Exploring morphological innovation and diversification: **Analysis of genes involved in gin-trap formation and antenna remodeling during metamorphosis in *Tribolium castaneum***

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Declaration

I hereby declare that this thesis has been written solely by myself and that it has not been submitted, in whole or in part, in any previous application for a degree. Except where stated otherwise by reference or acknowledgment, the work presented is entirely my own.

______________ Göttingen, 09.07.2017

Yonggang Hu

Dedication

This dissertation is dedicated to my families who give me understanding, encouragement and constant support.

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Table of Contents

1 General Summary 1

2. Chapter 1 3

A morphological novelty evolved by co-option of a pruned wing GRN and gene recruitment but without orphan genes in the red flour beetle 3

Author Contribution 3

Abstract 3

2.1 Introduction 5

2.2 Materials and Methods 9

2.2.1 Animals 9

2.2.2 Inverse PCR 9

2.2.3 Cloning and sequencing of *Tribolium* genes 9

2.2.4 dsRNA Synthesis 9

2.2.5 *Tribolium* injection 10

2.2.6 Image processing and documentation 10

2.3 Results 11

2.3.1 Characterization of the gin-traps and one gin-trap marking enhancer trap line in *Tribolium* 11

2.3.2 The elytra and gin-trap primordia originate from conserved developmental field 13

2.3.3 Gin-traps recruited only parts of the wing gene regulatory network 15

2.3.4 *Hox* genes regulate gin-trap formation 20

2.3.5 An unbiased RNAi screen reveals novel gin-trap and wing patterning genes 21

2.4 Discussion 25

2.4.1 A highly pruned wing GRN is re-deployed in gin-trap formation 25

2.4.2 GRN pruning relates to morphological differences 27

2.4.3 Recruitment and gain of function required for the evolution of a morphological innovation 28

2.4.4 Gin-trap evolution and serial homology 29

3. Chapter 2 31

Identification and expressional characterization of novel genes in patterning the adult antenna formation in *Tribolium castaneum* 31

Abstract 31

3.1 Introduction 33

3.1.1 Antennal structure and function 33

3.1.2 The development of antennae in *D. melanogaster* 35

3.1.3 Conserved and divergent aspects of antennal development across arthropods 38

3.1.4 *Tribolium castaneum* is emerging as a representative model for antennal patterning during matemorphosis 39

3.2 Materials and Methods 43

3.2.1 Animals 43

3.2.2 Selection of candidate phenotypes 43

3.2.3 Orthology and phylogenetic analysis 43

3.2.4 dsRNA Synthesis 44

3.2.5 *Tribolium* injection 44

3.2.6 Cloning and sequencing of *Tribolium* genes 44

3.2.7 RNA probe preparation 44

3.2.8 Antenna WMISH 44

3.2.9 Image processing and documentation 46

3.3 Results 47

3.3.1 Selection of phenotypes for investigation 47

3.3.2 Phylogeny of *Tc010410*, a gene without *Drosophila* ortholog 48

3.3.3 Phenotype confirmation by injection of non-overlapping dsRNA fragments 49

3.3.4 Three genes mainly promote the growth and elongation of the antenna along the entire PD axis 50

3.3.5 Two genes function specifically within the funicle region 53

3.3.6 Genes that affect the entire flagellum 54

3.3.7 Genes required primarily in the tarsus 54

3.3.8 Overview on antenna metamorphosis 56

3.3.9 Expression pattern of the novel genes in remodeling antenna 58

3.4 Discussion 61

3.4.1 Novel genes specified the antenna metamorphosis 61

3.4.2 Taxonomically restricted gene for the morphological trait of antenna. 62

3.4.3 Relatedness of antenna elongation and joint formation. 62

3.4.4 The evolution of serially homologous appendages 63

3.4.5 Outlook 65

4. References 67

5. Appendix 81

1 General Summary

How morphological traits originate and diversify is a central question in evolutionary biology. Insects are the most diverse group of animals on the planet and over 80% of insect species belong to the subgroup of holometabola. The shape of a holometabolous insect experiences a striking change during metamorphosis, which allowed the evolution of an overwhelming morphological diversity. Hence, this process provides excellent samples to study the evolution of morphological innovation and diversity. Among insects, the developmental and genetic mechanisms of epidermal patterning are well understood in the model organism, *Drosophila melanogaster*. However, this highly derived Dipteran species does not show a typical metamorphosis. *Drosophila* replaces all larval epidermal cells by imaginal cells to form the adult epidermis. Instead, most holometabolous insects re-use larval cells to generate the adult epidermis, In contrast to *Drosophila*, the red flour beetle, *Tribolium castaneum*, shows a more typical mode of metamorphosis. Importantly, unbiased large scale RNA interference screening (iBeetle-screen) in *Tribolium* allows identifying and investigating gene sets involved in the process of morphological innovation and diversification independently from *Drosophila* knowledge.

In the first part of this thesis, the gin-trap was used as a study case to explore how a morphologically novel structure evolved during metamorphosis in *Tribolium*. Firstly, the wing genes known from *Drosophila* were investigated for their potential functions in gin-trap formation. The results showed that a large part of the upstream genes but much few downstream genes of the wing gene network were co-opted into gin-trap formation. Secondly, novel genes required for gin-trap development were searched in the iBeetle database. Ten genes were confirmed for their functions in gin-trap formation, most of which were required for wing formation as well. The only gin-trap specific gene, *Tc-caspar*, which was recruited from another biological context, was required for establishment of the anterior-posterior symmetry of the gin-traps. This is an innovation to this structure. Taken together, these data

suggested that gin-traps evolved by co-option of a pruned wing gene regulatory network and a low level of gene recruitment from a distinct biological context.

In the second part, novel genes from iBeetle screen were identified and analyzed on antenna metamorphosis in *Tribolium*. Of the ten confirmed genes, half belonged to the new classifications which were not reported to be associated with antenna patterning in *Drosophila*. Interestingly, four genes were related to pre-mRNA splicing, indicating the potential role of this process for antenna remodeling. One taxonomically restricted gene was found to affect a specific region of the antenna. And then, I optimized a protocol for whole mount *in situ* hybridization of pre-pupal antennae and the expression patterns of novel genes showed that the expression patterns were consistent with a role of these genes in antenna remodeling. Finally, I compared the gene sets between antenna and leg development and verified a complex mix of divergence and constraint among these serially homologous appendages.

The data obtained in this thesis provide new insight into the morphological innovation and diversification during metamorphosis and are the basis for future studies.

2. Chapter 1

A morphological novelty evolved by co-option of a pruned wing GRN and gene recruitment but without orphan genes in the red flour beetle

Author Contribution

The results of this chapter were submitted to peer-reviewed journal. Christian Schmitt-Engel, Jonas Schwirz, Nadi Stroehlein, Tobias Richter and Upalparna Majumdar identified the gin-trap phenotypes in the larval screen. Besides this, all the experiments referred to this chapter were done exclusively by myself.

Abstract

The evolution of morphological novelties has been a driving force for adaptation and consequently for the geographic spreading of insects. Co-option of existing gene regulatory networks (GRNs), recruitment of additional genes and the evolution of orphan genes were suggested to contribute to the development of morphological novelties. However, the relative importance of these processes has remained enigmatic because the prevailing candidate gene approach is biased towards a conserved gene function. Here, I combine the classical candidate gene approach based on *Drosophila* knowledge with unbiased screening, which has recently become available in the red flour beetle *Tribolium castaneum*. I determine the genes and pathways involved in the development of the gin-traps, which are defensive structures found on pupae of some beetle taxa. I find that 70% of the components of the gin-trap GRN were required for wing formation as well, confirming the co-option of the wing GRN. However, significant pruning of downstream components of the network correlated with the non-wing like structure of the gin-traps. Unexpectedly, even some upstream components like *engrailed* and Dpp signaling were pruned

from the wing GRN. Only one gene was recruited from another biological context but it played a crucial role in establishing anterior-posterior symmetry of the gin-traps, which is an innovation of this structure. Unexpectedly, I did not uncover any orphan genes with a function in gin-trap formation. With this work, I provide a first quantification of the contribution of different processes to the evolution of morphological novelties.

2.1 Introduction

Insects are the most species-rich animal taxon on the planet, representing more than half of all living animals (Grimaldi and Engel, 2005). More than 80% of extant insect species belong to the holometabola, which show an overwhelming morphological diversity (Grimaldi and Engel, 2005). This suggests that metamorphosis was a key innovation in promoting insect diversity (Rainford et al., 2014). Holometabolous insects produce distinct larval and adult morphologies that allow the stages to explore different food sources and to adapt to different ecological habitats. Hence, to understand the success of holometabolous insects it is crucial to investigate how their morphology evolved during metamorphosis. During that process, a large part of larval cells is integrated into the adult animal while another part undergoes apoptosis. In addition, imaginal cells proliferate and differentiate to form parts of the adult structures. The relative contribution of larval versus imaginal cells varies from species to species and from organ to organ within one species (Snodgrass, 1954). The epidermis of *Drosophila melanogaster* and other dipterans represents an extreme case where all larval epidermal cells are replaced by imaginal cells (Fristrom and Fristrom, 1993; Snodgrass, 1954). Coleopterans are more typical for insects in that larval cells contribute significantly to the adult epidermis (Snodgrass, 1954; Truman and Riddiford, 2002).

Innovation and diversification during metamorphosis have been studied in a number of insect taxa. For instance, the genetic basis of wing pigmentation has been scrutinized in butterflies and flies (Arnoult et al., 2013; Gompel et al., 2005; Keys et al., 1999; Reed et al., 2011) and morphological evolution was investigated with respect to the fore- and hindwings in beetles (Tomoyasu et al., 2005, 2009), beetle horns (Moczek and Rose, 2009; Wasik et al., 2010) and genital lobes in Drosophilids (Glassford et al., 2015). However, studies in Drosophilids reveal mechanisms acting during the rather derived mode of metamorphosis based on imaginal discs. Most studies in other insects, on the other hand, have been based on a candidate gene approach because efficient large scale screening tools are lacking in those

non-model species. Hence, this approach leads to a bias towards the identification of conserved gene functions while the contribution of unexpected or even taxonomically restricted genes (orphan genes) (Khalturin et al., 2009) may go unnoticed.

The red flour beetle *Tribolium castaneum* is an excellent model system for morphological evolution during metamorphosis. First, beetles show an insect typical mode of metamorphosis based on large contribution of larval cells to the adult epidermis (Snodgrass, 1954). Second, RNA interference (RNAi) is very efficient and systemic (Brown et al., 1999; Bucher et al., 2002; Tomoyasu and Denell, 2004) and a number of transgenic and genome editing tools have become available (Berghammer et al., 1999; Gilles et al., 2015; Lorenzen et al., 2007; Schinko et al., 2010; Trauner et al., 2009). Importantly, in the ongoing genome wide iBeetle screen, randomly selected genes have been studied for both, embryonic and metamorphic phenotypes, which are documented in the iBeetle-Base (Dönitz et al., 2015; Schmitt-Engel et al., 2015). Hence, *Tribolium castaneum* has become a model system where unbiased large scale phenotypic screening is feasible. Due to the systemic nature of RNAi, genes acting during metamorphosis can be tested by injection of dsRNA into late larval stages avoiding potential lethality due to earlier functions (Schmitt-Engel et al., 2015; Tomoyasu and Denell, 2004).

In this work I focused on a structure called gin-trap, which is an epidermal outgrowth consisting of an anterior and a posterior part armoured with a denticular sclerotization, respectively (Hinton, 1946; Wilson, 1971). Gin-traps are located at the dorso-lateral side of the first-to-seventh abdominal segments in *Tribolium* (Fig. 2.1). Interestingly, they are found exclusively on pupae of coleoptera of the closely related families Tenebrionidae and Colydiidae. Functionally equivalent but morphologically different gin-traps (dorsal gin-trap) are found on the dorsal part of abdominal segments of Coleoptera and Lepidoptera. Gin-traps are defensive organs, which grasp the appendages of predators in response to mechanical stimulation of the otherwise helpless pupa (Eisner and Eisner, 1992; Hinton, 1946). The fact that lateral gin-traps are found only on a holometabola specific life stage (the pupa) of very few taxonomically related taxa strongly suggest that gin-traps evolved after the

radiation of holometabolous insects in the lineages leading to these coleopteran and lepidopteran taxa. Hence, they represent an excellent study case for the evolution of a morphological novelty. Based on the expression and function of the wing selector gene *vestigial* in gin-trap development and a homeotic transformation from gin-trap to wing like structures after *Hox* gene RNAi in the tenebrionid beetle *Tenebrio molitor*, it has been suggested that gin-traps are wing serial homologs (Ohde et al., 2013). However, it has remained unclear, how much of the wing gene regulatory network (GRN) was co-opted, how many novel genes and how many orphan genes were recruited during the evolution of this morphological novelty.

To tackle these questions, I first systematically tested orthologs of the *Drosophila* wing GRN for a role in gin-trap formation in *Tribolium* in a classical candidate gene approach. Then, I identified novel genes involved in gin-trap and wing formation in an unbiased way by mining the results of the *iBeetle* screen. My data reveal that gin-trap development is based on the co-option of about 70% of the wing GRN components. Network pruning profoundly changed the GRN where mainly downstream genes were removed. Unexpectedly, central upstream components of the wing GRN like *engrailed* and Dpp signalling were lost as well. I estimate that co-option accounts for about 10% of the gin-trap GRN while, surprisingly, I did not find the involvement of orphan genes.

2.2 Materials and Methods

2.2.1 Animals

Wild-type *San Bernadino* strain (*SB*) and enhancer trap lines (*pu11* and *GöGal41152*) were used and reared on whole-wheat flour supplemented with 5 % yeast powder at 32 °C for all experiments.

2.2.2 Inverse PCR

Genomic DNA was isolated from four adults by standard phenol–chloroform extraction and then separately digested by the restriction enzymes Bsp143 I and Hha I. After self-ligation at room temperature for 1 hour, inverse PCR was performed with the primer sets (see table A1 for primer sequences in appendix) and the amplified fragment was sequenced (LGC Genomics).

2.2.3 Cloning and sequencing of *Tribolium* genes

Homologs of *Ultrabithorax* (*Ubx*), *bursicon* (*burs*) and *partner of bursicon* (*pburs*) were isolated from pupal cDNAs of *Tribolium* by PCR (see table A1 for primer sequences), cloned into pJET1.2/blunt vector and their sequence was confirmed by sequencing (LGC Genomics).

2.2.4 dsRNA Synthesis

dsRNA was produced from cloned genes for: *Tc-spitz* (*Tc-spi*), *Tc-EGFR*, *Tc-serrate* (*Tc-ser*), *Tc-Delta* (*Tc-Dl*), *Tc-wingless* (*Tc-wg*), *Tc-engrailed* (*Tc-en*), *hedgehog* (*hh*), *Tc-cubitus interruptus* (*TC-ci*), *Tc-Decapentaplegic* (*Tc-Dpp*), *Tc-optomotor blind* (*Tc-omb*), *Tc-iroquois* (*Tc-iro*) and *Tc-achaete scute homolog* (*Tc-ASH*). Vectors with the following cDNAs were kindly provided by Yoshinori Tomoyasu: *nubbin* (*nub*), *Tc-serum response factor* (*Tc-srf*), *Tc-daughters against dpp* (*Tc-dad*), *Tc-apterous A* (*Tc-apA*), *Tc-apterous B* (*Tc-apB*), *Tc-disheveled* (*Tc-dsh*) and *Tc-spalt* (*Tc-sal*) (Clark-Hachtel et al., 2013; Tomoyasu et al., 2005, 2009). Templates were generated by PCR adding terminal T7 promoter sequences (see table A1 for sequences) and

dsRNA was synthesized by *in vitro* transcription (Megascript T7; Ambion). The dsRNA products were denatured in a 94 °C heating block for 5 min and then reannealed by slowly cooling down to room temperature. Specificity of the products was confirmed via agarose gel electrophoresis. dsRNAs targeting iBeetle candidate genes were ordered from Eupheria Biotech GmbH (Dresden) (see Table A2 for iBeetle numbers in appendix).

2.2.5 *Tribolium* injection

Injection were performed in penultimate or last larval stage (L6 or L7). dsRNAs were titrated from 100 ng/µL to 1 µg/µL according to different genes and approximately 0.5-0.7 µL of dsRNA solution was injected into least 10 larvae for each set of injections similar to the procedure in the iBeetle screen (Schmitt-Engel et al., 2015). After injection, the larvae were kept on flour at 32°C until pupation. For novel genes identified from iBeetle database, non-overlapping fragments (1 µg/µL) were injected to control for off-target effects (see Table A3 in appendix).

2.2.6 Image processing and documentation

Images were captured by using Zeiss Axioplan 2 microscope (dorsal and ventral of pupa and close-up of gin-trap), Leica M205 FA microscope (enhancer trap lines) and Zeiss LSM 510 laser scanning microscope (enhancer trap lines). Adjustments for brightness and contrast were done with Adobe Photoshop and figures were assembled in Adobe Illustrator.

2.3 Results

2.3.1 Characterization of the gin-traps and one gin-trap marking enhancer trap line in *Tribolium*

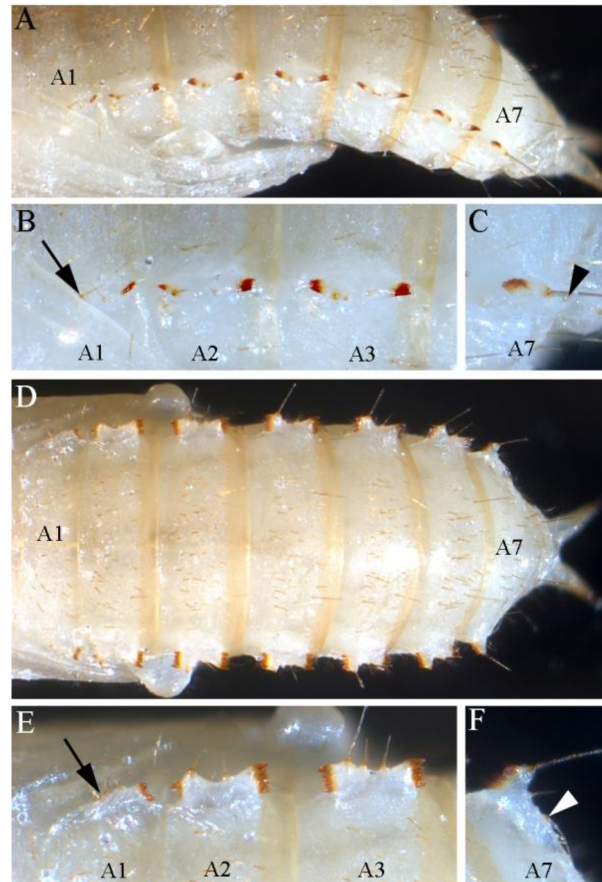

Figure 2.1 Morphology of the gin-traps in wild-type *Tribolium*. (A-C) Lateral view of gin-traps on abdominal segments (A) and close up of A1-A3 (B) and A7 (C), respectively. (D-F) Dorsal view of the gin-traps on abdominal segments and corresponding close up views. The anterior dentation on A1 (B and E, arrow) and posterior dentation on A7 (C and F, arrowhead) are absent.

In *Tribolium* gin-traps are located at the dorso-lateral side of the first-to-seventh abdominal segments (A1-A7) (Fig. 2.1). In A2-A6 the gin-traps show a mirror image anterior-posterior (AP) symmetry, but this symmetry is lost in A1 and A7 where the size of anterior or posterior gin-trap is drastically decreased, respectively (Fig. 2.1). Gin-traps are found exclusively at the pupal stage. In order to visualize the developing gin-traps prior to their emergence in the pupa I searched for enhancer

trap lines generated in a Gal4 enhancer trap screen (Bucher lab, unpublished). I characterized one line *GöGal41152*, which marked cells within the gin-traps but not its epidermis (Fig. 2.2). The first gin-trap marking signal was detected immediately after cessation of feeding at late 7th larval instar (L7) (Fig. 2.2B). Then the signal became stronger in the pre-pupal stage (Fig. 2.2C). The strongest gin-trap marking signal was detected within 2 days after molting to the pupa (Fig. 2.2D and E). Afterwards the signal declined gradually (Fig. 2.2G and H). No detectable signal could be found in the adult. It is unclear whether the cells that produce the gin-traps contribute to adult morphology. Inverse PCR identified the insertion site ~18.4 kb upstream of the *Tc033998* coding region (Fig. 2.2A). dsRNAs targeting *Tc033998* were injected to test whether its knockdown would affect gin-trap development. No morphological phenotypes were found in the pupa or the adult, which imply that this gene is not required for gin-trap development.

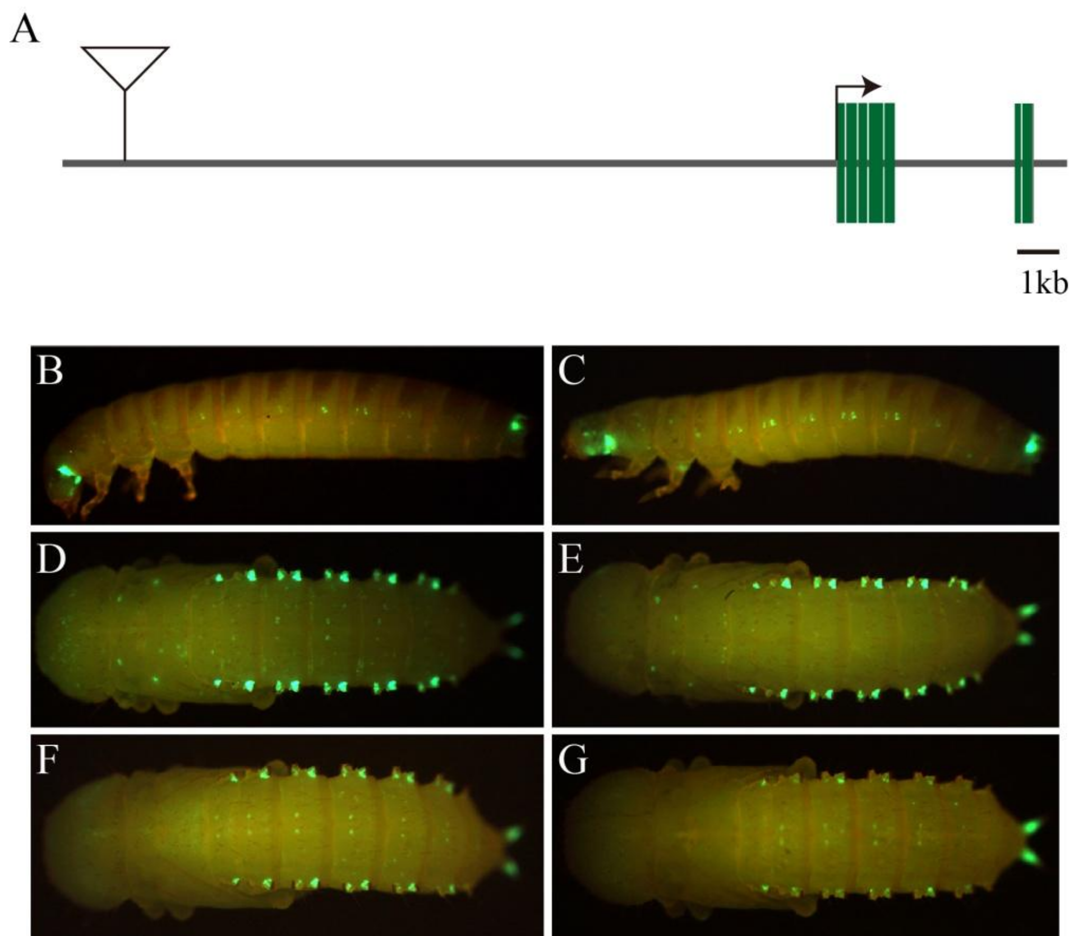

Figure 2.2 Characterization of the Gal4 enhancer trap line *GöGal41152*. (A) Insertion site of the construct upstream of gene *Tc033998*. dsRNA targeting Tc033998 did not elicit morphological

defects in the gin-traps. (B-F) Signal at different developmental stages. (B) First signal is detected immediately after cessation of feeding at late L7. (C) Pre-pupa (D) newly hatched pupa (E) 1-2 day old pupa (F) 3 day old pupa. (G) 4 day old pupa.

2.3.2 The elytra and gin-trap primordia originate from conserved developmental field

I asked whether wings and gin-traps develop at the same location in the respective segments by comparing the signal of *GöGal41152* with the enhancer trap line *pu11*, which marks wing anlagen (Tomoyasu et al., 2005) (Fig. 2.3A and B). Trachea, setae, sclerotized tergal margin were used as segmental cuticular landmarks to compare the location of gin-trap and wing primordia. The wing primordium was larger than the signal marking the gin-traps and it extended more to the ventral side relative to the cuticular landmarks (compare Fig. 2.3D-F with G-I). However, the gin-trap primordium is bent to the dorsal side within the pre-pupa while the wing primordium grows towards the ventral side (Fig. 2.3J). Taking this into account, it appears that both primordia originate from very similar regions in the respective segments (yellow line in Fig. 2.3), which is in line with the previous suggestion of serial homology of wings and gin-traps (Ohde et al., 2013).

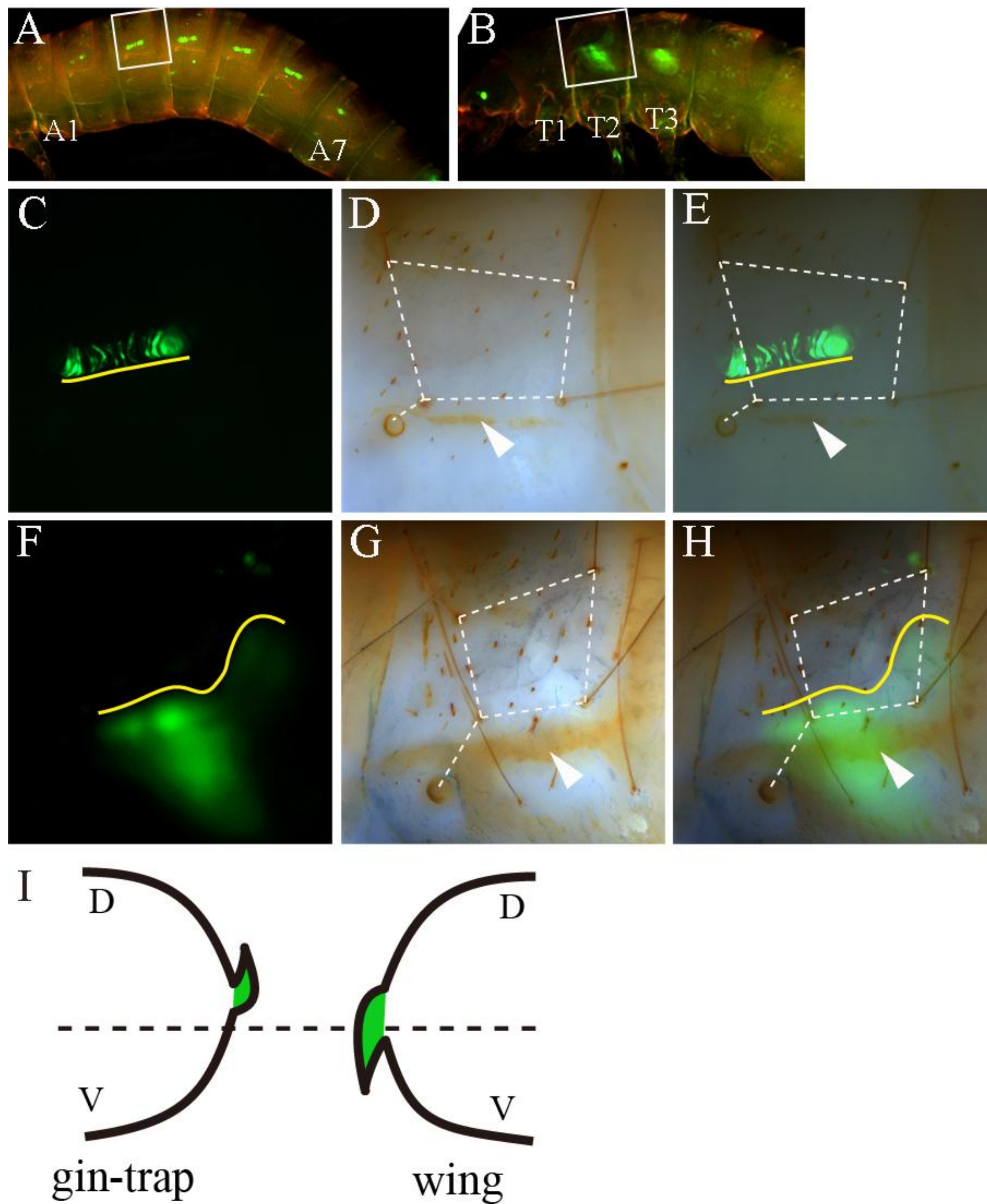

Figure 2.3 Morphology and segmental origin of gin-traps in *Tribolium*. (A) The transgenic line *GöGal41152* marks cells within the gin-traps from pre-pupal stages onwards (see white box). (B) The pu11 line marks wing primordia. (C-E) Location of the gin-trap primordium (C) relative to cuticular markers (D). (F-H) Location of the wing primordium relative to the same cuticular markers. Trachea, seta (D, E, G and H, dashed line) and sclerotized tergal margin (D, E, G and H, arrowhead) were used as landmarks to measure the relative position of gin-trap and wing. The yellow line indicates the proximal part of gin-trap and wing (C, E, F and H). Given the different projections of these outgrowths (I), the point of emergence is quite similar (see yellow lines). Dashed line (I) indicated position of tergal margin.

2.3.3 Gin-traps recruited only parts of the wing gene regulatory network

In order to test to what degree the development of gin-traps is based on the wing GRN, known homologs of wing patterning genes were knocked down in both wild-type and the *GöGal41152* line. I included genes known from *Drosophila* wing development, for which either a wing RNAi phenotype or wing specific expression had been described in *Tribolium* (Clark-Hachtel et al., 2013; Tomoyasu et al., 2005, 2009). In *Drosophila*, the wing imaginal disc is subdivided into anteroposterior (AP) and dorsoventral (DV) compartments (Morata, 2001 for review). The posterior *engrailed* (*en*) and the dorsal *apterous* (*ap*) selector genes activate the short-range signaling proteins Hedgehog (Hh) and Serrate (Ser), which in turn activate the expression of Decapentaplegic (Dpp) and Wingless (Wg), which are produced by the cells along the AP and DV compartment boundaries, respectively. Growth and patterning of the wing disc are then organized by long-range signaling by Dpp and Wg via the concentration-dependent regulation of downstream target genes. Previous studies showed that the wing gene network is largely conserved between *Tribolium* and *Drosophila* except for the divergent expression patterns of *dpp* and its target genes *optomotor-blind* (*omb*), *spalt* (*sal*) and *daughters against dpp* (*dad*), which are expressed at the distal tip of the AP boundary in *Tribolium* rather than along the entire AP border (Tomoyasu et al., 2005, 2009).

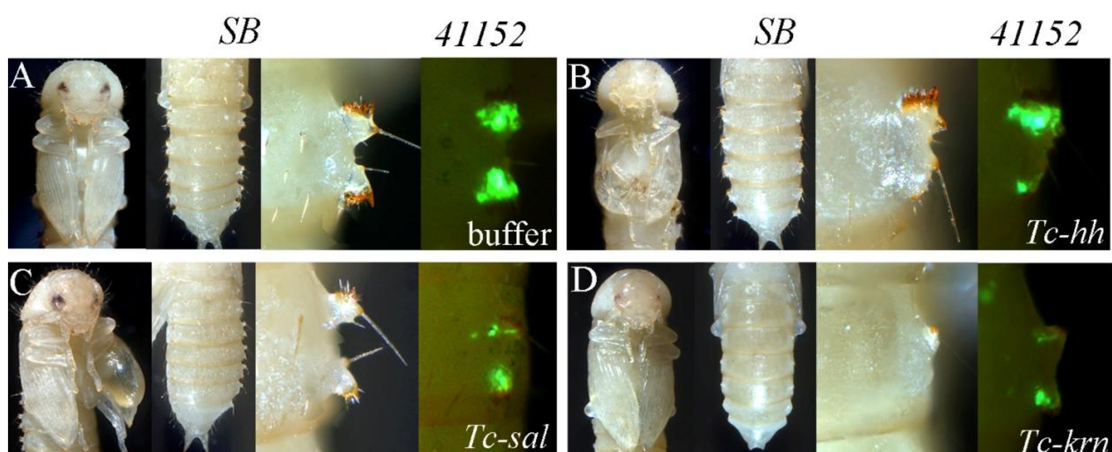

Figure 2.4 Function of candidate AP related wing genes in wing and gin-trap development. Panels show (from left to right) ventral view of a pupa showing the wings, dorsal view showing

the gin-traps, closeup of one gin-traps and the signal of the GöGal41152 transgenic line. (A) Negative control. (B-D) RNAi phenotypes of *Tc-hh*, *Tc-sal* and *Tc-krn*.

First, I tested components of the AP patterning system. Double knockdown of *Tc-engrailed* (*Tc-en)* and its paralog *Tc-invected* (*Tc-inv*) (Peel et al., 2006) resulted in an altered orientation and irregular surface of pupal elytra and about 50% of pupae and adult animals showed blistered elytra in the distal part (Fig. 2.8D and 2.9A). However, defects in gin-trap formation were not detected. Depletion of *Tc-hedgehog* (*Tc-hh*) resulted in deformation of elytra, walking legs and antennae, and the posterior part of the gin-traps was strongly reduced (Fig. 2.4B). Analysis of other members of the Hh pathway (*cubitus interruptus* (*ci*), *smoothened* (*smo*)) confirmed this result (Fig. 2.5). This asymmetric posterior requirement in gin-traps is contrasting the Hh function at the boundary between anterior and posterior compartments of the wing disc. Unexpectedly, *Tc-dpp*, which is the central AP morphogen of the wing and its target genes *Tc-omb* and *Tc-dad* were not involved in gin-trap formation (Fig. 2.9B-D). However, *Tc-sal* affected both anterior and posterior parts of the gin-traps (Fig. 2.4C). Depletion of *Tc-keren* (*Tc-krn*), the only activating EGF ligand present in *T. castaneum* genome (Tribolium Genome Sequnecing Consortium, 2008), resulted in the reduction of both parts of the gin-traps similar to the knock-down phenotypes of *Tc-EGFR* (Fig. 2.5A). In addition, *Tc-krn* RNAi affected the formation of veins in the elytra (Fig. 2.8B).

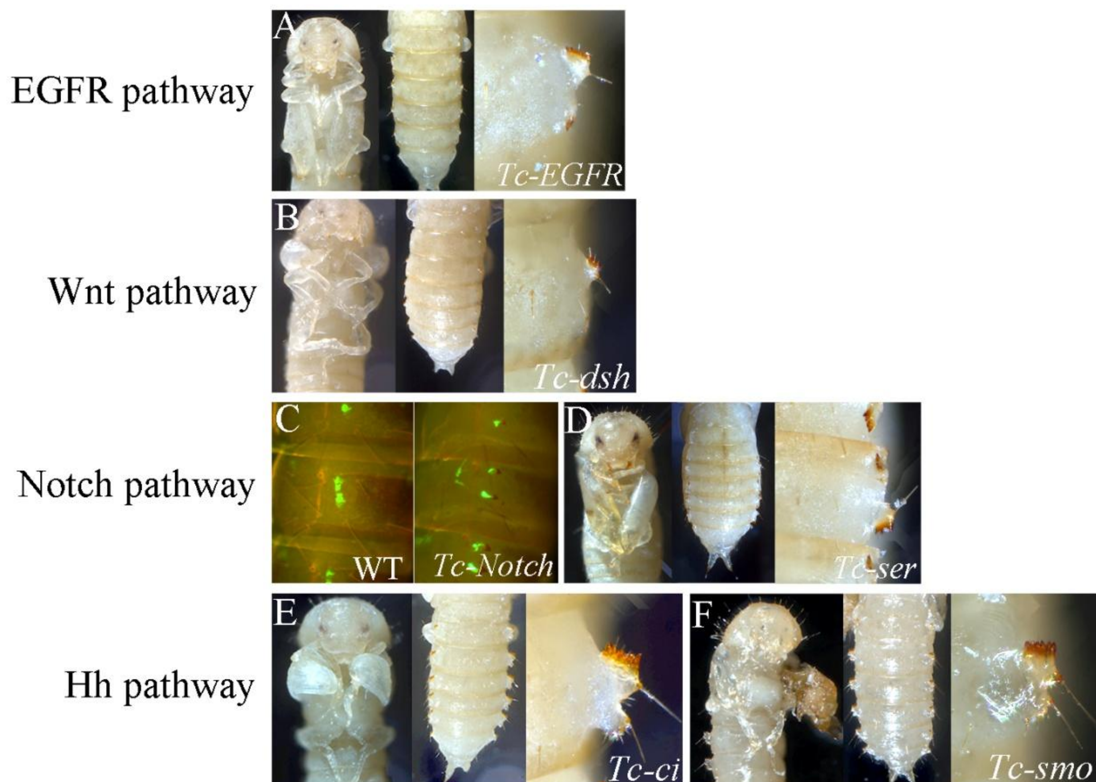

Figure 2.5 Effects of four different signaling pathways on gin-traps and wing morphology. (A) *Tc-EGFR* RNAi. (B) *Tc-dsh* RNAi. (C,D) RNAi phenotype of Notch pathway genes: *Tc-Notch* (C) and *Tc-Ser* (D). (E,F) RNAi of *hh* pathway genes: *Tc-ci* (D) and *Tc-smo* (E). Each panel shows ventral view pupal wing, dorsal view of the pupal abdomen and close-up of gin-trap on T3. *Notch* RNAi animals did not molt to pupae, hence the images were captured at the pre-pupal stage in line *GöGal41152* (C).

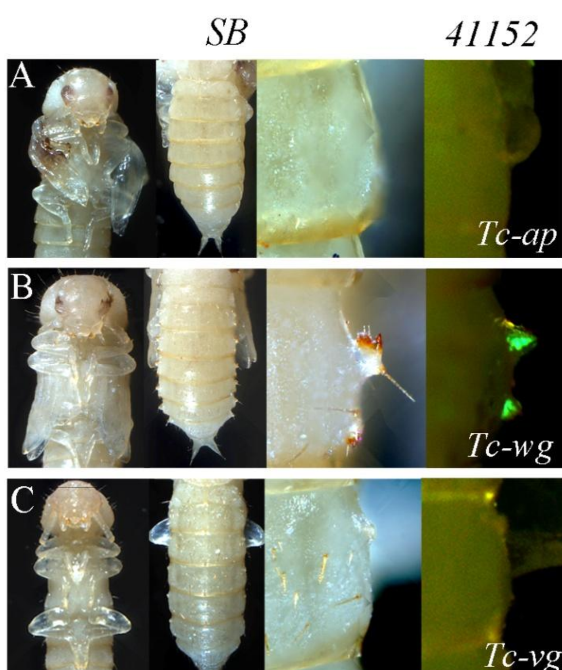

Figure 2.6 Function of candidate DV related wing genes in wing and gin-trap development. (A-C) RNAi phenotypes of *Tc-ap*, *Tc-wg* and *Tc-vg*. Panel as in Fig. 2.4.

Next, I tested components of wing DV patterning. *Tc-apterous (Tc-ap)* RNAi caused the complete absence of gin-traps, while its phenotype in wings was comparably moderate. The shape of elytra was deformed and their dorsal surface abnormal, sometimes showing necrosis (Fig. 2.6A). Depletion of the *Tc-Notch* receptor lead to death at the pre-pupal stage, but the size of the gin-trap signal was decreased in pre-pupa of the line *GöGal41152* (Fig. 2.5C). To further test the involvement of the *Notch* pathway, I knocked down the ligand *Tc-serrate* (*Tc-ser*). Gin-traps were moderately smaller and their orientation was irregular, while elytra became smaller and blistered (Fig. 2.5D). RNAi targeting the Wnt ligand *Tc-wingless (Tc-wg)* affected predominantly the posterior part of the gin-traps with minor alterations of the anterior parts (Fig. 2.6B). This was phenocopied by knocking down *Tc-disheveled* (*Tc-dsh*) another component of the Wnt signaling pathway (Fig. 2.5B). Knocking down of *Tc-vestigial* (*Tc-vg*) induced the complete deletion of gin-traps and wings (Fig. 2.5C), confirming the *vg* RNAi phenotype found in *T. molitor* (Ohde et al., 2013).

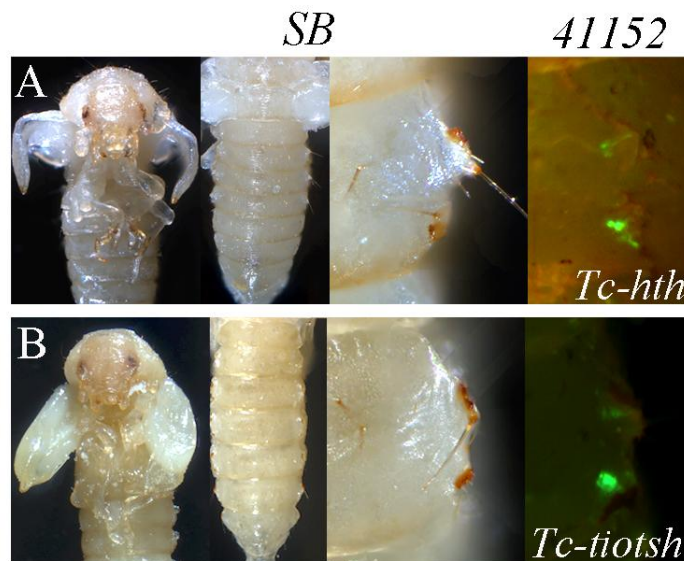

Figure 2.7 Effects of candidate PD wing genes on gin-traps and wing morphology. (A and B)RNAi phenotypes of *Tc-hth* and *Tc-tiotsh*. In the *GöGal41152* line, no RNAi larvae hatched after *Tc-hth* RNAi. Hence, the signal in the pre-pupa is shown. Panels as in Fig. 4.

In *Drosophila*, homothorax (*hth*) and *teashirt* (*tsh*) are expressed in the proximal part of wing disc to specify the notum (Azpiazu and Morata, 2000; Casares and Mann, 2000; Wang et al., 2000). RNAi of *Tc-hth* and *Tc-tiotsh* led to moderate phenotypes of

the gin-traps and deformation of elytra (Fig. 2.7).

The terminal wing specification genes *Dll*, *ASH*, *srf* and *iro,* which specify the margin, sensory, intervein and vein differentiation of wings, respectively, were not required for gin-trap formation (Fig. 2.8 and 2.9). Likewise, the wing pouch marker gene *nubbin* (*nub*) was not involved (Fig. 2.9).

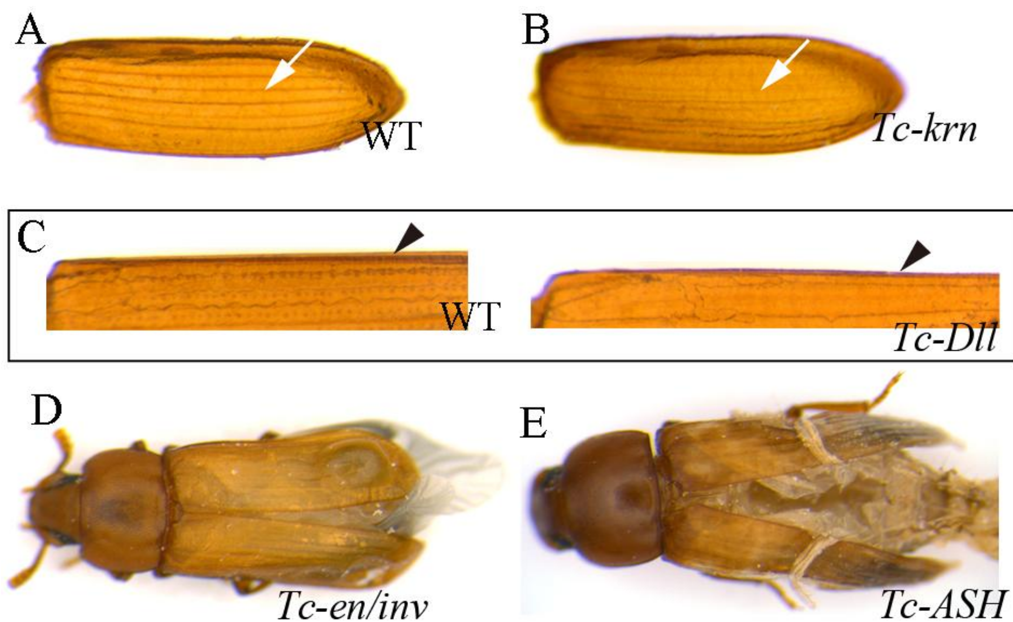

Figure 2.8 Effects of RNAi on *Tribolium* adult elytra. (A) Wild-type. (B,C) In both *Tc-krn* and *Tc-Gug* RNAi the vein pattern of the elytra was affected (arrow in A, B and C). (D) *Tc-Dll* RNAi. The margin of the elytra was altered after depletion of *Tc-Dll* (arrowheads). (E) *Tc-en/inv* double RNAi led to distal defects in the elytra. (F) *Tc-ASH* RNAi. The development of setae was affected in all epidermal tissues.

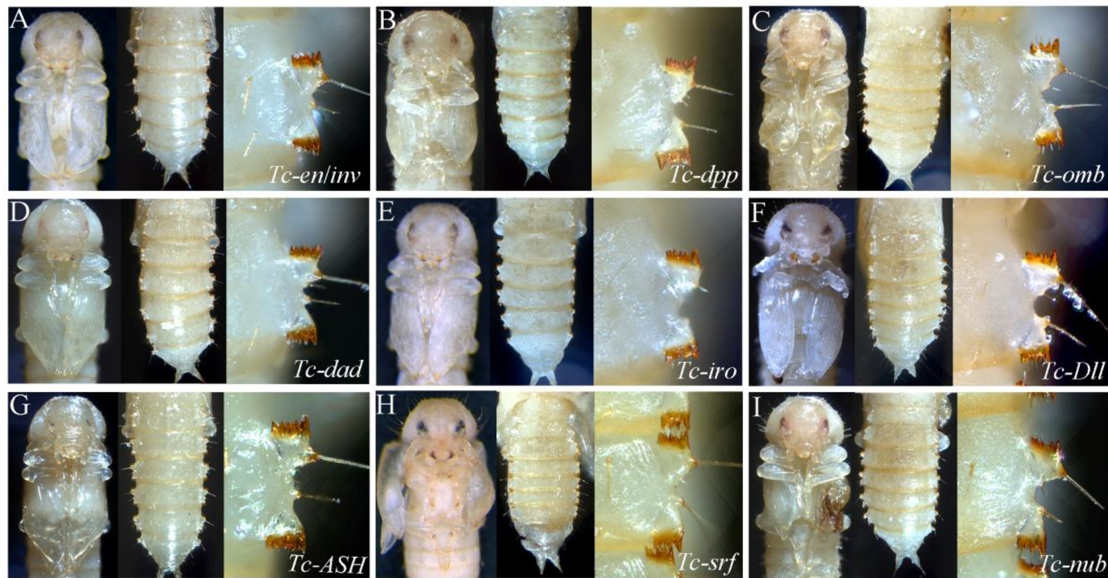

Figure 2.9 Wing genes without gin-trap phenotype. (A-I) No gin-trap phenotype was found for the following genes: *Tc-en/inv*, *Tc-dpp*, *Tc-omb*, *Tc-dad*, *Tc-iro*, *Tc-Dll*, *Tc-ASH*, *Tc-srf*, and *Tc-nub*. Panels as in Fig. 2.5.

2.3.4 *Hox* genes regulate gin-trap formation

Previous studies showed that Ubx and Abd-A regulated gin-trap formation in *T. molitor* (Ohde et al., 2013). Here I tested the function of Ubx, Abd-A and Abd-B in *Tribolium*. *In Tc-Ubx* RNAi pre-pupa, the gin-trap marker was absent and the wing marker appeared in the corresponding position (Fig. 2.10B and E), indicating the transformation from gin-trap to wing identity. In *Tc-Abd-A* RNAi pupae the gin-traps on the abdominal segments A2 through A6 were transformed into an identity more like that on A1, where the size of anterior gin-trap is largely reduced as it consists of the posterior part only (Fig. 2.10H). In strong phenotypes one protrusion formed posterior to gin-traps on A4-A7. Double knockdown of *Tc-Ubx* and *Tc-Abd-A* abolished the gin-trap marker in all abdominal segments while the *nubbin* wing marker appeared (Fig. 2.10C and F), which confirms a previous report (Tomoyasu et al., 2005). In *Tc-Abd-B* RNAi pupae, the portion gin-trap of A7 (only the anterior part is present in wild-type) was complemented by a posterior part and one more pair of intact gin-traps appeared on A8 (Fig. 2.10I).

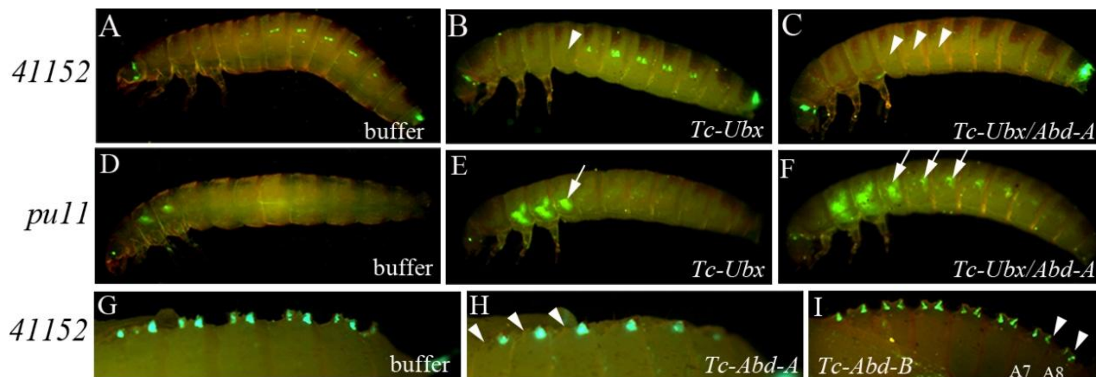

Figure 2.10 Effects of *Hox* genes on gin-trap formation. (A-F) Injected animals at the pre-pupal stage injected with buffer (A,D); *Tc-Ubx* dsRNA (B,E) or *Tc-Ubx/Tc-Abd-A* double dsRNA in the transgenic lines *GöGal41152* (A-C) or *pu11* (D-F). (G-I) Dorsal view on *GöGal41152* pupae injected with buffer (G), Abd-A (H) and Abd-B (I). Arrowhead in B and C showed the position of gin-trap where it grows. Ectopic signal wing marker is donated by arrow. Additional gin-traps formed in Tc-Abd-B RNAi pupae are donated by arrowhead.

2.3.5 An unbiased RNAi screen reveals novel gin-trap and wing patterning genes

Next, I wanted to assess the portion of genes that were recruited from other biological contexts to the gin-trap GRN and detect potentially involved orphan genes (i.e. beetle specific genes). Therefore, I searched for gin-trap phenotypes annotated with a penetrance of more than 50% at the iBeetle-Base (http://ibeetle-base.uni-goettingen.de) (Dönitz et al., 2015; Schmitt-Engel et al., 2015). The iBeetle project is a “first pass” screen, where false positive and off target phenotypes need to be considered. Hence, I repeated the injection of the *iBeetle* dsRNA fragments in the previous strain and in a different genetic background (*SB*) and analyzed the phenotypes induced by non-overlapping dsRNA fragments (Kitzmann et al., 2013; Schmitt-Engel et al., 2015). Ten phenotypes were confirmed (see Table A3 in appendix). Several of these genes were known to be involved in *Drosophila* wing patterning like *Tc-vg*, the EGFR pathway component *Tc-krn* as well as the Notch pathway component *mind bomb1* (*mib1*) confirming the involvement of these pathways (Fig. 2.11E). The *Hox* gene *Tc-Abd-A* was also tested by candidate gene approach above (Fig. 2.10). The size of the gin-traps was moderately decreased in RNAi targeting *Tc-Grunge (Tc-Gug;* in *Drosophila* also called *atrophin*) (Fig. 2.11D),

a nuclear repressor protein , which in *Drosophila* regulates EGFR, Hh signaling and Teashirt in wings and other tissues (Charroux et al., 2006; Erkner et al., 2002; Zhang et al., 2013). Size, orientation and surface of elytra was also abnormal when *Tc-Gug* was knocked down (Fig. 2.11D).

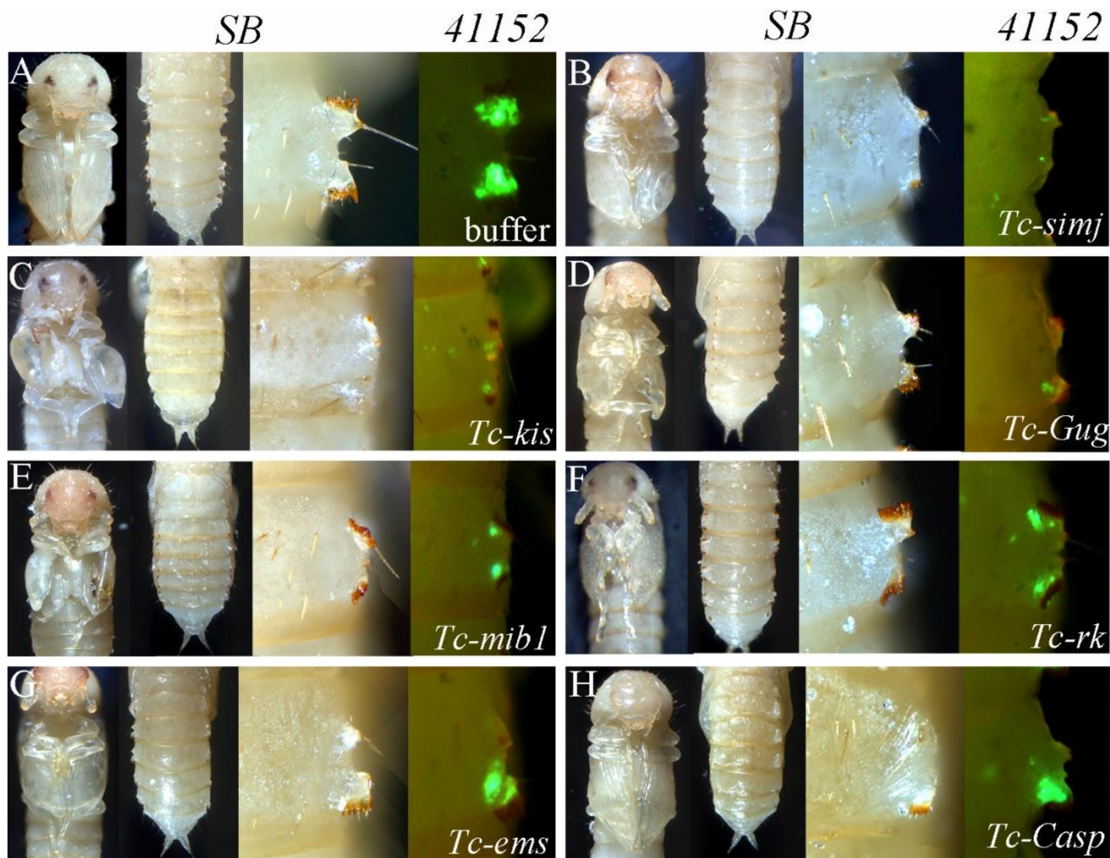

Figure 2.11 Genes identified by unbiased screening. (A) Negative control. (B-H) RNAi phenotype of *Tc-simj*, *Tc-kis*, *Tc-Gug*, *Tc-mib1*, *Tc-rk*, *Tc-ems* and *Tc-Casp*, respectively. Panels as in Fig. 2.4.

Figure 2.12 Confirmation of the involvement of the Bursicon signaling pathway in gin-trap and wing morphology. (A-C) Genes of the bursicon pathway *Tc-burs* RNAi (A), *Tc-pburs* RNAi (B) and *Tc-burs*/*Tc-pburs* double RNAi (C). Panels as in Fig. 2.5.

Four genes had not been connected to *Drosophila* wing development before but

showed phenotypes in both gin-traps and wings of *Tribolium*. In *Tc-rickets* (*Tc-rk*) RNAi gin-traps were slightly smaller in size and showed irregular orientation while the elytra showed a wrinkled surface as previously described (Fig. 2.11F) (Bai and Palli, 2010). *rk* is a G-protein coupled receptor involved in the bursicon signaling pathway involved in molting related behaviors and neuropeptide-induced tanning (Baker and Truman, 2002; Luo et al., 2005). I confirmed the involvement of this pathway in gin-trap formation by testing the hormones *Tc-bursicon* (*Tc-burs*) and *Tc-pbursicon* (*Tc-pburs*) (Fig. 2.12). Further, *Tc-simjang* (*Tc-simj*), *Tc-kisment* (*Tc-kis*), *Tc-empty-spiracles* (*Tc-ems*) affected both wings and gin-traps (Fig. 2.11B, C, G). Both *simj* and *kis* are involved in chromatin gene regulation (Daubresse et al., 1999; Terriente-Félix et al., 2011). The respective RNAi pupae showed reduced gin-traps and denticles (Fig. 2.11B and C). *Tc-ems* is a transcription factor and was required mainly for the anterior part of the gin-traps (Fig. 2.11G). Knockdown of these genes affected the wing pattern to different degree. The size, orientation or shape of the pupal wing was abnormal when *Tc-simj* and *Tc-ems* were knocked down (Fig. 2.11B and G) while the elytra were deformed and blistered in *Tc-kis* RNAi pupae (Fig. 2.11C).

One gene affected gin-traps but not wings. *caspar* (*casp*) is a repressor of the immune deficiency pathway (but not Toll signaling) (Kim et al., 2006). In *Tribolium* it was essential for the formation of the anterior part of the gin-traps (Fig. 2.11H).

2.4 Discussion

2.4.1 A highly pruned wing GRN is re-deployed in gin-trap formation

Co-option of existing GRNs (GRNs), recruitment of genes to a novel context and emergence of orphan genes have been suggested to contribute to the evolution of morphological novelties (Khalturin et al., 2009; Moczek, 2009; True and Carroll, 2002). However, the relative importance of the contribution of these processes remained obscure because unbiased screening tools had been missing in insects outside *Drosophila*. The ongoing genome wide RNAi screen iBeetle offers the unique possibility to determine the gene sets required for the development of novel structures. Consequently, my work goes beyond the classical candidate gene approach in that the involved genes are identified in an unbiased way. My results show that the main mechanism in the evolution of gin-traps was co-option of significant parts of the wing GRN (about 70%; 14 out of 20; each signaling pathway counted as one component, Fig. 2.13 and table 2.1). This figure might be an overestimation because it is based on the number of involved components but not on their regulatory interactions. If the interactions of conserved components are quite different an alternative interpretation would be an independent recruitment of that component or gain of a novel function. *Tc-ems* could be such a case because it has only mild defects in the wings but severe reduction of the anterior part of the gin-trap (Fig. 2.11G).

Further I find that an unexpected large portion of the wing GRN components were pruned during co-option (30%). These were mainly downstream genes in line with the morphological differences of wings and gin-traps. However, I also found that some upstream core components were pruned as well (i.e. *engrailed* and the Dpp pathway). This suggests a large degree of flexibility in the co-option of modules of a network (see Fig. 2.13) (Moczek, 2009; True and Carroll, 2002).

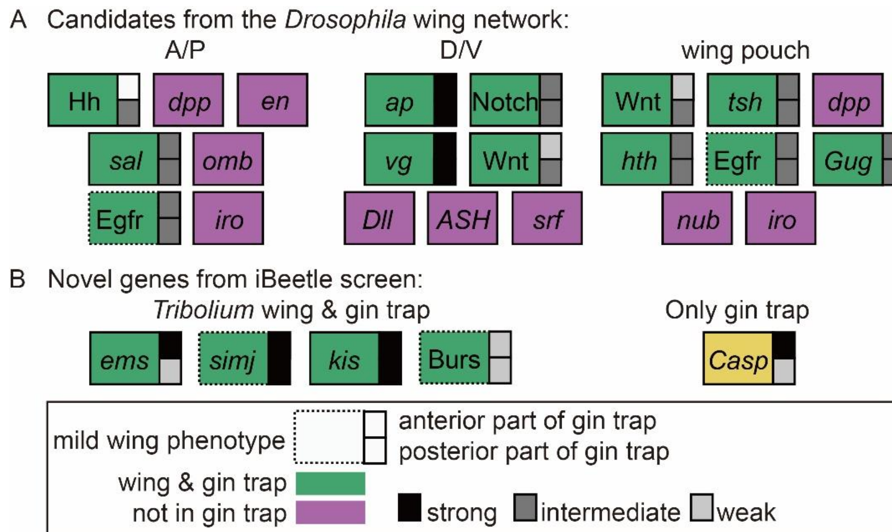

Figure 2.13 Components of the wing and gin-trap GRNs. (A) Many genes known from *Drosophila* wing development have a function in gin-trap development as well (green). Many downstream components but also the upstream components *dpp* and *en* did not show gin-trap phenotypes (purple). Components are arranged according to their approximate position in the network (upstream top row vs downstream bottom row) and their involvement in AP; DV and wing pouch formation. Based on (Tomoyasu et al., 2009) (B) The unbiased screen revealed novel genes required for wing and gin-trap development (green) and one gene, which was clearly recruited from another biological context (yellow).

It had been suggested that taxonomically restricted genes (i.e. genes that evolved only in a certain lineage) may be essential to the evolution of morphological diversity (Dai et al., 2008; Harpur et al., 2014; Khalturin et al., 2008). In contrast to this prediction, I did not find any such gene in the gin-trap GRN.

The unbiased screening revealed only *Tc-casp* as newly recruited component. *casp* is a negative regulator of the immune deficiency pathway in *Drosophila* and diverse anopheline species (Garver et al., 2009; Kim et al., 2006). Hence, this is a prime example of the recruitment of a gene from a completely different context. This work is based on the first part of the *iBeetle* screen where 4,480 randomly selected genes were scored for phenotypes during metamorphosis (28% of the gene set) (Schmitt-Engel et al., 2015). A similar portion of novel genes acting in wing and/or gin-trap GRNs is predicted to be present in the remainder of the genome. Under this assumption, the portion of genes newly recruited in the gin-trap GRN would be

around 10% (see Table 2.1 for calculation).

Surprisingly, I identified four genes involved in wing formation in *Tribolium*, which had not been connected to this process in *Drosophila*. This suggests that either the *Drosophila* GRN has not been comprehensively studied or that significant differences exist to the *Tribolium* GRN.

Table 3.1 Classification of genes

Candidate genes from the known wing gene regulatory network			
	only wing	**wing and gin-trap**	**only gin-trap**
pathways	Dpp	Hh[1] Wnt[2] Notch EGFR	
genes	*optomotor-blind* *srf* *nubbin* *engrailed/invected* *Distal-less*	*apterous* *vestigial* *teashirt* *homothorax* *spalt* *Grunge/atrophin*	
Novel genes identified in the iBeetle screen			
	only wing	**wing and gin-trap**	**only gin-trap**
pathways		Bursicon *(neuropeptide)*	*Caspar*[3] *(immunity)*
genes		*kismet* *simjang* *ems*[3]	
Notes [1] only posterior part of gin-trap affected [2] mainly posterior but also anterior part [3] only anterior part of gin-trap affected		In *Tribolium*, *Tc-iroquois* & *Tc-ASH* RNAi affected bristles on the entire cuticle – therefore not scored as specific wing phenotype.	
Genes/pathways affecting wing patterning: n=20 6 do not affect gin-traps (30%) 4 novel in wing patterning (20%)		**Genes/pathways affecting gin-traps:** n=15 1 affects only gin-traps (6.6%)	
Estimation of total number of co-opted genes: So far, 28% of the genome was screened for metamorphosis phenotypes. In this randomly selected gene set, I found 4 novel genes involved in wing and gin-trap formation and 1 gene active in gin-traps only. Screening the other 72% of the genome I expect at least another 8 genes of the former and 2 genes of the latter category. Together with the genes found by screening wing GRN candidates this would sum up to 25 genes required for gin-trap formation of which 3 would be co-opted (12%).			

2.4.2 GRN pruning relates to morphological differences

Compared to the wing with its asymmetry along AP, DV and PD axes and its complex vein pattern, the gin-traps are simple epidermal outgrowths decorated with spines

and setae. The large degree of pruning of downstream components of the re-deployed wing GRN reflects this simplification. Along the proximo-distal axis, the body wall patterning genes (*hth*, *tsh*, *EGFR*, *sal*) but not the distal wing patterning genes (*dpp*, *nub*, *omb*, *dad*) were required. This indicates that mainly the proximal part of the wing GRN was co-opted for the gin-traps (Fig. 2.13). Unexpectedly, I did not find pruning of upstream DV patterning genes despite the fact that gin-traps are symmetric along this axis. However, the results of Linz et al. (unpublished) suggest that the gin-traps are built by the dorsal but not the ventral component of the wings, which might explain the loss of DV polarity.

2.4.3 Recruitment and gain of function required for the evolution of a morphological innovation

Intriguingly, the gin-traps show mirror image symmetry along the AP axis while the walking legs, the wings and the underlying segments all have a clear AP asymmetry. Hence, the gin-trap GRN had to evolve a mechanism to realize this innovation. My results suggest that several modifications of the re-deployed wing GRN contributed. Firstly, it was pruned of *engrailed,* which is a key factor of posterior identity of segments, legs and wings. Indeed, the results of Linz et al. (unpublished) shows that gin-traps do not comprise *engrailed* positive cells. Hence, the development of symmetry did not have to overcome this very fundamental posterior identity. Secondly, the segment polarity genes *Tc-hh* and *Tc-wg* specify the posterior part of the gin-traps. Hh signaling, which emerges from *engrailed* positive cells in embryos, legs and wings, was required exclusively for the posterior part of the gin-trap (Fig. 2.4B). Segmental *wg* expression is located anterior to *en/hh* positive cells and was required predominantly for the posterior part of the gin-traps (Fig. 2.6B). Hence, two segment polarity genes active in the posterior half of each segment specify the posterior part of the gin-trap. Thirdly, a mirror image copy of the posterior part of the gin-trap needs to be specified independently of the highly conserved segmental AP asymmetry. Intriguingly, the only newly recruited gene that I found in my search, *Tc-casp*, affected predominantly the anterior part of the gin-traps (Fig. 2.11H). Hence, while recruitment was rare overall, it appeared to have been essential for the

formation of an innovation regarding gin-trap morphology. *Tc-ems* is the second component, which was required mainly for the anterior part. Interestingly, the *Tc-ems* wing phenotype was rather mild (Fig. 2.11G) while the phenotype in the anterior gin-trap was among the strongest that I observed. Apparently, *Tc-ems* has gained a novel upstream function in the anterior gin-trap GRN. Interestingly, the *Tc-Abd-A* RNAi phenotype showed a loss of the anterior part rather than a transformation. Hence, it appears that this gin-trap specific symmetry is under the control of an abdominal *Hox* gene. In summary, specification of an innovation (mirror image symmetry) involved the recruitment of one, and the gain of upstream functions of another component under the control of a region specific *Hox* gene.

2.4.4 Gin-trap evolution and serial homology

Based on *vg* expression in gin-trap and wing anlagen, absence of both structures in RNAi and homeotic transformations it was suggested that gin-traps are wing serial homologs (Ohde et al., 2013) or partial serial homologs (Clark-Hachtel and Tomoyasu, 2016; Tomoyasu et al., 2017). Likewise, I find signs of transformation in *Ubx*/*Abd-A* double RNAi. But I also note a complex regulation of gin-trap development where *Ubx* and *Abd-A* cooperate to repress the wing GRN while at the same time they seem to be required for the different parts of the gin-traps (Fig. 2.10 and 2.14). These data together with the location and the extensive overlap of components involved in wing and gin-trap GRNs are in line with the view of serial homology. However, I find a large degree of network pruning (about 30%) including upstream components and identify an innovation, which depends on a recruited gene and the gain of function of another component. Together with the lack of ventral and engrailed positive tissues in the gin-traps (Linz et al., unpublished) this amounts to a significant degree of divergence of both composition and GRN. Hence, I wonder in how far “serial homology” fully reflects this situation or whether the concept of “homocracy” (i.e. the fact of being regulated by the same genes) may be more appropriate (Nielsen and Martinez, 2003).

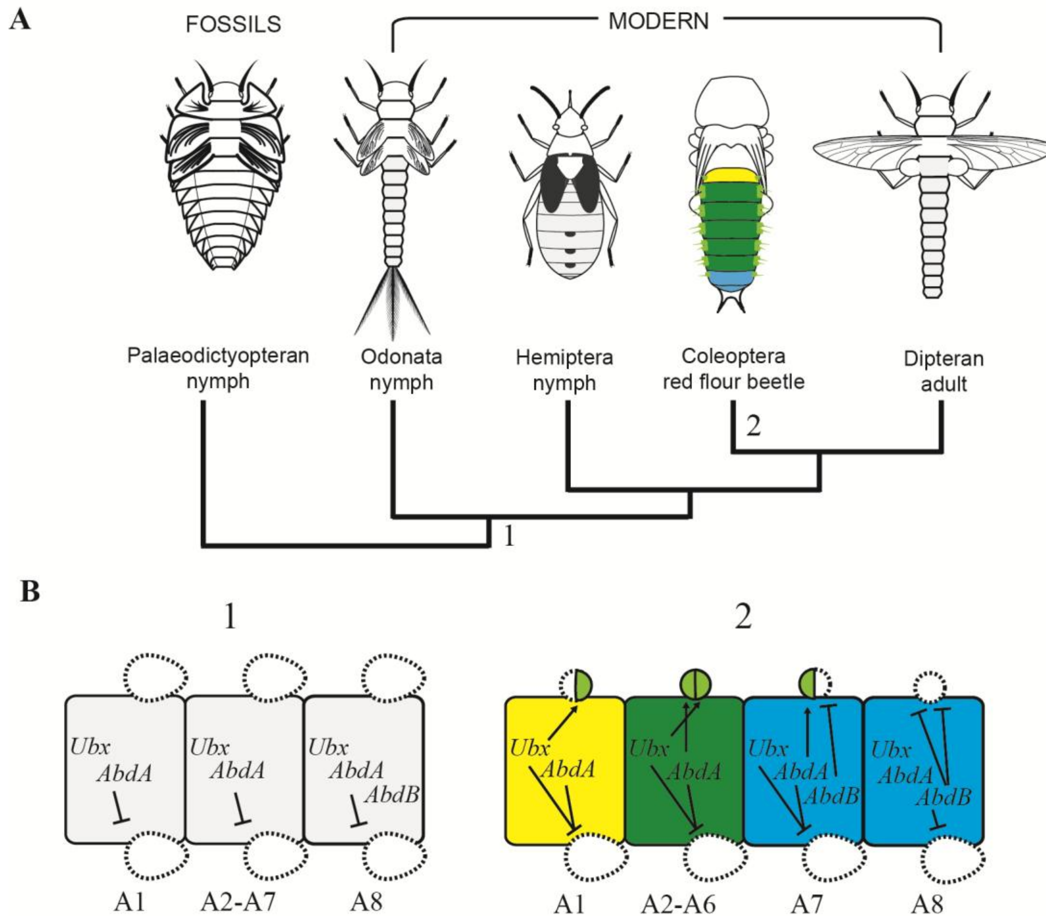

Figure 2.14 Model for *Hox* gene function on wings and gin-traps. (A) The fossil record shows early pterygotes with lateral structures on all thoracic and abdominal segments (Carroll et al., 1995). Whether these abdominal structures are serial homologs to wing remains disputed. (B) The wing network probably evolved initially without input from *Hox* genes because *Antp* is not required for wing formation in extant insects (Carroll et al., 1995). However, *Ubx* expressed in T3 modified the hindwings to varying degrees in some insect taxa (Abouheif and Wray, 2002; Tomoyasu et al., 2005; Warren et al., 1994; Weatherbee et al., 1998). Activation of the wing GRN network in abdominal segments was repressed by abdominal *Hox* genes (B 1). I propose that the first step for gin-trap evolution was a partial de-repression of the wing GRN leading to an outgrowth based on the co-opted wing GRN. Recruitment of novel genes and pruning and regulatory changes in the GRN led to the gin-trap morphology. For instance, co-option of *Tc-casp* and evolution of an upstream role of *Tc-ems* were probably required to generate the symmetry of gin-traps in an otherwise AP asymmetric segment. Note the asymmetric requirement of *Tc-Abd-A* and *Tc-Ubx* for anterior versus posterior part of the gin-traps (B 2). Part of Fig. 2.14A was redrawn from publication (Carroll et al., 1995).

3. Chapter 2

Identification and expressional characterization of novel genes in patterning the adult antenna formation in *Tribolium castaneum*

Abstract

Compared to the relatively conserved structure of legs, the morphology of antennae varies widely among insects. The antenna tends to maintain a distinct morphology in different developmental stages in most of Holometabola. Like most holometablous insects, but not *Drosophila*, the reduced larval antenna was remodeled into the fully annulated and more segmented form appropriate for the adult in *Tribolium*. Nonetheless, the developmental genetics of appendage formation are more completely described in *Drosophila* than in any other insects. However, *Drosophila* may not retain the ancestral state for appendage patterning. Candidate gene studies in *Tribolium* provide an insight into the likely ancestral mode of antenna development, which leaves novel genes unidentified.

In order to overcome this limitation, unbiased large scale RNAi screening revealed novel genes affecting antenna metamorphosis. In total, ten novel genes were confirmed to be involved in antenna remodeling in this study and new classifications of genes were found, which were not reported before. Surprisingly, it was revealed for the first time four genes involved in four pre-mRNA splicing affected the antenna patterning in RNAi resultants. These results suggest the essential role of this biological process for antenna metamorphosis, which is possibly mediated via the regulation of gene expression. One taxonomically restricted gene was shown to be specifically involved in antenna patterning in local region, implying that taxonomically restricted genes might be important for the evolution of lineage specific antenna morphology. However, this needs to be further

investigated in other Coleopteran species. Finally, the data presented here suggest a complex mix of divergence and constraint among serial appendages through functional comparisons between antenna and leg.

3.1 Introduction

3.1.1 Antennal structure and function

All insects possess a pair of antennae, but they may be greatly reduced, especially at the larval stage (Chapman, 2013). Insect antennae consist of three primary segments, a basal scape, a pedicel and a flagellum (Keil, 1999). The scape is mounted into a sclerotized region, often elevated from the head capsule, and pivoted on a single marginal projection, the antennifer (Fig. 3.1). Therefore the scape enables insects to move the antenna in all directions. The pedicel is flexibly connected to the distal end of the scape. Frequently the flagellum comprises a number of annuli known as flagellomeres, which are jointed to each other by membranes so that the flagellum generally moves as a whole because of the absent of intrinsic muscles in the flagellum. The antennae of insects are moved by applying internal muscles (levator and depressor muscles) connected to the scape. Likewise the flexible movement of pedicel is controlled by muscles (flexor and extensor muscles) connecting the scape and the pedicel (Fig. 3.2) (Chapman, 2013).

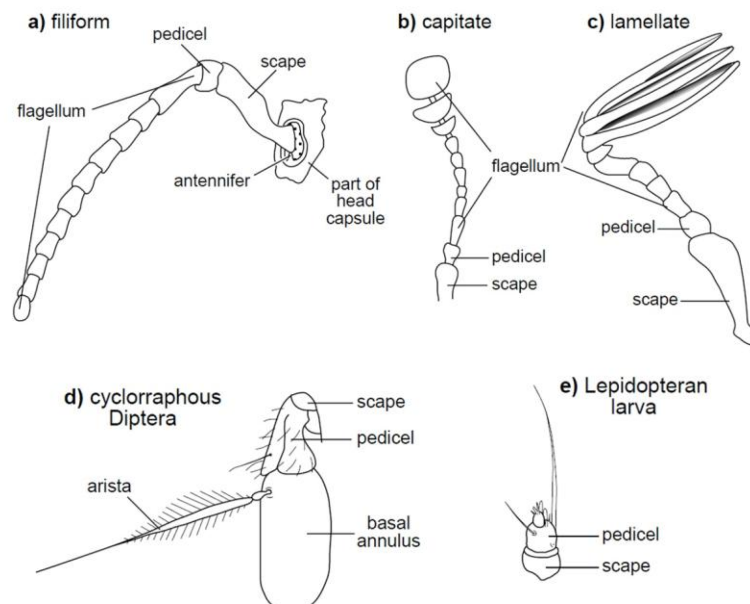

Figure 3.1 Different forms of antennae occurring in different insects (from Chapman, 2013).

The number of annuli is highly variable between species and often is of taxonomic

revelance. For example, adult Odonata possess five or fewer annuli while there are over 150 annuli in adult Periplaneta (Blattodea). The form of the antenna varies considerably according to its unction (Fig. 3.1). The surface area of antennae in some male moths, for instance, is increased by modifying the antenna into a plumose form, allowing a large number of sensilla to be accommodated on the antenna. Sexual dimorphism of antennae is a widespread phenomenon, which sometimes is associated with the occurrence of different kinds of sense organs. The antennae of the male are often more complex than those of the female, which often occurs where the male is attracted to or recognizes the female by her scent (Chapman, 2013; Schneider, 1964).

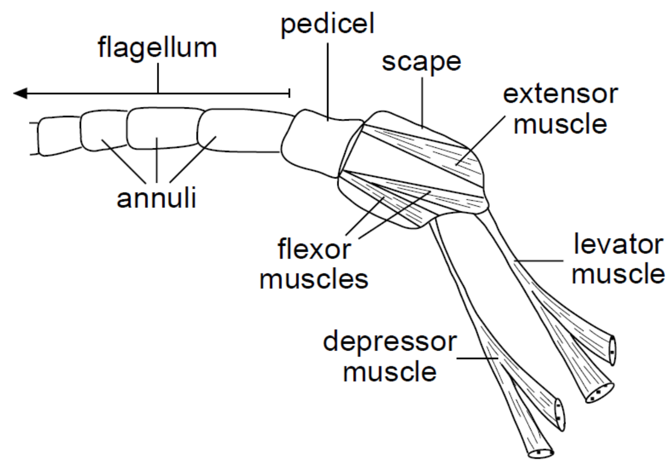

Figure 3.2 Typical insect annulated antenna. There are no muscles in the flagellum (from Chapman, 2013).

The antenna functions primarily as sensory structure, which is equipped with sensilla in most insects. Antennae are the primary olfactory organ of all insects and also serve as tactile sensors in some insects. They are also used for some other functions, like for instance, detecting wind speed and direction, heat and moisture and they serve even non-sensory functions, such as grasping prey in some insects (Zacharuk, 1985). The pedicel possesses a chordotonal organ, the Johnston's organ, which is important for measuring air speed in flying insects (Gewecke, 1974; Taylor and Krapp, 2007). Moreover, antennae are associated with the perception of near-field sounds in some insects, such as female *Drosophila melanogaster*, male mosquitoes and worker honey bees (Ai and Itoh, 2012; Boekhoff-Falk and Eberl, 2014; Göpfert and

Robert, 2001). In the cockroach, the long antennae are associated with their functions as feelers (Okada, 2016). Monarch butterflies are famous for their seasonal long-distance migrations. Antennae are necessary for proper time-compensated sun compass orientation in migratory monarch butterflies. Antennal clocks, existing in monarchs, likely provide the primary timing mechanism for sun compass orientation (Merlin et al., 2009). In water striders, males could use their modified hook-shaped antennae to grasp females for mating (Khila et al., 2012).

The development of antennae are quite different between hemimetabolous and holometabolous insects. The morphology of antennae of nymphal hemimetabolous insects is similar to that of the adult, but with fewer antennomeres. The number of antennomeres generally increases during the series of post-embryonic molts. In contrast, the antennae of larval holometabolous insects are usually considerably different from those of the adult. In larval Coleoptera and Lepidoptera, the antennae possess only three primary antennomeres. The larval antenna is modulated into fully annulated adult antenna during metamorphosis. However, the homology between the larval and the adult antennameres remains ambiguous (Svácha, 1992). In some larvae of Diptera the antennae are highly reduced (Chapman, 2013).

3.1.2 The development of antennae in *D. melanogaster*

Although the morphology of antenna varies wildly among insect species, our understanding of antennal development comes almost solely from studies of a single species - the fruit fly, *D. melanogaster*.

The antenna develops identity from a more anterior, *Hox*-free region. If the *Hox* gene is ectopic expressed in antenna, it overrides the antennal identity, like for instance the *Antennapedia* mutant in *Drosophila*, in which the antennae are transformed into second legs by the ectopic overexpression of the *Hox* gene *Antennapedia* (*Antp*) in the antennal segment (Schneuwly et al., 1987). Misexpression of the *Hox* genes *Sex combs reduced* (*Scr*), *Ultrabithorax* (*Ubx*) or *Abdominal-A* (*Abd-A*) in the antennal disc causes similar antenna-to-leg transformations (Yao et al., 1999). These transformations are induced through a common mechanism: suppression of the

transcription of the *homothorax* (*hth*) gene thereby preventing the nuclear localization of the Extradenticle (Exd) protein (Yao et al., 1999). Hypomorphic or null mutants of either the *hth* or the *Exd* gene induce a transformation of the entire antenna into leg (Casares and Mann, 1998, 2001; Gonzalez-Crespo and Morata, 1995; Rauskolb et al., 1995).

Furthermore, *Distal-less* (*Dll*) activates genes specifically to distal antenna identity and hypomorphic alleles of *Dll* cause the transformation of the distal antenna into leg while the proximal antennal regions retain antennal identity (Chu et al., 2002; Dong et al., 2000; Duncan et al., 1998; Galindo et al., 2002; Sunkel and Whittle, 1987). Loss-of-function of *spineless* (*ss*), a bHLH-PAS transcription factor-encoding gene, results in the transformation of the distal antenna to leg, whereas the identity of the proximal region is not affected, resembling *Dll* null mutations (Duncan et al., 1998). Co-expression of *exd*/*hth* and *Dll* activates *ss* expression in the developing distal antenna, specifying distal antenna identity. In contrast, the identity of the proximal antenna is specified by *exd* and *hth*, but not *Dll*, and this is not modulated through *ss*.

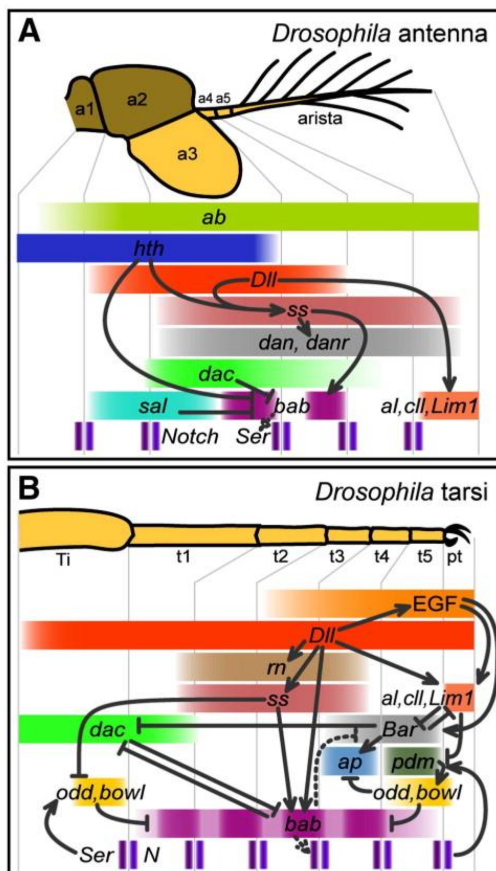

Figure 3.3 Patterning of the antenna and the distal leg in *Drosophila*. The regions of gene expression are mapped onto the adult antenna (A) and tarsus (B). The black lines indicate gene interactions: activation is denoted by arrows, repression by blunt-ended lines. The dashed lines show hypothesized interactions. (from Angelini, 2009)

Along the proximodistal (PD) axis, the adult fruit fly antenna consists of six antennomeres, scape (a1), pedical (a2) and flagellum (a3-a6). The terminal of the flagellum is modified into the arista (Fig. 3.3), which is a large bristle attached to the front part of antennae in some species of Diptera. Studies of leg imaginal disc development in *Drosophila* have shown that the PD axis of the leg imaginal disc is organized by gradients of secreted signaling proteins Wingless (Wg) and Decapentaplegic (Dpp) (Diaz-Benjumea et al., 1994; Lecuit and Cohen, 1997). *hth* and *Dll* are required for PD regional identity. Losing the function of either *hth* or *Dll* causes reduction or deletion of the proximal and distal domains, respectively (Casares and Mann, 1998; Cohen and Jürgens, 1989; Pai et al., 1998). *dachshund* (*dac*), which encodes a nuclear factor, expresses in a region between the *Dll* and *hth* domains in leg and antenna. Legs lack intermediate region along the PD axis in *dac* mutant (Mardon et al., 1994). In contrast, mutations in *dac* have very limited effects on antennal development (Dong et al., 2002). Consistent with the expectation that the targets of *Dll* or *hth* for PD patterning are expressed in both antenna and leg, the *Dll* targets, *bric a brac* (*bab*), *aristaless* (*al*), and *BarH1*/*BarH2*, are expressed and required in both the distal antenna and leg (Campbell and Tomlinson, 1998; Godt et al., 1993; Kojima et al., 2000). In contrast, some genes, like for instance *spalt* (*sal*), *cut* (*ct*) and *atonal* (*ato*), are identified to be antenna-specific targets of *Dll* and/or *hth* (Dong et al., 2002). Therefore, the PD axis is specified through interactions with *exd/hth* and *Dll* and their targets.

The formation of joints is a complex process involving changes in cell shape, in the adhesion between cells and in the distribution of filamentous actin and extracellular matrix proteins (Kojima, 2004). The Notch pathway has been shown to be fundamental for joint formation, which is mediated by the interaction of the Notch pathway and other genes (Fig. 3.3). In the proximal part of each segment, *fringe* (*fng*) which encodes a modulator of Notch activity is expressed and required for proper joint formation. Several downstream genes of Notch pathway, including *odd-skipped* (*odd*) family genes, *odd*, *bowl*, *sister of odd and bowl* (*sob*), and *drumstick* (*drm*), are known to mediate the Notch function in leg development. Notch pathway activity

appears to function similarly in leg and antennal development (Rauskolb and Irvine, 1999).

However, Dipterans are derived in many aspects of appendage development, and comparative genetic and developmental studies have suggested that the gene functions and interactions involved in *Drosophila* appendage patterning are a mixture of derived and ancestral ones (Angelini and Kaufman, 2005a; Ober and Jockusch, 2006).

3.1.3 Conserved and divergent aspects of antennal development across arthropods

Genetic and developmental studies in *Drosophila* have provided insights into the mechanisms that produce the appendages in this species, which has been of great value, and has enabled researchers to take a comparative approach to the study of developmental process in other insects and arthropods. By comparing what is known of appendage development in *Drosophila* with that in other species, a more complete understanding of insect appendage development and evolution is emerging. First, *Hox* genes are not expressed in the antennal segment in all investigated arthropod species (Brown et al., 2002; Hughes and Kaufman, 2002; Jager et al., 2006; Janssen and Damen, 2006; Manuel et al., 2006; Sharma et al., 2012; Shippy et al., 2008). The antennal segment identity is repressed by *Hox* genes. Second, similar to the *Drosophila* antenna disc but different from the legs, extensive overlap of the expression domains of *exd/hth* and *Dll* is found during embryogenesis in chelicera of spiders (Abzhanov and Kaufman, 2000; Pechmann and Prpic, 2009; Prpic et al., 2003), the harvestman *Phalangium opilio* (Sharma et al., 2012), and the mite *Archegozetes longisetosus* (Barnett and Thomas, 2013) and the antennae of insects *Tribolium castaneum* (Jockusch et al., 2004) and *Oncopeltus fasciatus* (Angelini and Kaufman, 2004). However, discrete expression patterns of these genes are found in the developing antenna of millipede *Glomeris marginata* (Prpic and Tautz, 2003) and the insects *Gryllus bimaculatus* (Mito et al., 2008; Ronco et al., 2008) and *Schistocerca americana* (Jockusch et al., 2004). This discrete expression patterns of *exd/hth* and *Dll* are similar to their expression in the leg in these species.

Therefore, the extensive overlapping expression of these genes in developing antenna is not conserved among arthropods.

3.1.4 *Tribolium castaneum* is emerging as a representative model for antennal patterning during matemorphosis

Like most holometabolous insects but in contrast to the limbless maggot of *Drosophila*, the red flour beetle, *Tribolium castaneum*, possesses well-developed antennae in both larva and adult. Interestingly, they display drastically different morphologies one another. At the larval stage, the *Tribolium* antenna comprises three primary antennomeres: scape, pedicle and a non-annulated flagellum. The adult antenna consists of eleven antennomeres. The flagellum is subdivided into nine flagellar articles, including a six-annulated funicle and a three-annulated club. The total number of antennomeres is fixed to eleven antennomeres in most but not all of Coleoptera (Minelli, 2005, 2017). The reduced larval antenna is remodeled into the fully annulated and more segmented adult form during metamorphosis, indicating that the antennal tissue undergoes substantial re-patterning during this process. Given these differences, the question raises of how the identity of antenna is specified in *Tribolium*, what genes are involved in PD patterning and in the remodeling process that form a morphologically distinct antenna during metamorphosis.

In the last decade some insights into *Tribolium* antenna remodeling during metamorphosis were gained by candidate gene studies. The *Tribolium* ortholog of *spineless* (*Tc-ss*) is required for identity specification of the larval and adult antennae (Shippy et al., 2009; Smith et al., 2014; Suzuki et al., 2009; Toegel et al., 2008). Depletion of *Tc-ss* by RNAi during either embryonic or adult development induces the transformation of the distal portion of the antennae to legs, which suggests that at least this aspect of patterning is conserved between larval and adult antennae (Shippy et al., 2009; Toegel et al., 2008). Loss-of-function of *Tc-Dll* resembles the phenotypes of *Tc-ss* knock-down (Smith et al., 2014; Suzuki et al., 2009). Hence, the functions of *Dll* and *ss* in specifying antennal identity are conserved between *Drosophila* and *Tribolium*. In addition, genes with conserved functions comparing to

Drosophila include EGF signaling for antenna growth, and Notch signaling for antenna growth, joint formation and sensory bristle development (Angelini et al., 2009; Smith et al., 2014; Suzuki et al., 2009).

On the other hand, notable differences of gene functions were also found between *Tribolium* and *Drosophila*. In *Tribolium*, *Tc-exd/hth* is required for the proximal identity of antenna. Respective RNAi cause the homeotic transformation of proximal antenna (scape and pedicel) toward distal leg identity, which differs from their functions for identity specification throughout the antenna in early instar of *Drosophila* (Smith et al., 2014). *Tc-Lim1* is required only for the formation of the scape-pedicel joint in contrast to the function of promoting arista growth in *Drosophila* (Tsuji et al., 2000). *Tc-Odd-skipped-related* is required for the development of the entire funicle while *Tc-dachshund* (*Tc-dac*), *Tc-spalt* (*Tc-sal*), *Tc-rotund* (*Tc-rn*) and *Tc-ss* affect only small regions. And *Tc-bab*, *Tc-al*, *Tc-apterous* (*Tc-ap*) and Tc-*pdm* contribute to joint formation within the club (Angelini et al., 2009).

These studies provided us with new insights into the likely ancestral model of antenna development and into the genetic changes correlating with the evolution of diverse antenna morphologies. However, all of these studies are based on the candidate gene approach inspired from the highly derived *Drosophila* situation, which has obvious limitations. Firstly, the candidate gene approach does not identify genes required for processes that are not represented in *Drosophila*. Furthermore, it leads to a bias towards the study of conserved gene functions and contribution of novel genes may go unnoticed.

The ongoing unbiased iBeetle screen aims to overcome the limitation of the candidate gene approach to reveal the function of genes for embryogenesis and metamorphosis (Schmitt-Engel et al., 2015). For each genome-annotated gene, the corresponding dsRNA is designed for injection. Two screens are performed in parallel by injection of dsRNAs at different developmental stages. In the "larval injection screen", penultimate instar larvae (L6) are injected and then morphological defects are checked both at pupal and at adult stages, which reveals genes involved

in the process of metamorphosis. In the "pupal injection screen", their offspring embryos are analysed for muscle and cuticle phenotypes as readouts for defects in embryogenesis (Schmitt-Engel et al., 2015). All the phenotypes got from these two screens are documented in iBeetle database (http://ibeetle-base.uni-goettingen.de/) (Dönitz et al., 2015).

Combined with other strengths of the *Tribolium* model system, for instance, ease of culture, short generation time, systemic RNAi effect (Brown et al., 1999; Bucher et al., 2002; Tomoyasu and Denell, 2004) and available transgenic and genome editing tool (Berghammer et al., 1999; Gilles et al., 2015; Lorenzen et al., 2007; Schinko et al., 2010; Trauner et al., 2009), *Tribolium* has emerged as an excellent model system for morphological evolution during metamorphosis.

The aim of this project is to take advantage of the unbiased iBeetle screen to identify and analyze novel genes affecting antenna remodeling during metamorphosis in *Tribolium*, which represents a more typical model of metamorphosis than *Drosophila* (Snodgrass, 1954). For some confirmed candidates, the expression patterns in the developing antenna were determined by using the optimized whole mount *in situ* hybridization (WMISH). This work laid the foundation for further studies on the regulatory network of antenna development.

3.2 Materials and Methods

3.2.1 Animals

Animals of wild-type strain *San Bernadino* (*SB*) were used and reared as described in section 2.2.1.

3.2.2 Selection of candidate phenotypes

To identify novel antenna patterning genes, potential candidates were selected based on antenna phenotypes found in the iBeetle screen (Schmitt-Engel et al., 2015). Phenotypes were searched at the iBeetle database (http://ibeetle-base.uni-goettingen.de/) (Dönitz et al., 2015). The following search terms were used: developmental stage: "adult" or "pupa"; morphological structure: "antenna"; Penetrance: >30% (to get more candidate phenotypes). In order to focus on novel genes involved in antenna development, the candidate phenotypes were selected if they meet one of the two following criteria. Firstly, the *Drosophila* ortholog of the iBeetle target gene was not known to be involved in antenna or leg development. Secondly, the iBeetle target gene had no ortholog in *Drosophila*. The primary identification of *Drosophila* ortholog was based on reciprocal BLAST hits.

3.2.3 Orthology and phylogenetic analysis

The iBeetle ID number (e.g. iB_00548) of each candidate was searched in the *Tribolium* genome browser (http://bioinf.uni-greifswald.de/gb2/gbrowse/tcas5/) to retrieve the translated protein sequence of the corresponding gene. Protein sequences were used as query to find homologs through the blastp algorithm in the Reference Protein Database of *Drosophila melanogaster*, *Mus musculus* and *Tribolium castaneum* at NCBI (National Center for Biotechnology Information, https://blast.ncbi.nlm.nih.gov/Blast.cgi) (Altschul et al., 1990). The protein sequences of the three best hits were chosen for alignments and phylogenetic analysis. All protein sequences were aligned using the ClustalW alignment algorithm and phylogenetic trees were constructed using the neighbor-joining method as

implemented in MEGA6 (Tamura et al., 2013). Detailed information on the function of the *Drosophila* orthologs was retrieved from Flybase (http://flybase.org/).

3.2.4 dsRNA Synthesis

dsRNAs targeting the genes identified from the iBeetle screen were ordered from Eupheria Biotech GmbH (Dresden, Germany) (see Table 3.1 for iBeetle ID numbers).

3.2.5 *Tribolium* injection

Injections were performed in the penultimate or last larval stage (L6 or L7). The injection method was described in detail in section 2.2.5. Non-overlapping fragments were injected to control for off-target effects (see Table 3.2).

3.2.6 Cloning and sequencing of *Tribolium* genes

Please refer to the procedure in section 2.2.3. The primers for cloning are listed in Table A1. All amplified fragments were cloned into pJET1.2 vector.

3.2.7 RNA probe preparation

Sense and anti-sense Digoxigenin (DIG) labelled RNA probes were synthesized with the DIG RNA labelling mix (Roche Diagnostics GmbH, Manheim, Germany) from gel extraction of PCR products, which were amplified with antisense primers with attached T7 promoter sequence. The T7 RNA polymerase (Roche Diagnostics GmbH, Manheim, Germany) was used for *in vitro* synthesis of RNA probes according to manufacturer's instructions. The RNA probes were dissolved in resuspension buffer [50% deionized formamide, 5× saline sodium citrate (SSC, pH4.5), 20 ug/ml heparin, 0.1% Tween-20] and stored at -20°C until use.

3.2.8 Antenna WMISH

The protocol for antenna whole mount *in situ* hybridization (WMISH) was based on previous methods (Suzuki et al., 2009; Tomoyasu et al., 2009) with a few modifications.

Pre-pupae at the desired stage were identified based on the position of larval eye in the head capsule. After cease of feeding, when the eyes were still in the anterior of the head capsule, this was defined as early pre-pupal stage. When the eyes reached to the middle and posterior of the head capsule, they were at mid- and late pre-pupal stages, respectively (personal communication with Takahiro Ohde). Pre-pupae from these three developmental stages were dissected in chilled phosphate-buffered saline [PBS: 137mM NaCl, 2.683mM KCL, 1.764mM KH_2PO_4, 8.101mM Na_2HPO_4, pH7.4]. The head region was cut off and fixed in 3.7% formaldehyde (FA) at room temperature for 25min. After three washes (5min each) in PBS, the antennae were then dissected out of the larval cuticle. The dissected antennae were washed three times with PTw (0.1%Tween-20 /PBS) and then dehydrated through 25%, 50%, 80% and 100% methanol series in PTw. The tissues can be stored in 100% methanol for months until use. Prior to use, the tissues were rehydrated through washing in 80%, 50% and 25% serial methanol/PTw and then rinsed three times in PTw. A 5min proteinase K (10 µg/ml in PTw) digestion was followed by washes in PTw with 2 mg/ml glycine. After two washes (5min each) in PTw, the tissues were post-fixed in 3.7% FA for 20min. The tissues were then rinsed several times in PTw and subsequently transferred to 65°C pre-warmed HybeB buffer [50% formamide, 5 × SSC (pH5.5), 1% SDS], and then to the 65°C pre-warmed HybeA buffer [50% formamide, 5 × SSC (pH5.5), 1% SDS, 100 µg/ml yeast RNA, 50µg/ml heparin, 200µg/ml sonicated salmon sperm DNA]. After at least 1h of incubation in HybeA buffer at 65°C, the probe was added. Prior to use, the probe was heated to 95°C for 3min, then cooled on ice for 2 min and pre-warmed to 65°C.

After incubation with the RNA probe for 14-20h, the probe was removed and the tissues were washed three times (10min each) at 65°C with pre-warmed HybeB buffer. After one wash with 1 : 1 mix of HybeB : MABT buffer (100mM maleic acid, 150mM NaCl, 0.1% Tween-20, pH7.5), then three washes (10min each) at 65°C with MABT buffer, and three additional washes in MABT buffer at room temperature, the tissues were blocked for 1h in blocking buffer [2% blocking reagent (Roche Diagnostics GmbH, Manheim, Germany) in MABT buffer]. Tissues were incubated overnight at 4°C with anti-DIG-alkaline phosphatase (AP) fab fragments (Roche

Diagnostics GmbH, Manheim, Germany) at a concentration of 1:2000. After washing with PTw buffer several times and then with NB buffer (100mM Tris-HCl, 50mM $MgCl_2$, 100mM NaCl, 0.1% Tween-20), the color reaction was performed using NBT/BCIP as the substrate. Tissues were rinsed in PTw buffer to stop color reaction. Finally, the tissues were mounted on slides in glycerol for observation and image capture.

3.2.9 Image processing and documentation

Images were captured by using a Zeiss Axioplan 2 microscope. Adjustments for brightness and contrast were performed in Adobe Photoshop and figures were assembled in Adobe Illustrator.

3.3 Results

3.3.1 Selection of phenotypes for investigation

To find novel genes involved in antenna patterning in *Tribolium*, I searched for antenna phenotypes in the pupal and adult stages at the iBeetle database and retrieved the detailed information (e.g. protein sequence, phenotypes, ortholog in *Drosophila*, etc.). The genes, which had no orthologs in *Drosophila*, or the *Drosophila* orthologs of which has not been described to affect antenna or leg development, were selected. To preliminarily identify the putative orthologs of the selected genes, reciprocal BLASTp was used for identification. Ten novel genes with antenna phenotypes from the iBeetle database were selected based on these criteria (Table 3.2). To verify the ortholog information from iBeetle database and the reciprocal BLASTp results, the orthologs of these ten genes were further examined by phylogenetic analysis (Fig. A1 in appendix). The results of the detailed phylogenetic analysis were identical to the annotation in iBeetle database and the reciprocal BLASTp results. Nine of these ten genes had orthologs *in Drosophila* while one of them did not. Unexpectedly, only half of them, including *Tc-zld*, *Tc007176*, *Tc013909*, *Tc010410* and *Tc007176*, encoded transcription factors or signaling pathway related proteins according to the protein domain analysis at NCBI Conserved Domain Database (https://www.ncbi.nlm.nih.gov/Structure/cdd/wrpsb.cgi) (Marchler-Bauer and Bryant, 2004; Marchler-Bauer et al., 2017). This proportion was considerably lower than previous studies in *Tribolium* where all of antenna related genes encoded either transcription factors or signaling pathway related proteins (Angelini et al., 2009; Beermann et al., 2001; Ober and Jockusch, 2006; Prpic et al., 2001; Shippy et al., 2009; Smith et al., 2014; Toegel et al., 2008). Several classifications of proteins had never been reported to affect antenna development, including enzymes (Tc-Usp39 and Tc-DNApoly-delta), WD 40 repeats containing proteins (Tc-WDR79 and Tc-rig) and Tc005331. The ortholog of Tc005331 in *Mus* is a survival of motor neuron-related-splicing factor while the information about its *Drosophila* ortholog is lacking.

Genes involved in antenna metamorphosis

Table 3.1 Novel genes investigated in this study.

iBeetle number	Target gene	Symbol	Protein class (NCBI Conserved Domain Database)	Ortholog in *Drosophila melanogaster*
iB_00548	*Tc003290*	*Tc-Usp39*	Ubiquitin protease	*Ubiquitin specific protease 39* (*Usp39*)
iB_00794	*Tc004992*	*Tc-DNApol-delta*	DNA polymerase	*DNA-polymerase-delta* (*DNApol-delta*)
iB_01136	*Tc007176*	*Tc007176*	Zn-finger TXF	*CG6769*
iB_02889	*Tc002221*	*Tc-WDR79*	WD 40 repeat domain	*WD repeat domain 79 homolog* (*WDR79*)
iB_06249	*Tc008111*	*Tc-rig*	WD 40 repeat domain	*rigor mortis* (*rig*)
iB_06393	*Tc005331*	*Tc005331*	Tudor superfamily	*CG17454*
iB_02334	*Tc014798*	*Tc-zld*	Zn-finger TXF	*zelda* (*zld*)
iB_01220	*Tc007562*	*Tc007562*	FGF receptor activating protein	*CG3876*
iB_05521	*Tc013909*	*Tc013909*	Homeobox TXF	*vismay*, *achintya*
iB_01684	*Tc010410*	*Tc010410*	Zn-finger TXF	*no ortholog*

Abbreviations: FGF, fibroblast growth factor; TXF, transcription factor.

3.3.2 Phylogeny of *Tc010410*, a gene without *Drosophila* ortholog

Tc010410 is the only gene without ortholog in *Drosophila*. To detect whether this gene is lineage-specific, the protein sequences of its best hits (query coverage >50% and identity value >24%) among insects were used for constructing a phylogenetic tree. Interestingly, the homologs of *Tc010410* and *Tc012726* (the closest paralog of *Tc010410* in *Tribolium*) were restricted to lineages of Coleoptera and Hymenoptera (Fig. 3.4). Although *Tc010410* cannot be classified as an orphan gene according to the strict definition (i.e. genes without detectable sequence similarity in the genomes of other organisms) (Khalturin et al., 2009), it clearly has a lineage-restricted distribution, which opens the possibility that this gene was recruited recently to antenna development. The orthologs of *Tc032650* and *Tc007057* as outgroups were present in a wider range of insect lineages.

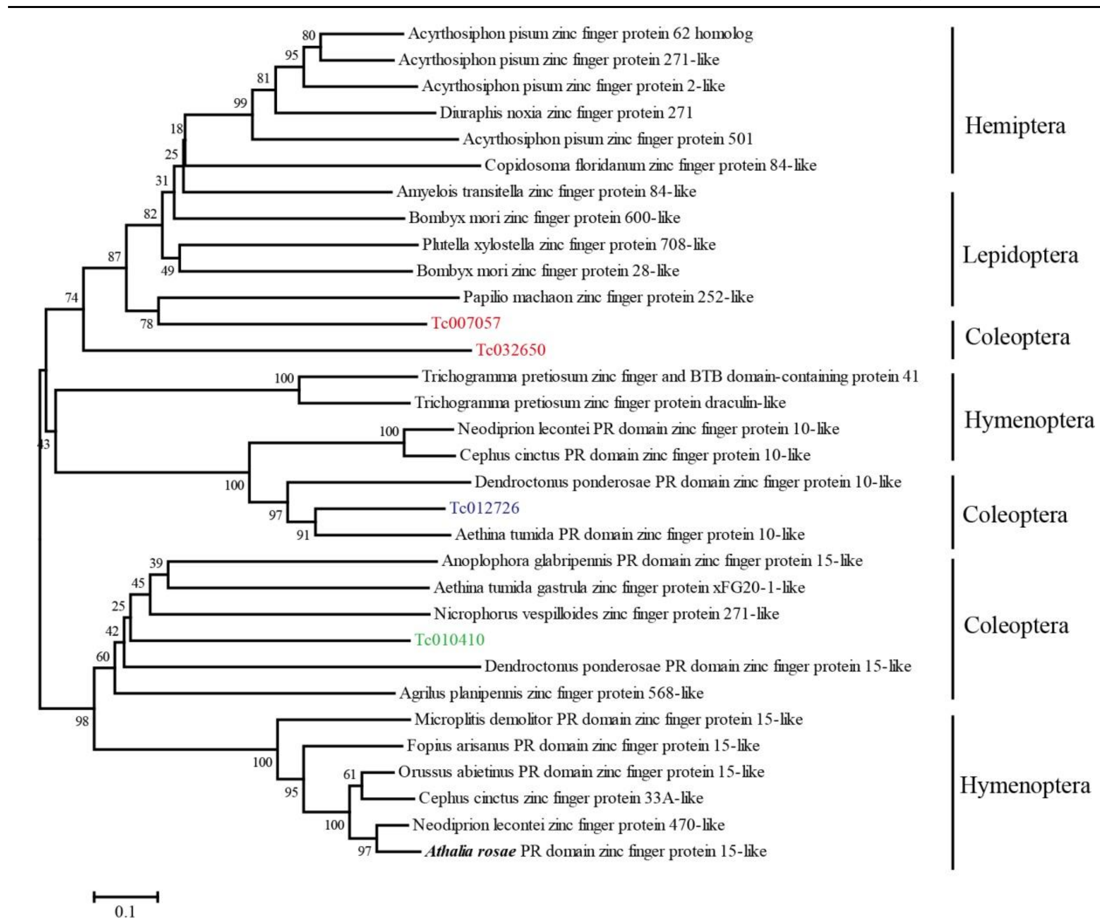

Figure 3.4 Phylogenetic tree of gene *Tc010410* and its *Tribolium* paralogs *Tc012726*, *Tc032650* and *Tc007057*. The numbers on the branching points show reliability values of 1000 bootstrap repetitions. *Tc010410* is shown in green, *Tc012526* is shown in blue, *Tc032650* and *Tc007057* are shown in red.

3.3.3 Phenotype confirmation by injection of non-overlapping dsRNA fragments

To avoid potential off-target effects and to further confirm the phenotypes, both iBeetle fragment and a non-overlapping fragment (NOF) were injected into larvae of *San Bernardino* (*SB*) strain. dsRNAs were titrated to different concentrations according to different genes to avoid lethality before pupation. The antenna phenotypes of all ten genes were confirmed with at least 50% phenotypic penetrance (Table 3.2). Moreover, three of them were found to affect the leg development as well (Table 3.2). Hence, most genes were specifically involved in the patterning of antenna, but not its ventral serial homology-leg.

Table 3.2 Reproducibility and frequency of phenotypes.

iBeetle number	size (bp)	Concentration (µg/µl)	Number of injected animals	Survival to pupa	Phenotypic penetrance	
					antenna	leg
iB_00794	472	0.2	19	58%	73%	36%
iB_00794_2	278	0.5	11	18%	100%	100
iB_00548	331	0.2	21	48%	100%	58%
iB_00548_2	260	0.2	10	30%	100%	66%
iB_02334	476	1	20	95%	100%	100%
iB_02334_2	225	1	15	40%	100%	67%
iB_02334_3	236	1	13	46%	100%	83%
iB_01684	409	1	23	100%	100%	0
iB_01684_2	190	1	12	92%	55%	0
iB_06249	471	0.5	25	96%	100%	0
iB_06249_2	254	1	12	50%	100%	0
iB_02889	476	0.5	20	55%	91%	0
iB_02889_2	280	0.2	13	69%	67%	0
iB_06393	483	0.5	18	72%	85%	0
iB_06393_2	226	0.2	13	77%	50%	0
iB_05521	515	1	20	85%	100%	0
iB_05521_2	580	1	13	77%	100%	0
iB_01220	491	0.5	22	82%	100%	0
iB_01220_2	146	1	12	67%	75%	0
iB_01136	470	1	13	77%	100%	0
iB_01136_2	244	1	11	45%	100%	0

Phenotype penetrance: proportion of pupae with antenna phenotype in the individuals which could pupate.

3.3.4 Three genes mainly promote the growth and elongation of the antenna along the entire PD axis

Previous studies showed that the growth and elongation along the PD axis of the antenna are mainly promoted by *Tc-krn*, *Tc-ser*, and *Tc-Dll* (Angelini et al., 2009). Here three novel genes, *Tc-zld*, *Tc-DNApoly-delta*, and *Tc-005331*, were identified to be involved in this process since RNAi targeting on each of them induced severe reduction in the length of antenna (Fig. 3.5B-D). RNAi against *Tc-zld*, a zinc finger

transcription factor involved in zygotic genome activation in *Drosophila* (Liang et al., 2008), resulted in severe reduction in antenna length (Fig. 3.5B). The three antennomeres of the club fused and the overall number of antennomeres was reduced. The scape-pedicel joint was eliminated, but the pedicel-flagellum and flagellum-club joints were relatively unaffected. Phenotypes generated by RNAi against *Tc-DNApoly-delta* showed a similar reduction in length and number of articles (Fig. 3.5C). In weak phenotypes the defects appeared only in the funicle region. In severely affected *Tc-DNApoly-delta* knock-down animals, the antenna was strongly reduced and most of joints failed to form. Depletion of *Tc-007562* closely resembled the phenotype seen in *Tc-DNApoly-delta* RNAi individuals. In severely affected knock-down animals, the flagellum became shorter and wider compared with wild-type and the flagellar joints were absent. However, the scape-pedicel and pedicel-flagellum joints formed normally (Fig. 3.5D). *Tc-zld and Tc-DNApoly-delta* were the only genes which affected the development of scape and pedicel in addition to flagellum.

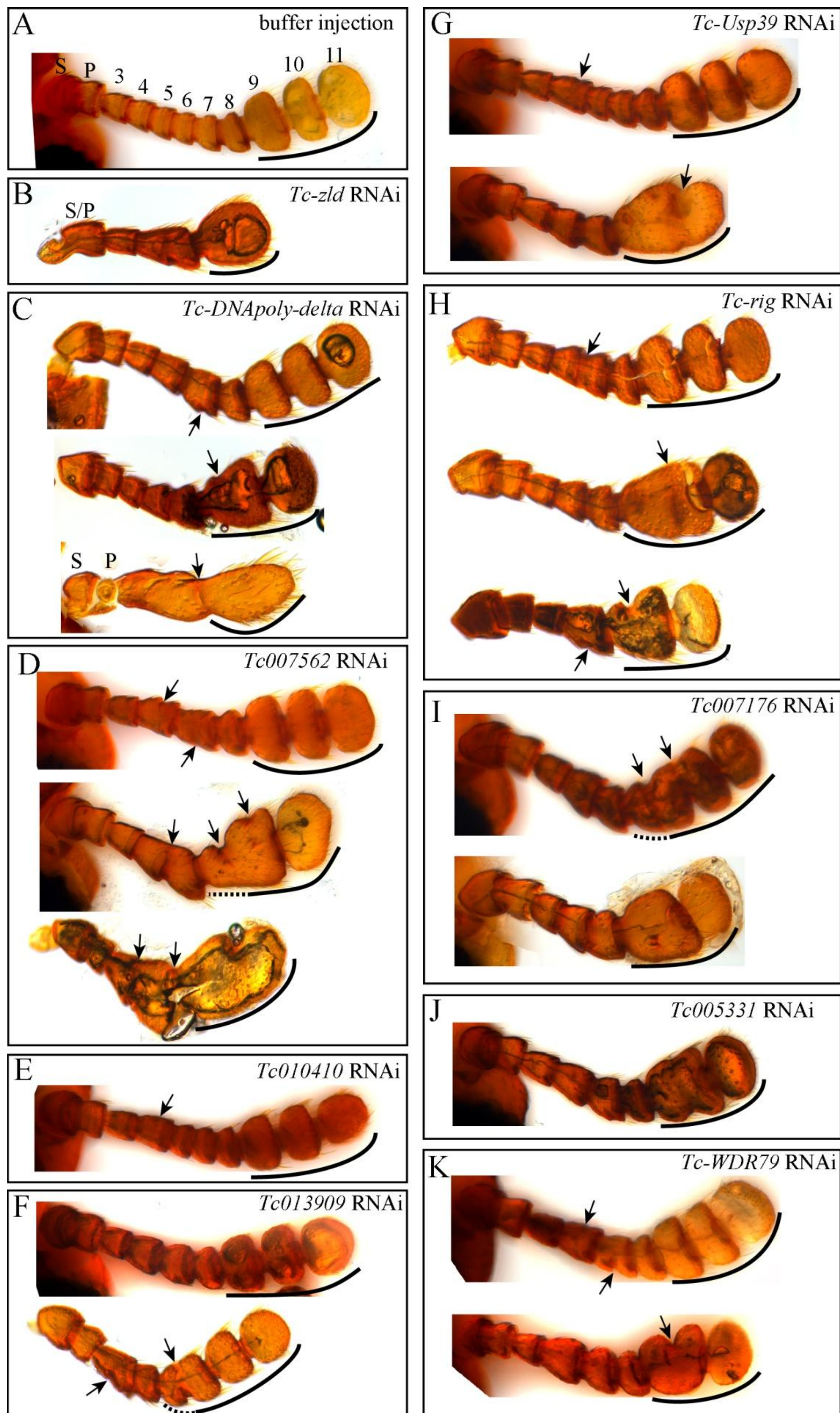

Figure 3.5 Antennal RNAi phenotypes. All antennae are oriented with the distal region to the right. (A) Control treatments were indistinguishable from wild-type beetles. Wild-type antenna are composed of scape (S), pedicel (P) and flagellum which is subdivided into six articles of the funicle and three articles of the club. These club articles are enlarged and equipped with a ring of macrochaetes. The club is marked by a black line. (B) *Tc-zld* RNAi caused a severe reduction of

antenna length and number of articles. It affected all parts of the antenna. (C) Depletion of *Tc-DNApoly-delta* induced a reduction of antenna length and a failure of joint formation. Mild phenotypes showed a fusion of the articles of the funicle. Strong phenotypes showed a severe reduction in length and lack of most joints. Arrows mark the fusion of articles. The phenotype affected all parts of the antenna. (D) RNAi against *Tc007562* caused a reduction of antenna length and a fusion of joints of club and funicle. Mild phenotypes showed only fusion of funicle articles without affecting on the length. Strong phenotypes showed severe reduction of length and fusions in the funicle region in addition to fusions within the club. However, the scape and pedicel appeared to be unaffected. The region where the identity is uncertain is marked with dashed black line. (E) *Tc010410* RNAi caused only a fusion of the joint between funicle articles 4-5. (F) *Tc013909* RNAi reduced antenna length and article number. Strong phenotypes showed a fusion of adjacent articles. (G) Depletion of *Tc-Usp39* induced a reduction in length and article numbers of funicle and fusions within the club. The 4/5 joint was mostly reduced in mild phenotypes. Strong phenotypes showed s drastic reduction in length and numbers of articles in addition to fusions within the club. (H) RNAi against *Tc-rig* showed a reduction in length and fusions of funicle articles in weak phenotypes. In moderate and strong phenotypes, the proximal two club articles fused in addition to strong reduction in length and the articles number within funicle. (I) *Tc007176* RNAi caused reduction in length and article number of the flagellum and partial fusion of adjacent flagellum articles. (J) *Tc005331* RNAi resulted in reduction in length and articles number within funicle and partly fusion of proximal two club articles. (K) In weak phenotypes of Tc-WDR79 RNAi, the 4/5 and 6/7 joints were largely reduced. In strong phenotypes, fusion of proximal club region was found in addition to the reduction in length and fusions of adjacent articles within funicle.

3.3.5 Two genes function specifically within the funicle region

Among the ten confirmed antenna related genes, two genes specifically affected the region of the funicle. RNAi against *Tc010410*, encoding a repeated C2H2 Zn-finger transcription factor, resulted in the fusion of 4-5 articles but no other defects (Fig. 3.5E). In mild phenotypes of *Tc013909* RNAi, the reduction of antenna length was accompanied by the reduction of the number of funicle articles, and in strong phenotypes, additional articles were fused or lost from the funicle area and the formation of funicle-club joint was partially affected (Fig. 3.5F). This gene was predicted to encode a transcription factor with the homeobox KN domain conserved from fungi to human and plants (Bürglin, 1997; Mukherjee and Bürglin, 2007; Mukherjee et al., 2009).

3.3.6 Genes that affect the entire flagellum

RNAi revealed a function for several genes in the development of both the funicle and the club, including *Tc-Usp39*, *Tc-rig*, *Tc-007176*, *Tc-005331* and *Tc-WDR79* (Fig. 3.5G-K). Fusion of the entire club was only found in *Tc-Usp39* RNAi phenotypes (Fig. 3.5G). RNAi against the remaining four genes caused fusions of the proximal two club articles (9-10), but the distal club remained unaffected (Fig. 3.5H-K). In all cases, there was no substantial effect on the size of the club. The overall reduction in length was achieved by the reduction in article number and length in the funicle region. In severely affected individuals, a greater reduction in length was observed for *Tc-Usp39* and *Tc-rig* than for *Tc-005331* and *Tc-WDR79* (Fig. 3.5G, H, J, K).

Among all these ten genes, phenotypes affecting the entire antenna were observed only by two genes (*Tc-zld* and *Tc-DNApoly-delta*), which were also the genes affecting scape and pedicel development in addition to the flagellum. Two genes (*Tc010410* and *Tc013909*) were found to function specifically within the funicle region. For the remaining six genes, phenotypes affecting the entire flagellum were observed in knock-down animals. Fusion or loss of articles was the most frequently observed phenotypes in RNAi individuals, usually leading to reduction in length and/or deformation of antennae in specific region.

3.3.7 Genes required primarily in the tarsus

As serially homologous structures, leg and antenna share many aspects of developmental patterning within a single species (Abzhanov and Kaufman, 2000; Angelini and Kaufman, 2005a; Angelini et al., 2012a; Beermann et al., 2001; Palopoli and Patel, 1998; Panganiban et al., 1997; Prpic and Damen, 2009). In contrast, divergent developmental genetic processes have been found as well with respect to the development of diverse appendage morphologies along the PD axis across body segments (Angelini and Kaufman, 2005b; Angelini et al., 2012a; Jockusch et al., 2004; Ronco et al., 2008; True and Haag, 2001). To test the conserved and diverged aspects of the developmental processes between antenna and leg, I also examined the functions of these novel antenna related genes in leg patterning in *Tribolium*.

Depletion of three genes yielded phenotypes that were restricted to the tarsal region, whereas most genes played no roles in leg development during metamorphosis.

RNAi targeting the transcription factor *Tc-zld* induced leg defects that were most pronounced in the proximal tarsus (Fig. 3.6B and Table 3.2). The phenotypes included fusions and reduction of tarsomeres, leading to the reduction of tarsal length. Incomplete fusion of proximal tarsomeres occurred in the prothoracic and mesothoracic tarsus while in the metathoracic tarsi, the tarsomeres 1-3 were fused completely. The joint between tarsomeres 4-5 (or 3-4 in the metathoracic tarsi) was still present but mildly affected.

Reduction of *Tc-Usp39* function also resulted in the loss of joints, fusion of adjacent tarsomeres and reduction of length (Fig. 3.6C). Fusion occurred throughout all tarsal regions in prothoracic tarsi. This was different from the phenotypes in mesothoracic and metathoracic tarsi where the defects were restricted to the proximal tarsomeres, while the two distal tarsomeres were comparably less affected. Depletion of *Tc-DNApoly-delta* produced incompletely fused tarsomeres throughout all thoracic tarsi in addition to an irregular expansion of the proximal region of tarsus in metathoracic segment (Fig. 3.6D).

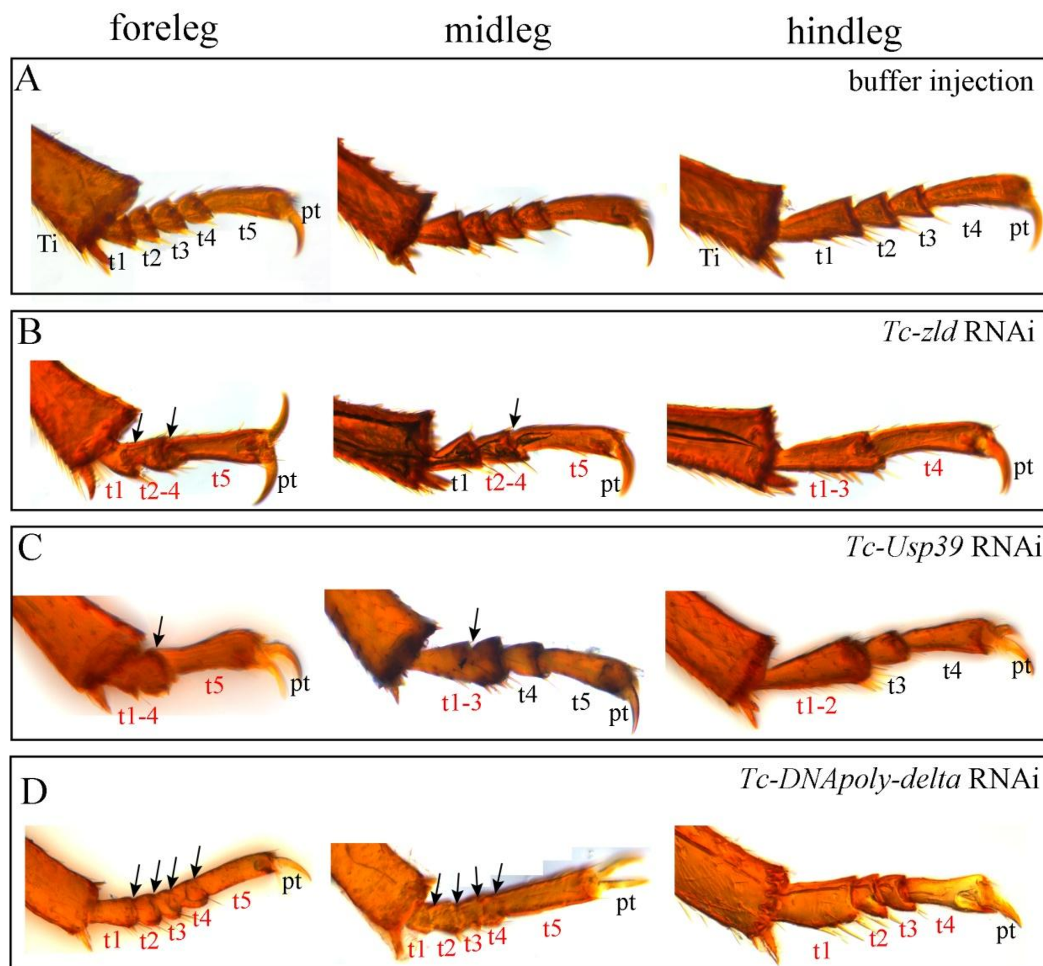

Figure 3.6 RNAi effects on adult tarsi. (A) Buffer injected individuals showed wild-type morphology. There are five tarsomeres in a normal tarsus of the pro- and mesothoracic legs, whereas metathoracic legs have four tarsomeres. (B) Depletion of *Tc-zld* produced reduction and fusion in the proximal tarsus (black arrows). (C) *Tc-Usp39* RNAi caused severe reduction and fusion in proximal tarsi of foreleg and moderate fusion of proximal tarsi of mid- and hindlegs. (D) RNAi targeting *Tc-DNApoly-delta* eliminated most of the joints in the tarsi. The joint and shape of tarsus in hindleg were mildly deformed. Structures with defect are labeled in red. Abbreviations: Ti, tibia; t1-5, tarsomeres 1-5; pt, pretarsus.

3.3.8 Overview on antenna metamorphosis

How the antennal morphology gradually changes during metamorphosis is still unknown. To visualize this process, the remodeling antennae were dissected out from the head capsule at different pre-pupal stages. After ceasing of feeding the final instar larvae develop into the pre-pupal stage. The larval eyes under the larval cuticle migrate gradually to the posterior of the head capsule before the adult ommatidia

appear. Based on the position of larval eyes the pre-pupal stage is divided into three stages: early, mid and late (Fig. 3.7A-C) (personal communication with Takahiro Ohde). At the early pre-pupal stage, the size of antenna was comparably smaller and with an elongated distal part. Then, the proximal-middle region of antenna gradually expanded and the distal region contracted, became rounded and probably integrated into the pupal antenna during growth and remodeling of the antenna. The antenna morphology at late pre-pupal stage was very similar to that of pupae.

In order to describe the development of the antenna based on gene expression patterns, a WMISH protocol for developing antennae was optimized by referring to previous methods established for antennae, legs and wings (Suzuki et al., 2009; Tomoyasu et al., 2009) (see details in materials and methods). The transcription factor *Tc-Dll* and the Notch ligand *Tc-ser* were used as positive controls for the functionality of the ISH and to describe development, because the expression pattern of *Tc-Dll* in antenna was studied before and the phenotypes of *Tc-ser* RNAi indicated an function in formation of all joints and elongation of antenna (Angelini et al., 2009; Suzuki et al., 2009).

Tc-Dll was expressed in middle to distal regions of antennae with different expression intensity along the proximal-distal axis (Fig. 3.7G-H), which was identical to the expression pattern reported previously (Suzuki et al., 2009) and in line with its phenotypes all antennal segments distal to the scape are missing (Angelini et al., 2009).

In the antenna, *Tc-ser* expression was detected throughout most of the antenna, but it was particularly prominent in the presumptive joints. Expression was absent from the distal elongated part of the antenna at the early pre-pupal stage (Fig. 3.4D). At the late pre-pupal stage, the expression of *Tc-ser* expanded to the distal antenna (Fig. 3.4F). This expression pattern of *Tc-ser* was in line with its function in the antenna, where the antenna was severely shortened and the joints were completely absent (Angelini et al., 2009).

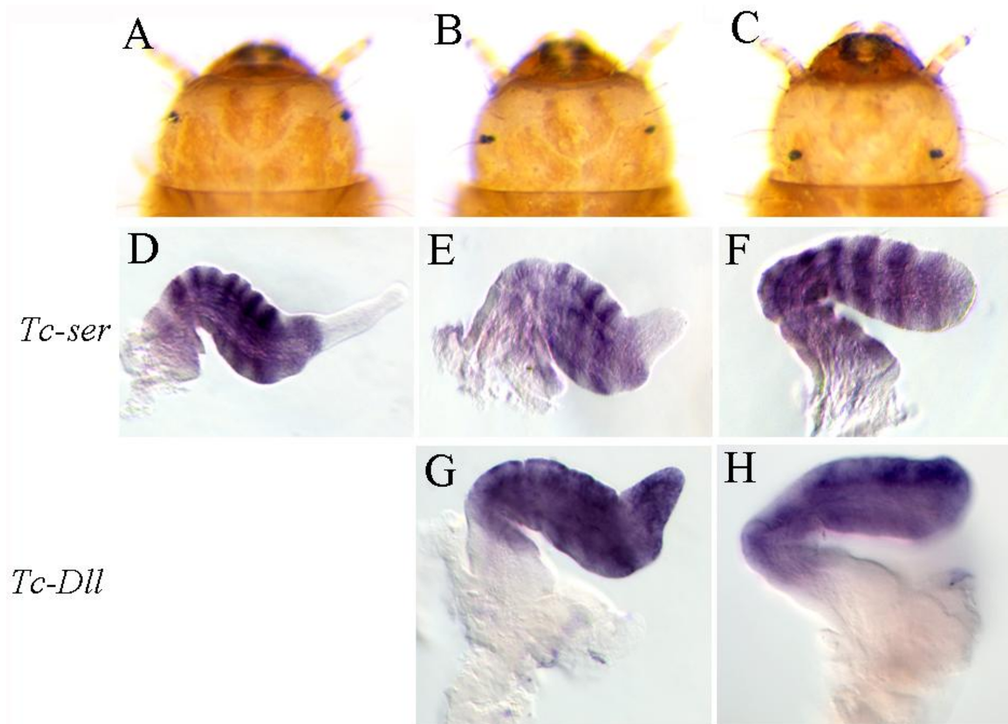

Figure 3.7 Expression of *Tc-ser* and *Tc-Dll* in pre-pupal antenna. (A-C) Staging of the pre-pupa into early (A), mid (B) and late (C) according to the position of the eyes. (D-F) The expression of *Tc-ser* in the antenna throughout these stages. (G-H) The expression pattern of *Tc-Dll* in the antenna. See details in section 3.3.8.

3.3.9 Expression pattern of the novel genes in remodeling antenna

I detected the expression patterns of four of the novel genes during the pre-pupal stage. At the early pre-pupal stage, *Tc-zld* was mainly expressed in the proximal and middle region of the antenna but not in the more fine distal part (Fig. 3.8A). Then the expression expanded throughout the antenna in the mid pre-pupal stage (Fig. 3.8B). Ultimately, the expression was prominent in the presumptive joints of distal club (Fig. 3.8C), which probably correlated with its RNAi phenotype: the club region was completely fused (Fig. 3.5B). Former studies showed that joint formation within antennae was mediated by Ser-Notch signaling in *Tribolium* (Angelini et al., 2009, 2012a, 2012b). The expression of *Tc-zld* in a subset of the presumptive joints suggests that Ser-Notch signaling may interact locally with *Tc-zld* to specify the specific joints. The reduction of antenna length and joint formation in *Tc-zld* RNAi and its expression patterns in expanding areas of the antenna suggest that *Tc-zld* activity could be necessary for cell divisions. This would be in line with its function

during embryonic development in *Drosophila*. *Dm-zld* encodes a key activator of the early zygotic genome and its deficiency disrupts the pattern of mitotic waves in preblastoderm embryos and causes improper chromosome segregation in *Drosophila* (Liang et al., 2008; Staudt et al., 2006).

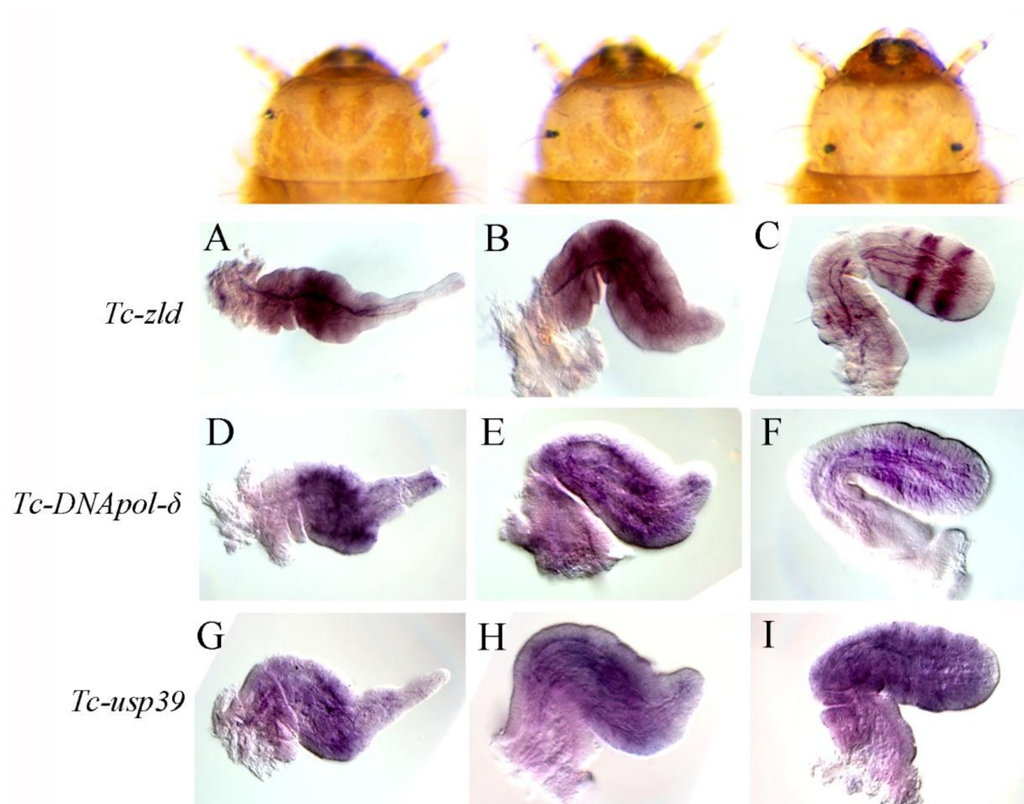

Figure 3.8 Expression of newly identified genes in pre-pupal antenna. A-I: The expression of *Tc-zld* (A-C), *Tc-DNApoly-delta* (D-F), and *Tc-usp39* (G-I) in the antenna throughout three pre-pupal stages, respectively.

Tc-DNApoly-delta and Tc-usp39 showed similar expression patterns (Fig. 3.8D-F and G-I). They were mainly expressed in the middle region of the antenna at the early pre-pupal stage and then in middle and distal regions at mid- and late pre-pupal stages (Fig. 3.8E, F, H, I). This result indicated that both genes have a regionalized function in remodeling antenna.

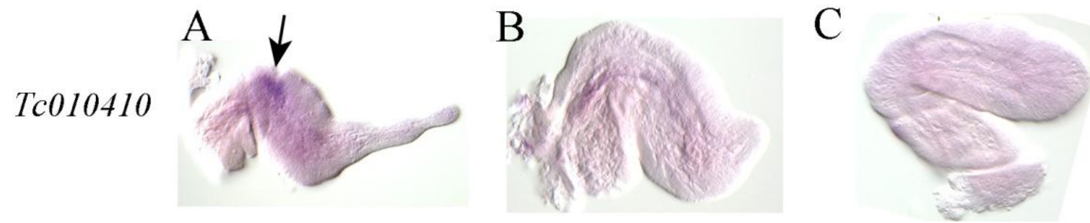

Figure 3.9 The expression of *Tc010410* in metamorphic antenna. A-C: Expression patterns of *Tc010410* in antenna at the early (A), mid (B) and late (C) pre-pupal stages. Arrow marks the position with relatively strong intensity of expression.

Strong expression of *Tc010410* at the early pre-pupal stage was restricted to a small area in the middle antenna (Fig. 3.9A). I did not find any detectable expression at the mid- and late pre-pupal stages (Fig. 3.9B, C). The expression reflected a specific function in the middle region of the antenna. This might correlate with the very localized RNAi phenotype where only the articles 4 and 5 were fused. Based on these results and the *Tc-ser* staining, it appears that this region is specified to form joint between articles 4-5 (Fig. 3.5E).

For these three genes, the region of expression appeared to correlate well with functions. For none of them, a long-range signaling (like Dpp and Wg) was likely (Nellen et al., 1996; Neumann and Cohen, 1997; Zecca et al., 1996).

3.4 Discussion

3.4.1 Novel genes specified the antenna metamorphosis

The developmental genetics of appendage formation are more completely described in *Drosophila* than in any other insects. However, *Drosophila* maintains a highly derived developmental system (Truman and Riddiford, 1999) and may not retain the ancestral state for appendage patterning. Comparative studies in *Tribolium* provided insight into the likely ancestral mode of antenna development by the candidate gene approach (Angelini et al., 2009, 2012a, 2012b; Jockusch et al., 2004; Ober and Jockusch, 2006; Smith et al., 2014; Suzuki et al., 2009). During antenna metamorphosis, different genes were involved in different processes of the remodeling process: from the maintenance of the antenna identity through the elongation and differentiation of antennomere to joint formation (Angelini et al., 2009; Smith et al., 2014; Suzuki et al., 2009). The majority of the antenna related genes revealed so far are transcription factors and the remaining genes encode components of signaling pathways, for instance, Ser, a Notch ligand (Artavanis-Tsakonas et al., 1995) and Krn, the only activating EGF ligand present in *T. castaneum* genome (Tribolium Genome Sequnecing Consortium, 2008).

In this study, novel classes of genes involved in antenna patterning during metamorphosis were revealed. Surprisingly, the orthologs of four genes, including *USp39* (also known as *U4/U6.U5 tri-snRNP-associated protein 2* in *Mus*), WDR79 (also known as *telomerase Cajal body protein 1* in *Mus*), *Tc005331*, *rig*, were reported to participate in the same biological process: precursor messenger RNA (pre-mRNA) splicing via spliceosome (Gubitz et al., 2002; Lau et al., 2009; van Leuken et al., 2008; Makarova et al., 2001; Meister et al., 2001; Mount and Salz, 2000; Talbot et al., 1998; Workman et al., 2015). Pre-mRNA splicing is a critical step in the posttranscriptional regulation of gene expression, providing significant expansion of the functional proteome of eukaryotic organisms with limited gene numbers (Keegan et al., 2001; Maniatis and Tasic, 2002). This was the first time that pre-mRNA splicing related genes were revealed to be involved in antenna metamorphosis, suggesting

this progress of gene regulation might be of importance in appendage diversification.

zld is an example for a gene with different functions in different species. As a member of the C2H2 zinc finger transcription factor, it is strongly expressed in mitotically dividing cells and its activity is necessary for normal mitotic cell divisions in *Drosophila* (Liang et al., 2008; Staudt et al., 2006). During postembryonic development, gain- and loss-of-function experiments showed that *zld* is essential for proper wing development but not antenna (Giannios and Tsitilou, 2013). In contrast, *Tc-zld* RNAi showed its function specifically in antenna and leg but not in the wing of *Tribolium*, reflecting the divergent roles in these two species. Interestingly, the *Drosophila* antenna is shortened and has fewer antennomeres than the *Tribolium* antenna. It is tempting to speculate that the loss of *zld* function in *Drosophila* antenna development was involved in the reduction of this antenna.

3.4.2 Taxonomically restricted gene for the morphological trait of antenna.

Taxonomically restricted genes were demonstrated to be important for the evolution of lineage specific traits and drive morphological specification, thus enabling organisms to adapt to changing conditions (Harpur et al., 2014; Johnson and Tsutsui, 2011; Khalturin et al., 2008, 2009) . Here, one taxonomically restricted gene (*Tc010410*) was found to have a role in antenna metamorphosis. The orthologs of this gene are restricted to the order of Coleoptera and Hymenoptera, which might indicate the specific role in these species. Despite the fact that the number of antennomeres is highly variable between insect species, the total number of antennomeres is largely but not universally fixed within the group of Coleoptera, most of which have antenna of 11 antennomeres (Minelli, 2005). The specific expression and corresponding function in remodeling the antenna might reflect a lineage specific evolutionary process of antenna in Coleoptera, which needs to be further tested in other Coleopteran species.

3.4.3 Relatedness of antenna elongation and joint formation.

Previous studies revealed three developmental processes of antenna metamorphosis in *Tribolium*: (1) maintenance of antenna identity by the activation of *Tc-Dll* and *Tc-ss* (Angelini et al., 2009; Lee et al., 2013; Smith et al., 2014). (2) Growth and elongation of antenna promoted by several factors (e.g., *Tc-krn*, *Tc-Dll*, *Tc-ser*) (Angelini et al., 2009; Smith et al., 2014). (3) Joint formation is initiated through ser-Notch signaling (Angelini et al., 2009). The results presented here revealed two more factors (*Tc-zld*, *Tc-DNApoly-delta*) for the second process. RNAi targeting these two genes resulted in severe reduction in the length of the antenna, suggesting they act to promote growth and elongation along the PD axis. The remaining genes also affected the growth of the antenna but to lesser degree. Interestingly, most of the genes found here promoted both growth and joint formation either in the entire antenna or within specific areas, which was in line with a previous study that growth and joint formation were linked developmentally in the funicle (Angelini et al., 2009). A general inter-relatedness of antenna growth and joint formation was observed in RNAi phentypes of most genes. Fusion of antennomeres or reduction of the number of antennomeres was one of the most common phenotypes observed, but most phenotypes were observed in specific regions.

In *Drosophila*, Notch signaling pathway mediates the joint formation (Bishop et al., 1999; Rauskolb and Irvine, 1999), which is conserved in *Tribolium* (Angelini et al., 2009). This model has been proposed as a defining characteristic of arthropods (Prpic and Damen, 2009). Elimination of *Tc-ser*, the Notch ligand, leads to the loss of joints and severe reduction of the length. The functional study of the novel genes here raises the possibility that they appears to interact with Notch signaling locally to promote antenna growth and guide the joint formation, leading to the coupling of two processes: antenna elongation and joint formation within specific areas.

3.4.4 The evolution of serially homologous appendages

The most remarkable feature of arthropod body plans is that they are formed by a series of appendage-bearing segments. Appendages have diversified both along the body axis within species and between species. Understanding the evolutionary

developmental basis of this variation is essential for addressing questions about the evolutionary diversification of limbs. Serial homologs are similar morphological structures that are repeated at different positions within an organism's body plan (Owen, 1848). Among arthropods, ventral appendages share many, but not all, aspects of developmental patterning (Abzhanov and Kaufman, 2000; Angelini and Kaufman, 2005b; Angelini et al., 2012a, 2012b; Beermann et al., 2001; Jockusch et al., 2000; Palopoli and Patel, 1998; Prpic and Damen, 2009; Rogers et al., 2002; Smith et al., 2014). These studies support the dependent model for the evolution of developmental mechanisms controlling serially homologous appendages, which states that changes to the patterning network of one appendage in a certain species would also cause changes in the patterning network of other serially homologous appendages in the same species. This leads to a greater similarity in patterning of serial homologs in different segments within one species than of direct homologous appendages between species. This model assumes that pleiotropic functions act as a strong constraint.

This appears to be true for only three of ten genes (*Tc-zld*, *Tc-DNApoly-delta* and *Tc-Usp39*), which affected the patterning of serial homologs (antenna and leg) (Fig. 3.5 and 3.6). In the case of *zld*, differences were observed between species. *zld* is required for the patterning and development of wing imaginal disc in *Drosophila* (Giannios and Tsitilou, 2013). Although it is expressed in the eye-antenna imaginal disc (Staudt et al., 2006), no morphological defect of the antenna was reported. By contrast, *zld* involved in both antenna and leg metamorphosis in *Tribolium*. This is in line to the model above. However, the majority of these ten novel genes showed specific functions in antennae but not legs, which suggests that gene functions may evolve independently among serial appendages. Hence, the independent model is supported to a limited extent in contrast to that there is a significant portion of appendage diversification may relate to the function of appendage specific genes. This model may have been overlooked because of the prevailing candidate gene approach. Additionally, the taxonomically restricted gene with antenna specific function suggests a recent change in developmental patterning that affect only one serial homolog.

The functional comparisons between antenna and leg suggest a complex mix of divergence and constraint among appendages. And the study of antenna patterning in *Tribolium* probably reflects a relatively representative model among insects. However, comparative studies in diverse species and serial homologs will help us to elucidate the evolutionary developmental mechanisms of morphological diversification. Therefore, insect typical antenna development could be studied in *Tribolium* first (because of the possibility of RNAi screen) and then compared to other speicies (e.g. *Gryllus* and others).

3.4.5 Outlook

Whether the functions of novel genes presented here are evolutionary ancestral need to be further studied. One or more hemimetabolous insects, like *Blattella germanica* or *Gryllus bimaculatus*, should be selected to test the function of these genes in patterning appendage formation. Due to their basal position within the phylogeny, they would provide insight into the putative ancestral situation of appendage formation. Further, other beetles and holometabolous insects (e.g. horn beetles, hymenopterans) should be studied to determine the ancestral state of development during metamorphosis.

What is the developmental mechanism of the novel genes for antenna patterning was not studied in this work. In the case of *zld*, it binds specifically to the "TAGteam" sites to activate transcription of the early zygotic genome, including many genes essential for cellularization, sex determination and pattern formation in *Drosophila* (Bosch et al., 2006; Liang et al., 2008). The further work should test how *Tc-zld* directs the antenna remodeling during metamorphosis in *Tribolium*. To identify which genes are regulated by *Tc-zld*, RNA-seq and Assay of Transposase-Accessible Chromatin with high throughput sequencing (ATAC-seq) can be carried out to screen out target genes with more specificity. From all the candidate genes, the previously confirmed *Tribolium* antenna genes would be tested firstly as potential target genes of *Tc-zld*.

It is generally accepted that the larval and adult limb are developmentally coupled in terms of morphology and patterning in most insect species (Lee et al., 2013; Nagel,

1934; Tanaka and Truman, 2005; Truman and Riddiford, 1999, 2002). However, there are plenty of questions that remain to be answered: What is the fate of larval limb epidermis? Specifically, which part of the larval limb epidermis undergoes apoptosis and which part contributes to the adult limb? How is the larval epidermis remodeled to contribute to the adult epidermis? To answer these questions, EdU cell proliferation labeling and apoptosis caspase antibody staining approaches can be combined to detect the cellular dynamic during metamorphosis. The cell division can be compared with and without RNAi of antenna related genes as well. Additionally, to which extent does the re-patterning phase of the *Tribolium* antenna during metamorphosis rely on the same genes that are used during embryonic antenna patterning? The genes involved in embryonic antenna patterning can be tested the function for antenna remodeling during the process of metamorphosis.

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5. Appendix

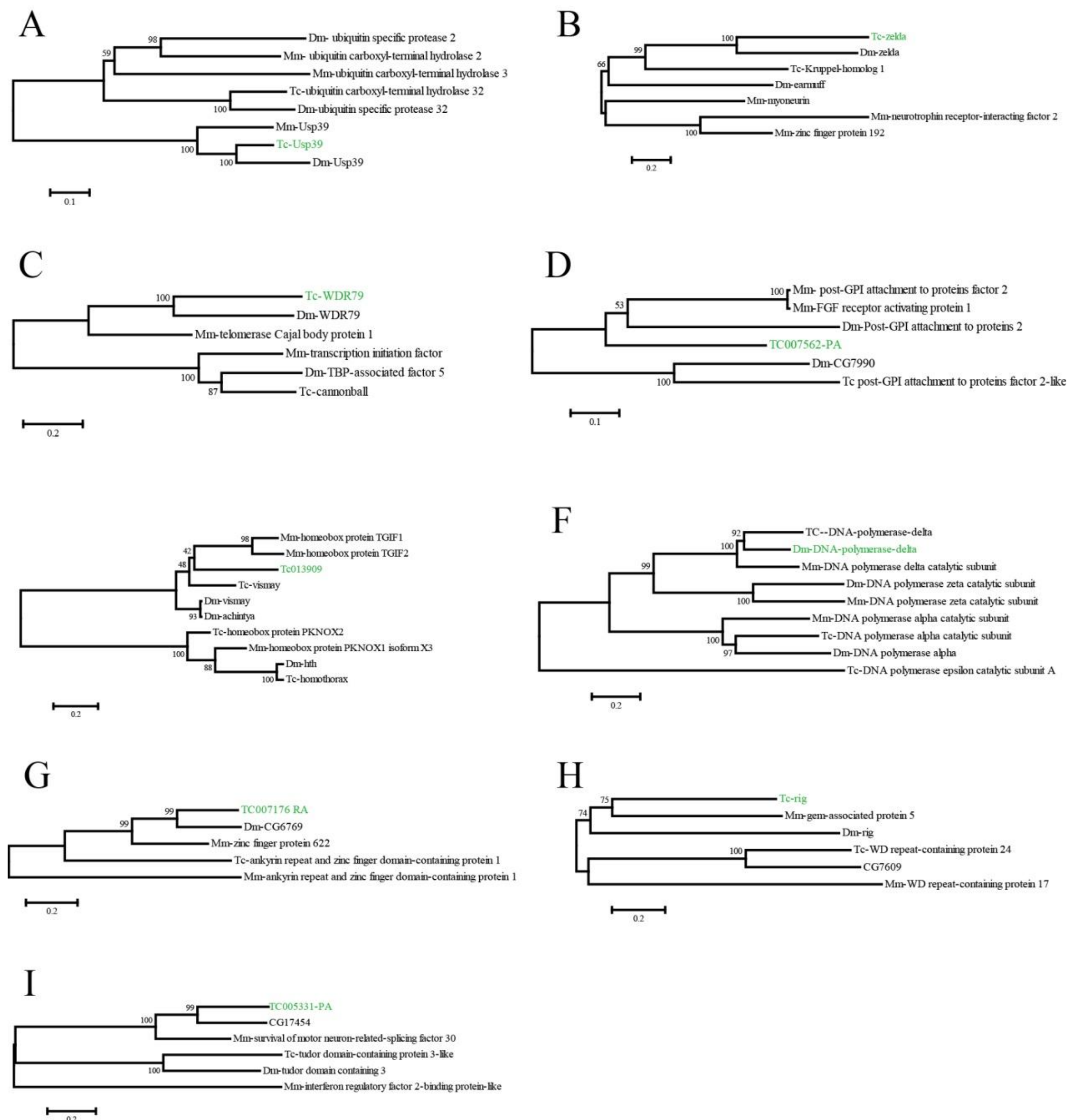

Figure A1 Phylogenetic tree of homologs of nine novel genes. The number on the branching points are reliability values of 1000 times bootstrap. The nine novel genes were notated in blue color. Abbreviation: Tc, *Tribolium castaneum*; Dm, *Drosophila melanogaster*; Mm, *Mus musculus*.

Appendix

Table A1 Primer sequences used in this study.

primer name	Sequence	use
Tc-bursF	ACTCTTACTTCGAGCAGAGC	Cloning of the region used for RNAi
Tc-bursR	CGATTTTCTTATCGAGCATG	
Tc-pburF	ACAGAGAATATGCAATGGGGAGG	Cloning of the region used for RNAi
Tc-pburR	TCGGCTGAAATCGCCACACT	
Tc-UbxF	ACTCTTACTTCGAGCAGAGC	Cloning of the region used for RNAi
Tc-UbxR	GGTGTATCTGCGTGCCAAC	
T7-pJETF	GAATTGTAATACGACTCACTATAGGCGA CTCACTATAGGGAGAGC	amplify the template for *in vitro* transcription to generate dsRNA
T7-pJETR	TAATACGACTCACTATAGGAAGAACATC GATTTTCCATGGCAG	
T7	GAATTGTAATACGACTCACTATAGG	
T7-sp6	TAATACGACTCACTATAGGATTTAGGTG ACACTATAGA	
T7-T3	TAATACGACTCACTATAGGAATTAACCCT CACTAAAGGG	
iPCR5'F1	GACGCATGATTATCTTTTACGTGAC	1st round PCR after Bsp143I treatment
iPCR5'R1	TGACACTTACCGCATTGACA	
iPCR5'F2	GCGATGACGAGCTTGTTGGTG	2nd round PCR after Bsp143I treatment
iPCR5'R2	TCCAAGCGGCGACTGAGATG	
iPCR5'Seq	CGCGCTATTTAGAAAGAGAGAG	sequencing
iPCR3'F1	CAACATGACTGTTTTTAAAGTACAAA	1st round PCR after HhaI treatment
iPCR3'R1	GTCAGAAACAACTTTGGCACATATC	
iPCR3'F2	CCTCGATATACAGACCGATAAAAC	2nd round PCR after HhaI treatment
iPCR3'R2	TGCATTTGCCTTTCGCTTAT	

iPCR3'Seq	CGATAAAACACATGCGTCAAT	Sequencing
Tc-Usp39F	CAGTACATTGGACGTTAGCAC	Cloning of the region used for RNA probe generation
Tc-Usp39R	TCGCCACTAGATCATAGACC	
Tc-DNApol-delta F	AAACGCAATCTTCCGCCTCCG	Cloning of the region used for RNA probe generation
Tc-DNApol-delta R	AAACGCAATCTTCCGCCTCCG	
TC010410F	TGTTACTTGCCGAATGTTGC	Cloning of the region used for RNA probe generation
TC010410R	TCTATCTCGAAATTGAAATGGTC	
Tc-zldF	CACCGACTCCTGCCTATCACC	Cloning of the region used for RNA probe generation
Tc-zldR	GCTGTTGTTGGCGTCCTCCT	

Table A2 Summary of iBeetle ID number, target gene and dsRNA length.

iBeetle number	Target gene	dsRNA length
iB_03555	*Tc-krn*	510bp
iB_04438	*Tc-rk*	493bp
iB_04697	*Tc-simj*	559bp
iB_02268	*Tc-kis*	470bp
iB_04931	*Tc-vg*	434bp
iB_04737	*Tc-casp*	513bp
iB_05098	*Tc-ems*	481bp
iB_05634	*Tc-mib1*	476bp
iB_05728	*Tc-Gug*	495bp
iB_06451	*Tc-Abd-A*	509bp
iB_03099	*Tc-Abd-B*	480bp
iB_04526	*Tc-hth*	530bp
iB_05191	*Tc-tiotsh*	471bp
iB_06279	*Tc-inv*	535bp
iB_04091	*Tc033998*	498bp
	Tc-burs	591bp
	Tc-pburs	363bp
	Tc-Ubx	268bp
	Tc-spi	469bp
	Tc-EGFR	974bp
	Tc-ser	610bp
	Tc-Notch	310bp
	Tc-wg	1100bp
	Tc-en	885bp
	Tc-hh	1148bp
	Tc-smo	961bp
	Tc-ci	1351bp
	Tc-dpp	1129bp
	Tc-omb	418bp
	Tc-iro	1043bp
	Tc-ASH	762bp
	Tc-nub	685bp
	Tc-srf	872bp
	Tc-dad	655bp
	Tc-apA	684bp

Tc-apB	798bp
Tc-dsh	525bp
Tc-sal	930bp

Table A3 Summary of off-target control

					gin-trap		wing	
iBeetle number	RNAi target	Beetle strain	number of injected larvae	survival to pupal stage	phenotype	penetrance	phenotype	penetrance
iB_03555	*Tc-krn*	*SB*	10	7	size largely decreased	100.0%	slightly smooth elytra surface of pupa, rids or vein developed abnormally and number of setea was less in adult elytra	100.0%
iB_03555	*Tc-krn*	*D17×het*	10	9	size largely decreased	100.0%	slightly smooth elytra surface of pupa, rids or vein developed abnormally and number of setea was less in adult elytra	100.0%
iB_03555 NOF	*Tc-krn*	*SB*	10	7	size largely decreased	100.0%	slightly smooth elytra surface of pupa, rids or vein developed abnormally and number of setea was less in adult elytra	100.0%
iB_03555 NOF	*Tc-krn*	*D17×het*	10	5	size largely decreased	100.0%	slightly smooth elytra surface of pupa, rids or vein developed abnormally and number of setea was less in adult elytra	100.0%
iB_04931	*Tc-vg*	*SB*	10	4	absent	100.0%	size largely decreased or absent	100.0%

iB_04931	*Tc-vg*	*D17×het*	10	6	absent	100.0%	size largely decreased or absent	100.0%
iB_04931 NOF	*Tc-vg*	*SB*	10	3	absent	100.0%	size largely decreased or absent	100.0%
iB_04931 NOF	*Tc-vg*	*D17×het*	10	5	largely decreased or absent	100.0%	size largely decreased or absent	100.0%
iB_04697	*Tc-simj*	*SB*	10	6	largely decreased	83.3%	smaller and a little deformed	83.3%
iB_04697	*Tc-simj*	*D17×het*	10	10	largely decreased	100.0%	size smaller and distal part more rounded	70.0%
iB_04697 NOF	*Tc-simj*	*SB*	10	2	largely decreased	100.0%	size smaller and orientation irregular	100.0%
iB_04697 NOF	*Tc-simj*	*D17×het*	12	9	largely decreased	100.0%	size smaller and orientation irregular	100.0%
iB_04737	*Tc-Casp*	*SB*	9	5	largely decreased, some showed anterior gin-trap had stronger phenotype than posterior gin-trap	100.0%	no phenotype	0.0%

iB_04737	*Tc-Casp*	*D17×het*	10	8	largely decreased, some showed anterior gin-trap had stronger phenotype than posterior gin-trap	100.0%	no phenotype	0.0%
iB_04737 NOF	*Tc-Casp*	*SB*	10	4	anterior gin-trap largely decreased, posterior gin-trap slightly decreased	100.0%	no phenotype	0.0%
iB_04737 NOF	*Tc-Casp*	*D17×het*	9	3	anterior gin-trap largely decreased, posterior gin-trap slightly decreased	66.7%	no phenotype	0.0%
iB_05728	*Tc-Gug*	*SB*	10	2	largely decreased	100.0%	elytra size a little smaller, smooth surface	100.0%
iB_05728	*Tc-Gug*	*D17×het*	10	1	largely decreased	100.0%	elytra size a little smaller, smooth surface	100.0%
iB_05728 NOF	*Tc-Gug*	*SB*	15	5	largely decreased	80.0%	elytra size a little smaller, smooth surface	80.0%
iB_05728 NOF	*Tc-Gug*	*D17×het*	10	5	largely decreased	100.0%	elytra size a little smaller, smooth surface	100.0%

iB_05634	*Tc-mib1*	*SB*	10	2	moderately smaller	100.0%	mostly absent	100.0%
iB_05634	*Tc-mib1*	*D17×het*	8	7	moderately smaller, orientation irregular	100.0%	largely decreased or absent	100.0%
iB_05634 NOF	*Tc-mib1*	*SB*	12	1	mildly or moderately smaller and orientation irregular	100.0%	largely decreased	100.0%
iB_05634 NOF	*Tc-mib1*	*D17×het*	10	2	slightly smaller, orientation irregular	100.0%	largely decreased or absent	100.0%
iB_04438	*Tc-rk*	*SB*	10	2	slightly smaller, orientation irregular	100.0%	a little shorter, wrinkled surface	100.0%
iB_04438	*Tc-rk*	*D17×het*	10	7	slightly smaller, orientation irregular	100.0%	a little shorter, orientation irregular	100.0%
iB_04438 NOF	*Tc-rk*	*SB*	10	10	slightly smaller, orientation irregular	100.0%	a little shorter, wrinkled surface	100.0%
iB_04438 NOF	*Tc-rk*	*D17×het*	10	6	slightly smaller, orientation irregular	100.0%	a little shorter, wrinkled surface	100.0%

iB_05098	*Tc-ems*	*SB*	17	15	size decreased, anterior gin-trap largely decreased or absent, posterior gin-trap moderately or largely smaller	100.0%	elytra a little shorter	33.3%
iB_05098	*Tc-ems*	*D17×het*	20	5	anterior gin-trap size largely decreased	100.0%	elytra a little shorter and surface irregular	80.0%
iB_05098 NOF	*Tc-ems*	*SB*	10	9	shape irregular, anterior gin-trap smaller and less scleritized jaw	44.4%	no phenotype	0.0%
iB_05098 NOF	*Tc-ems*	*D17×het*	12	8	size decreased, anterior gin-trap size largely decreased	100.0%	no phenotype	0.0%
iB_02268	*Tc-kis*	*SB*	18	18	moderately or largely decreased	100.0%	a little shorter, some showed blistered elytra	100.0%
iB_02268	*Tc-kis*	*D17×het*	20	13	moderately decreased	100.0%	slightly shorter	100.0%
iB_02268 NOF	*Tc-kis*	*SB*	18	17	moderately or largely decreased	100.0%	slightly shorter	100.0%
	Tc-kis	*D17×het*	20	15	moderately	100.0%	slightly shorter	100.0%

					decreased			
iB_06451	*Tc-Abd-A*	*SB*	10	7	anterior gin-trap largely decreased, proximal part of gin-traps a little expanded	100.0%	no phenotype	0.0%
iB_06451	*Tc-Abd-A*	*D17×het*	10	8	anterior gin-trap largely decreased, proximal part of gin-traps a little expanded	87.5%	no phenotype	0.0%
iB_06451 NOF	*Abd-A*	*SB*	16	12	anterior gin-trap largely decreased, proximal part of gin-traps a little expanded	100.0%	no phenotype	0.0%
iB_06451 NOF	*Abd-A*	*D17×het*	10	3	anterior gin-trap largely decreased	100.0%	no phenotype	0.0%

NOF: non-overlapping fragment of dsRNA; phenotype penetrance: proportion of pupae with phenotype in the individuals which could pupate; *D17×het* is the beetle strain used for iBeetle screen.