germ embryos of Tribolium castaneum." Nature 367: 460-463.

Natarajan L., N. E. Witwer and D. M. Eisenmann (2001). “The divergent Caenorhabditis elegans beta-catenin proteins BAR-1, WRM-1 and HMP-2 make distinct protein interactions but retain functional redundancy in vivo“. Genetics 159: 159-172.

Neumann-Silberberg, F. S. and T. Schupbach (1993). “The Drosophila dorsoventral pattering gene gurken produces a dorsally localized RNA and encodes a TGF alpha-like protein.” Cell 75: 165-174.

Niwa, N., Y Inoue, A. Nozawa, M. Saito, Y. Misumi, H. Ohuchi, H. Yoshioka and S. Noji (2000). “Correlation of diversity of leg morphology in Gryllus bimaculatus (cricket) with divergence in dpp expression pattern during leg development.” Development 127 (29): 4373-81.

Ober, K. A. and E. L. Jokusch (2006). „The roles of wingless and decapentaplegic in axis and appendage development in the red flour beetle Tribolium castaneum.“ Dev Biol 294 (2): 391-405.

O’Neill, E. M., I. Rebay, R. Tjian and G.M. Rubin (1994). “The activities of two Ets-related transcription factors required for Drosophila eye development are modulated by the Ras/MAPK pathway.” Cell 78: 137-147.

Rauskolb, C., K. M. Smith, M. Pfeifer and E. Wieschaus (1995). “extradenticle determines segment identities throughout Drosophila development.“ Development 121: 3663-3673.

Regier, J. C., J. W. Shultz, A. Zwick, A. Hussey, B. Ball, R. Wetzer, J. W. Martin and C. W. Cunningham (2010). “Arthropod relationships revealed by phylogenomic analysis of nuclear protein-coding sequences.” Nature 463 (7284): 1079-83

Rieckhof, G. E., F. Casaeres, H. D. Ryoo, M. Abu-Shaar and R. S. Mann (1997). “Nuclear translocation of extradenticle requires homothorax, which encodes an

extradenticle-related homeodomain protein.” Cell 91: 171-183.

Panganiban, G., L. Nagy and S. B. Carroll (1994). “The role of the Distal-less gene in the development and the evolution of insect limbs.” Curr Biol 4 (8): 671-5.

Pechmann, M. (2011). “Comparative studiesof appendage and segment formation in arthropods.” Dissertation, Georg-August-University Göttingen.

Penton, A. and F. M. Hoffmann (1996). “Decapentaplegic restricts the domain of wingless during Drosophila limb pattering.” Nature 382: 162-165

Perrimon, N. (1994). “Signalling pathways initiated by receptor protein tyrosine kinases in Drosophila.”. Curr Opin Cell Biol 6: 260-266.

Perrimon, N. and L. A. Perkins (1997). “There must be 50 ways to rule the Signal: The case of the Drosophila EGF receptor.” Cell 89: 13-16.

Prpic, N. M., R. Jansen, B. Wigand, M. Klingler and W. G. Damen (2003). “Gene expression in spider appendages reveal reversal of exd/hth spatial specificity, altered gap genes dynamics, and suggests divergent distal morphogen signalling.“ Dev Biol 264 (1): 119-40.u8iu8

Prpic, N. M. and D. Tautz (2003). “The expression of the proximodistal axis pattering genes Distal-less and dachshund in the appendages of Glomeris marginata (Myriapoda: Diplopoda) suggests a special role of these genes in pattering head appendages.” Dev Biol 260 (1):97-112.

Prpic, N. M. (2004). “Vergleichende Studien zur Gliedmaßenentwicklung bei Arthropoden.” Dissertation, University of Cologne.

Prpic, N. M. and W. G. M. Damen (2008). “Arthropod appendages: a prime example for the evolution of morphological diversity and innovation.” Evolving Pathways: Key Themes in Evolutionary Development Biology” Cambridge University Press.pp. 381-398.

Reich, A. and B. Z. Shilo (2002). “Keren, a new ligand of the Drosophila epidermal growth factor receptor, undergoes two modes of cleavage.” EMBO J. 21: 4287-4296.

Rousso, T., J. Lynch, S. Yogev, S. Roth, E. D. Schejter and B. Z. Shilo (2010). “Generation of distinct signalling modes via diversification of the Egfr ligand-processing cassette.” Development 137: 3427-3437.

Rudledge, B. J., K. Zhang, E. Bier, Y. N. Jan and N. Perrimon (1992). “The Drosophila spitz gene encodes a putative EGF-like growth factor involved in dorsal-ventral axis formation and neurogenesis.” Genes Dev 6: 1503-1517.

Ruppert, E. E., R. S. Fox & R. D. Barnes (2004). *Invertebrate Zoology* (7th ed.). Brooks/Cole. ISBN 0030259827.

Sanchez-Salazar, J., M. T. Pletcher, R. L. Bennet, S. J. Brown, T. J. Dandamudi, R. E. Denell and J. S. Doctor (1996). “The Tribolium decapentaplegic gene is similar in sequence, structure and expression to the Drosophila dpp gene.” Dev Genes Evol 206: 237-246.

Sanicola, M., J. Sekelsky, S. Elson and W. M. Gelbart (1995). “Drawing a stripe in Drosophila imaginal discs: negative regulation of decapentaplegic and patched expression by engrailed.” Genetics 139: 745-756.

Schaeper, N. D., N. M. Prpic and E. A. Wimmer (2009). “A conserved function of the zinc finger transcription factor Sp8/9 in allometric appendage growth in the milkweed bug Oncopeltus fasciatus.” Dev Genes Evol 219: 427-435.

Schnepp, B., G. Grumbling, T. Donaldson and A. Simox (1996). “Vein is a novel component in the Drosophila epidermal growth factor receptor pathway with similarity to the neuregulins.” Genes Dev 10: 2302-2313.

Schnepp, B., T. Donaldson, G. Grumbling, S. Ostrowski, R. Schweitzer, B. Z.

Schweitzer and A. Simox (1998). “EGF domain swap converts a drosophila EGF receptor activator into an inhibitor.“ Genes Dev 12, 908-913.

Schoppmeier, M and W. G. Damen (2001). “Double-stranded RNA interference in the spider Cupiennius salei: the role of Distal-less is evolutionary conserved in arthropod appendage formation.” Dev Genes Evol 211 (2): 76-82.

Schweitzer, R., M. Shaharabany, R. Seger and B. Z. Shilo (1995). “Secreted Spitz triggers the DER signalling pathway and is a limiting component in the embryonic ventral ectoderm determination.” Genes Dev 9: 1518-1529.

Schweitzer, R. and B. Z. Shilo (1997). “A thousand and one roles fort he Drosophila EGF receptor.“ Trends Genet 13: 191-196.

Shilo, B. Z. and E. Raz (1991). “Developmental control by the Drosophila EGF receptor homolog DER.” Trends Genet 7: 388-392.

Shilo, B. Z. (2002). “signalling by the Drosophila epidermal growth factor receptor pathway during development.” Experimental Cell Research 284: 140-149.

Shilo, B. Z. (2005). “Regulation the dynamics of EGF receptor signalling in space and time.” Development 132: 4017-4027.

Snodgrass, R. E. (1935). “Principles of Insect Morphology.” McGraw-Hill Book Co., Inc, New York.

Svacha, P. (1992). “What are and what are not imaginal discs: reevaluation of some basic concepts (Insecta, Holometabola).” Dev. Biol. 154: 101-117.

Svendsen, P. C., A. Formaz-Preston, S. M. Leal and W. J. Brook (2009). “The Tbx20 homologs midline and H15 specify ventral fate in the Drosophila melanogaster leg.” Development 136 (16): 2689-93.

Theisen, H., T. E. Haerry, M. B. O'Connor and J- L. (1996). "Development territories created by mutual antagonism between Wingless and Decapentaplegic. "Development 122 (12): 3939-48.

Tribolium Genome Sequencing Consortium (2008)."The genome of the model beetle and pest Tribolium castaneum." Nature 452: 949-955.

Vachon, G., B. Cohen, C. Pfeifle, M E. McGuffin, J. Botas and S. M. Cohen (1992). "Homeotic genes of the Bithorax complex repress limb development in the abdomen of the Drosophila embryo through the target gene Distal-less." Cell 71 (3): 437-50.

van der Zee, M., O. Stockhammer, C. von Levetzow, R. Nunes da Fonseca and S. Roth (2006). „Sog/Chordin is required for ventral-to-dorsal Dpp/BMP transport and head formation in a short germ insect." Proc Natl Acad Sci USA 103 (44): 16307-12.

Wilder E. L. and N. Perrimon (1995). "Dual functions of wingless in the Drosophila leg imaginal disc." Development 121: 477-488.

Wimmer E. A., H. Jäckle, C. Pfeifle, S. M. Cohen (1993). "A Drosophila homologue of human SP1 is a head-specific segmentation gene." Nature 366: 690-94.

Wimmer, E. A., G. Frommer, B. A. Purnell, H. Jäckle (1996). "buttonhead and D-SP-1: a novel Drosophila gene pair." Mech Dev 59: 53-62.

Wu, J. and S. M. Cohen (1999). "Proximodistal axis formation in the Drosophila leg: subdivision into proximal and distal domains by Homothorax and Distal-less." Development 126 (1): 109-17.

Wu, J. and S. M. Cohen (2000). "Proximal distal axis formation in the Drosophila leg: distinct functions of teashirt and homothorax in the proximal leg." Mech Dev 94 (1-2): 47-56.

Zak, N. B., R. J. Wildes, E. D. Schejter, E. Raz and B. Z. Shilo (1990). "Localization

of the DER/flb protein in embryos: implications on the faint little ball lethal phenotype.” Development 109: 865-874.

6 Appendix

6.1 List of Abbreviations

cDNA	complementary DNA
DNA	deoxyribonucleic acid
dsRNA	double-stranded RNA
EGFP	enhanced green fluorescent protein
eRNAi	embryonic RNA interference
GEKU-Screen	pBac mediated insertional mutagenesis screen performed by laboratories in Göttingen, Erlangen, Kansas and the United States Department of Agriculture
Ko	Keilin's Organ
pRNAi	parental RNA interference
RNA	ribonucleic acid
RNAi	RNA interference
PCR	polymerase chain reaction
wt	wildtype

Species names

C. salei (Cs)	*Cupiennius salei*
D.melanogaster (Dm)	*Drosophila melanogaster*
G. bimaculatus (Gb)	*Gryllus bimaculatus*
T. castaneum (Tc)	*Tribolium castaneum*
O. fasciatus (Of)	*Oncopeltus fasciatus*

Gene names

btd	*buttonhead*
cll	*clawless*
dac	*dachshund*

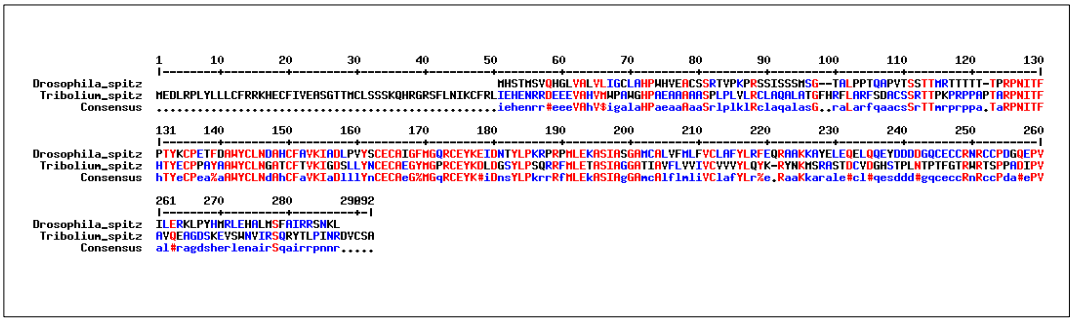
Dll	*Distal-less*
dpp	*decapentaplegic*
EGFR	*Epidermal growth factor receptor*
en	*engrailed*
esg	*escargot*
fz-1	*frizzled-1*
hh	*hedgehog*
hth	*homothorax*
omb	*optomotor-blind*
pnt	*pointed*
spi	*spitz*
tsh	*teashirt*
wg	*wingless*

6.2 Sequences

6.2.1 *Tribolium castaneum spitz*

For cloning *Tc'spitz* fragments specific primers were designed and gene fragments were isolated from cDNA by standard PCR. The cloned fragment was sequenced by Macrogen (Korea) by using standard T7 (TAATACGACTCACTATAGG) and Sp6 (GATTTAGGTGACACTATAGA) primers.
Fragment length: 469 bp
Primer forward: CTACTGCACGGCCTAACATC
Primer reverse: GGAACGTGATCAGGTCGC

CTACTGCACGGCCTAACATCACATTCCACACGTACGAATGTCCGCCGGCGTACGCGG
CCTGGTACTGCCTTAACGGAGCAACGTGCTTTACCGTCAAAATTGGCGATTCCCTCC
TATACAATTGCGAGTGTGCAGAAGGTTACATGGGTCCTAGATGTGAATATAAAGATT
TAGACGGTTCATATTTACCGTCACAACGAAGGTTCATGTTGGAAACGGCGAGTATAG
CCGGTGGGGCGACGATAGCCGTTTTTTTAGTGGTGATAGTGTGCGTCGTTGTGTATC
TGCAGTATAAACGCTATAATAAAATGAGTCGTGCGAGCACGGACTGCGTGGACGGAC
ACAGCACCCCTTTGAACACGCCGACCTTCGGCACCCGATGGCGGACGAGTCCGCCGG
CGGATATCCCGGTGGCGGTGCAAGAAGCGGGCGACTCGAAGGAGGTCAGTTGGAACG
TGATCAGGTCGCA



Alignment of *Drosophila* and *Tribolium spitz* protein sequences.
Performed with Multalin (http://multalin.toulouse.inra.fr/multalin/cgi-bin/multalin.pl).

6.2.2 *Tribolium castaneum Epidermal growth factor receptor*

For cloning *Tc'Egfr* fragments specific primers were designed and gene fragments were isolated from cDNA by standard PCR. The cloned fragments were sequenced by Macrogen (Korea) by using standard T7 (TAATACGACTCACTATAGG) and Sp6 (GATTTAGGTGACACTATAGA) primers.

6.2.2.1 Cloned sequence of *Tc'Egfr*

Fragment length: 976 bp
Primer forward: TGCATTGGAACAAACGGC
Primer reverse: GGGTTGTACTGTGATAGAAGGC
The following clone was used for embryonic RNAi experiments and in-situ-hybridisation.

TGCATTGGAACAAACGGCCGCATGTCCGTCCCCTCAAACCGCGAGCACCACTACCGC
AACCTGAAGGACCGCTACACCAACTGCACCTACGTGGACGGCAACCTGGAGCTGACC
TGGCTCCAGGACGAGAACCTGGACTTGAGCTTCCTCCAATACATCCGTGAAGTAACG
GGTTACGTCCTCATCTCGCACGTGGACATCAAGCGCATCGTCTTGCCCCGCCTCCAA
ATCATCCGCGGTCGCACCCTCTTCAAGATGAACGTCCGCAACGAGGAGTTCGCCCTC
TTGGTCATCCTCTCCAAGATGTACACCTTGGAGTTGCCCGCCCTCCGGGATGTCCTC
ATCGGAAACGTTGGAGTTTTCAACAATTACAACCTTTGCCACTTCAAGACCATCAAC
TGGAAGGAAATCATCACGGACCCCAAGAGCAAGTACGTCTTCGTCTACAACTTCACC
TCCCCCGAACGCGACTGTCCCCCATGTCACAAGAACTGTGAGAAAGGGTGTTGGGGG
GAAGGCGAAGAAAACTGCCAGAAGTTTTCCAAGGAGAACTGCAGTCCACAGTGTTAC
CAAGGGCGGTGTTTCGGGCCGAACCCACGGGAGTGCTGCCACTTGTTTTGTGCGGGA
GGTTGTACCGGACCTAAGCAAAGTGATTGTATCGCCTGTCGGAACTTTTACGACGAT
GGGGTTTGCACACAGGAGTGCCCCCCTATGAAAATTTACAGCCCCATTACGTACTCC
TGGCAGGATAACCCTAACGGGAAGTACGCTTACGGTGCCACGTGTGTGAAAAACTGC
CCTGAACATTTACTCAAAGACAATGGGGCTTGCGTCCGGTCCTGCCCCCCTGACAAG
AAGGCCCATGAAGGGGCGTGTGTCCCTTGCAACGGCCCGTGCCCCAAAACCTGCCGC
GTTGATACTTTCATCCATTCTGGGAACATTGACACCTTCAAGGGTTGTACTGTGATA
GAAGGCA

6.2.2.2 Cloned sequence of *Tc'Egfr_2*

Fragment length: 1096 bp

Primer forward: CATCTCGCACGTGGACATCAAG

Primer reverse: GAAGCGACTCAATGCCGGGAC

The following clone was used for embryonic RNAi in order to test for off-target effects.

CATCTCGCACGTGGACATCAAGCGCATCGTCTTGCCCCGCCTCCAAATCATCCGCGG
TCGCACCCTCTTCAAGATGAACGTCCGCAACGAGGAGTTCGCCCTCTTGGTCATCCT
CTCCAAGATGTACACCTTGGAGTTGCCCGCCCTCCGGGATGTCCTCATCGGAAACGT
TGGAGTTTTCAACAATTACAACCTTTGCCACTTCAAGACCATCAACTGGAAGGAAAT
CATCACGGACCCCAAGAGCAAGTACGTCTTCGTCTACAACTTCACCTCCCCCGAACG
CGACTGTCCCCCATGTCACAAGAACTGTGAGAAAGGGTGTTGGGGGGAAGGCGAAGA
AAACTGCCAGAAGTTTTCCAAGGAGAACTGCAGTCCACAGTGTTACCAAGGGCGGTG
TTTCGGGCCGAACCCACGGGAGTGCTGCCACTTGTTTTGTGCGGGAGGTTGTACCGG
ACCTAAGCAAAGTGATTGTATCGCCTGTCGGAACTTTTACGACGATGGGGTTTGCAC
ACAGGAGTGCCCCCCTATGAAAATTTACAGCCCCATTACGTACTCCTGGCAGGATAA
CCCTAACGGGAAGTACGCTTACGGTGCCACGTGTGTGAAAAACTGCCCTGAACATTT
ACTCAAAGACAATGGGGCTTGCGTCCGGTCCTGCCCCCCTGACAAGAAGGCCCATGA
AGGGGCGTGTGTCCCTTGCAACGGCCCGTGCCCCAAAACCTGCCGCGTTGATACTTT
CATCCATTCTGGGAACATTGACACCTTCAAGGGTTGTACTGTGATAGAAGGCAATAT
CCTGATTTTGCAAAACACCTTCGAGGGGTACCAACACTTCTACCCCAACTACACCTT
CGGGGCCAGGTACCCCCGGATGCACCCCGACCGCTTGGAGGTCTTTAGCACCCTGAA
GGAGGTCACGGGGCATATCAACGTTCAAGCGTACCATTCCGATTTTACCAATTTGTC
GTACTTTAGAAACTTGGAAGTGATCGGTGGTCGCTCACTTTCAGACTACTTCACTTC
GCTGTATATTGTTAAATCGTCGCTGAAATCGCTCGAGTTGCGGTCGTTGAAGCGACT
CAATGCCGGGACA

6.2.2.3 Cloned sequence of *Tc'Egfr_3*

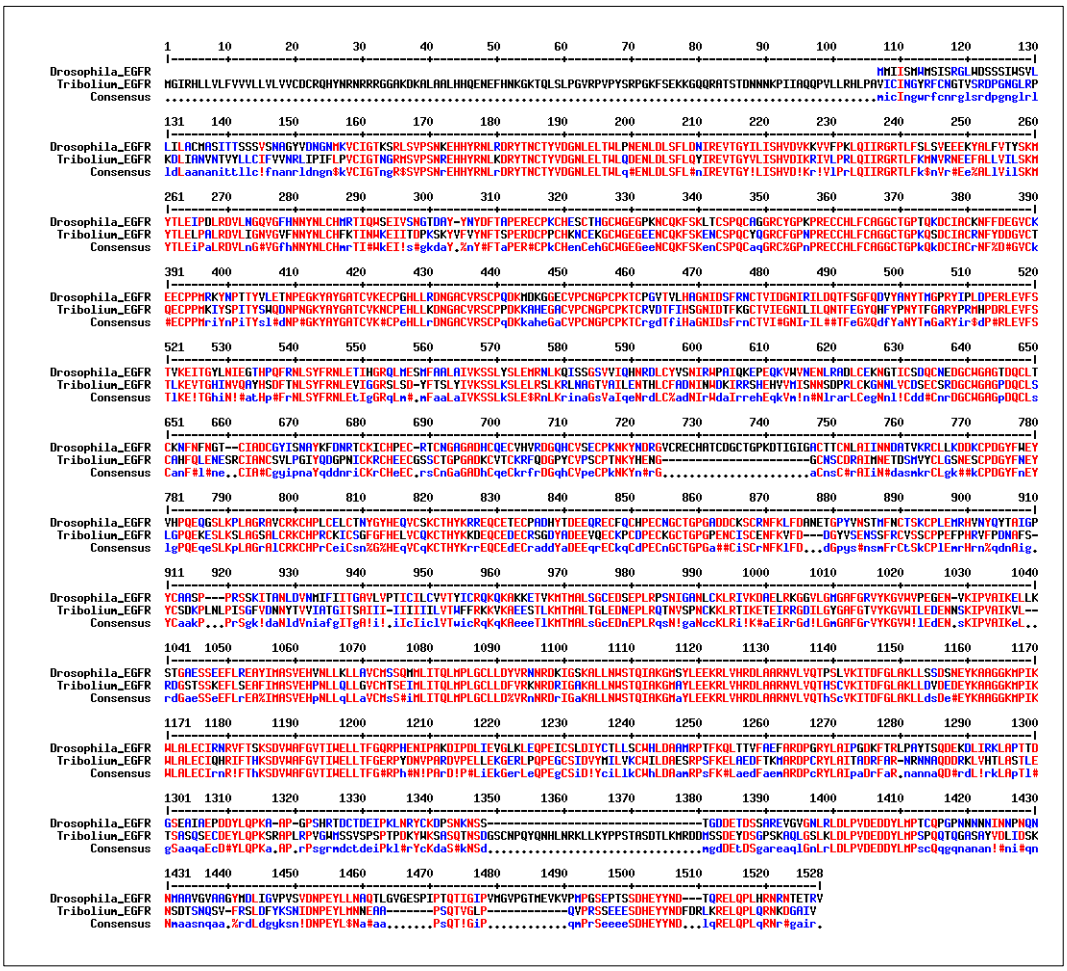
Fragment length: 847 bp

Primer forward: GTCGTGTGCCCACTTCCAGC

Primer reverse: CAATAACTACACTGTGGTGATCGC

The following clone was used for embryonic RNAi in order to test for off-target effects.

GTCGTGTGCCCACTTCCAGCTCGAGAATGAGAGTCGGTGTATTGCCAACTGCTCCGT
TTTGCCAGGGATTTACCAAGACGGTCCCAACATTTGCAAGCGCTGCCACGAAGAATG
CGGCTCTTCGTGCACAGGTCCAGGTGCCGACAAATGCGTCACTTGCAAGCGCTTCCA
GGATGGTCCCTACTGCGTACCATCCTGTCCCACTAACAAGTACCACGAAAACGGTCA
GTGCAAGAGCTGCCACGCCAATTGCGTCGGGGGATGCACCGGTCCCACCAACATTGT
TGGTTTAGGCGGCTGTAACTCGTGCGACCGTGCTATCATGAACGAAACCGACTCCAT
GGTCTATTGCTTGGGCAGCAACGAGTCCTGTCCTGACGGCTACTTCAACGAGTACTT
GGGGCCGCAGGAGAAGGAGTCGCTCAAATCGCTGGCGGGCTCAGCGCTGTGCCGGAA
ATGCCATCCAAGATGCAAGATCTGTTCCGGCTTCGGTTTCCACGAACTAGTCTGCCA
AAAATGCACCCACTACAAGAAAGACGAGCAATGCGAAGACGAATGTCGTTCCGGTGA
CTACGCAGACGAAGAAGTCCAAGAATGCAAACCTTGCGACCCTGAATGTAAAGGCTG
CACTGGTCCAGGTCCCGAAAACTGTATTTCCTGCGAAAATTTTAAAGTTTTCGACGA
CGGATACGTTTCCGAAAACTCTTCCTTCCGCTGCGTGTCCTCATGCCCACCTGAATT
CCCACACCGGGTTTTCCCCGACAATGCCTTCTCCTACTGTTCCGACAAACCCTTGAA
CTTGCCCATCTCTGGTTTCGTCGACAATAACTACACTGTGGTGATCGCA



Alignment of *Drosophila melanogaster* and *Tribolium Egfr* protein sequences. Performed with Multalin (http://multalin.toulouse.inra.fr/multalin/cgi-bin/multalin.pl).

6.2.3 *Tribolium castaneum pointed*

6.2.3.1 Cloned sequence of Tc'pointed

For cloning *Tc'pointed* fragments specific primers were designed and gene fragments were isolated from cDNA by standard PCR.. The cloned fragments were sequenced by Macrogen (Korea) by using standard T7 (TAATACGACTCACTATAGG) and Sp6 (GATTTAGGTGACACTATAGA) primers.

Fragment length: 926 bp

Primer forward: GACCGCTTTATTTGCATTG
Primer reverse: CGAAGATGAACTACGAGAAGC

This following clone was used for embryonic RNAi experiments and in-situ-hybridisation.

GACCGCTTTATTTGCATTGTTCTATTGTCGCAGTTGGCCGCGATTCAGCTAATTCGA
TTGTCCGTTGCAGGTGGGTGCGGTCAATTGAGGAGTCCGGCGTGTCCCCCCGCCGAC
GAGATCCGGGAGGAAGGCTCACCGCCGCCGCCGGGACCTCCCTCGGGGTTCCTGCAC
ATACAAAGAAGTCCTGCCTACCACCATCTCAAGGATGGTTTCAAGAGTTCGTGTTCG
CCGAACGAATCTGGCAATTCGGCGCCGGTATGGACAACCTGCAATCGATGAACAGCG
AAGATCAATCTCACCTCTTCCAAACGTCACATTATTCGCCCGAAGAGCACGAATACC
AAGCTCTGGAGGCAGGCCACCAACCCCAATACTTGGAAAGTTCGCCCGAATTCTACG
CTGCCAACAACCTAATCGAGTCCAAGTACCACCCGCACTCTTACGTCAAAAACTACG
CCAGAGGAACGGGGAGGTATACCGATGGTTACGGCGATACCTATGGTTCCCCCTACG
ACGGAACCCCTTTCCAGACCGTTCCCAGTGCTAACAATGGTGCTCCAGAGCAATGGG
CCCACAATCACGATCTCGGTTCCATAGCGCATGCGCACACGCATCCCGCCTTTTTAT
CAGCCGGGATGCAAGGGAGGGATCCCATCAATCCACTGGGACCAGACACCAAACCTA
TGCTCCAAAATGGGATGATCACGGGATATCCCAATAGCGGCGCGCCGGGAGGTCCCT
GCTTCACAGGTTCGGGTCCTATCCAGTTGTGGCAATTCCTGCTCGAACTCCTAACGG
ACAAATCCTGCCAAACGTTTATCTCCTGGACCGGCGACGGCTGGGAGTTTAAGCTAA
CAGACCCCGACGAGGTGGCCCGACGCTGGGGCATTCGCAAGAACAAGCCGAAGATGA
ACTACGAGAAGCA

6.2.3.1 Cloned sequence of *Tc'pointed*_2

Fragment length: 606 bp
Primer forward: GTAATCGAGAGTGAGAGTGCG
Primer reverse: CGATGTGCGTTCCAGACTTG
This following clone was used for embryonic RNAi in order to test for off-target effects.

GTAATCGAGAGTGAGAGTGCGCCAATGTTTTGGGCCGAGTGCGTACTTATGGTCATA
GGAATAAAAGGGATAAAGCGGAGTTGTTTTCCTGCCGCGCTATTCCGGGCGATGGAC

CAAAATAGACCAGTGACAGTGTTTTAGAGCAAAAATTTCGGAAAATCGCAATGTAGT
GGGTGCTGTGCTGTGCGATGCTTAATTTATTTACGTGTATATACCGACCCAGGTGCC
GCCACTGACGCCGGGCACGAACAAGACCATGGCCGAGGCGCTGAAAGCGTCTTTCGC
ATCGTGGGAAAAGGAACAAATACGATTAAACATAACCAAAGATCCTCGCCAATGGAC
GGAGAACCACGTGGCCCACTGGCTGCAATGGGCCGCGAAAGAGTTCTCCCTCGAATG
CATCCCTTTGCACCAGTTCCGGATGAAGGGCAAGGACATCTGCGCCATGGGCAAAGA
CGCGTTCGCCGCTCGCGCCCCAGCATTCGTCGGCGACATCCTCTGGGAGCACTTGGA
GTTGTTGCAGAAAGATGTCGAGAAGGAACGCTCATTGACGAACAATGTCGTCCAGGG
GAACATCTACAGCCCGATGTGCGTTCCAGACTTGA

6.2.3.3 Cloned sequence of *Tc'pointed_3*

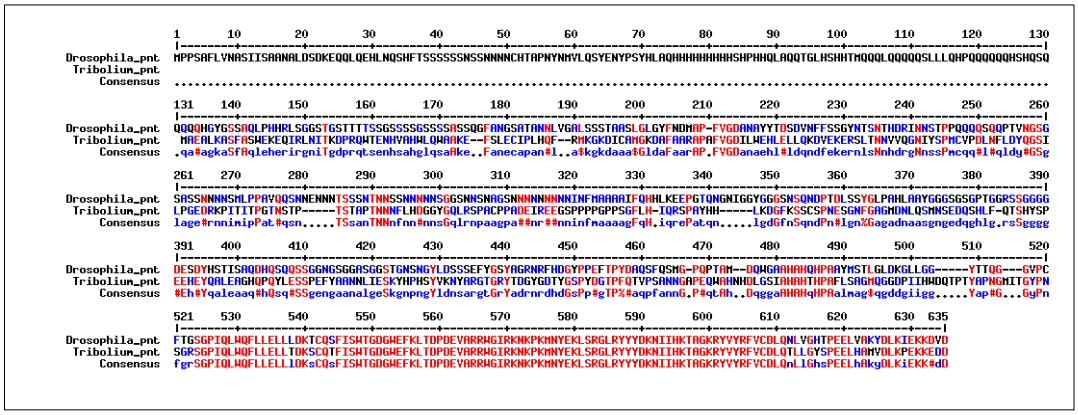
Fragment length: 722 bp

Primer forward: CGCCCGAAGAGCACGAATACC

Primer reverse: CACGCAATGGTAGATCTCAAGCC

This following clone was used for embryonic RNAi experiments in order to test for off-target effects.

CGCCCGAAGAGCACGAATACCAAGCTCTGGAGGCAGGCCACCAACCCCAATACTTGG
AAAGTTCGCCCGAATTCTACGCTGCCAACAACCTAATCGAGTCCAAGTACCACCCGC
ACTCCTACGTCAAAAACTACGCCAGAGGAACGGGGAGGTATACCGATGGTTACGGCG
ATACCTATGGTTCCCCCTACGACGGAACCCCTTTCCAGACCGTTCCCAGTGCTAACA
ATGGTGCTCCAGAGCAATGGGCCCACAATCACGATCTCGGTTCCATAGCGCATGCGC
ACACGCATCCCGCCTTTTTATCAGCCGGGATGCAGGGAGGGGATCCCATCATCCACT
GGGACCAGACACCAACCTATGCTCCAAATGGGATGATCACGGGATATCCCAATAGCG
GACGTTCGGGTCCTATCCAGTTGTGGCAATTCCTGCTCGAACTCCTAACGGACAAAT
CCTGCCAAACGTTTATCTCCTGGACCGGTGACGGCTGGGAGTTTAAACTAACAGACC
CCGACGAGGTGGCCCGACGCTGGGGCATTCGCAAGAACAAGCCGAAGATGAACTACG
AGAAGCTTTCGCGAGGCCTTCGGTACTACTACGATAAAAACATTATCCACAAGACTG
CCGGGAAGCGTTACGTCTATCGCTTCGTCTGCGACTTACAGACCTTGTTAGGGTATA
GTCCGGAGGAGCTGCACGCAATGGTAGATCTCAAGCCA



Alignment of *Drosophila* and *Tribolium pointed* protein sequences.
Performed with Multalin (http://multalin.toulouse.inra.fr/multalin/cgi-bin/multalin.pl).

6.2.4 *Tribolium castaneum decapentaplegic*

Cloning of *Tc'dpp* has been performed by Nico Posnien.

Fragment length: 1130

GTATCGCGATGCGTTTGAACATGCTGATATACCTCATAGTGGCATGTTGTTGGGGTA
AATCTCTCTCGATACCTCAACAAATACTCAATGAATTCAAATCGACACTGTTGCCCC
TTTTCGGACTCAAAGAACAGCCGAAAATCGAAGGCAAAGTGCAAGTACCAGAAGCTT
TGAAGAAGATCTACAACATTCAGAATAACTTCGAGTACGACACAGCATCCTTGCCCC
TTCCGGGACTCTACACCAAAAGTGCCAACACCATACGGAGCTTCACCCATGTGGAAA
GTCCAATCGATGAAAAGTTCGTGCACCCTCACCGCTTCCGCTTGAAATTCAATATTT
CATCAATTCCTCGACACGAAAAACTCACAGCGGCCGAAATAAAACTAACCCGTGAAA
CGGCCAAAAACGCATCACACCCCTTTCAACGTGTCTTAGTCCACGACATTCTCCAAC
CTGGTGTCAAGGGCCTCCACGGCCCCATCACCCGAGTGATTGACTCAAAAGTGGTCG
ACTCTCGAAAAAACACCACCGTGAGCATCGACGTCTTCCCGGCAGTGGCTCGCTGGA
TGCAAGACCCCAAAACCAACCACGGCATCCTCATTGTGGTTTACTCCATTGGGGCGA
AAAAAATCACCCCCAGAGAAACACCTAAGGCTGCGGCGCGACACAGCCCCCCCACAG
TGGTACCAACACCAACCCCTTCTCTTCACCTACACCGACGATGGTAAGAACCAGCAA
AGGACAGGGACGGAGCTGACGAAAATGCGGCCCAAACGACAGAGTTCGCGCAGGCAC
CGGAAAAATCTGAAAGACCCCTGCCGGAGACGGCAGATGTACGTCGATTTCGGTTCC
GTGGGGTGGAACGACTGGATCGTGGCCCCCTTGGGCTACGACGCGTATTATTGCGGG

GGCGAGTGCGAGTACCCCATTCCAGACCACATGAACACGACGAACCACGCCATAGTC
CAGAGCCTGGTCAACTCGATGAAGCCGAAGGAGGTGCCAGGGCCCTGCTGCGTGCCG
ACGCAGCTGGGGCAGATGTCCATGCTGTATCTGGGCAGCGACGGCAGCGTCATCCTA
AAAAACTACAAGGAGATGGTGGTGGTGGGGTGCGGCTGCCGATAGTA

6.2.5 *Tribolium castaneum wingless*

Cloning of *Tc'wg* has been performed by Johannes Schinko.
Fragment length: 1082 bp

GAATTCGGCTATCCACCTTTCGTCGGCCCATGGCACCCTGCGACGGAAGCAGCGTCG
CCTGGCTCGGGAAAATCCCGGCTTGTTGGTGGCTCTACATAAAGGTGCCAATAATGC
GATTCACGAGTGCCAGCATCAGTTTCGCAATCAGCGCTGGAACTGTTCGACGAGAGC
CTTCCCTCGAGGAAAGAACTTGTTCGGAAAAATAGTCGATAAAGGATGCAGGGAAAC
TGCCTTCATTTATGCGATAACGAGTGCTGCGGTGACCCACGCGATCGCCAGAGCTTG
CAGCGAAGGTTCGATAGATACGTGCAACTGCGAAACGCACTACAAAGGAAGGCCGCA
TGTTTCTGGGAACGGCGGCGGGGCCGCCGTTGCCGGCGTCAGAGATTTCGAATGGGG
CGGCTGTTCGGATAATATTGGGTTCGGATTCACGGTCAGTCGGGAGTTTGTCGACGC
CGGCGAAAGGGGCAAAACCATTCGAGAAAAGATGAATTTGCACAACAACGAGGCCGG
AAGATGGCACGTAAAGGATCAGATGCGTCAAGAATGTAAATGCCACGGAATGTCCGG
TTCTTGCACTATTAAGACTTGTTGGATGCGACTTCCTCCGTTTCGAGTGATCGGCGA
CCTCCTCAAAGACCGCTTCGACGGCGCTTCTCATGTCGCCGCCTCCGGCCACCACCG
AAACAACAACAACGCGCACCAGAACCGTCCACCGAAGAACCCCAAGCTGAACGCGAT
CTCCAGTAACAGCATCCACAGTAAGAGGGAGAATCGCCGCAAGCACAAGTACGGCTT
CCAGTTGAAACCGTTCAACCCCGAGCACAAACCCCCCGGAACCAAGGATCTGGTCTA
CTACGAGATGTCGCCCGGCTTCTGCGAGAAGAACCCGAAGCTGGGGATTCAAGGGAC
GCATGGGCGGCTGTGCAACGACACCTCGATGGGTGTCGATGGGTGCGATATCATGTG
CTGCGGACGGGGGTATCGTACCCAGGAAGTCGTCGTATTCGAGAGATGCAACTGTAC
GTTTCATTGGTGCTGTGAAGTGAAGTGCGATGTGTGTGGATCCAAGCCGAATTCA

7 Acknowledgment/ Danksagung

Mein ganz besonderer Dank gilt Herrn Dr. Nikola-Michael Prpic-Schäper für die hervorragende und engagierte Betreuung meiner Arbeit. Ich danke ihm nicht nur für das entgegengebrachte Vertrauen selbständig diese Arbeit in seinem Labor anfertigen zu dürfen, sondern auch für seine Unterstützung, Hilfsbereitschaft und seine Motivation während der gesamten Dauer der Anfertigung dieser Arbeit.
Ebenso danke ich Jun. Prof. Dr. Gregor Bucher und Prof. Dr. Ernst A. Wimmer für kritische Diskussionen und Anmerkungen zu meiner Arbeit während der Labor-Seminare und für jegliche Unterstützung, wann immer es nötig war.

Meinen Kollegen der gesamten Abteilung danke ich für die äußerst schöne Arbeitsatmosphäre und stete Hilfsbereitschaft und Unterstützung. Mein besonderer Dank gilt allen jetzigen und ehemaligen Mitglieder des Labor 3a/b für die besonders schöne gemeinsame Zeit, die tolle Zusammenarbeit und die vielen interessanten wissenschaftlichen und vor allem „nicht-wissenschaftlichen“ Gespräche. Im Speziellen möchte ich mich bei Matthias, Beni, Nico, Monique und meinen früheren Kollegen Johannes und Anna bedanken, die mir alle immer mit großer Anteilnahme und unterstützend zur Seite standen.

Ganz besonders möchte ich meinen Eltern und meinen Bruder Stefan danken, die mir dieses Studium erst ermöglicht haben und mir während der langen Zeit immer unterstützend und ermutigend zur Seite gestanden haben.

Ich möchte hier auch meiner Freundin Anne danken, die mich vom ersten Tag meines Studiums bis heute durch alle Höhen und Tiefen begleitet hat.

Zuletzt möchte ich ganz besonders meinem Freund Michael danken. Danke, dass du diese manchmal sehr anstrengende Zeit geduldig mit mir durchgestanden hast, immer an mich geglaubt hat und mich in schweren Zeiten immer wieder zum Lachen gebracht hat.