Genome-wide RNAi screening and the analysis of candidate genes for dorsoventral patterning in *Tribolium castaneum*

Inaugural–Dissertation

zur

Erlangung des Doktorgrades

der Mathematisch-Naturwissenschaflichen Fakultät

der Universität zu Köln

vorgelegt von

Van Anh Dao

aus Hanoi, Vietnam

Köln 2014

Berichterstatter/in: Prof. Dr. Siegfried Roth

P.D. Michael Kroiher

Tag der letzten mündlichen Prüfung: 25.06.2014

Contents

Abstract

The iBeetle project is a genome-wide RNAi screen to systematically identify genes involved in oogenesis, embryonic and larval development in the red flour beetle, *Tribolium castaneum*. In the course of this thesis the general screening phase was followed by the investigation of phenotypes suggesting the impairment of dorsoventral (DV) axis formation.

Our detailed analysis revealed one component acting upstream of Toll receptor, *Tc-nudel*, and two downstream components, *Tc-pelle* and *Tc-tube-like-kinase*. *Tc-tube-like-kinase* is of particular interest because it has homology to vertebrate IRAK 4. The kinase domain, which is observed in Tc-Tube-like kinase, must have been lost during evolution in the lineage leading to *Drosophila*. The requirement of the kinase domain for proper DV patterning in *Tribolium* is still unclear.

Knockdown of *Tc-SoxNeuro* resulted in a phenotype resembling the loss of *Tc-sog*, which suggests a function of *Tc-SoxNeuro* in BMP signaling. *Drosophila SoxNeuro* is involved in early CNS development but seems not to function in BMP signaling in establishing ectodermal fate. *Tc-SoxNeuro* potentially fulfills a vertebrate-like function in establishing the BMP gradient, in agreement with previous work on BMP signaling in *Tribolium* suggesting a vertebrate-like network structure.

Besides the analysis of candidate genes found in the iBeetle screen, an independent investigation was carried out in order to find the missing components of the perivetelline serine protease cascade in *Tribolium*. TC015295 is the putative *Tc-gastrulation defective*. The *snake*-related TC004160 might function like Persephone in immunity. TC002112 is *Tc-easter* and, at the same time, *Tc-spätzle processing enzyme* that functions in immunity. This study suggests that the upstream Toll signaling machinery in *Tribolium*, the more basally branching insect, is simpler than that in *Drosophila*, and the divergence of the upstream Toll signaling machinery observed in *Drosophila* is a derived feature.

CHAPTER 1: General introduction

The proper development of a fertilized egg into a multicellular embryo is a fascinating process. This process poses many difficult and interesting questions. For example, how cells and tissues “conceive” their fate, how the positional information is accurately established, how a new morphological structure of an embryo can be formed in the context with other already-completed structures (for example in the case of abdomen formation in short-germ insect), how an embryo "counts" the segment number, etc. However, the most intriguing and crucial questions of modern developmental biology would be which genes are involved in the embryonic development and whether their functions are conserved during evolution.

Over the last decades, the flour beetle *Tribolium castaneum* has become an important insect model organism for developmental biology, second only to the best studied insect – the fruit fly *Drosophila melanogaster*. *Tribolium* is of particular interest in developmental biology because its short-germ type of embryogenesis represents that of basal insect linages. The gene regulatory network governing DV patterning in *Drosophila* is one of the best understood systems in early embryonic development. Previous studies in *Tribolium* have opened up many questions about the mechanisms setting up DV axis in this organism. The ultimate purpose of this thesis is to understand the gene regulatory network underpinning DV axis formation in *Tribolium castaneum*.

1.1 Dorsoventral patterning in *Drosophila melanogaster*

In insects, both body axes, the anterior-posterior (AP) axis that goes from mouth to anus, and the dorsoventral axis that goes from back to belly are already established during oogenesis. Some signaling pathways and gene regulatory networks that govern this axis formation are highly conversed throughout the animal kingdom. In insects, the signals that induce the polarity establishment are localized mRNA sources that are maternally provided (Morisato and Anderson, 1995). Symmetry is broken when the oocyte nucleus moves to the dorsal side of the egg chamber. From here the nucleus sends a germline-to-soma signal to the surrounding follicle cells. This process employs the *tgf-*α like Gurken protein (Roth and Schüpbach, 1994). The polarity signals are localized to the ventral side of the follicular epithelium. Subsequently, they are transferred back to the embryo by successive activations of a proteolytic cascade in the perivitelline space (Moussian and Roth, 2005). In response to these signals, Toll signaling pathway is activated and restricted to the ventral side of the embryo where mesoderm and neuroectoderm fates are determined. Toll signaling represses the expression of BMP signaling. As a result, it is restricted to the dorsal side. BMP plays a key role in patterning extraembryonic tissues and dorsal ectoderm (Moussian and Roth, 2005; O'Connor et al., 2006; Roth, 2003). The three signaling processes that set up the formation of DV axis in the fly *Drosophila melanogaster*, including EGF signaling, Toll signaling and BMP signaling, are depicted in Fig 1.

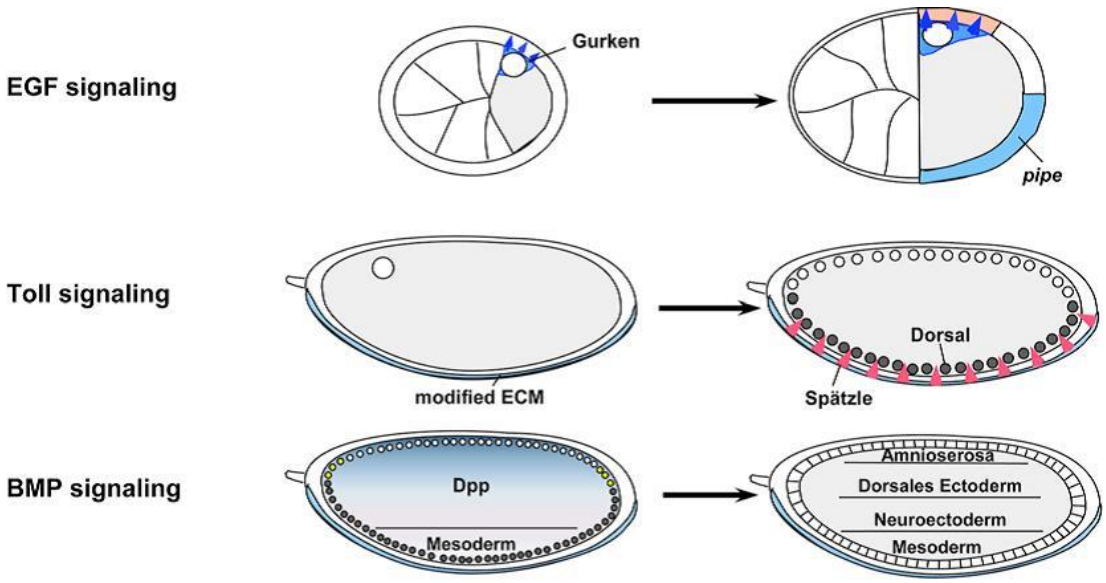

Figure 1 *Three essential steps of DV patterning in Drosophila*
EGF signaling occurs in oogenesis stage 9. Oocyte nucleus habors at the anterior dorsal region of the egg chamber. Gurken protein is secreted and activates EGF receptor at the follicle cells surrounding the oocyte. Toll signaling is activated in the ventral side of the blastoderm stage embryo, which leads to the Spätzle activation. Dorsal is transported into the nuclei in the gradient manner along the DV axis. BMP signaling is activated in the dorsal side of the embryo. Mesoderm, ectoderm and amnioserosa are formed in response to the gene regulatory network controlled by Dorsal.

The *Drosophila* ovariole is essentially a tube-like structure of a number of egg chambers that are connected to each other (Fig 2). The anterior tip of the ovariole consists of the germarium, which is comprised of the germ line and the somatic stem cells. Each egg chamber is a structure containing 16 germ cells. Out of 16 germ cells, only one cell develops into an oocyte, the other 15 become nurse cells. The egg chamber is surrounded by a monolayer epithelium of somatic follicle cells (Lin and Spradling, 1993). Already during the midstage of oogenesis, long before the egg is laid, three specific mRNAs are localized within the oocyte, which are the key factors to initiate the formation of both the AP and the DV axes of the future embryo (Riechmann and Ephrussi, 2001; Van

Eeden and St Johnston, 1999). One of which is Gurken, an ovarian-specific TGF-α protein, that plays a key role in the establishment of DV axis (Neuman-Silberberg and Schupbach, 1993). The other two, Oskar and Bicoid, are components of the AP patterning system (Cha et al., 2001; Kugler and Lasko, 1995; St Johnston and Nüsslein-Volhard, 1992). At stage 9 of oogenesis, the oocyte nucleus moves from the central posterior asymmetrically to the anterior dorsal region of the egg chamber. It has been proved that this movement of the oocyte nucleus defines the dorsal side of the future embryo (Roth, 2003). From here, Gurken protein is secreted to the overlying somatic follicle cells. Gurken is the ligand that activates the EGF receptor in the follicular epithelium (Schüpbach and Roth, 1994; Sen et al., 1998) (Fig 2).

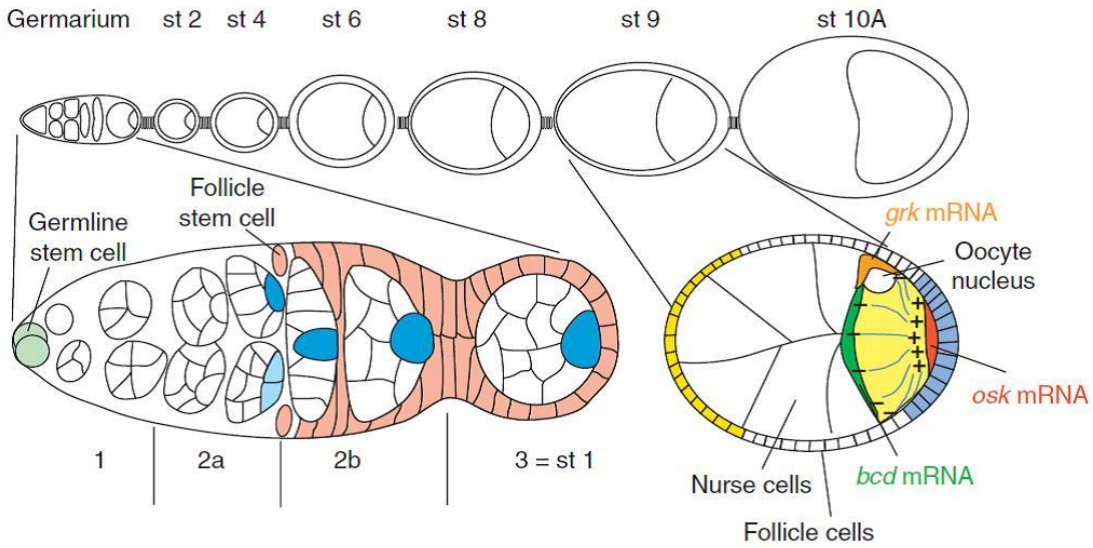

Figure 2 The Drosophila ovariole

Germarium at the anterior tip and aging egg chambers. Nuclear movement is the first symmetry break event that occurs at stage 7 oogenesis. (Roth and Lynch, 2009).

The EGF receptor is activated at the highest levels in the follicle cells that are close to the oocyte nucleus. In the follicular epithelium, EGF receptor activates and represses a number of target genes in a concentration-dependent manner. With regard to the DV axis patterning process, the main outcome of the

activation of EGF receptor is the restriction of *pipe* expression to a ventral domain comprised 40% of the egg chamber circumference (Sen et al., 1998). The sulfotranferease Pipe presumably modifies extra cellular matrix (ECM) components, which are present in the vitelline membrane (Zhang et al., 2009). As a result, the ECM components are only modified in the ventral side of the egg chamber. Pipe, together with two other components, Windbeutel (Wbl) and Slalom (Sll), transfer cues into the perivitelline space between the follicular epithelium and the plasma membrane. The universal sulfate donor for sulfotransferase is 3'-phosphoadenisine 5'-phosphosulfate (PAPS). Slalom is important to transport PAPS from the cytoplasm to the Golgi (Lüders et al., 2003; Zhu et al., 2005). *wbl* is expressed uniformly in the follicular epithelium. It is suggested that *wbl* is required for the localization of Pipe to the Golgi. In the knockdown of *wbl*, Pipe remained in the endoplasmic reticulum (ER) (Konsolaki and Schüpbach, 1998; Nilson and Schüpbach, 1998; Sen et al., 1998). The Pipe-sulfated signals, after egg deposition, trigger a proteolytic cascade in the perivitelline space (more information about this proteolytic cascade can be found in the Chapter 3, section 3.1 of this thesis). The outcome of this process is the activation of Toll receptor in the ventral side of the blastoderm embryo. This gradient is stable throughout 14 nuclear division cycles, when most embryonic patterning takes place, and shows a similar shape at lower level during the next five syncytial divisions (Kanodia et al., 2009; Liberman et al., 2009).

Toll signaling pathway is conserved throughout the animal kingdom. Besides its function in DV patterning, Toll signaling pathway plays an important role in innate immunity in insects and mammalians (Brennan and Anderson, 2004; Imler et al., 2004; Kawai and Akira, 2007; Kim and Kim, 2005; Kumar et al., 2009), where it induces the expression of antimicrobial peptides or inflammatory cytokines in response to microbial challenge (Khush and Lemaitre, 2000; Lemaitre et al., 1996). The proteolytic cascade resembles the blood

clotting and complement activating cascades of vertebrates (Krem and Cera, 2002).

Activation of Toll receptor stimulates the import of the NF-κB transcription factor Dorsal into the nuclei of the blastoderm stage embryo (Moussian and Roth, 2005). The intracellular domain of Toll receptor interacts with two adaptors, dMyd88 and Tube. Subsequently, the Toll-dMyd88-Tube complex employs the protein kinase Pelle to form a heterotetramer. Pelle undergoes auto-phosphorylation and detaches from the complex (Horng and Medzhitov, 2001; Hu et al., 2004; Stathopoulos and Levine, 2002; Sun et al., 2004). The IκB homolog Cactus in the absence of Toll signaling binds Dorsal. Cactus is phosphorylated by Pelle and releases Dorsal into the cytoplasm (Drier et al., 2000, 1999; Edwards et al., 1997; Israël, 2000; Karin and Ben-Neriah, 2000). Then, Cactus is degraded by proteasome (Bergmann et al., 1996; Reach et al., 1996). Dorsal also needs to be phosphorylated to enter the nucleus (Drier et al., 2000, 1999). Dorsal bind to Tamo and is transported into the nucleus through nuclear membrane pores formed by DNTF-2 and Mbo (Minakhina et al., 2003; Uv et al., 2000). A nuclear Dorsal gradient with peak level at the ventral side is established (Roth et al., 1989) (Fig 3).

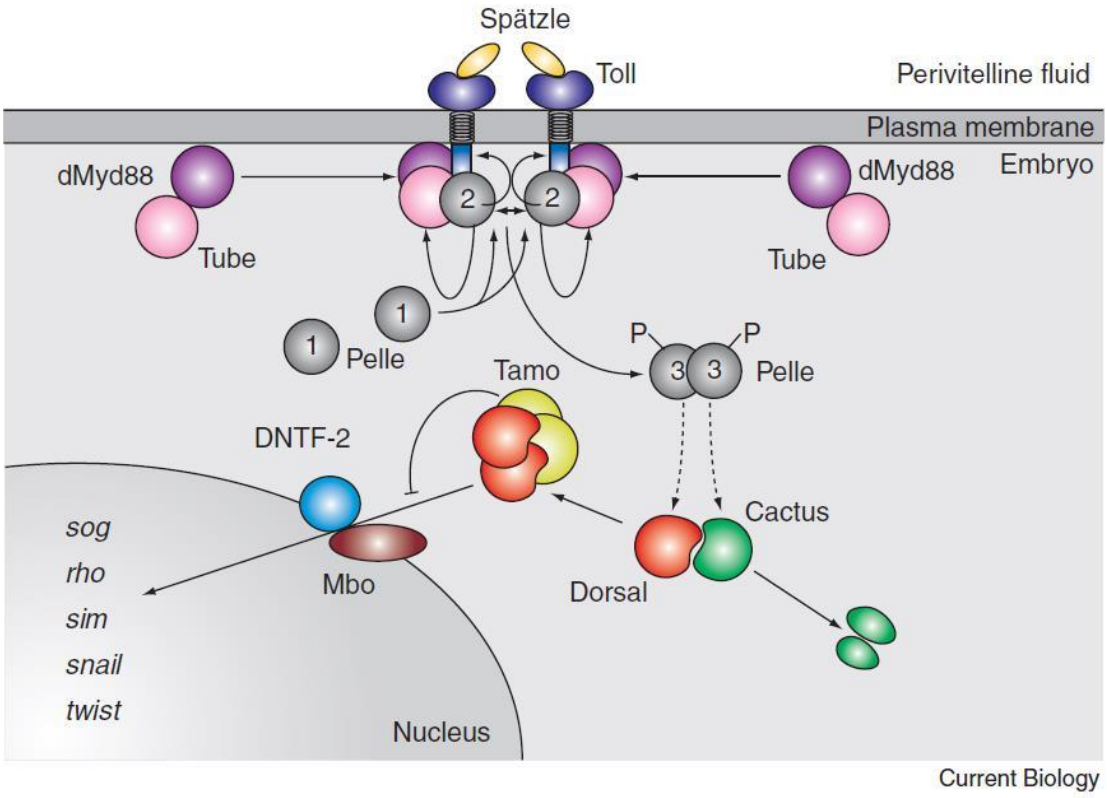

Figure 3. Nuclear Dorsal uptake.

The intracellular domain of the activated Toll receptor interacts with two adaptors dMyd88 and Tube. Subsequently, the kinase Pelle joins the Toll-Tube-dMyd88 complex and undergoes phosphorylation. Pelle is released from the complex and phosphorylates Cactus to release Dorsal. Dorsal binds to Tamo and enters the nucleus through a nuclear pole containing DNTF-2 and Mbo. (Moussian and Roth, 2005).

Establishing the nuclear gradient of Dorsal initiates the gene regulatory network that regulates the specification of cell fates along DV axis. In *Drosophila*, the Dorsal gradient is stable and most of the target genes depend directly or indirectly on it for their proper positional expression (Markstein et al., 2004, 2002; Papatsenko and Levine, 2005; Stathopoulos et al., 2002). Dorsal acts as a morphogen, activating or repressing target genes in a concentration-dependent manner (Rushlow et al., 1989; Steward, 1989). The highest nuclear concentration of Dorsal is found in the 18-20 ventral most cells of the embryo,

where *twist* and *snail* are expressed, which regulate the formation of mesoderm after invagination (Rusch and Levine, 1996). Flanking the mesoderm are two stripes of mesectoderm where *single minded* (*sim*) is expressed. Dorsal accumulates at low levels in the region where the neuroectoderm will form with the expression of *rhomboid* (*rho*) and *short gastrulation* (*sog*) and a number of other genes (Markstein et al., 2004; Stathopoulos and Levine, 2005; Stathopoulos et al., 2002). Nuclear Dorsal uptake does not reach the dorsal side of the embryo where BMP signaling is activated to regulate the establishment of dorsal ectoderm and extraembryonic tissues. BMP signaling is inhibited in the ventral side by the Dorsal downstream target *sog*. On the other hand, the complex Sog-BMP is transported from ventral to dorsal, where it is cleaved by the metalloprotease Tolloid (Tld). Consequently, BMP molecules are released and activated in the dorsal hemisphere of the embryo, where Sog levels are low (Meinhardt and Roth, 2002) (Fig 4).

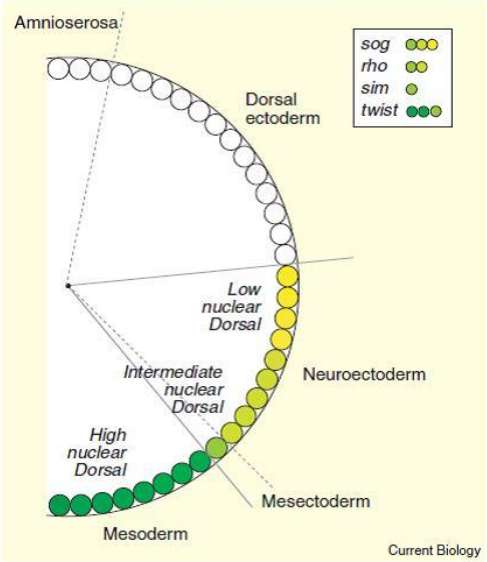

Figure 4. Dorsoventral fatemap

Highest nulear Dorsal levels are in the ventral most part of the embryo, where twi and sna are expressed, which control the formation of mesoderm. Intermediate nuclear Dorsal level leads to the activation of sim, which is responsible for mesectoderm establishment. sog and rho determine the neuroectodermal fate. Dorsal is absent in the dorsal side, where BMP is active. BMP patterns the dorsal ectoderm and the amnionserosa.

The nuclear Dorsal gradient shape is not merely formed by the expression of *pipe* in the follicular epithelium. Pipe is expressed uniformly in a domain that comprises 40% of the egg circumference with sharp borders in the lateral sides. Moreover, expanding the *pipe* domain by mutants affecting *grk* and other EGF

signaling components did not simply lead to the expansion of *dorsal* expression domain. Instead, the Dorsal gradient splits, resulting in two lateral peaks in the embryo (Moussian and Roth, 2005). Thus, there must be some self-regulatory mechanisms that transform the uniformly expressed *pipe* domain in the ovary into a graded domain of Dorsal nuclear uptake in the embryo (Morisato and Anderson, 1994), but the exact nature of this mechanism is still obscure. It has also been suggested that there might be another source of DV polarity information besides *pipe* (Zhang et al., 2009). Egg chambers expressed *pipe* ubiquitously in the follicle cells still developed into embryos with some degree of DV polarity. Supporting this hypothesis, earlier findings have shown that BMP signaling molecules expressed during oogenesis might have a direct input into Toll signaling in the embryo (Schinko et al., 2010).

The question of how the gradient of Dorsal can provide the positional information for the expression of its downstream target genes in sharp domain is not trivial. The most essential factors that determine the sensitivities of Dorsal target genes are the affinities and arrangement of Dorsal-binding sites in their enhancers. Enhancers of genes expressed ventrally possess low-affinity binding sites, while those that interact with Dorsal on the lateral sites must be more sensitive to the low levels and are composed of high-affinity binding sites (Hong et al., 2008; Stathopoulos and Levine, 2004). However, the expression of target genes in sharp domains is not merely archived by the sensitivities of Dorsal-binding sites. Additional factors are involved with which Dorsal cooperates such as maternally provided Zelda, basic helix-loop-helix (bHLH) proteins, and at least one unidentified factor to achieve the proper expression of genes in the dorsal side (Crocker et al., 2008; Liberman et al., 2009).

The gene regulatory network that governs dorsoventral axis formation in *Drosophila melanogaster* has been intensively studied and is one of the best understood systems in embryogenesis (Levine and Davidson, 2005). It is the gold standard for comparative studies of early embryonic patterning.

1.2 DV patterning in *Tribolium castaneum* – State of the Art

The flour beetle *Tribolium castaneum* has developed into an important insect model organism. *Tribolium* undergoes short germ development, as found in ancestral insects, while *Drosophila melanogaster* shows the derived mode of long germ development. Therefore, it is of particular interest to understand the mechanisms of development in *Tribolium*. It was shown that the hierarchical mechanism of the *Drosophila* gene regulatory network (Anderson et al., 1985; St Johnston and Nüsslein-Volhard, 1992), in which the Toll signaling serves as a sole conductor for DV patterning is not representative for more basally branching insects like *Tribolium* (Nunes da Fonseca et al., 2008). Comparative studies between these two insect species in particular, and among other insects in general would provide insights into the evolution of insect development.

The DV patterning in *Tribolium* follows the same process and uses many similar components like in *Drosophila*. However, many differences between *Tribolium* and *Drosophila* have been discovered, especially during the early stages. Previous work in *Tribolium* has opened many questions for investigations. Fig 5 illustrates the DV patterning processes in *Tribolium*, with regards to the unidentified components and pathways.

In *Drosophila*, there are three TGFα-like ligands; one of them is the ovarian specific ligand Gurken. In contrast, there is only one TGFα in *Tribolium* (*Tc-TGFα*). There is only one protease of the rhomboid family (*Tc-rho*) for TGFα ligand activation in *Tribolium*; meanwhile, there are three different ones in *Drosophila*. *Tc-TGFα* and *Tc-rho* are expressed in the oocyte, while the EFG receptor (*Tc-egfr*) is expressed in the follicle epithelium. Knockdown of all three genes showed variable DV defects during oogenesis and embryogenesis (Lynch et al., 2010). These data suggest that EGF signaling is also used in setting up the early DV polarity in *Tribolium*, and that the process also occurs via germline-to-soma signaling. However, *Tc-TGFα* mRNA is distributed evenly

in the oocyte. Therefore, it is currently unclear how the germline-to-soma signal is localized in *Tribolium*.

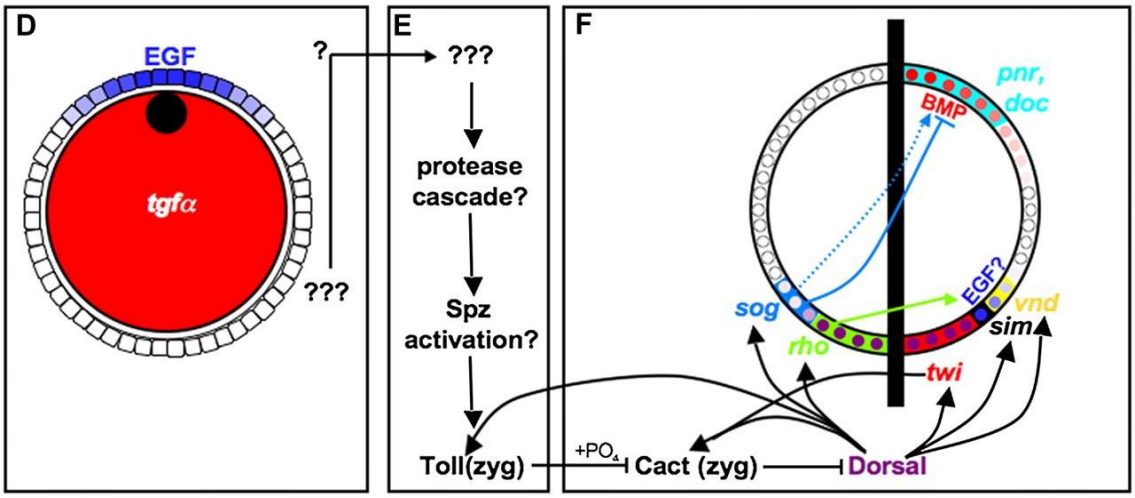

Figure 5. DV patterning in Tribolium.

Left: EGF signaling occurs in the follicular epithelium overlying the oocyte nucleus. tgfα mRNA is not localized. The target of EGF signaling are unidentified and how the signals are transmitted to the embryo is unclear.

Middle: The component of the protease cascade are not found and how, or if at all this protease cascade is restricted ventrally is not known.

Right: Feedback regulation of Dorsal to Cactus and Toll. The left half of the cross section shows genes that are expressed before gastrulation. The right half shows genes that are downstream of Dorsal, directly or indirectly. (Lynch and Roth, 2011).

A group of downstream targets of *Tc-Gurken*, including *Tc-kekkon, Tc-rhomboid* and *Tc-pipe*, has also been investigated in *Tribolium*. Surprisingly, *in situ* hybridization showed no expression of these genes in the follicular epithelium. The possibility that *Tc-pipe* expression escaped detection is considered, since experience from *Drosophila* shows that follicle cell *in situ* hybridization is difficult. RNAi knockdown has been performed for *Tc-pipe* using several different constructs. But it failed to show DV patterning defects (Lynch et al., 2010).

In conclusion, at the moment, we know that *Tc-Gurken* is involved in inducing early DV signal in the follicle epithelium. However, we do not know how this signal is transmitted to the embryo. Apparently, *Tribolium* uses a different mechanism than *Drosophila*.

Tc-Toll has been identified and analyzed (Maxton-KuchenmeisterMaxton-Kü et al., 1999). However, the perivitelline proteolytic cascade, which leads to the activation of the Toll receptor, is not yet identified in *Tribolium*. This is a topic of my PhD and is written in detail in the Chapter 3 of this thesis.

A gradient of nuclear Dorsal distributed along the dorsoventral axis in *Tribolium* embryo has been observed (Chen et al., 2000). *Drosophila* Dorsal covers the whole anterior-posterior length of the blastoderm embryo, and is stable through the early embryonic stages before gastrulation. The Dorsal gradient in *Tribolium*, in contrast to *Drosophila*, is very dynamic. Nuclear accumulation is first uniform at the nuclear stage 9 when the nuclei reach the periphery. It progressively becomes narrower and eventually it is refined to a thin ventral stripe along the ventral midline and disappears before gastrulation (Fig 6). Like in *Drosophila*, the formation of the Dorsal gradient in *Tribolium* is dependent on the inhibitor Cactus (Tc-Cact); however, the pattern of regulation is essentially different. *Tc-cact* is expresses zygotically, not maternally as in the case of *Drosophila*. At the early stages, *Tc-cact* is ventrally expressed in a dynamics similar to that of Dorsal, indicating that *Tc-cact* is a target of Dorsal. At the later stages, *Tc-cact* is activated by the Dorsal-target gene, *Tc-twi*. This up-regulation of *Tc-cact* by *Tc-twi* in the late blastoderm and gastrulation stages terminates the nuclear uptake of Dorsal. In conclusion, *Tribolium* uses zygotic negative feedback loops to generate the dynamic Dorsal gradient. (Chen et al., 2000; Nunes da Fonseca et al., 2008).

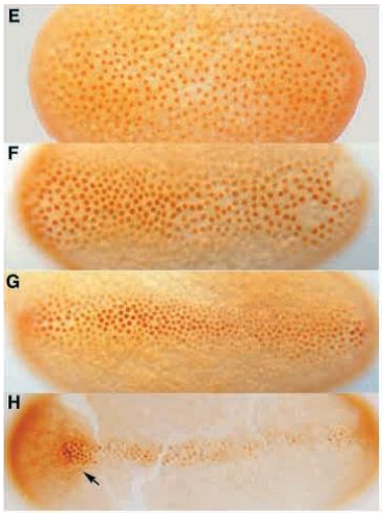

Figure 6. Dorsal gradient dynamic in Tribolium

Nuclear Dorsal uptake begins at nuclear cycle 9. At first, Dorsal is accumulated in a ventral span that makes up 40% of the egg circumference. This ventral Dorsal domain gradually shrinks ventrally and in the end is refined to a sharp stripe along the ventral midline. It disappears before gastrulation (Chen et al., 2000).

The gene regulatory network downstream of Dorsal, which is hugely complex in *Drosophila*, has partially been revealed in *Tribolium*. The establishment of BMP signaling in *Tribolium* seems to be much simpler than in *Drosophila*. It depends entirely on Sog, the Dpp-binding secreted protein (Van der Zee et al., 2008; Zee et al., 2006). In *Drosophila*, Dpp gradient is determined by the inputs from several components downstream of Dorsal (Jaźwińska et al., 1999). *Drosophila twisted gastrulation* (*tsg*) has two paralogs, *crossveinless* and *schrew*, and *tolloid* has one paralog, *tolkin*. While in *Tribolium*, there is only one *twisted gastrulation* and one *tolloid* in the genome. In *Tribolium*, only Dpp and the receptor Thickvein are required in BMP activation. While in *Drosophila*, one more ligand, Screw and one more receptor, Saxophone are needed (Nunes da Fonseca et al., 2008). The *Tribolium* model is closer to vertebrates, in which BMP gradient also depends largely on secreted BMP inhibitors, with Chordin playing a major role (De Robertis and Kuroda, 2004).

In *Tribolium*, the head primordium is not only an anterior fate like in *Drosophila*, but also a ventral fate. Van de Zee et al. (2006) showed that in the absence of *Dpp*, the embryo is ventralized, and the head anlage is expanded. When *sog* function is abolished, BMP signaling is expressed ectopically along the border between embryonic and extraembryonic regions, which leads to the

deletion of the head. This cross-talk between AP and DV axis in *Tribolium* is a typical feature of early embryonic patterning in vertebrate (De Robertis, 2008).

Tribolium is a short germ insect. Less evolutionary derived insects like *Tribolium* undergo short germ development, where the anterior part of the body, comprised of head and thorax, is formed first from the germ anlagen before gastrulation, and the remaining posterior part are specified during a later secondary phase from the growth zone. In contrast, derived insects, such as *Drosophila*, undergo long germ development, where all body segments develop almost simultaneously (Fig 7). The long germ mode of embryogenesis is found only in holometabolous insects, it is therefore considered a derived mode of embrogenesis. All hemimetabolous insects and some of the holometabolous insects develop the short germ embryogenesis (Davis and Patel, 2002).

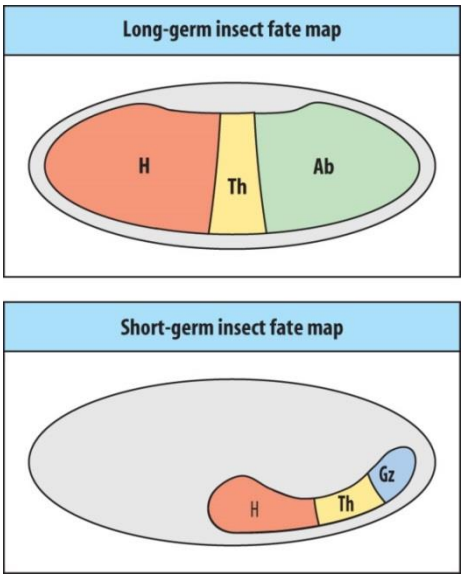

Figure 7. Long germ vs. short germ insect fate map

H: head. Th: thorax. Ab: Abdomen. Gz: growth zone. Long germ insect develop all body sections almost at the same time while short germ insect develop the head and the thorax first, the abdomen is derived later from the growth zone. (Principles of development, L.Wolpert)

The mechanisms of DV axis formation in the growth zone in *Tribolium* are mainly unclear, as there is no corresponding process in *Drosophila*. The growth zone is the structure typical for short germ development that gives rise to the abdomen fate. In *Tribolium*, the growth zone is composed of two cell layers. The inner mesenchymal layer presumably develops into mesoderm and the outer epithelial layer probably gives rise to the neuroectoderm and the dorsal ectoderm of the abdominal segments (Handel et al., 2005; Nunes da Fonseca

et al., 2008). The initial development of the growth zone relies on Toll signaling. In the absence of Toll, the embryo ends up having only one cell layer in the growth zone and the abdominal segments are dorsalized (Van der Zee et al., 2008). However, the mechanisms and molecules that are involved in this process remain to be identified.

Aim of this thesis

I started my PhD as a screener in the iBeetle project. iBeetle is a genome-wide RNAi screening to systematically identify genes involved in oogenesis, embryonic and larval development in the red flour beetle *Tribolium castaneum*. During the screen, we have identified some phenotypes indicating potential DV polarity disruptions. The detailed analysis of these genes is discussed in Chapter 2 of this thesis.

Chapter 3 of this thesis deals with the perivitelline serine protease cascade upstream of Toll signaling activation in *Tribolium*. The missing components of this serine protease cascade were identified by means of RNAi knockdown and phylogenetic analysis.

CHAPTER 2: iBeetle - Genome-wide RNAi screening in *Tribolium castaneum*

Researches on developmental biology in particular, and in biology in general, face the problem of being biased towards a limited number of model organisms. The fruit fly *Drosophila melanogaster* is so far the best studied insect model. However, the flour beetle *Tribolium castaneum* is more representative for insect embryonic development than *Drosophila*. The ultimate aim of the iBeetle project is to develop *Tribolium castaneum* into a powerful insect model organism for developmental biology.

2.1 INTRODUCTION

2.1.1 Overview of the iBeetle project

The iBeetle project is the genome-wide RNAi screening in *Tribolium castaneum*. The iBeetle screening aims to knockdown all the genes of *Tribolium* by RNAi and to analyze the embryonic phenotypes. Better understanding of the developmental mechanism in this model organism provides another comparative platform, besides *Drosophila melanogaster*, for developmental biology in insects.

The iBeetle project started in September 2010 and is going on until September 2016. It employs, in total, two postdocs, ten PhD students and two technicians. The PhD students, who joined the project, participated in the screen in the first 16 months of their PhD. After the screen, PhD students can choose the phenotypes of interest for the detailed analysis.

iBeetle is the collaboration of the University of Cologne, the University of Göttingen, the University of Erlangen, the University of Greifswald and MPI Dresden.

2.1.1.1 *Tribolium castaneum* as a screening platform

Before, developmental biology in *Tribolium* was mainly based on candidate gene approach, meaning analyzing the orthologous genes of *Drosophila* in *Tribolium*. This approach has been very successful for a number of developmental processes. Conserved features such as segmentation, AP axis formation, have been largely revealed. In the meanwhile, however, many new questions have been raised. *Tribolium* represents an ancient insect species, its embryogenesis has “typical-insect” features; meanwhile *Drosophila* is a more highly derived insect. Therefore, some developmental processes in *Tribolium* cannot be found in *Drosophila*, such as the development of extra embryonic tissues, larval legs, non-involuted head, etc… These fundamental developmental differences suggest divergent embryogenesis mechanisms and open many basic biological questions for functional investigations.

Apparently, these processes require another unbiased approach that does not rely on the knowledge about *Drosophila*. In addition, it has been shown that many genes are present in *Tribolium* and vertebrates but not in *Drosophila* (Richards et al., 2008). Thus, *Tribolium* is more suitable for comparative studies between phyla than *Drosophila* (especially between chordates and anthropods). Intensive developmental studies on *Tribolium* are highly desired to broaden our knowledge on the mechanisms of evolution and overcome the prevailing biased case where most of what is known about the mechanisms of development is from *Drosophila*.

The molecular techniques for developmental biology in *Tribolium* have vastly increased in recent years. These advances include: chemical mutagenesis screens (Maderspacher et al., 1998), transgenesis (A. J. Berghammer et al., 1999; Pavlopoulos et al., 2004), and insertional mutagenesis and enhancer trap systems (Lorenzen et al., 2007). Several hundred enhancer trap lines are already available (mainly generated by the

GEKU Consortium). In addition, heat shock mediated misexpression systems (Schinko et al., 2012), transgenic RNAi (Ntini and Wimmer, 2011) and *in vivo* imaging lines (Schinko et al., 2010) have recently been established. The *Tribolium* genome sequence has been published in 2008 (Richards et al., 2008), opening the opportunities for functional genomics of embryogenesis. Importantly, *Tribolium* is exceptionally amenable to reverse genetics by RNAi - the simple but powerful tool for gene knockdown that has been more and more widely used recently (Bucher et al., 2002; Tomoyasu et al., 2008).

2.1.1.2 *Tribolium castaneum* is exceptionally amenable to reverse genetics via RNAi

RNAi screening is a powerful reverse-genetic approach to large-scale functional analysis. Gene functions of a given process, structure or pathways are identified via the phenotypes associated with the gene knockdown. *C.elegans*, *Drosophila* and mammalian culture cells have been the most-used system for RNAi screening. Knockdown of genes by RNAi has been applied to the *Tribolium castaneum* system for over 10 years (Bucher et al., 2002). In some organisms, for example, *C. elegans* and *Tribolium*, but not in *Drosophila*, the RNAi response is systemic. The signal for the knockdown is transmitted from the source to the entire body and can be transmitted to the next generation (Bucher et al., 2002; Hunter et al., 2006; Tomoyasu and Denell, 2004; Tomoyasu et al., 2008). Therefore, it is possible to inject dsRNA at different stages of development in order to temporally disrupt functions of a gene (Schinko et al., 2008).

Since the RNAi knockdown effect is transferred from injected females to their offspring (parental RNAi, pRNAi), RNAi embryos can be obtained without being damaged by the injection procedure. pRNAi knockdown can also affect all individuals of an egg collection (Bucher et al., 2002). Moreover, pRNAi is less laborious than embryonic injections, which is a big advantage for a large-scale

screening. In *Tribolium*, RNAi phenotypes have been observed in the following tissues: embryonic ectoderm, muscles and gut; adult ovary, testis, germ line cells in developing gonads; nervous system, including eyes and epidermal sense organs; metamorphosing tissues including thoracic and abdominal epidermis, wing and eye discs, larval appendages and flight musculature. RNAi also works with sex determination genes, and even against components of the RNAi pathway itself, including its systemic aspects (Tomoyasu et al., 2008). Finally, in *Tribolium*, RNAi does not spread along a gene transcript beyond the sequence of the trigger dsRNA, which allows RNAi against specific splice variants (Arakane et al., 2005).

2.1.1.3 Advantages of a reverse genetic RNAi screen over classical forwards genetic screens

RNAi screen does not demand expenses and labors to maintain the transgenic strains, which are required in classical genetic screens. In our screen, the obtained data is stored in an electronic database that is accessible via web interface. Additional studies can be carried out anywhere and anytime without the need to exchange specific stocks or reagents.

The identity of the knockdown gene is immediately known in RNAi screen, thus we can avoid the additional work of positional cloning of the affected gene.

RNAi phenotypes are usually more penetrant than classical genetic screen phenotypes (over 80% compared to 25%).

2.1.2. Aims

The iBeetle genome-wide screening aims to overcome the restriction of the candidate gene approach and to develop *Tribolium* into a new platform for developmental biology.

Novel fields of research that potentially be opened as benefit from the iBeetle project are the processes that cannot be easily – if not impossibly –

studied in *Drosophila*, either because they are absent in *Drosophila*, or they are hard to study due to technical reasons. For example:

- Migration of muscle precursor tissues in embryonic leg development (not present in *Drosophila*).
- Odoriferous gland development and its role in pest control (not present in *Drosophila*).
- Extraembryonic tissues development and its role in immune defense (not present in *Drosophila*).
- Metamorphosis patterning and hormonal control of metamorphosis (*Drosophila*'s pupa enclosed in opaque puparium that hampers visualization, which is not the case in *Tribolium*).

For the reason that candidate gene approach got exhausted for several processes, we aim to overcome this limitation and complete our knowledge gaps about development in *Tribolium*. There is evidence that essential factors, to name a few, are missing in:

- Dorsoventral patterning (e.g. upstream components of Toll pathway, localization of the germ line-to-soma signals)
- Segmentation from the growth zone (what short germ specific genes have we not known of so far?)
- Anterior-posterior patterning (e.g. what maternal morphogens are localized in *Tribolium* given that *bicoid* is missing and *nanos* is not localized?)

Furthermore, many *Tribolium* homologs of *Drosophila* have no compatible functions (e.g. *fuzi tarazu, knirps, hunchback, hairy*), suggesting that there might be genes in *Tribolium* that have no detectable phenotypes in *Drosophila*. Even in *Drosophila*, many processes are still open to investigation. Examples are: adult leg development, somatic stem cell lineages in oogenesis, embryonic head development, and adult muscle development. Thus, independent work on identification of these factors is required in *Tribolium*.

Another main focus of the iBeetle project is to develop *Tribolium* as a complementary screening platform, i.e. identifying genes that have been missed in *Drosophila* by searching for *Tribolium* orthologs.

2.1.3 Searching for DV phenotypes from the screen

Within the frame of the iBeetle project, my PhD thesis aimed to identify the missing links of the early DV patterning, which failed to be revealed by candidate gene approach (Lynch and Roth, 2011). The iBeetle screening was an unbiased process, therefore we were opened to all phenotypes which hold potential for new components involved in all three signaling pathways of DV patterning in *Tribolium.* Knockdown of genes involved in the establishment of DV patterning in *Tribolium* lead to diverse phenotypes. Previous work in *Tribolium* has revealed some of the typical DV phenotypes. Based on these phenotypes, we developed criteria to search for DV phenotype from the screen.

DV patterning is a process that occurs early during development. Knockdown of genes involved in early DV patterning results in phenotypes that are not specific. For example, phenotypes resulted from *egfr* knockdown are recognized as cuticles with only disorganized anterior segments, the posterior segments including thorax and abdomen are missing (Fig 8). It is not possible to distinguish these DV phenotypes from AP phenotypes. Thus, it is important to keep an eye on severe patterning defects, even if they seemed not to be DV-like.

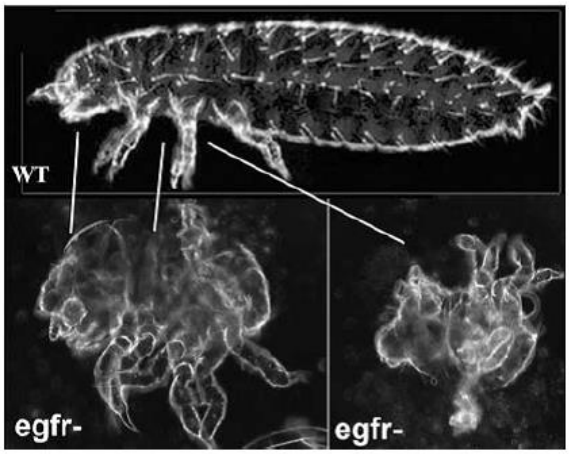

Figure 8 *egft RNAi phenotypes.*
Top: Wildtype embryo. Bottom: egfr RNAi embryos, left: embryo lacking the abdomen, right: embryo lacking the abdomen and T3 thoracic segment.

Often genes involved early DV patterning lead to embryos lacking cuticle formation, referred to as empty egg phenotype. These are the genes acting in ovarian EGF signaling, Toll signaling and early BMP signaling. Knockdown of these genes terminates development at very early stages, often before gastrulation and cuticle secretion. However, empty egg phenotypes can also result from malfunction of house-keeping genes or from a lack of fertilization. Due to the multiple causes of empty egg phenotypes, we expected a big number of empty egg phenotypes showing up in the screen. DV patterning phenotypes can be distinguished most of the time by the presence of differentiated tissues. However, it is challenging to score tissue differentiation during the screen, either by cuticle preparation or by muscle preparation with fluorescence. Subsequent screen with nuclei stain, such as Fuchsin staining, is necessary in this case to eliminate the unfertilized egg phenotypes. Thus, it is important to take all genes leading to a lack of cuticle formation for a second round of analysis with nuclei stain.

Dorsalized or ventralized phenotypes lead to cuticles with disorganized “tube-like” structure. In these phenotypes, AP axis formation took place, but DV patterning is not accomplished. Ventralized phenotypes can be further identified

by the presence of rows of denticle belts. Dorsalized phenotypes can be further characterized by the loss of head. These phenotypes are important to identify component downstream of Dorsal that contribute to the establishment of fate map along the DV axis. Weakly ventralized phenotypes, in which only the most dorsal fate such as amnion and dorsal ectoderm is lost, can be characterized by dorsal open or inside-out cuticles (Fig 9).

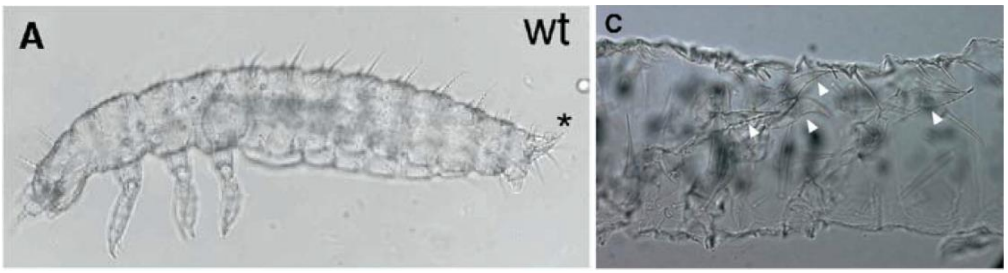

Figure 9 *inside-out phenotype caused by zen-2 RNAi knockdown.*
Left: wildtype embryo. Right: inside-out phenotype, appendages and bristles are inverted inside the cuticle (van der Zee et al., 2005).

Knockdown of genes involved in the patterning of mesoderm results in almost normal cuticle formation. *twist* RNAi leads to phenotype with almost normal morphology, except that embryos are slightly bent in the thoracic region and has a protruding hindgut (Fig 10). These types of phenotype are not always easy to spot in the screen; however, subsequent screen with muscle-specific GFP will reveal the loss of musculature, indicating the malformation of mesoderm.

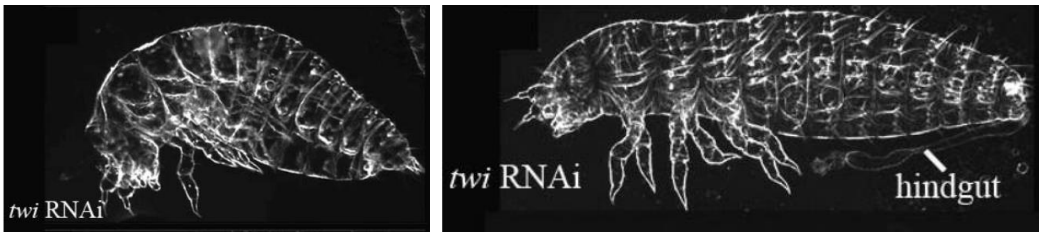

Figure 10 *twi RNAi phenotype.*
Left: embryo with bent thorax, right: embryo with protruding hindgut.

If the genes are mainly involved in the formation of the growth zone, the knockdown phenotype can be fairly easily spotted by the cuticle structure that has normal head and thorax and abnormal abdomen. One example of this phenotype is *saxophone* RNAi, the abdomen in this case is ventralized and has smaller DV circumference (Fig 11). Since the mechanisms of growth zone development are largely unknown in *Tribolium*, we expected more of this type of phenotype in the screen.

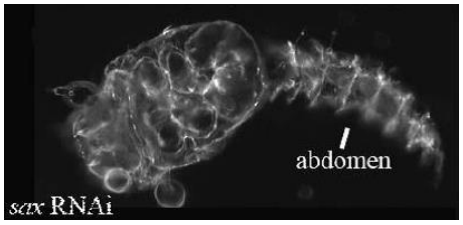

Figure 11 *sax RNAi phenotype, the abdominal circumference is narrowed.*

2.2. MATERIALS AND METHOD

In order to cover all developmental processes in embryogenesis and metamorphosis, the screen was performed at both pupal stage and larval stage. Late larval injection allows discovering the phenotypes affecting metamorphosis, stink gland development and oogenesis. Late pupal injection leads to embryonic phenotypes of metamorphosis and oogenesis in the offspring. In the pupal screening, phenotypes were inspected based on embryonic cuticles and fluorescent-labeled musculature structure by making use of transgenic imaging lines (See section 2.2.3). In the larval screen, phenotypes were inspected based on pupal and adult morphology.

DV patterning is an early embryonic process, most of the phenotypes would be revealed in the pupal screen. Therefore, I participated in the pupal screen.

2.2.1 Time and place

The screen is divided into two phases. The first phase, which I took part in, was from September 2010 until December 2011. The second phase is going on, starting from September 2013. Each screening phase covers half of the *Tribolium* genome.

The screen took place in two screening centers: (1) Department of Developmental Biology, University of Erlangen and (2) GZMB, Department of Developmental Biology, University of Göttingen. There were two screening teams in each center that perform and analyze either larval or pupal screen in parallel. I joined the Göttingen team.

2.2.2 The screening procedure

The iBeetle screen was designed to optimize the quantity of injections. As I took part in the pupal screen, I will introduce the workflow of the pupal screen in this thesis.

Overall, the screen was organized into bundles. Each bundle consists of 110 genes. Injections and analysis of a bundle were finished within five week. Each bundle was organized into five repetitions. In each repetition, 25 injections were carried out including 22 new *Tribolium* genes, 2 buffer injections and 1 positive control. The overall workflow of a bundle is illustrated in Fig 12.

Injected beetles were kept in the 25 vial system (A. Berghammer et al., 1999) and incubated at 30^0C, 40-60% humidity. This 25 vial system assists embryo collection and synchronizes condition for stock keeping.

A repetition was performed as following:

- *Injection:* for each gene, corresponding dsRNAs were injected into 10 female pupae of the pBA19 strain. dsRNAs were used at 1 μg/μl concentration. Three days later, the hatched adults were transferred into vials and crossed with 4 males of the black strain. The number of hatched female was scored in order to discover lethal genes and to judge egg production.

- *Cuticle preparation*: The first egg lay was collected on day 9 after injection. The quantity of egg production was scored and compared with that of wildtype. The number of living females was also scored for lethality control. This first egg lay was kept in the 25 well block system with the 300 μm meshes and incubated at 30ºC for 4 more days. Hatched larvae were sorted out by the meshes and discarded. Only unhatched embryos were subjected to cuticle preparation, which was carried out on day 13. Cuticle preparation was carried out as described in Chapter 3, Section 3.2.6 of this thesis.

- *Muscle (fresh) preparation*: The second egg lay was collected on day 11 after injection. Analysis was carried out on the same day. Embryos were

embedded on Voltalef Oil and analyzed under fluorescent microscopes to observe musculature defects. The number of living females was also scored.

- *Ovary preparation*: the quantity of egg production was checked one more time on day 13. If egg production was reduced to 50% or less compared to wildtype situation, the females were dissected.

- *Cuticle analysis*: cuticle preparations were analyzed on the week 5.

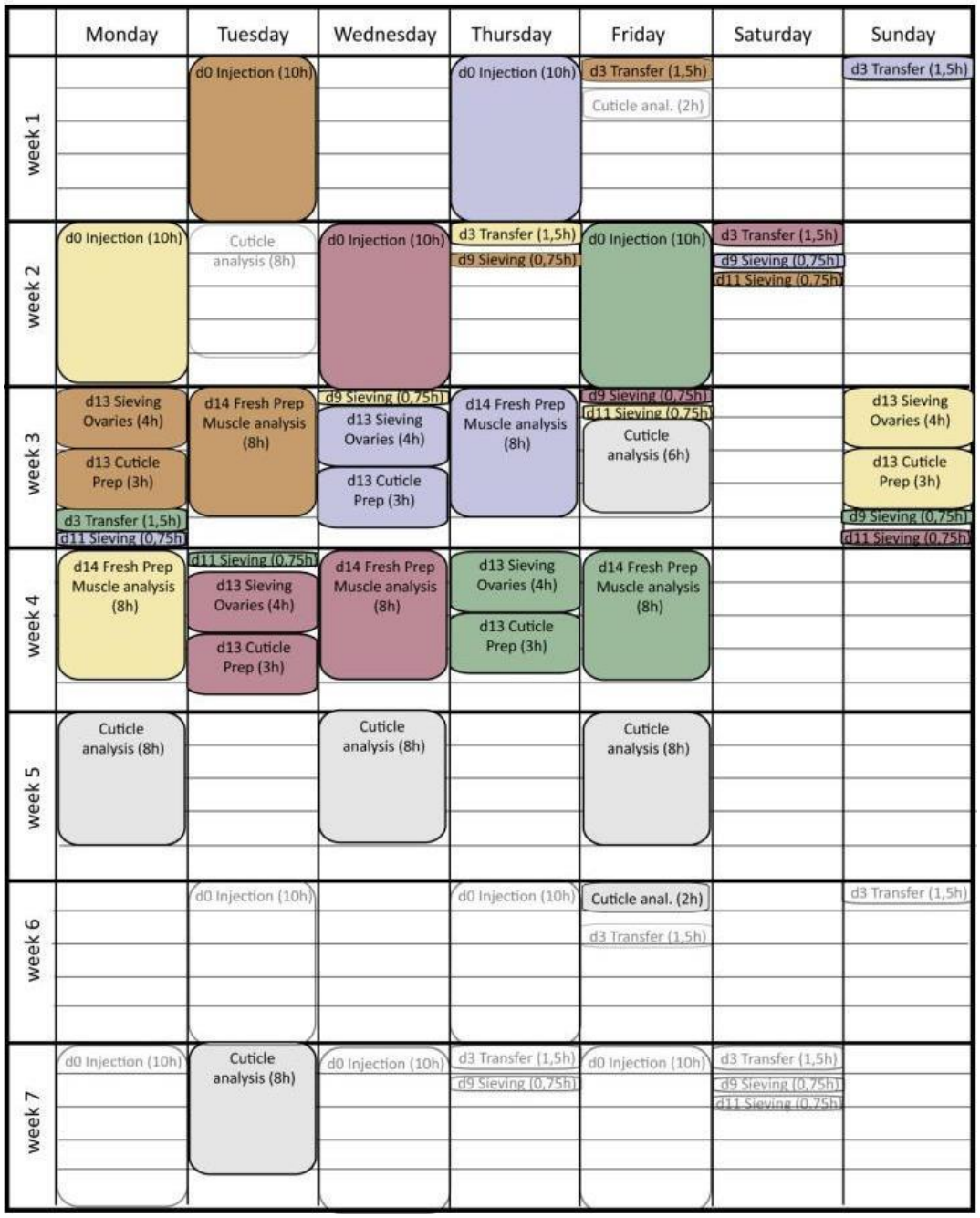

Figure 12 Workflow for pupal injection

24 injections are performed in parallel in one repetition. Different steps of one repetition are illustrated in the same color. The length of the boxes reflects the time required for each step. Five repetitions are organized into the time frame of 5 weeks. The subsequent injections starts in week 6 (open grey boxes). This timetable is designed in

a way that a second screener can perform the same work with a 2 week offset using the same equipment.

2.2.3 Beetle strains

In the pupal screen, the beetle strain pBA19 was used for injection. The pBA19 strain contains the insertion of the pBac{EGFP}. This insertion point is in the 3' UTR of an *actin* gene, resulting in expansion of the EGFP expression domain in muscle tissue throughout the body.

Injected females of the pBA19 strain were crossed to males of the black mutation. Benefit of the black strain is the possibility to visually distinguish females and males. It was a great help to score the number of living injected females.

2.2.4 Generation of dsRNAs

The templates for dsRNAs (i.e. iBeetle-Library) were generated using both cDNA and PCR based strategies. Most of the templates were produced through an EST (Expressed Sequence Tag) screen. The rest of genes that were not covered by this approach were subsequently generated by PCRs based on *in silico* gene prediction.

cDNA based template generation

Normalized cDNA from two developmental stages (embryonic and larval/pupal) were used. About 40,000 EST sequences were generated that collapsed into 7,500-10,000 clusters. One sequence was selected from each cluster.

PCR based template generation

For genes not covered by the cDNA bacsed template generation, templates for dsRNAs will be produced directly from cDNA using gene-specific primers.

Template generation and dsRNA production were carried out by the company Eupheria Biotech GmbH.

2.2.6 Positive controls

Positive controls were used to assess the sensitivity of the screen. One positive control is added into a repetition spontaneously. Positive controls are genes with known functions and phenotypes. There are 38 genes used as positive controls in the pupal screen (Appendix A). The identity of positive control was hidden to the screeners during the screen.

2.2.7 The iBeetle-Base

iBeetle screen was expected to produce a huge amount of data. Therefore, this data would be best stored electronically, for the matter of convenience for the screeners and to monitor data synchronously. The iBeetle-Base is an electronic database implemented by the iBeetle team to support organizing experiments and searching for phenotypic data. The iBeetle-Base includes the repetition manager function to organize the injections, the feature manager function to collect and monitor phenotypic data and the search function to find annotated phenotypes.

Data produced during the screen was entered directly into the database. The server is located in Göttingen and was accessed via internet interface. Each screener has an account of the database, by which they can access the dsRNA library, follow the screening procedure and annotate phenotypes.

Complete information for each dsRNA is summarized up and made accessible via the search function.

2.2.7.1 Repetition manager

The repetition manager is a module of the iBeetle-Base that functions like an electronic Lab note. The repetition manager stores information for all delivered iBeetle dsRNAs, including iB number, position in the 96 well plates with IDs. To start a new injection repetition, a screener activate a set of 22 new genes from a 96 well plate (Fig 13). The database automatically suggests a set of genes, normally continuing from the last screened repetition, and adds buffer controls as the first and the last injections. This automated suggestion could be manually changed (for example, in case a gene needed to be re-injected). The order of genes in the repetition could also be altered anytime during the experiment if needed. After a repetition is activated in the database, the new dataset is visualized in the 96 well plate in different color to help screeners keep track of the current injection. At the same time, the 25 well system in which beetles are kept is also displayed with indication of the position of the current injection (Fig 13).

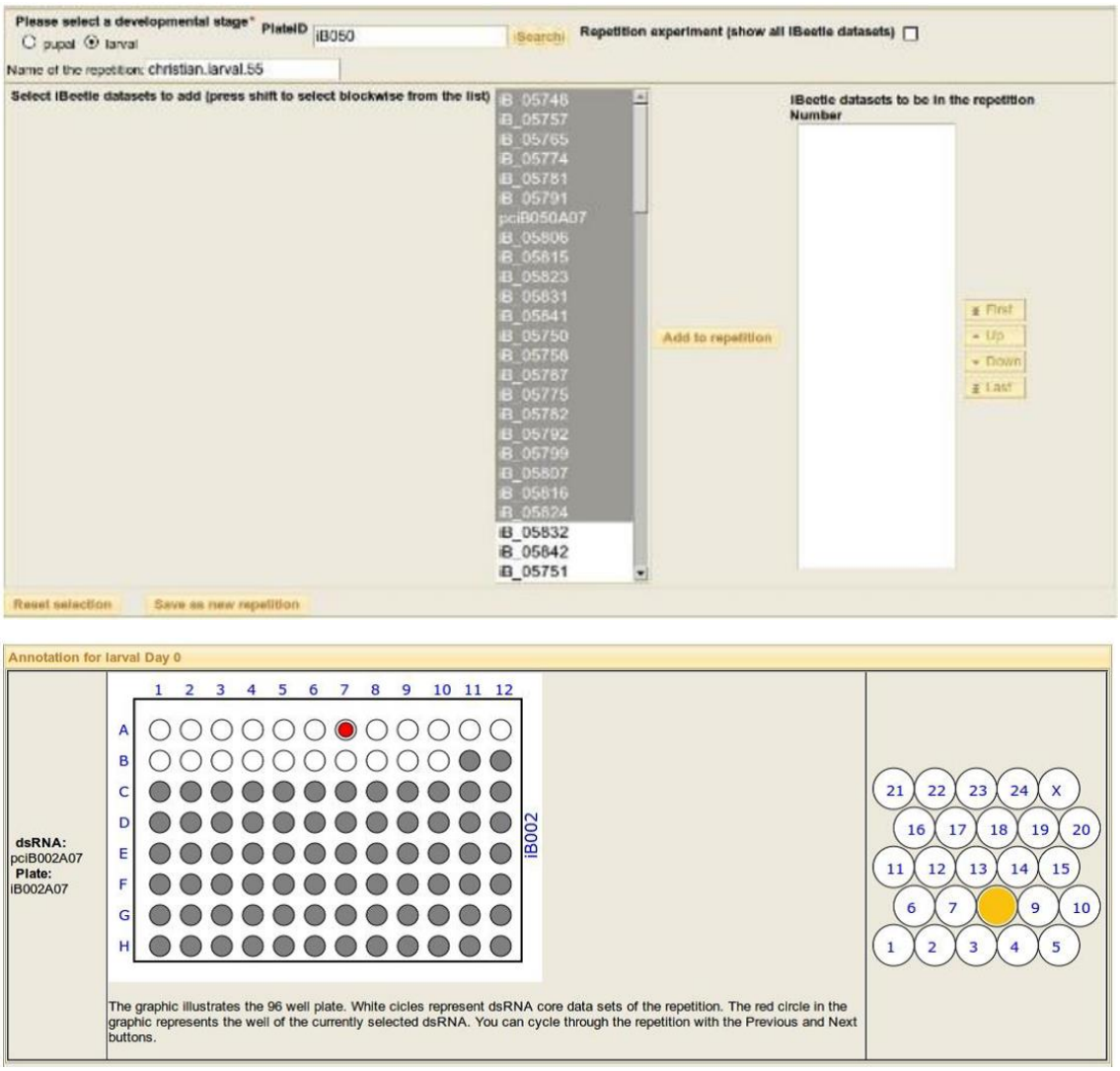

Figure 13 *Repetition manager*

Upper: a screenshot of the repetition manager. Repetition managet helps organize 21 genes and 2 buffer controls into one repetition.

Lower: a currently open dataset. Left panel: the 96 well plate in which dsRNAs are stored. Right panel: the 25 well system in which the injected beetles were kept. Current injection is displayed in different colors.

During the injection step, technical remarks, such as pupae younger/older than usual, amount of dsRNA injected more/less than usual, needle clogged,

the whole repetition injected earlier/later, etc… can be noted down, for better judgement of the injection quality and phenotypic strength in some cases. The following experiment days, i.e. day 3 – beetle transfer or day 9 and day 11 – egg colletion are displayed in new interface (Fig 13). All important information, such as number of hatched females, number of living females, quantity of egg production are mandatory fields to annotate.

2.2.7.2 Phenotype annotation

The aspects to be annotated are called features, i.e. phenotypic aspects or morphological structures to be screened. The features are organized in dropdown menus. Vocabulary for each specific structure is displayed in the dropdown menu only for that structure. The respective features for each screening day are determined to be displayed for annotation. For example, the feature “pupal abdomen” was only displayed on those days when it was to be monitored.

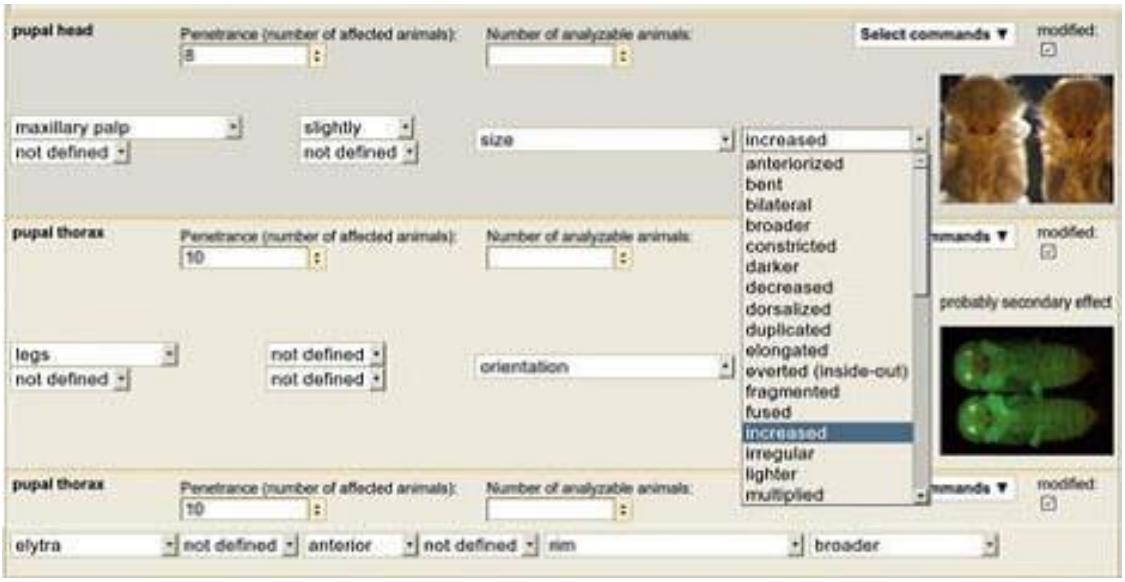

Figure 14 *Data entry interface*

An example of annotating a phenotype using the given vocabulary and uploaded pictures documenting the respective phenotype.

A phenotype was annotated according to the EQM system (Entity, Quality, Modifier). For example, a phenotype “maxillary palp” (E), “size” (Q), “increased” (M) can be annotated using dropdown menus with given vocabulary (Fig 14). The phenotypic penetrance can be annotated as the estimated proportion of cuticles showing the phenotype, i.e. <30%, 30-50%, 50-80% or >80%. Free notes/comments were possible but screeners were encouraged to primarily use the given vocabulary for the sake of searchability. Phenotypes are additionaly documented by pictures (Fig). Pictures uploaded in the database were automatically tagged with the current iBeetle number, the corresponding feature and the respective screening day.

Finally, the dataset of one iB number was completed with the iB fragment and primer sequences and with additional data such as the date of injection, screener and screening center.

2.2.7.3 Search function

The search function of the iBeetle-Base is free to access at http://ibeetle-base.uni-goettingen.de/. iBeetle search is keyword-based. Upon typing into the “Morphological structure” field, a selection of anatomical terms is suggested. The penetrance of the respective phenotype (over 30%, 50% or 80%) can be set. The outcome number of datasets for the chosen phenotype is displayed immediately at this point, giving a quick overview of the amount of genes leading to the respective phenotype. This helps to quickly decide whether the search criteria should be relaxed or strengthened. It is possible to search for multiple aspects in parallel or to ignore some aspects by combining the searches with AND, OR, or NOT (Fig 15).

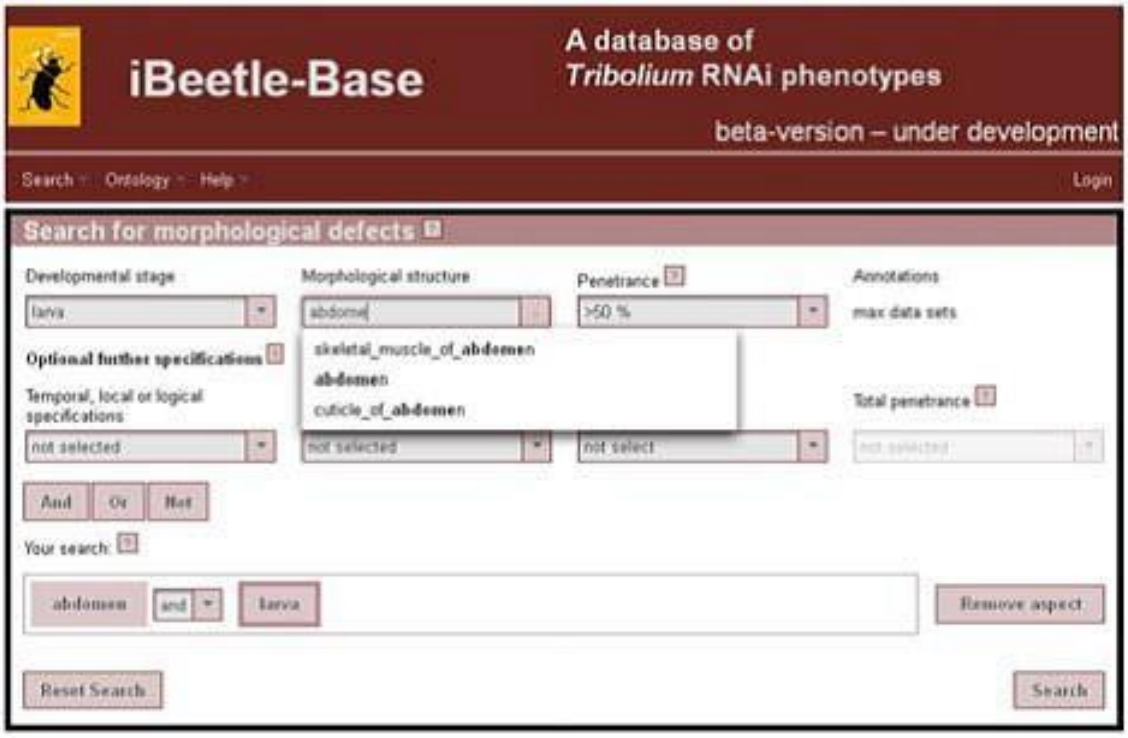

Figure 15 *An example of a morphological search*

The developmental stage (larval/pupal) and the morphological structure can be chosen from dropdown list. The penetrance can be set to >30%, >50% or >80%. Nature of the change can be implied in the dropdown boxes. Several searches can be combined by the terms "and", "or", or "not".

2.3 RESULTS AND DISSUSION

Overall, I have screened 890 genes. The screen took more time (16 months) than expected (12 months) due to the extension of the training phase, which means less time for detail analysis. We, screeners, find this training phase necessary, especially to get used to working with the iBeetle database and the heavy workload.

From the database, we have found 25 candidate genes showing potential DV phenotypes. Re-screening using different beetle strains and different RNA fragments has narrowed down the list to 10 most promising phenotypes. Detailed analysis of these candidates has revealed components of the Toll and BMP signaling pathways and one gene involved in growth zone patterning or morphogenesis.

2.3.1 Criteria for the search of DV phenotypes

There are several criteria we used to search for DV phenotypes in the database. For empty egg phenotypes, only highly penetrant phenotypes were chosen as potential candidates. A cutoff of 80% penetrance gave 68 candidates (Appendix B).

Potential dorsalized or ventralized phenotypes, which were often annotated as “fragmented”, “structures missing” or “cuticle remnants”, usually appeared as background phenotypes at low penetrance. Therefore, only highly penetrant phenotypes were considered as candidates. There were 31 phenotypes found with a cut-off of 50 % penetrance. Same concept was true for inside-out phenotypes. We encountered this class of phenotypes quite often also in buffer injections and wildtype (personal communication with other screeners). However, we kept in mind that inside-out phenotypes could also be accompanied by stronger phenotypes such as empty eggs or fragmented cuticles, or weaker phenotypes such as dorsal open. We took a cut-off of 50%

for inside-out phenotypes. After all, there were 63 candidates in this selection. No instance with normal cuticles and loss of musculature was observed. We carefully looked at the detailed annotations for better overview of the phenotypic strength. True phenotypes were determined based on the consistency and penetrance of the defects. For example, phenotypes with diverse defects, phenotypes with defects indicating AP disruption rather than DV were eliminated from the list. Using this method, the list of abnormal cuticle phenotypes was reduced to 46 (Appendix C). In total, we had 114 potential candidates for DV patterning genes, 68 empty eggs and 46 abnormal cuticles.

We further looked at gene annotations of these 114 genes. The iB fragments were loaded into the NCBI Blast (http://blast.ncbi.nlm.nih.gov/Blast.cgi) to search for orthologs of *Drosophila*, vertebrates or other insects. Empty eggs caused by the knockdown of typical house-keeping genes were not taken. Genes involved in common biological processes like metabolism were excluded. Kinases and phosphatases, transcription factors, protein-protein interaction factors, etc… are the most interesting. This strategy resulted in a list of 25 genes (Table 1). These genes were subjected to the re-screening.

Table 1 *25 potential candidates for Tribolium DV patterning*

#	iB_#	Phenotype	Gene Info
1	iB_05549	similar to *sog* RNAi	SoxNeuro
2	iB_00095	inside-out	fatty acid-CoA ligase
3	iB_00641	inside-out	Gef26
4	iB_01094	inside-out	RhoGAP68F
5	iB_01775	empty eggs	Cysteine proteinase-1
6	iB_01901	empty eggs, remnant inside-out	pelle 2
7	iB_02469	empty eggs, remnant inside-out	pelle
8	iB_03838	empty eggs	Neprilysin 4
9	iB_04007	empty eggs	serpin 4

10	iB_05768	empty eggs	Tumour suppressor protein
11	iB_00081	fragmented head-thorax missing	eclair
12	iB_00567	empty eggs	Ras guanyl exchange factor
13	iB_00676	fragmented abdomen constricted	membrane-targeted protein
14	iB_00917	abdomen inside-out, constricted	serpin 43A
15	iB_02340	abdomen inside-out, constricted	Nipped-A
16	iB_02419	empty eggs	Tango2
17	iB_02466	remnant inside-out	serine protease H158
18	iB_03900	empty eggs	Scraps
19	iB_05566	inside-out	smell impaired 35A
20	iB_06060	inside-out, diverse	klaroid
21	iB_01908	empty eggs	C-terminal LisH motif
22	iB_03968	inside-out	serine-type carboxypeptidase
23	iB_03370	empty eggs	γ-soluble NSF attachment
24	iB_04191	empty eggs	actin binding
25	iB_05623	inside-out, remnant	serine/threonine phosphatase

2.3.2 High number of empty egg phenotypes and the technical limits in the first funding period of the iBeetle project

During the screening, we have realized that we encountered empty egg phenotypes quite often. Empty egg phenotypes represented early development disturbance, which includes early DV polarity effects. However, with the techniques we used in the screen, it was very difficult to score the presence of embryonic tissues (which indicates early development) and the absence of embryonic tissues (which indicates the involvement of fundamental house-keeping genes, unfertilized eggs or genes required for cuticle secretion). Because interesting axis formation and segmentation genes can also lead to empty phenotypes, it is necessary to apply another technique for better

assessment of this class of phenotypes. This is also one of the aspects to be improved for the second funding period.

The main aim of my research focused on the missing components of the Toll signaling pathway. Therefore, it is essential to select the right empty egg phenotypes for the detailed analysis. Among the 68 empty egg phenotypes found from the database, we have chosen 12 most interesting genes based on gene information (Table 1). In the screen, we have also found *Tc-nudel* (iB_03095), an upstream component of Toll signaling that transmits the ovarian signals to the embryo (LeMosy et al., 1998). Knockdown of *Tc-nudel* resulted in a high penetrant, totally empty egg phenotype instead of a dorsalized phenotype as in the case of Toll knockdown. This result suggested that all iB genes that resulted in empty egg phenotype should be taken in a second round of screen.

In the rescreening, besides the standard process like cuticle preparation and muscle preparation, we also performed fuchsin stainings. Fuchsin stainings were performed as described in the Chapter 3. Egg collections comprised a broad range of embryonic stages. With fuchsin stanings, we were able to evaluate whether the empty egg phenotypes were due to (1) lack of embryonic development (unfertilized eggs or eggs which do not support development due to general maternal defects), (2) abortion of embryonic development caused by defects in cell division, cellularisation or non-specific cell death (Fig 16), or (3) severe patterning defects like complete dorsalization, ventralisation or abnormal positioning of the DV axis (Fig 16).

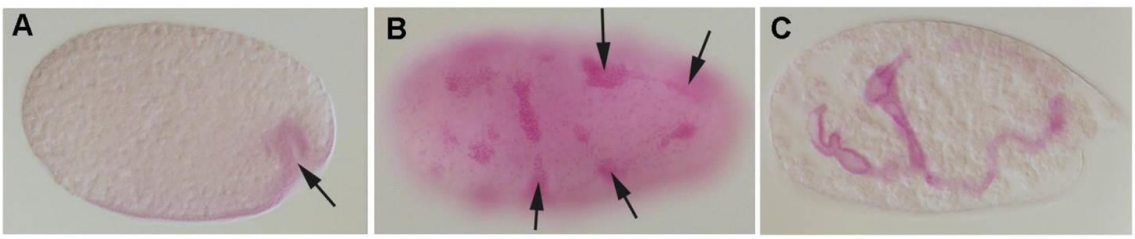

Figure 16 *Fuchsin staining of some empty egg phenotypes.*

(A) Wildtype embryo during gastrulation, the germ rudiment locates at the posterior ventral region, arrow indicates invagination at the posterior pole. (B) Knockdown of neprilysin 4, nuclear condensations observed throughout the blastoderm (arrows). (C) Knockdown of serpin 4 resulted in hollow epithelial tubes running through the yolk, which might indicate DV axis formation defects.

The results of our Fuchsin stainings showed that two of the empty egg phenotypes (iB_04191 and iB_01908) completely lack embryonic development. Three phenotypes (iB_03838, iB_01775, iB_04007) were due to early development defects including irregular cellularisation. Some of these early defect phenotypes include potential DV patterning defects.

2.3.3 The complexity of DV cuticle phenotypes and the interference of background defects

Next to the high number of empty egg phenotypes, which hold potential for early defects during DV axis formation, we had to face some other problems while searching for DV cuticle phenotypes. Cuticle phenotypes indicating DV patterning disruption can range variably, which made our selection quite big at the beginning. Moreover I have learned from the iBeetle screen that the cuticle phenotypes were usual not consistent but often appeared in a range of phenotypic series, which can be well explained by RNAi time or strength effects. Therefore, it is sensible to not rely only on high penetrant phenotypes, but rather broaden the criteria to not miss any false positives. On the other hand, it happened from time to time that knockdown of a gene resulted in a striking phenotype at the first glance, but on the same slide can be found a diverse class of phenotypes which do not seem to relate to the phenotype we are interested in, which might indicate that the gene is pleiotropic. Thus, critical reading of the annotations is very important.

Another crucial point is that the description of a phenotype depends heavily on the screener who annotated it. Even though the annotation to iBeetle database was made with keywords based on a vocabulary developed by iBeelte team, personal opinions and subjective perspectives cannot be taken too lightly. In most cases, there are more than one correct ways to annotate a phenotype; for example, “leg – length – reduced”, “leg – shortened”, or “leg – length – shortened”. iBeetle phenotypes were documented additionally by pictures, which were a great help in understanding the phenotypes.

Some features of the cuticle phenotypes are often caught in buffer injections, which also occurred from time to time at some level in iB specific gene injections. Several phenotypes are characterized as background defects, e.g. parts of the head missing, leg shortened, etc…, and also inside-out cuticle and dorsal opened, which are DV phenotypes. They were often very few, but it was not possible to rule out that they occurred with higher penetrance. Here, it depends totally on the screener who wants to annotate those background-like phenotypes or not. It is possible that we have false negatives or false positives in a massive genetic screen like the iBeetle screen.

For those reasons, strict searching criteria did not seem to work for DV cuticle phenotype. It was my favor at the beginning to look for highly penetrant cuticle phenotype. However, in the end I loosened the searching criteria and started with a big number of candidates. Further refinement of the list was done with speculations of gene information.

2.3.4 Reproducibility, strain specificity and off target defects

RNAi gene knockdown is imperfect in a way that the strength and the penetrance phenotype depend on the degree of gene knockdown. RNAi in *Tribolium* has been shown to be efficient even when injected at very low concentration, i.e. 0,001 μg/μl, 1000 fold dilution compared to our RNAi concentration (Miller et al., 2012). In our screen, we have tested if the 1 μg/μl

dsRNA concentration would not induce the full phenotype. For 94 genes we injected both dsRNAs of 1 μg/μl and 3 μg/μl concentration. Only in 14.9 % the phenotypic strength was strongly enhanced in high concentrated dsRNAs. Most probably our injections were sufficient to produce the full knockdown phenotypes.

In RNAi gene knockdown, it is important to keep in mind that the results can be distorted by off target effects. Injected dsRNAs might hit not only the target gene but also unrelated genes due to partial sequence identity (Kulkarni et al., 2006; Ma et al., 2006). Off target effects can also well happened due to the matching of a few siRNAs, out of many more that originate from the large dsRNA fragments we inject. Therefore, a portion of phenotypes that could not be reproduced with non-overlapping fragments is expected in our rescreen.

Strain specific effects were true not only in our screen in *Tribolium*. Influence of genetic background on mutant phenotypes was observed for *E.coli,* rice, *C.elegance*, mouse and *Drosophila* (Cao et al., 2007; Dworkin et al., 2009; Félix, 2007; Lander and Schork, 1994; Linder, 2006; Remold and Lenski, 2004; Threadgill et al., 1995). A study followed up the recovering of *Tc-importin α1* in iBeetle screen proposed that one possibility for strain specific phenotypes is the difference of maternal input in different strains (Kitzmann et al., 2013). Strain specific effects are challenging issues while searching for specific phenotypes of interest and might confuse the assessment of phenotypes. Therefore, it is critical to relax the searching criteria and count on gene information while working with the iBeetle database.

The iBeetle screen was designed with the concept of minimizing false negatives with the tradeoff of increased false positive results. The last injection of each injection day was either a positive control or a buffer injection. The buffer injections were used to statistically assess the potential of negative annotations. Indeed only 2.6 % of those buffer injections were annotated with phenotypes. In our screen, wildtype embryos could be easily recognized by the hatch rate and the number of animals on slide (the block system allowed

separation of hatch larvae and unhatched embryos, only unhatched embryos were embedded on slides). This system made it easy for screeners to quickly determine the no phenotype situations. However, there was a higher risk of having false positives, for example, due to the nature of the gene, the sensitivity of RNAi or genetic background. False positive annotation due to technical issues (for example lethality caused by bad injection, bad stock…) can always be traced back by data stored in the database.

In the first funding period, which I took part in, the template production was based exclusively on gene predictions that were supported by RNA sequence data. It means those genes are expressed at above-average levels. In fact, there was a strong bias towards conserved genes in the gene set screened, i.e. 82% in the iBeetle screen compared to 59% in the official gene set. Moreover, data from our screen showed that knockdown of conserved genes is more likely to produce phenotypes (60%). It seems like our dataset is biased towards genes that have orthologs in other species, which are more promising to show phenotypes. A portion of unknown, novel or beetle specific genes were not screened in the first funding period. We expect more specific phenotypes to show when iBeetle screen reaches the whole genome coverage, especially for the processes that we know so far only in *Tribolium*.

2.3.6 The rescreen of the 25 candidates

To confirm the phenotypes of the 25 candidates in our final list, we performed the rescreen. The rescreen was designed to eliminate the phenotypes caused by strain specificity and off target effects. We carried out the rescreening as following: non-overlapping fragments were injected into pBA19 females to check for off target effects; the same fragments used in the screen were injected in to San Bernardino (SB) strain to check for strain specific effects. Since the future analysis work would be performed on the wildtype SB

strain, it is necessary to check the phenotype in this platform. dsRNAs were purchased from the company.

Our rescreening resulted in 12 genes that did not show an RNAi phenotype in the SB strain and 3 genes that could not be confirmed with non-overlapping fragments for dsRNA synthesis. The remaining 10 genes were confirmed (Table 2).

Table 2 *List of 10 successfully rescreened candidates*

	Cuticle	**Fuchsin**
iB_00095	inside-out loss of head segment	head defects
iB_01094	inside-out	growth zone abnormal
iB_01775	empty eggs	cellularisation defects some potential DV defects
iB_01901	empty eggs remnants inside-out	dorsalized
iB_01908	empty eggs ovary defects	cellularisation defects
iB_02469	empty eggs remnants inside-out	dorsalized
iB_03838	empty eggs	cellularisation defects
iB_04007	empty eggs	cellularisation defects some potential DV defects
iB_04191	empty eggs	no embryo
iB_05549	*sog*-like phenotype	germ band defects

Indeed, almost half of our candidates were not confirmed in the SB strain. This data suggested a relatively big genetic background difference between SB and pBA19, and that genetic background is essential in RNAi screening. Meanwhile, the off target effects observed in our rescreen were quite small, indicating that gene predictions and template production in iBeetle were reliable. The reproducibility of phenotypes in our candidate selection was lower than data from the whole iBeetle project (40 % versus 80-90 %). It seems that DV phenotypes are susceptible to false positives, probably due to the wide range of defects that can be interpreted as DV phenotypes. There is no simple solution

for this problem; it takes just more effort to rescreen more genes in order to find out the true DV phenotypes.

2.3.7 Potential DV patterning genes

2.3.7.1 Pelle and Tube-like kinase

These two genes share a highly penetrant severely dorsalized phenotype. This phenotype was characterized by hollow epithelial tubes running through the yolk, which closely resembles the *dorsal* or *Toll* knockdown embryos (Fig 17). One of those is the protein kinase Pelle (iB_02469), which is the key downstream component of Toll signaling. The second phenotype resulted from the knockdown of another protein kinase which is related to *Drosophila* Tube (iB_01091). We therefore refer iB_01901 to *Tribolium* Tube like kinase.

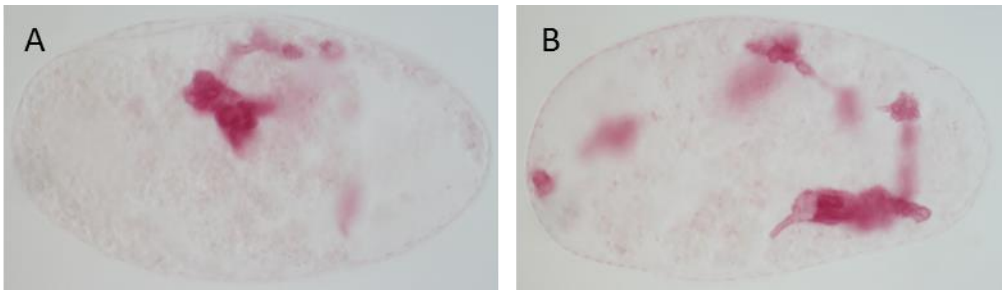

Figure 17 *pelle (A) and tube-like kinase (B) RNAi knockdown*
The hollow tube-like structure is observed as in Toll or dorsal knockdown.

Pelle and Tube-like kinase are components of the Toll signaling pathway. *Drosophila* Pelle is composed of an N-terminal death domain required for interaction with MyD88 and a C-terminal kinase domain that phosphorylates downstream targets (Galindo et al., 1995; Shelton and Wasserman, 1993). *Drosophila* Tube contains only a death domain, which explains its function in protein-protein interaction as an adaptor (Letsou et al., 1993). Tube death domain acts as a link between the death domain of MyD88 and Pelle (Sun et al., 2002; Xiao et al., 1999). A study based largely on bioinformatic analysis has revealed that *Drosophila tube* emerged from an ancestral protein with both a

death domain and a kinase domain (Towb et al., 2009). This study has also pointed out that *Drosophila* Tube is orthologous to the human IRAK 4, the protein kinase that functions in Toll signaling in vertebrate innate immunity (Suzuki et al., 2002; Towb et al., 2009).

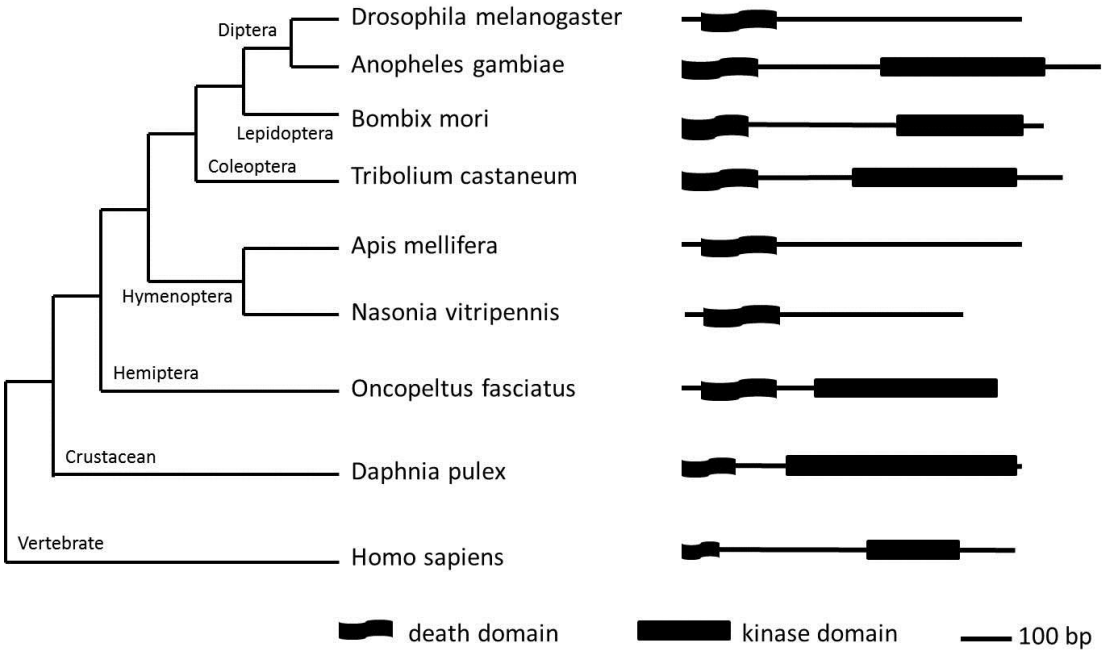

Figure 18 *Scale diagram of Tube or Tube like kinase protein structures*

Fig 18 shows a scale diagram of several structural Tube or Tube like kinase proteins through the animal kingdom. Indeed, many *Drosophila* Tube orthologs are protein kinases. *Tribolium* Tube like kinase also contains a kinase domain. However, the function of this kinase domain in Toll signaling, if it has function at all, is still unclear. The loss of the kinase domain occurred multiple times independently in insect evolution, as seen in some certain linages such as *Drosophila, Apis* and *Nasonia*. However the kinase domain is observed in a more basal branching insect, *Oncopeltus fasciatus*. Interestingly, the function of *Drosophila* Tube and Pelle can be combined in one single chimeric protein (Towb et al., 2009). If the kinase domain is not necessarily needed for proper

DV axis formation, it would indicate that the system is already prone to evolutionary change, as seen in *Drosophila*.

2.3.7.2 SoxNeuro

The SRY-related high-mobility-group box (Sox) transcription factors are known for their function as regulators of cell fate decisions during development. Sox factors fall into 8 subfamilies. There are 20 *Sox* genes in vertebrates and 9 *Sox* genes in insects (Lefebvre et al., 2007). In *Drosophila*, there are 4 members of the class B Sox genes that have been functionally characterized, including *Dichaete* and *SoxNeuro* (Phochanukul and Russell, 2010). *Dichaete* and *SoxNeuro* act redundantly in *Drosophila*. They are involved in early CNS development but seem not to function in BMP signaling in establishing ectodermal fate.

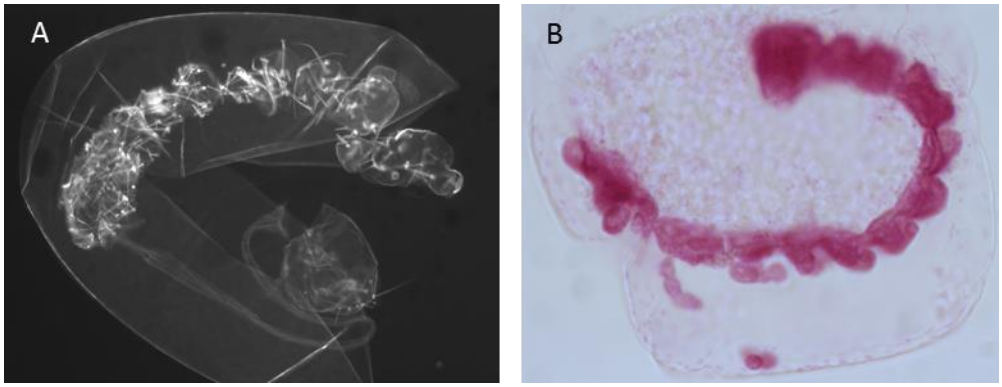

Figure 19 *SoxNeuro RNAi knockdown*
(A) Cuticle prep. (B) Fuchsin staining.

In our screen, *Tc-SoxNeuro* was identified by a *sog*-like dorsalized phenotype (Fig 19). This *sog*-like phenotype suggested that *Tc-SoxNeuro* might be involved in interpreting the BMP gradient during early development. *Tc-SoxNeuro* is expressed during early blastoderm stages in a broad central domain (Fig 20 A and A'). During gastrulation, *Tc-SoxNeuro* is repressed in the presumptive mesoderm, but is expressed in all ectodermal cells except for the

future growth zone region (Fig B and B', C and C'). During the extended germband stage, *Tc-SoxNeuro* continues to be up-regulated in the neuroectoderm and remains absent in the growth zone (Fig 20 D and D'). These *in situ* hybridization results support our hypothesis that *SoxNeuro* is involved in BMP signaling in *Tribolium*.

Besides the DV phenotype, *SoxNeuro* parental RNAi also leads to sterility in the injected females. Egg production was tremendously reduced due to ovary defects. In *Drosophila*, *SoxNeuro* regulates the expression of *shavenbaby*, a most downstream target of the signaling cascades that pattern the ventral epidermis. *shavenbaby* is also maternally provided, which is expressed during oogenesis and often referred to as *ovo*. *shavenbaby/ovo* is required in the female germ line for proper oogenesis (Garfinkel et al., 1994; Overton et al., 2007; Payre et al., 1999).

The analysis of the *Tc-SoxNeuro* phenotype is hampered by this problem. We have tried several different injection conditions but none of them could minimize sterility yet still resulted in strong embryonic phenotypes. Injection in adult female beetles with lower RNAi concentration still lead to nearly complete sterility; moreover the minor phenotypic penetrance turned into wildtype after one or two egg lays.

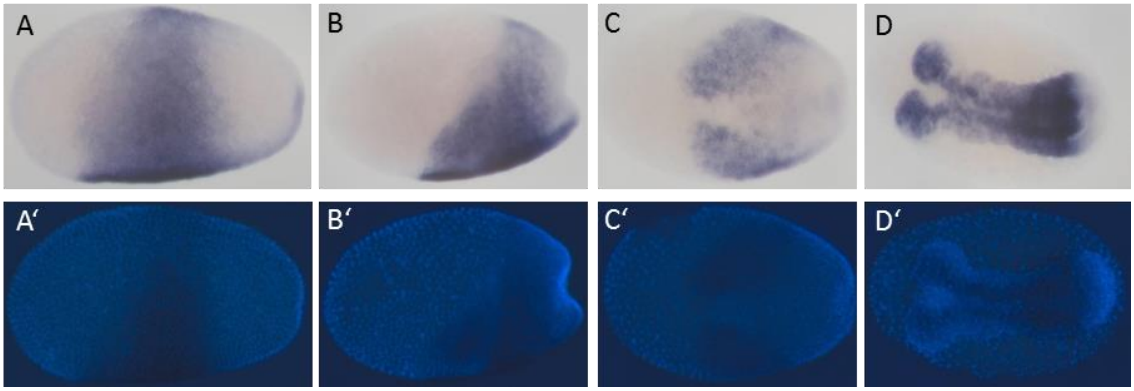

Figure 20 *Tc-SoxNeuro expression.*

(A-D) In situ hybridization for Tc-SoxNeuro. (A'-D') DAPI of the same embryos. (A) Lateral view of uniform blastoderm stage. (B) Lateral view of differentiated blastoderm stage. (C) Same embryo as in B from ventral view. (D) Early germband stage.

Recently, a study in zebrafish has shown a systematic knockdown of all four B1 Sox genes, which resulted in severe abnormalities in early dorsoventral patterning. This dorsoventral phenotype is related to the reduced expression of *bmp2b/7* (Okuda et al., 2010). Previous work on BMP signaling in *Tribolium* suggested that the early function of BMP in DV patterning shares more similarity with vertebrates than with *Drosophila* (Nunes da Fonseca et al., 2010; Van der Zee et al., 2008; Zee et al., 2006). Taken together, *Tc-SoxNeuro* potentially fulfills a vertebrate-like function in establishing the BMP gradient in *Tribolium*.

2.3.5.3 Acyl-CoA Synthetase

Knockdown of an enzyme belonging to Acyl-CoA Synthetase (ACS) family led to a series of head loss phenotype (Fig 21). Previously, it has been shown that *Tribolium* head is not only an anterior structure but also a ventral structure. Knockdown of *Tc-short gastrulation*, the BMP antagonist, resulted in progressive loss of head structures (Kotkamp et al., 2010); (Jonas Schwirz, PhD Thesis unpublished).

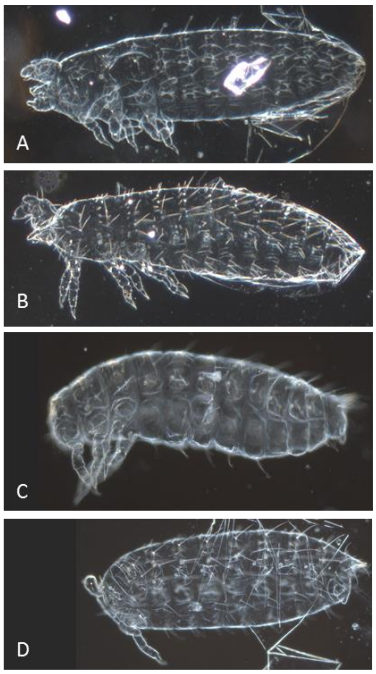

Figure 21 *Acyl-CoA sythentase RNAi. (A-D) Progressive loss of anterior structures upon ACS knockdown. (A, B) Incomplete deletion of head structures. (C) Complete head loss. (D) Deletion of head, T1 and T2.*

In *Drosophila*, a homolog of the vertebrate acyl-CoA synthetase long chain 4 (ACSL4) acts like a gap gene in embryonic segmentation (Zhang et al., 2011). However, knockdown of ACSL4 in zebrafish leads to a dorsalized phenotype, meaning to reduce BMP activity (Renisch, 2009). In *Tribolium*, it seems that the acyl-CoA synthetase gene we have identified up-regulates BMP activity. It is particularly interesting to understand the function of ACS as a component of BMP signaling.

CHAPTER 3: The perivitelline serine protease cascade

From the iBeetle screening, we have found *Tc-nudel*, the component upstream of Toll, and two components acting downstream of Toll, *Tc-pelle* and *Tc-tube-like kinase*. Besides analyzing the candidate genes from the screen, I independently searched for the missing components of the serine protease cascade from *Tribolium* genome. These components were identified by means of RNAi knockdown and phylogenetic analysis.

3.1 INTRODUCTION

The perivitelline proteolytic cascade acting upstream of Toll plays a crucial role in establishing DV axis during embryonic development. The proper activation of this proteolytic cascade induces signal transduction from the vitelline membrane to the perivitelline fluid, and triggers the activation of Toll receptor in the ventral side of the embryo. This cascade is comprised of four serine proteases, i.e. Nudel (Ndl) (Stein et al., 1991), Gastrulation defective (Gd) (Konrad et al., 1998), Snake (Snk) (DeLotto and Spierer, 1986) and Easter (Ea) (Chasan and Anderson, 1989). Successive activations of these serine proteases generate a gradient of the diffusible ligand Spätzle (Spz) (Morisato and Anderson, 1994; Schneider et al., 1994). Ventral confinement of this proteolytic cascade additionally requires the Easter inhibitor Serpin27A (Spn27A) (Misra et al., 1998) (Fig 22).

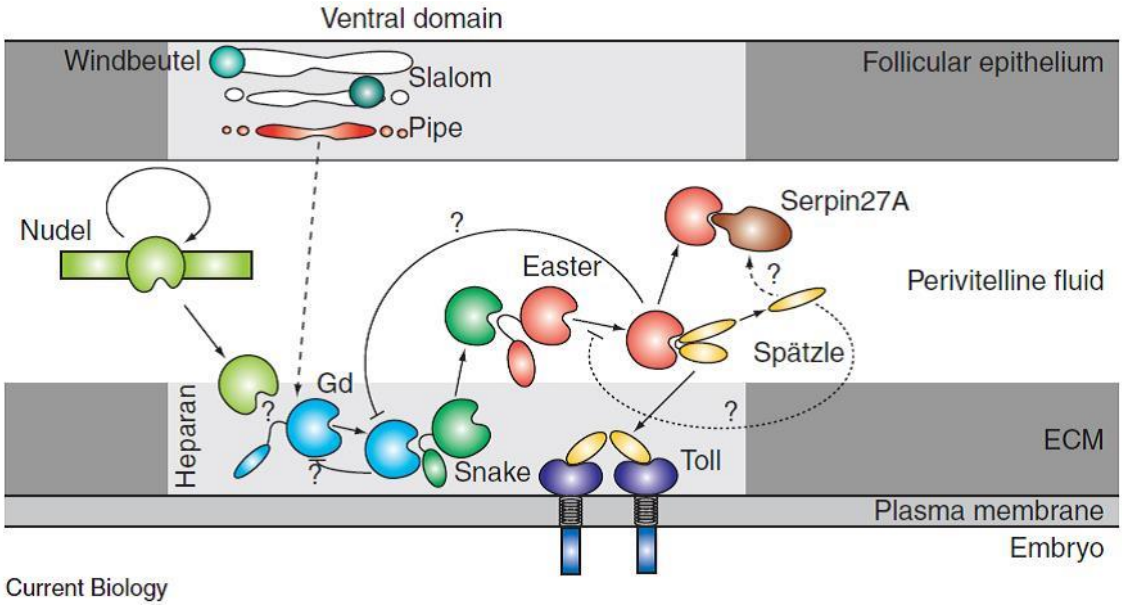

Figure 22 Toll activation in the ventral side of the embryo.

Pipe is expressed in the ventral follicular epithelium. Windbeutel is required for the export of pipe from ER to Golgi. Slalom transports the sulfate donor PAPS to the Golgi. Pipe modifies an ECM component, which is inserted into the vitelline membrane, and activates the proteolytic cascade in the perivitelline space, the space between the vitelline membrane, a product of the follicle cells, and the plasma membrane surrounding the embryo. Nudel is autocatalytically processed in an active form and cleaves Gd. Activated Gd cleaves and activates Snk. Snk in turns cleaves and activates Ea. In the end, Ea cleaves and activates the ligand Spz. Spz binds and activates Toll receptors (Moussian and Roth, 2005).

Nudel is expressed in all follicle cells and is activated by autocatalytic function and by an unknown factor (LeMosy et al., 2001, 1998). *nudel* encodes a very large protein. It structurally resembles extracellular matrix proteins with two central serine protease domains. Ndl possesses several low-density lipoprotein domains that are important for the structural formation of the eggshell (LeMosy and Hashimoto, 2000). So far, the substrate of Ndl remains unclear, but it has been suggested by three dimensional computer modelling that Ndl may bind and cleave Gastrulation defective (Rose et al., 2003). This is

the first of the three sequential serine protease actions that process Spätzle. However, it has been shown that exogenously injected Gd was able to trigger Toll signaling in a *ndl* mutant background (Han et al., 2000). These findings suggested that Ndl alone is not necessarily needed to activate Gd. Moreover, Ndl activity is not restricted to the ventral side but rather ubiquitous along the DV axis and Nudel also plays a role in eggshell biogenesis (LeMosy and Hashimoto, 2000). Taken together, these findings raised a possibility that Nudel acts indirectly in DV patterning by modifying the extracellular matrix.

Gd is initially expressed in the oocyte. It is secreted as an inactive precursor and accumulates in the ventral side of the perivitelline fluid (Chasan et al., 1992). It has long been proposed that Gastrulation defective acts to process Snake (Dissing et al., 2001; LeMosy et al., 2001). But very recently, they proved that Gastrulation defective has a second function in facilitating the Pipe-independent ventral processing of Easter by Snake (Cho et al., 2012). Genetic analysis of the *gd* locus suggested that the proteolytic activity of Gd cleaves Snake and the second function of Gd to facilitate the cleavage of Easter by Snake depends upon the integrity of the prodomain (Cho et al., 2012; Ponomareff et al., 2001). When perivitelline fluid containing GFP-tagged wild-type Gd from donor embryos was transplanted in the perivitelline space of recipient embryos, Gd-GFP was observed to accumulate in the ventral region. This localization depends on both Pipe activity and the prodomain function of Gd. These findings suggest that Gd interacts with Pipe-sulfated molecules, which brings about the ventral process and activation of Easter by Snake. Therefore, Gd seems to be an essential component of the proteolytic cascade that has input into the localization of the ventral signals (Cho et al., 2012).

Both *snake* and *easter* encode trypsin-like serine proteases with a signal peptide at their amino termini. They are expressed in the germline as inactive zymogens. Activation of Snake and Easter requires a cleavage reaction that releases the catalytic region in the C-terminus from the prodomain located in the N-terminal segment (DeLotto and Spierer, 1986; Jin and Anderson, 1990).

The processing of Easter by Snake is partially controlled by the inhibitor Serpin27A. Serpin27A acts as a suicide substrate that rapidly binds and inactivates Easter once it is cleaved (Chang and Morisato, 2002; Dissing et al., 2001; Hashimoto et al., 2003; Hoskins et al., 2002). By binding to activated Easter, Serpin27A prevents it from diffusing to lateral and dorsal regions (Chang and Morisato, 2002). Therefore, Easter activity is confined to the ventral region of the perivitelline space. The significant role of Easter inhibition by Serpin27A is demonstrated by the completely ventralized phenotypes in the progeny produced by females homozygous for a Serpin27A mutation (Hashimoto et al., 2003; Ligoxygakis et al., 2003).

In *Tribolium*, little is known about the proteolytic cascade. Most components, except for *Tc-nudel* that has been identified in the iBeetle screening, remain unidentified. *Tc-spätzle* has been discovered by Nadine Frey and Dr. Jeremy Lynch. Two fragments, *Tc-spzb2* and *Tc-spzb3*, were proposed to be the ligands of the Toll receptor. Knockdown of these *Tc-Spz* fragments lead to dorsalized phenotype, similar to that of *Toll* knockdown (J. Lynch and S. Roth, unpublished results).

Aims

The aim of this project was to identify the missing components of the serine protease cascade in *Tribolium castaneum*.

3.2 MATERIALS AND METHOD

3.2.1 *Tribolium* stock maintainance

For usual stock keeping, flour was mixed as following: “Diamant Weizen Mehl Extra Type 405” and “Diamant Dunkles Weizen Mehl Type 1050” to the ratio of 2:1, 0.1 g Fumagilin B /kg flour and 30 g baker’s yeast /kg flour. For egg collection, we used “Diamant Instant Mehl Type 405” supplemented with 0.1 g Fumagilin B /kg flour. For refine egg collection, flour was pre-sieved to get rid of the particles larger than 300 µm. Fumagilin B (Medivet Pharmaceuticals Ltd) was used to protect beetles from sporozoan infection (Bucher, 2009). Flour was kept at least one night at -20°C before using and sieves were kept at 60°C to prevent infections.

Beetles were kept in the plastic food boxes with proper air ventilation. Beetle boxes were placed in incubator at 30°C and 40% to 60% humidity. Every week beetles were separated from old flour using sieves with 710 µm wide meshes and transferred into a new box with fresh flour.

To collect embryos, first adult beetles were separated from flour by 710 µm sieve; the flour containing eggs was subsequently obtained by using 300 µm sieves. Embryos were then used directly for experiments or incubated further at 30°C to obtain a specific developmental stage.

3.2.2 Embryo fixation

Tribolium eggs were first washed from dirt and left over flour with tap water. To dechorionize the chorion, eggs were kept for 2.5 min in the 50% DanKlorix (Colgate-Palmolive). Chlorine was then washed off thoroughly by tap water.

Embryos were fixed for at least 60 min in 3 ml PBS, 2 ml 10% formadehyde and 5 ml heptane. After fixation, the aqueous phase was

discarded. Embryos were devitellinized by a methanol (MeOH) shock. 5 ml of MeOH was added and embryos were shaken vigorously by hand for 30 sec. Devitellinized eggs that sank down to the bottom of the bottle were collected and stored in MeOH at -20°C.

3.2.3 RNA isolation from *Tribolium* eggs/ovaries and cDNA synthesis

RNA isolation

RNA was isolated from *Tribolium* eggs/ovaries by using TRizol reagent. Eggs/ovaries were homogenized in TRizol using a pestle. The reaction was then centrifuged for 10 min, 12000Xg, 4°C to obtain the supernatant. RNA was purified by phenol/chloroform. After a centrifuge for 15 min, 12000Xg, 4°C, the upper aqueous phase containing RNA was collected into a new tube. Precipitation of RNA was done by 40 μl 3M NaAc pH5.2 and 800 μl EtOH. After incubation for at least 30 min at -80°C, RNA can be obtained as a pellet by a centrifuge for 15 min, 12000Xg, 4°C. The pellet was washed by 500 μl 70% EtOH. RNA pellet was then resuspended in water.

cDNA synthesis

cDNA was synthesized using SuperScriptVILO cDNA Synthesis Kit (Life Technologies) followed the manufacture's protocol.

3.2.4 Primer design

Primers were designed using the online version of the program Primer 3 (http://bioinfo.ut.ee/primer3/) (Koressaar and Remm, 2007). The process was done as following:

Paste source sequence into the program.

Change the parameter “Product Size Rangers” to 500-800 bp.

Press “pick primers” button.

By default, several primer sets will be provided. Usually the first suggestion has the best quality.

To make templates for dsRNA and *in situ* probes, we used Universal primers. For the forward primer, the sequence ggccgcgg was added to the 5' end. For the reverse primer, the sequence cccggggc was added to the 5' end.

Primers used are listed in the Appendix D.

3.2.5 Synthesis of dsRNA and *in situ* hybridization probes

dsRNA and *in situ* probes were synthesized using a two-step PCR.

First PCR

PCR reaction mix	Thermal cycles
12.5 μl REDTagReadyMix (Sigma-Aldrich)	1) 5 min at 94°C
1 μl 5 mM forward primer	2) 30 sec at 94°C; 30 sec at 55°C;
1 μl 5 mM reverse primer	1 min at 72°C; 35 cycles
1 μl embryonic/ovarian cDNA	3) 5 min, 72°C
9.5 μl water	

Second PCR

To make template for a labeled antisense probe, the gene specific forward primer and the 3' T7 universal primer were used. To make dsRNA, both the 5' T7 and 3' T7 Universal primers were used. PCR reaction mix and thermal cycles were the same as the first PCR. Template was the first PCR products.

5' T7 universal primer sequence: gagaattctaatacgactcactatagggccgcgg.

3' T7 universal primer sequence: agggatcctaatacgactcactataggcccggggc.

Making dsRNA

dsRNA was made using the Ambion T7 Megascript Kit. Reaction was mixed as followed:

- 2 μl each of the NTPs

- 2 μl T7 transcription buffer
- 2 μl T7 polymerase

8 μl of PCR template was added to the mixture and the reaction was incubated at 37°C for 4 hrs. Reaction was stopped by adding 115 μl RNAse–free water and 15 μl ammonium acetate stop solution. dsRNA purification was performed with 500 μl Phenol:Chloroform. The upper phase containing dsRNA was collected and precipitated by 500 μl isopropanol. After incubation at –20°C for at least 30 min, dsRNA can be obtained by centrifuge at full speed at 4°C for 15 minutes. The pellet was washed by 300 μl 70% ethanol (EtOH) and resuspended in 20 μl RNAse–free water. dsRNA concentration was checked on spectrophotometer.

Making Dioxygenin (DIG)-labelled probes for *in situ* hybridization (ISH)

DIG-labelled probes were made with the T7 reaction master mix (Using Ambion T7 Maxiscript kit) like following:

- 8 μl RNAse – free water
- 2 μl transcription buffer
- 2 μl DIG – labeling mix
- 2 μl T7 RNA polymerase

6 μl PCR templates were added and reaction was incubated at 37°C for 2-4 hrs. The reaction was stopped 30 μl water and 50 μl 2x stop solution. 5 μl tRNA and 10 μl Lithium Chloride were added. RNA was precipitated by 300 μl 100% EtOH for at least 30 min at –20°C. RNA was then obtained by centrifuge at 4°C 14000 rpm for 15 minutes. RNA pellet was washed by 300 μl 70% EtOH and resuspended in 100 μl hybridization wash solution.

3.2.6 RNAi injection

Pupal injection

Female pupae were collected shortly before hatching and the dorsal side of the terminal segment was fixed on a microscope slide by double-sided tape. dsRNA was diluted to 1μg/μl for injection. Micro-needles were made from borosilicate glass capillaries (Kwik-Fil™) using a needle puller (Sutter Instrument Co). Micro-needles containing dsRNA solution was inserted into pupae between the third and fourth abdominal segment. The amount of dsRNA injected should fill the abdomen. Injected pupae were transferred to flour. After three days, the successfully eclosed adult females were crossed with male beetles (2 females : 1 male).

Adult beetle injection

Virgin female beetles were anesthetized on ice for about 5 to 10 min. When the beetles stopped moving, the abdominal dorsal part was affixed on a microscope slide using double-sided tape. Micro-needles containing dsRNA solution were injected into body cavity between the third and fourth abdominal segment. Once the beetles recover from being anesthetized, they were transferred to flour. Male beetles were added in the next day (2 females : 1 male).

3.2.7 *In situ* hybridization

DAY ONE:

Embryos stored in MeOH were brought to PBST 1x slowly (first 2:1 MeOH/PBST then 1:2 MeOH/PBST). In the end, embryos were washed 2 times with PBST. Embryos were then brought to hybridization solution (first 1:1 PBST:HybWash then 100 % HybWash, each time for 10 min at RT). Embryos were pre-hybridized to avoid unspecific bindings by incubating with PreHyb Solution at least 1 hr at 60^0C. After pre-hybridization, embryos were incubated with 2 μl *in situ* hybridization probe overnight at 63^0C.

DAY TWO:

Embryos were washed with HybWash solution, 3 quick washes followed by 4 long washes 30 min each. Afterwards, embryos were brought back to PBST (first 10 min 2:1 HybWash:PBST, 55^0C then 10 min 1:2 HybWash:PBST, 55^0C). In the end, embryos were kept in PBST at RT for 10 min. To prevent unspecific bindings, embryos were incubated in blocking solution (100 μl of 1 mg/mL bovine serum albumin (BSA) and 30 μl of 60 mg/ml normal goat serum (NGS) in 1 ml PBST). After blocking, embryos were treated with antibodies. Anti-DIG-AP (Roche) was used (diluted 1:5000 in blocking solution). Incubation was performed overnight at 4^0C.

DAY THREE:

Antibodies were removed by washing 3x 15 min each in PBST. Embryos were brought to AP buffer pH9.5. Color reaction was performed with NBT/BCIP solution (10 μl NBT/BCIP in 500 μl AP buffer). After the staining was ready, reaction was stopped by adding PBST. Background staining was removed by washing with EtOH 100%. Embryos were embedded onto VECTASHIELD Mounting Medium with 4',6-diamidino-2-phenylindole (DAPI) (Vector Laboratories).

HybWash Solution (for 50 ml)

25 ml Formamide
12.5 ml SSC 20x
50 μl Heparin (50mg/mL)
250 μl 20% Tween
Water up to 50 ml

AP-buffer for NBT/BCIP

1 ml 1 M Tris, pH 9.5
500 μl 1 M $MgCl_2$
320 μl 3 M NaCl
100 μl 20% Tween
Water up to 10 ml

PreHyb Solution

HybWash Solution
500 μl salmon sperm (100ug/mL)

PBT

0.1% Tween in PBS

3.2.8 Fuchsin staining

Dechorionized eggs stored in MeOH were transferred into Sol. B and incubated at RT for 1 hr. Embryos were washed 4 times 20 min each with 70% EtOH. EtOH was replaced by 2N HCl and embryos were incubated for 10 min at 60°C. Embryos were then washed with water and 70% EtOH and stained for 30 min with alkaline Fuchsin solution. Fuchsin solution was washed off with 95% EtOH. Embryos were brought to 1 ml Sol. C and let stand until embryos sank down. Sol. D was used to embed embryos.

Sol. B

4,0 ml 95% EtOH

0,5 ml 100% Acetic acid

0,8 ml 10% Formaldehyde

Alcoholic Fuchsin solution

100 mg Pararosanilin (Sigma)

16 ml EtOH

4 ml H_2O

0,2 ml concentrated HCl

Sol. C

100% EtOH : Sol. D = 50 : 50

Sol. D

Benzyl benzoat : Benzyl alcohol = 4 : 1

3.2.9 Hoechst staining

Hoechst staining is a quick and simple way to visualize nuclear DNA. Embryos after fixation were incubated with Hoechst staining solution (1:1000 in PBST) for 15 min at RT. Unbound dye was removed by washing with PBST for 3 times, each time for 5 min. We used Hoechst 33258, pentahydrate (bis-benzimidine) from Invitrogen. Embryos were mounted into 70% glycerol and studied under fluorescent microscope.

3.2.10 Cuticle preparation

Eggs were collected in egg baskets and further incubated at 30°C for 3 days. Eggs were dechorionized in 50% DanKlorix and washed thoroughly by tap water. Embryos were embedded into Hoyer's Medium : Lactic acid 1 : 1. After one night at 60ºC, soft tissues were digested by lactic acid. Cuticles can now be observed and studied.

3.2.11 Microscopy

Embryos were embedded onto microscope slides using two spacers made from cover slips. Optical microscopes were used to take pictures. Pictures were processed with AxioVision software (Zeiss). Further procession of pictures was done with Adobe Photoshop CS4 without any changes of the specimens.

3.2.12 Phylogenetic analysis

Phylogenetic analysis was generated using the software MEGA6 with maximum likelihood phylogeny program.

(http://www.megasoftware.net/mega.php)

3.3 RESULTS AND DISCUSSION

To investigate whether the upstream components of Toll activation are conserved between *Drosophila melanogaster* (Diptera) and *Tribolium castaneum* (Coleoptera), I analyzed the respective genes using the sequenced genome of *Tribolium* available on the *Tribolium castaneum* Assembly 3.0 annotation (unpublished at http://bioinf.uni-greifswald.de/gb2/gbrowse/*Tribolium*/).

The BLAST (Basic Local Alignment Search Tool) revealed *Drosophila*-related sequences of all serine proteases belonging to the perivitelline proteolytic cascade. For phylogenetic analysis, the related sequences of *Nasonia vitripennis* (Hymenoptera) and *Oncopeltus fasciatus* (Hemiptera) were provided by Dr. T. Buchta and Y. T. Chen, respectively. Data from other species were identified by the NCBI BLAST. The BLAST and phylogenetic analysis were performed using full protein sequences of all species.

Functional analysis of the candidates discovered as *Drosophila*-related sequences was performed by pRNAi gene knockdown.

3.3.2 *Tc-nudel* is very conserved

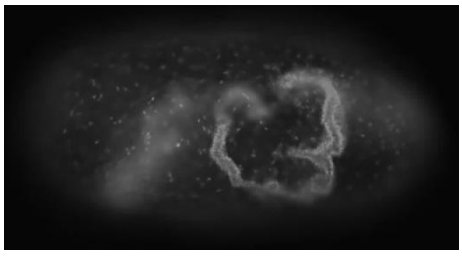

Figure 23 *Tc-nudel knockdown*
This phenotype is characterized by a tube-like structure of cuticles running through the yolk

Protein BLAST analysis using *Drosophila* Nudel as the query sequence revealed only one *Tribolium* ortholog (TC000870), indicating that *nudel* is

conserved between these two species. Knockdown of *Tc-nudel* led to the phenotype typical for the loss of components involved in Toll signaling (Fig 23). *Tc-nudel* knockdown embryos are fragile and usually do not survive bleaching and/or fixation, as Nudel is one component of the eggshell structure (LeMosy and Hashimoto, 2000).

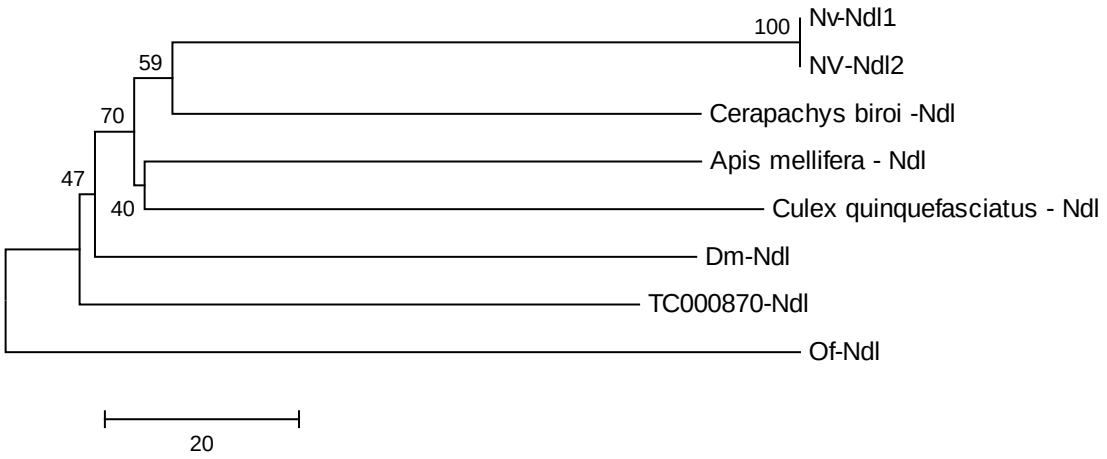

Figure 24 *Phylogenetic analysis of Nudel*
This tree describes the relationship between nudel orthologs in defferent insects. Dm – Drosophila melanogaster. Nv – Nasonia vitripennis. Of – Oncopeltus fasciatus

The phylogenetic analysis of *nudel* orthologs shows that Tc-Ndl and Of-Ndl are leaves on the same branch. This branch is an independent clade to other analyzed Hymenopteran and Dipteran species. In this tree, Dm-Ndl is most closely related to Cl-Ndl (Diptera), and three Hymenoptera species are grouped together in one clade, which is more closely related to Diptera than to *Tribolium* (Fig 24).

3.3.4 TC015295 as putative *gastrulation defective*

Protein BLAST for Gastrulation defective-related sequences revealed many *Tribolium* homologs similar to *Drosophila*'s Gd. 11 first hits were subjected to further detailed analysis. Fig 25 shows the phylogenetic analysis of

these 11 sequences and the query sequence of *Drosophila*'s Gd, together with the *Nasonia*'s Gd and *Oncopeltus*'s Gd. Interestingly, TC015295 (Gd1) and TC015297 are grouped together with the Gd from all investigated species. All the other *Tribolium* Gds (Gd3 – Gd11) are phylogenetically separated from this clade. However, our parental RNAi experiments failed to show a phenotype for TC015295 and TC015297, as well as for the other 9 potential Gds homologs.

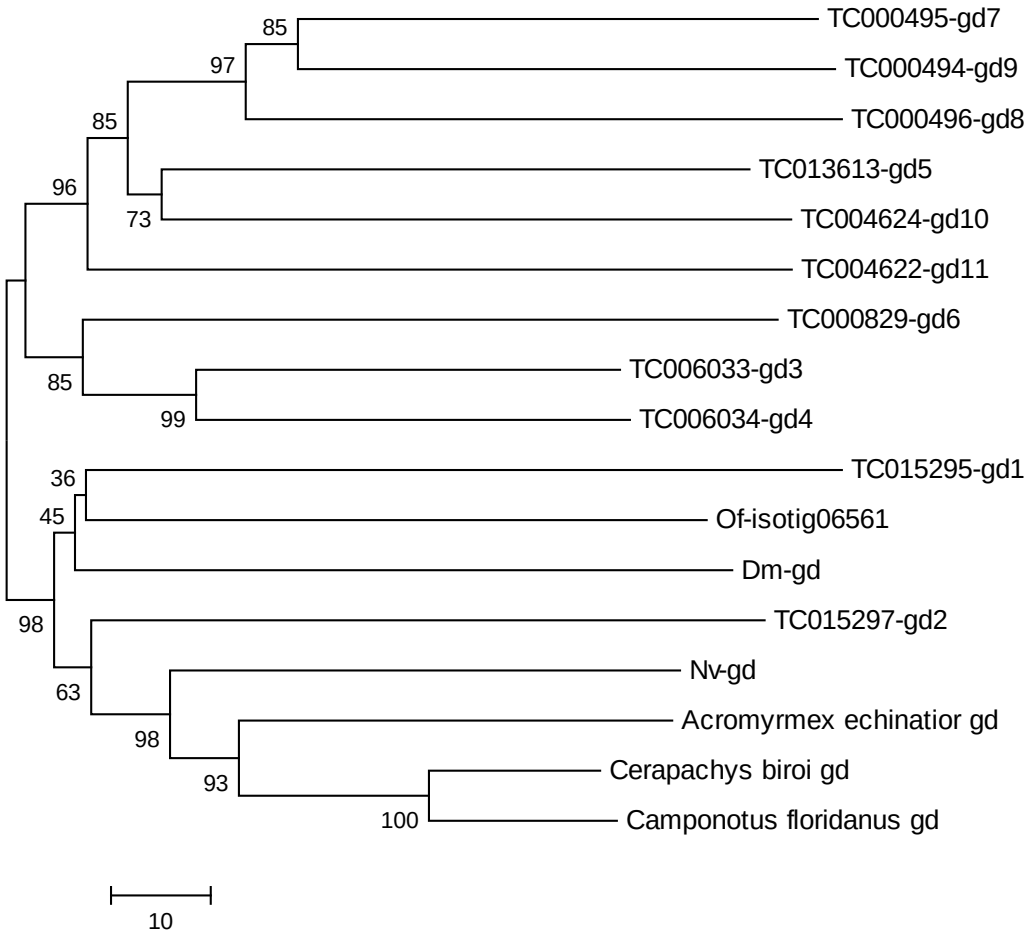

Figure 25 *Phylogenetic analysis of Gastrulation defective*

The tree describes the relationship between the 11 Tribolium Gastrulation defective homologs and the query sequence of Drosophila Gd, together with Nasonia (Nv) Gd and Oncopeltus Gd (Of).

Given the fact that Gastrulation defective has a particular protein structure, atypical for serine proteases (Konrad et al., 1998), it is very likely that Gd is conserved between species. Our phylogenetic analysis revealed two putative *Tribolium* Gd homolog that are most closely related to the investigated Hemipteran, Dipteran and Hymenopteran Gds. Our results suggested that TC015295 and TC015297 are potentially *Tribolium gastrulation defective*.

3.3.4 *Snake*-related TC004160 might function like Persephone in immunity

Similar to Easter, BLAST analysis of Snake related sequences revealed many homologous sequences in *Tribolium*. The first 8 most closely related hits were subjected to our functional analysis. A phylogenetic tree containing the 8 serine proteases, *Drosophila* Snake, *Nasonia* Snake and *Oncopeltus* Snake does not give a convincing hint of a true functional ortholog in *Tribolium* (Fig 26). We therefore performed RNAi gene knockdown for all candidates. None of the candidates gave a phenotype in the offspring. However, RNAi injection of TC004160 (Snk1) resulted in dead pupae, and injected females died gradually within 15 days after injection. We further carried out adult injection and obtained a similar phenomenon, i.e. injected adult females died within 9 days after injection.

Previously, a study based largely on bioinformatic analysis (Zou et al., 2007) suggested that TC004160 (serine protease P44) might function like *Drosophila* Persephone (LeMosy and Hashimoto, 2000). Our knockdown lethality phenotype also suggests a function of TC004160 in immunity. Our results, together with Zou et al.'s study provide strong evidences that TC004160 acts as Persephone in *Tribolium* innate immunity. On the other hand, it cannot be ruled out that TC004160 might also function in DV patterning, since no offspring could be obtained from our parental RNAi experiments. It is possible that during evolution, one serine protease was employed for the Toll signaling pathway(s) in both embryonic patterning and innate immunity in *Tribolium*.

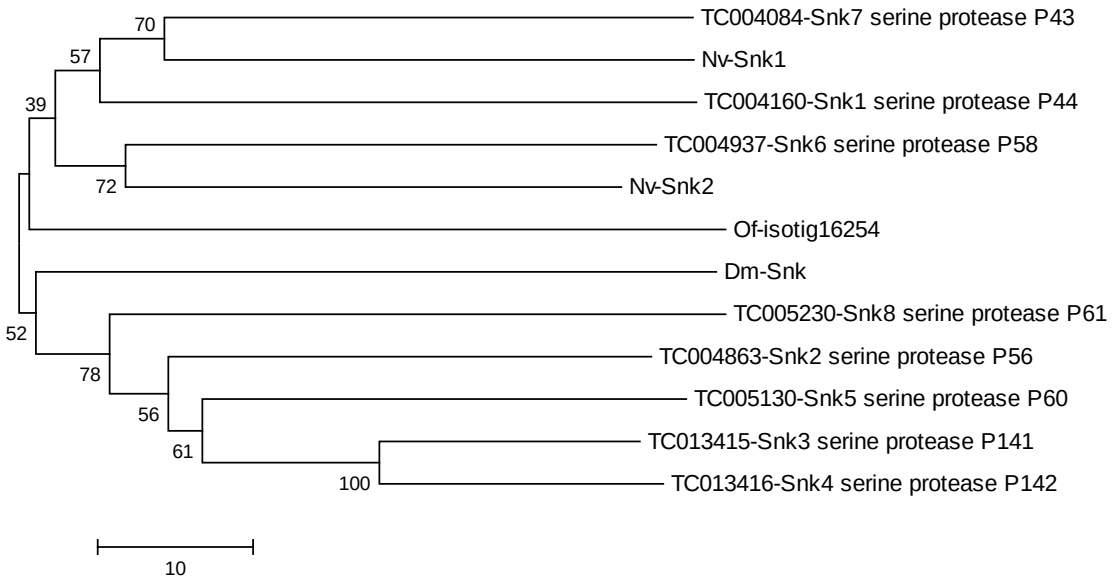

Figure 26 *Phylogenetic tree of Snake*

The tree describes the relationship between the 8 Tribolium Snake homologs and the query sequence of Drosophila Snake, together with Nasonia (Nv) Snake and Oncopeltus Snake (Of).

3.3.1 TC002112 as *Tribolium easter*

Protein BLAST against *Drosophila easter* revealed many related *Tribolium* orthologs, indicating that there are many serine proteases similar to *easter* in the *Tribolium* genome. The 10 most closely related sequences were subjected to further analysis. A phylogenetic analysis including the sequences of the 10 candidates together with the *Dm-easter* is presented in Fig 27. Dm-Ea is a leave of a branch that is grouped with a clade including Tc-Ea1, Tc-Ea2, Tc-Ea7, Tc-Ea8, together with *Nasonia*'s and *Oncopeltus*'s Easter. The other 6 *Tribolium* serine proteases (Tc-Ea3, Tc-Ea4, Tc-Ea5, Tc-Ea6) belong to an outgroup. This phylogenetic tree shows that four *Tribolium* serine proteases are more homologous to Easters of the three investigated species than the other six. However, this phylogenetic tree was insufficient to reveal the functional

Tribolium serine protease *easter* since it did not suggest a promising candidate. Therefore, we performed pRNAi for all 10 candidates.

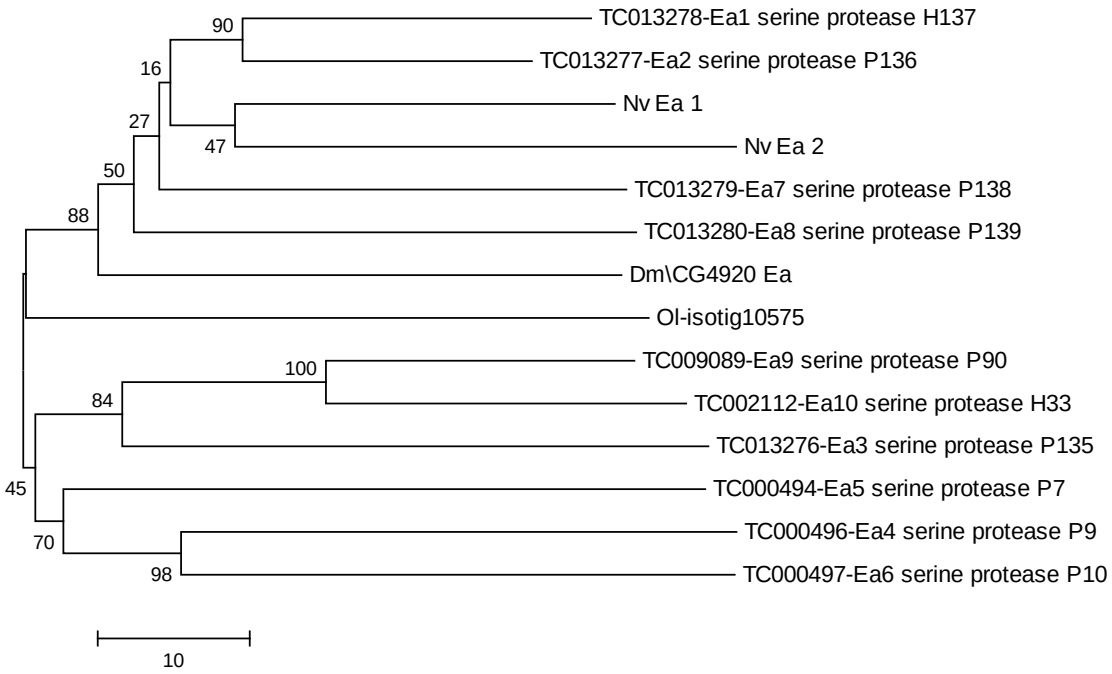

Figure 27 *Phylogenetic tree of Easter*

The tree describes the relationship between the 10 Tribolium Easter homologs and the query sequence of Drosophila Easter, together with Nasonia and Oncopeltus Easter.

Surprisingly, the last candidate, TC002112 (Ea10) showed RNAi knockdown effects. *TC002112* RNAi led to a tube-like phenotype typical for knockdown of genes involved in Toll signaling (Fig 28). We further performed ISH for *TC002112* knockdown embryos with the mesodermal markers *twist* and *sog*. In both cases, *TC002112* expression is abolished, indicating a loss of mesoderm formation in these embryos. Moreover, DAPI pictures (Fig 28 F and I) show that the border between germ rudiment and the serosa is straightened up, indicating that the DV polarity is disrupted. In the wildtype situation, this border is oblique (Zee et al., 2006). The RNAi phenotype, along with the ISH results allow the conclusion that TC02112 is *Tribolium easter*.

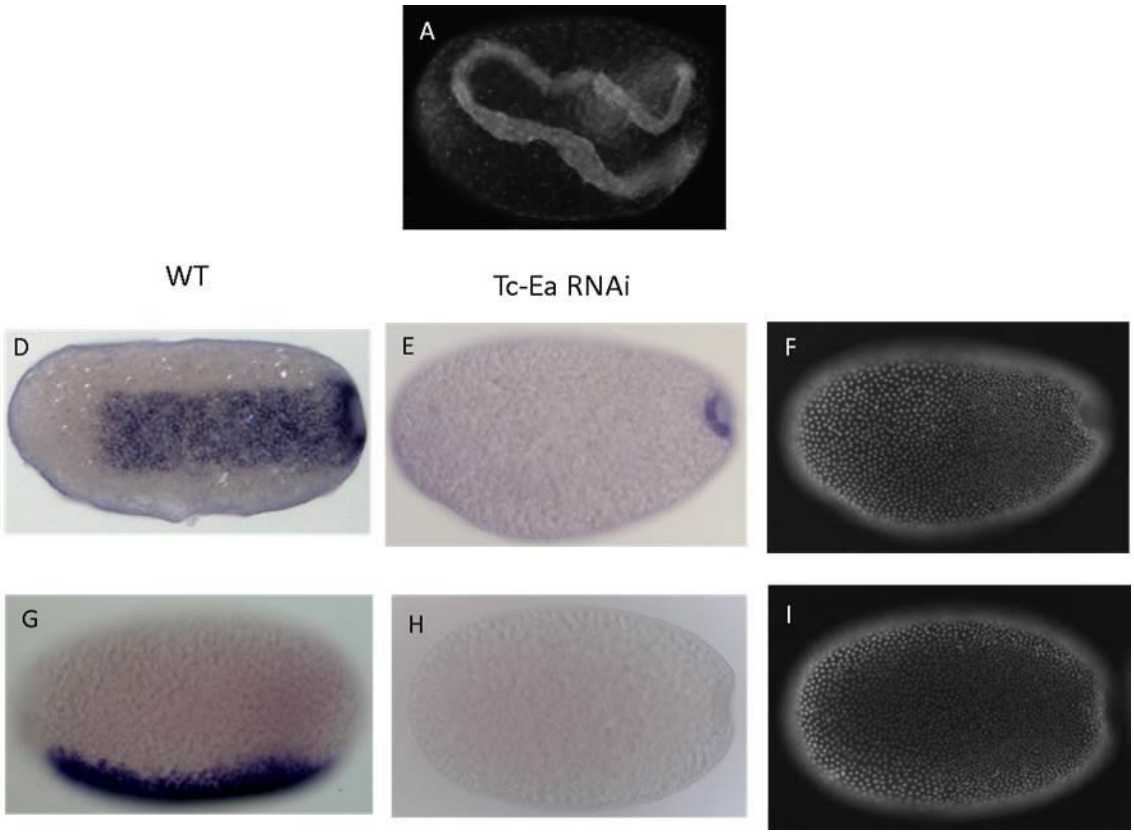

Figure 28 *Tc-ea RNAi knockdown and ISH on Tc-ea knockdown embryos.*
(A) Tc-ea RNAi knockdown is characterized by the tube-like phenotype.
(D, G) ISH with twi and sog on wildtype embryos. (E, F) ISH with twi on Tc-ea knockdown embryos. (H, I) ISH with sog on Tc-ea knockdown embryos. In both cases, the expressions of the marker genes are lost. DAPI pictures (F, I) show the oblique of the border between germ rudiment and serosa.

Easter belongs to the trypsin-like serine protease family. Its structure is representative of a typical serine protease, which consists of clip and chymotrypsin-like SP domains (Piao et al., 2007). The serine protease family is very diverse and has multiple biological functions, which makes it difficult to unravel the true functional ortholog using the method of BLAST search and phylogenetics. Interestingly, a protein BLAST search using Tc-Ea as a query sequence revealed the related *Drosophila* Spätzle processing enzyme as the best *Drosophila* hit. Spätzle processing enzyme is the serine protease involved

in the proteolytic cascade upstream of Toll receptor in the innate immunity (Jang et al., 2006).

We further analyzed the phylogenetic relationships of Tc-Ea with other Easters belonging to other insects. Fig 29 shows the phylogenetic relationship among Tc-Ea, Dm-Ea, Dm-Spz processing enzyme and some Easters from other insect species. Tc-Ea is grouped with three Hymenopteran species, *Acromyrmex echinatior, Apis mellifera* and *Cerapachys biroi*. These four species are in the sister branch with a Lepidoptera species, *Bombyx mori*. Tc-proclotting enzyme is grouped with the Easter proteases from three Hymenopteran species, *Nasonia vitripennis, Harpegnathos saltator* and *Camponotus floridanus*. Meanwhile, Dm-Ea belongs to a sister branch grouped with Dm-Spz processing enzyme and *Musca domestica*'s Easter (Diptera). This phylogenetic analysis shows that both Tc-Ea and Tc-proclotting enzyme are closest orthologs to Hymenoptera's Easters, and they are phylogenetically separated; while Dm-Ea and Dm-Spz processing enzyme are more closely related to each other, and all three Dipteran species are grouped together in one clade in this phylogenetic tree.

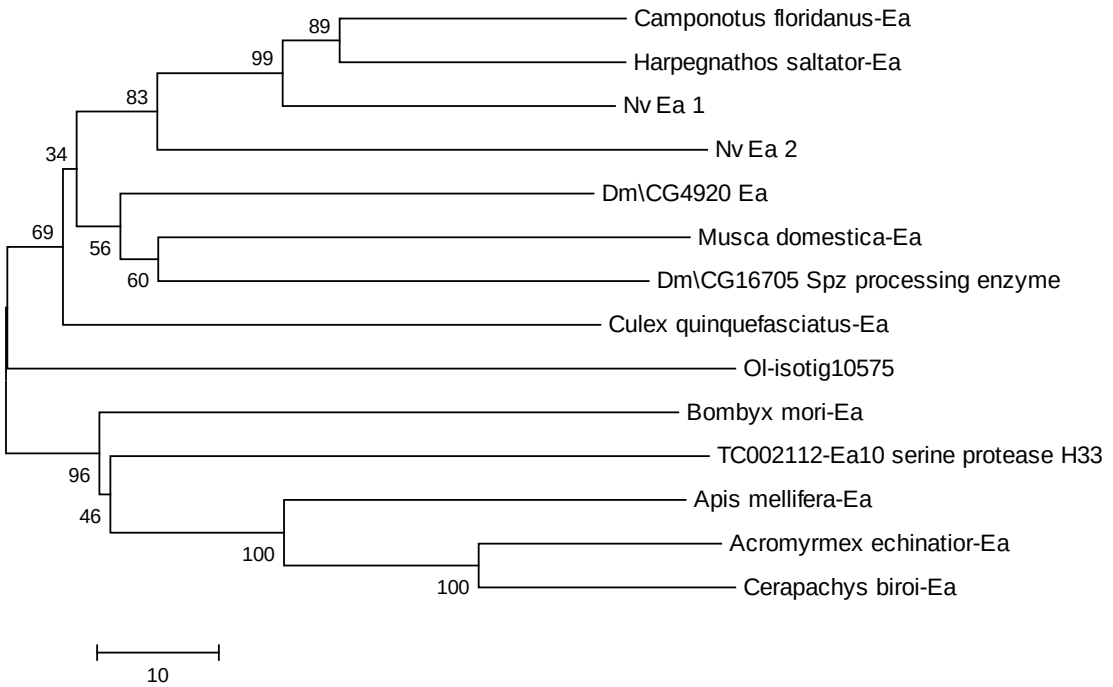

Figure 29 *Phylogenetic analysis of Easter*
The tree describes the relationship between Easter orthologs in different insects, including two serine proteases that function in innate immunity in Drosophila and Tribolium.

Previously, bioinformatic investigations led to a proposal that TC002112 is orthologous to *Drosophila*'s Spätzle processing enzyme, and for that reason given the name SPE2112 (Zou et al., 2007). Experimental evidences to conclude TC002112's function in immunity are lacking. Our RNAi experiments did not show any immune effect in the injected animals. However, it is possible that TC002112 acts as both Easter and Spätzle processing enzyme, cleaving the ligand for the activation of Toll receptor in DV patterning and, at the same time, in immunity.

Based on our results, it is possible to draw the conclusion that, in *Tribolium* some components of the serine protease cascade leading to Toll activation in immunity are employed in embryonic patterning. Meanwhile, in *Drosophila*, these two processes do not share any components (El Chamy et al., 2008; Gottar et al., 2006; Kambris et al., 2006; van der Zee et al., 2006). This would indicate that the upstream Toll signaling machinery in *Tribolium*, the more basally branching insect, is simpler than that in *Drosophila*. It would also lead to an interpretation that during evolution, some components of the perivitelline cascade, which was initially used to trigger immune responses against pathogens, were taken over to pattern the DV axis in early embryonic development. The divergence of the upstream Toll signaling machinery would be a derived feature, as seen in *Drosophila*. At this point, it would be very interesting to intensively investigate the upstream components of the Toll receptor in other basally branching insects.

REFERENCES

Anderson, K. V, Jürgens, G., Nüsslein-Volhard, C., 1985. Establishment of dorsal-ventral polarity in the *Drosophila* embryo: genetic studies on the role of the Toll gene product. Cell 42, 779–789. doi:10.1016/0092-8674(85)90274-0

Arakane, Y., Muthukrishnan, S., Kramer, K.J., Specht, C.A., Tomoyasu, Y., Lorenzen, M.D., Kanost, M., Beeman, R.W., 2005. The *Tribolium* chitin synthase genes TcCHS1 and TcCHS2 are specialized for synthesis of epidermal cuticle and midgut peritrophic matrix. Insect Mol. Biol. 14, 453–463. doi:10.1111/j.1365-2583.2005.00576.x

Berghammer, A., Bucher, G., Maderspacher, F., Klingler, M., 1999. A system to efficiently maintain embryonic lethal mutations in the flour beetle *Tribolium castaneum*. Dev. Genes Evol. 209, 382–389. doi:10.1007/s004270050268

Berghammer, A.J., Klingler, M., Wimmer, E.A., 1999. A universal marker for transgenic insects. Nature 402, 370–371. doi:10.1038/46463

Bergmann, A., Stein, D., Geisler, R., Hagenmaier, S., Schmid, B., Fernandez, N., Schnell, B., Nüsslein-Volhard, C., 1996. A gradient of cytoplasmic Cactus degradation establishes the nuclear localization gradient of the dorsal morphogen in *Drosophila*. Mech. Dev. 60, 109–123. doi:10.1016/S0925-4773(96)00607-7

Brennan, C.A., Anderson, K. V, 2004. *Drosophila*: the genetics of innate immune recognition and response. Annu. Rev. Immunol. 22, 457–483. doi:10.1146/annurev.immunol.22.012703.104626

Bucher, G., Scholten, J., Klingler, M., 2002. Parental RNAi in *Tribolium* (Coleoptera). Curr. Biol. 12, R85–6.

Cao, Y., Ding, X., Cai, M., Zhao, J., Lin, Y., Li, X., Xu, C., Wang, S., 2007. The expression pattern of a rice disease resistance gene xa3/xa26 is differentially regulated by the genetic backgrounds and developmental stages that influence its function. Genetics 177, 523–533. doi:10.1534/genetics.107.075176

Cha, B.J., Koppetsch, B.S., Theurkauf, W.E., 2001. In vivo analysis of *Drosophila* bicoid mRNA localization reveals a novel microtubule-dependent axis specification pathway. Cell 106, 35–46. doi:10.1016/S0092-8674(01)00419-6

Chang, A.J., Morisato, D., 2002. Regulation of Easter activity is required for shaping the Dorsal gradient in the *Drosophila* embryo. Development 129, 5635–45.

Chasan, R., Anderson, K. V, 1989. The role of easter, an apparent serine protease, in organizing the dorsal-ventral pattern of the *Drosophila* embryo. Cell 56, 391–400. doi:10.1016/0092-8674(89)90242-0

Chasan, R., Jin, Y., Anderson, K. V, 1992. Activation of the easter zymogen is regulated by five other genes to define dorsal-ventral polarity in the *Drosophila* embryo. Development 115, 607–616.

Chen, G., Handel, K., Roth, S., 2000. The maternal NF-kappaB/dorsal gradient of *Tribolium castaneum*: dynamics of early dorsoventral patterning in a short-germ beetle. Development 127, 5145–5156.

Cho, Y.S., Stevens, L.M., Sieverman, K.J., Nguyen, J., Stein, D., 2012. A Ventrally Localized Protease in the *Drosophila* Egg Controls Embryo Dorsoventral Polarity. Curr. Biol. 22, 1013–1018. doi:10.1016/j.cub.2012.03.065

Crocker, J., Tamori, Y., Erives, A., 2008. Evolution acts on enhancer organization to fine-tune gradient threshold readouts. PLoS Biol. 6, 2576–2587. doi:10.1371/journal.pbio.0060263

Davis, G.K., Patel, N.H., 2002. Short, long, and beyond: molecular and embryological approaches to insect segmentation. Annu. Rev. Entomol. 47, 669–699. doi:10.1146/annurev.ento.47.091201.145251

De Robertis, E.M., 2008. Evo-Devo: Variations on Ancestral Themes. Cell. doi:10.1016/j.cell.2008.01.003

De Robertis, E.M., Kuroda, H., 2004. Dorsal-ventral patterning and neural induction in Xenopus embryos. Annu. Rev. Cell Dev. Biol. 20, 285–308. doi:10.1146/annurev.cellbio.20.011403.154124

DeLotto, R., Spierer, P., 1986. A gene required for the specification of dorsal-ventral pattern in *Drosophila* appears to encode a serine protease. Nature 323, 688–692. doi:10.1038/323688a0

Dissing, M., Giordano, H., DeLotto, R., 2001. Autoproteolysis and feedback in a protease cascade directing *Drosophila* dorsal-ventral cell fate. EMBO J. 20, 2387–2393. doi:10.1093/emboj/20.10.2387

Drier, E.A., Govind, S., Steward, R., 2000. Cactus-independent regulation of Dorsal nuclear import by the ventral signal. Curr. Biol. 10, 23–26. doi:10.1016/S0960-9822(99)00267-5

Drier, E.A., Huang, L.H., Steward, R.C.-P., 1999. Nuclear import of the *Drosophila* Rel protein Dorsal is regulated by phosphorylation. Genes Dev 13, 556–68 ST – Nuclear import of the *Drosophila* Rel .

Dworkin, I., Kennerly, E., Tack, D., Hutchinson, J., Brown, J., Mahaffey, J., Gibson, G., 2009. Genomic consequences of background effects on scalloped mutant expressivity in the wing of *Drosophila melanogaster*. Genetics 181, 1065–1076. doi:10.1534/genetics.108.096453

Edwards, D.N., Towb, P., Wasserman, S.A., 1997. An activity-dependent network of interactions links the Rel protein Dorsal with its cytoplasmic regulators. Development 124, 3855–3864.

El Chamy, L., Leclerc, V., Caldelari, I., Reichhart, J.-M., 2008. Sensing of “danger signals” and pathogen-associated molecular patterns defines binary signaling pathways “upstream” of Toll. Nat. Immunol. 9, 1165–1170. doi:10.1038/ni.1643

Félix, M.A., 2007. Cryptic Quantitative Evolution of the Vulva Intercellular Signaling Network in Caenorhabditis. Curr. Biol. 17, 103–114. doi:10.1016/j.cub.2006.12.024

Galindo, R.L., Edwards, D.N., Gillespie, S.K., Wasserman, S.A., 1995. Interaction of the pelle kinase with the membrane-associated protein tube is required for transduction of the dorsoventral signal in *Drosophila* embryos. Development 121, 2209–2218.

Garfinkel, M.D., Wang, J., Liang, Y., Mahowald, A.P., 1994. Multiple products from the shavenbaby-ovo gene region of *Drosophila melanogaster*: relationship to genetic complexity. Mol. Cell. Biol. 14, 6809–6818.

Gottar, M., Gobert, V., Matskevich, A.A., Reichhart, J.M., Wang, C., Butt, T.M., Belvin, M., Hoffmann, J.A., Ferrandon, D., 2006. Dual Detection of Fungal Infections in *Drosophila* via Recognition of Glucans and Sensing of Virulence Factors. Cell 127, 1425–1437. doi:10.1016/j.cell.2006.10.046

Han, J.H., Lee, S.H., Tan, Y.Q., LeMosy, E.K., Hashimoto, C., 2000. Gastrulation defective is a serine protease involved in activating the receptor toll to polarize the *Drosophila* embryo. Proc. Natl. Acad. Sci. U. S. A. 97, 9093–9097. doi:10.1073/pnas.97.16.9093

Handel, K., Basal, A., Fan, X., Roth, S., 2005. *Tribolium castaneum* twist: gastrulation and mesoderm formation in a short-germ beetle. Dev. Genes Evol. 215, 13–31. doi:10.1007/s00427-004-0446-9

Hashimoto, C., Kim, D.R., Weiss, L.A., Miller, J.W., Morisato, D., 2003. Spatial regulation of developmental signaling by a serpin. Dev. Cell 5, 945–50.

Hong, J.-W., Hendrix, D. a, Papatsenko, D., Levine, M.S., 2008. How the Dorsal gradient works: insights from postgenome technologies. Proc. Natl. Acad. Sci. U. S. A. 105, 20072–20076.

Horng, T., Medzhitov, R., 2001. *Drosophila* MyD88 is an adapter in the Toll signaling pathway. Proc. Natl. Acad. Sci. U. S. A. 98, 12654–12658. doi:10.1073/pnas.231471798

Hoskins, R. a, Smith, C.D., Carlson, J.W., Carvalho, a B., Halpern, A., Kaminker, J.S., Kennedy, C., Mungall, C.J., Sullivan, B. a, Sutton, G.G., Yasuhara, J.C., Wakimoto, B.T., Myers, E.W., Celniker, S.E., Rubin, G.M., Karpen, G.H., 2002. Heterochromatic sequences in a *Drosophila* whole-genome shotgun assembly. Genome Biol. 3, RESEARCH0085.

Hu, X., Yagi, Y., Tanji, T., Zhou, S., Ip, Y.T., 2004. Multimerization and interaction of Toll and Spätzle in *Drosophila*. Proc. Natl. Acad. Sci. U. S. A. 101, 9369–9374. doi:10.1073/pnas.0307062101

Hunter, C.P., Winston, W.M., Molodowitch, C., Feinberg, E.H., Shih, J., Sutherlin, M., Wright, A.J., Fitzgerald, M.C., 2006. Systemic RNAi in Caenorhabditis elegans. Cold Spring Harb. Symp. Quant. Biol. 71, 95–100. doi:10.1101/sqb.2006.71.060

Imler, J.-L., Ferrandon, D., Royet, J., Reichhart, J.-M., Hetru, C., Hoffmann, J.A., 2004. Toll-dependent and Toll-independent immune responses in *Drosophila*. J. Endotoxin Res. 10, 241–246. doi:10.1179/096805104225005887

Israël, A., 2000. The IKK complex: An integrator of all signals that activate NF-κB?, in: Trends in Cell Biology. pp. 129–133. doi:10.1016/S0962-8924(00)01729-3

Jang, I.H., Chosa, N., Kim, S.H., Nam, H.J., Lemaitre, B., Ochiai, M., Kambris, Z., Brun, S., Hashimoto, C., Ashida, M., Brey, P.T., Lee, W.J., 2006. A Spätzle-processing enzyme required for toll signaling activation in *Drosophila* innate immunity. Dev. Cell 10, 45–55. doi:10.1016/j.devcel.2005.11.013

Jaźwińska, A., Rushlow, C., Roth, S., 1999. The role of brinker in mediating the graded response to Dpp in early *Drosophila* embryos. Development 126, 3323–3334.

Jin, Y.S., Anderson, K. V, 1990. Dominant and recessive alleles of the *Drosophila* easter gene are point mutations at conserved sites in the serine protease catalytic domain. Cell 60, 873–881. doi:10.1016/0092-8674(90)90100-S

Kambris, Z., Brun, S., Jang, I.-H.H., Nam, H.-J.J., Romeo, Y., Takahashi, K., Lee, W.-J.J., Ueda, R., Lemaitre, B., 2006. *Drosophila* Immunity: A Large-Scale In Vivo RNAi Screen Identifies Five Serine Proteases Required for Toll Activation. Curr. Biol. 16, 808–813. doi:10.1016/j.cub.2006.03.020

Kanodia, J.S., Rikhy, R., Kim, Y., Lund, V.K., DeLotto, R., Lippincott-Schwartz, J., Shvartsman, S.Y., 2009. Dynamics of the Dorsal morphogen gradient. Proc. Natl. Acad. Sci. U. S. A. 106, 21707–21712. doi:10.1073/pnas.0912395106

Karin, M., Ben-Neriah, Y., 2000. Phosphorylation meets ubiquitination: the control of NF-[kappa]B activity. Annu. Rev. Immunol. 18, 621–663. doi:10.1146/annurev.immunol.18.1.621

Kawai, T., Akira, S., 2007. TLR signaling. Semin. Immunol. doi:10.1016/j.smim.2006.12.004

Khush, R.S., Lemaitre, B., 2000. Genes that fight infection: What the *Drosophila* genome says about animal immunity. Trends Genet. doi:10.1016/S0168-9525(00)02095-3

Kim, T., Kim, Y.-J., 2005. Overview of innate immunity in *Drosophila*. J. Biochem. Mol. Biol. 38, 121–127. doi:10.5483/BMBRep.2005.38.2.121

Kitzmann, P., Schwirz, J., Schmitt-Engel, C., Bucher, G., 2013. RNAi phenotypes are influenced by the genetic background of the injected strain. BMC Genomics 14, 5. doi:10.1186/1471-2164-14-5

Konrad, K.D., Goralski, T.J., Mahowald, A.P., Marsh, J.L., 1998. The gastrulation defective gene of *Drosophila melanogaster* is a member of the serine protease superfamily. Proc. Natl. Acad. Sci. U. S. A. 95, 6819–6824. doi:10.1073/pnas.95.12.6819

Konsolaki, M., Schüpbach, T., 1998. windbeutel, a gene required for dorsoventral patterning in *Drosophila*, encodes a protein that has homologies to vertebrate proteins of the endoplasmic reticulum. Genes Dev. 12, 120–131. doi:10.1101/gad.12.1.120

Koressaar, T., Remm, M., 2007. Enhancements and modifications of primer design program Primer3. Bioinformatics 23, 1289–1291. doi:10.1093/bioinformatics/btm091

Kotkamp, K., Klingler, M., Schoppmeier, M., 2010. Apparent role of *Tribolium* orthodenticle in anteroposterior blastoderm patterning largely reflects novel functions in dorsoventral axis formation and cell survival. Development 137, 1853–1862. doi:10.1242/dev.047043

Krem, M.M., Cera, E.D., 2002. Evolution of enzyme cascades from embryonic development to blood coagulation. Trends Biochem. Sci. doi:10.1016/S0968-0004(01)02007-2

Kugler, J.-M., Lasko, P., 1995. Localization, anchoring and translational control of oskar, gurken, bicoid and nanos mRNA during *Drosophila* oogenesis. Fly (Austin). 3, 15–28. doi:10.1016/0092-8674(95)90324-0

Kulkarni, M.M., Booker, M., Silver, S.J., Friedman, A., Hong, P., Perrimon, N., Mathey-Prevot, B., 2006. Evidence of off-target effects associated with long dsRNAs in *Drosophila melanogaster* cell-based assays. Nat. Methods 3, 833–838. doi:10.1038/nmeth935

Kumar, H., Kawai, T., Akira, S., 2009. Toll-like receptors and innate immunity. Biochem. Biophys. Res. Commun. doi:10.1016/j.bbrc.2009.08.062

Lander, E.S., Schork, N.J., 1994. Genetic dissection of complex traits. Science 265, 2037–2048. doi:10.1126/science.8091226

Lefebvre, V., Dumitriu, B., Penzo-Méndez, A., Han, Y., Pallavi, B., 2007. Control of cell fate and differentiation by Sry-related high-mobility-group box (Sox) transcription factors. Int. J. Biochem. Cell Biol. doi:10.1016/j.biocel.2007.05.019

Lemaitre, B., Nicolas, E., Michaut, L., Reichhart, J.M., Hoffmann, J.A., 1996. The dorsoventral regulatory gene cassette spatzle/Toll/Cactus controls the potent antifungal response in *Drosophila* adults. Cell 86, 973–983. doi:10.1016/S0092-8674(00)80172-5

LeMosy, E.K., Hashimoto, C., 2000. The nudel protease of *Drosophila* is required for eggshell biogenesis in addition to embryonic patterning. Dev. Biol. 217, 352–361. doi:10.1006/dbio.1999.9562

LeMosy, E.K., Kemler, D., Hashimoto, C., 1998. Role of Nudel protease activation in triggering dorsoventral polarization of the *Drosophila* embryo. Development 125, 4045–4053.

LeMosy, E.K., Tan, Y.Q., Hashimoto, C., 2001. Activation of a protease cascade involved in patterning the *Drosophila* embryo. Proc. Natl. Acad. Sci. U. S. A. 98, 5055–5060. doi:10.1073/pnas.081026598

Letsou, A., Alexander, S., Wasserman, S.A., 1993. Domain mapping of tube, a protein essential for dorsoventral patterning of the *Drosophila* embryo. EMBO J. 12, 3449–3458.

Levine, M., Davidson, E.H., 2005. Gene regulatory networks for development. Proc. Natl. Acad. Sci. U. S. A. 102, 4936–4942. doi:10.1073/pnas.0408031102

Liberman, L.M., Reeves, G.T., Stathopoulos, A., 2009. Quantitative imaging of the Dorsal nuclear gradient reveals limitations to threshold-dependent patterning in *Drosophila*. Proc. Natl. Acad. Sci. U. S. A. 106, 22317–22322. doi:10.1073/pnas.0906227106

Ligoxygakis, P., Roth, S., Reichhart, J.-M., 2003. A serpin regulates dorsal-ventral axis formation in the *Drosophila* embryo. Curr. Biol. 13, 2097–102.

Lin, H., Spradling, A.C., 1993. Germline stem cell division and egg chamber development in transplanted *Drosophila* germaria. Dev. Biol. 159, 140–152. doi:10.1006/dbio.1993.1228

Linder, C.C., 2006. Genetic variables that influence phenotype. ILAR J. 47, 132–140.

Lorenzen, M.D., Kimzey, T., Shippy, T.D., Brown, S.J., Denell, R.E., Beeman, R.W., 2007. piggyBac-based insertional mutagenesis in *Tribolium castaneum* using donor/helper hybrids. Insect Mol. Biol. 16, 265–275. doi:10.1111/j.1365-2583.2007.00727.x

Lüders, F., Segawa, H., Stein, D., Selva, E.M., Perrimon, N., Turco, S.J., Häcker, U., 2003. Slalom encodes an adenosine 3'-phosphate 5'-phosphosulfate transporter essential for development in *Drosophila*. EMBO J. 22, 3635–3644. doi:10.1093/emboj/cdg345

Lynch, J. a, Peel, A.D., Drechsler, A., Averof, M., Roth, S., 2010. EGF signaling and the origin of axial polarity among the insects. Curr. Biol. 20, 1042–1047. doi:10.1016/j.cub.2010.04.023

Lynch, J. a, Roth, S., 2011. The evolution of dorsal-ventral patterning mechanisms in insects. Genes Dev. 25, 107–118. doi:10.1101/gad.2010711

Ma, Y., Creanga, A., Lum, L., Beachy, P.A., 2006. Prevalence of off-target effects in *Drosophila* RNA interference screens. Nature 443, 359–363. doi:10.1038/nature05179

Maderspacher, F., Bucher, G., Klingler, M., 1998. Pair-rule and gap gene mutants in the flour beetle *Tribolium castaneum*. Dev. Genes Evol. 208, 558–568. doi:10.1007/s004270050215

Markstein, M., Markstein, P., Markstein, V., Levine, M.S., 2002. Genome-wide analysis of clustered Dorsal binding sites identifies putative target genes in the *Drosophila* embryo. Proc. Natl. Acad. Sci. U. S. A. 99, 763–768. doi:10.1073/pnas.012591199

Markstein, M., Zinzen, R., Markstein, P., Yee, K.-P., Erives, A., Stathopoulos, A., Levine, M., 2004. A regulatory code for neurogenic gene expression in the *Drosophila* embryo. Development 131, 2387–2394. doi:10.1242/dev.01124

Maxton-KuchenmeisterMaxton-Kü, Handel, K., Schmidt-Ott, U., Roth, S., JackleJäckle, H., Maxton-Küchenmeister, J., Jäckle, H., 1999. Toll homolog expression in the beetle *Tribolium* suggests a different mode of dorsoventral patterning than in *Drosophila* embryos. Mech. Dev. 83, 107–114. doi:10.1016/S0925-4773(99)00041-6

Meinhardt, H., Roth, S., 2002. Developmental biology: sharp peaks from shallow sources. Nature 419, 261–262.

Miller, S.C., Miyata, K., Brown, S.J., Tomoyasu, Y., 2012. Dissecting Systemic RNA Interference in the Red Flour Beetle *Tribolium castaneum*: Parameters Affecting the Efficiency of RNAi. PLoS One 7. doi:10.1371/journal.pone.0047431

Minakhina, S., Yang, J., Steward, R., 2003. Tamo selectively modulates nuclear import in *Drosophila*. Genes Cells 8, 299–310. doi:10.1046/j.1365-2443.2002.00634.x

Misra, S., Hecht, P., Maeda, R., Anderson, K. V, 1998. Positive and negative regulation of Easter, a member of the serine protease family that controls dorsal-ventral patterning in the *Drosophila* embryo. Development 125, 1261–7.

Morisato, D., Anderson, K. V, 1994. The spätzle gene encodes a component of the extracellular signaling pathway establishing the dorsal-ventral pattern of the *Drosophila* embryo. Cell 76, 677–88.

Morisato, D., Anderson, K. V, 1995. Signaling pathways that establish the dorsal-ventral pattern of the *Drosophila* embryo. Annu. Rev. Genet. 29, 371–399. doi:10.1146/annurev.genet.29.1.371

Moussian, B., Roth, S., 2005. Dorsoventral axis formation in the *Drosophila* embryo--shaping and transducing a morphogen gradient. Curr. Biol. 15, R887–R899. doi:10.1016/j.cub.2005.10.026

Neuman-Silberberg, F.S., Schupbach, T., 1993. The *Drosophila* dorsoventral patterning gene gurken produces a dorsally localized RNA and encodes a TGFα-like protein. Cell 75, 165–174. doi:10.1016/0092-8674(93)90688-M

Nilson, L.A., Schüpbach, T., 1998. Localized requirements for windbeutel and pipe reveal a dorsoventral prepattern within the follicular epithelium of the *Drosophila* ovary. Cell 93, 253–262. doi:10.1016/S0092-8674(00)81576-7

Ntini, E., Wimmer, E.A., 2011. Unique establishment of procephalic head segments is supported by the identification of cis-regulatory elements driving segment-specific segment polarity gene expression in *Drosophila*. Dev. Genes Evol. 221, 1–16. doi:10.1007/s00427-011-0354-8

Nunes da Fonseca, R., van der Zee, M., Roth, S., 2010. Evolution of extracellular Dpp modulators in insects: The roles of tolloid and twisted-gastrulation in dorsoventral patterning of the *Tribolium* embryo. Dev. Biol. 345, 80–93. doi:10.1016/j.ydbio.2010.05.019

Nunes da Fonseca, R., von Levetzow, C., Kalscheuer, P., Basal, A., van der Zee, M., Roth, S., 2008. Self-regulatory circuits in dorsoventral axis formation of the short-germ beetle *Tribolium castaneum*. Dev. Cell 14, 605–615. doi:10.1016/j.devcel.2008.02.011

O'Connor, M.B., Umulis, D., Othmer, H.G., Blair, S.S., 2006. Shaping BMP morphogen gradients in the *Drosophila* embryo and pupal wing. Development 133, 183–193. doi:10.1242/dev.02214

Okuda, Y., Ogura, E., Kondoh, H., Kamachi, Y., 2010. B1 SOX coordinate cell specification with patterning and morphogenesis in the early zebrafish embryo. PLoS Genet. 6, 36. doi:10.1371/journal.pgen.1000936

Overton, P.M., Chia, W., Buescher, M., 2007. The *Drosophila* HMG-domain proteins SoxNeuro and Dichaete direct trichome formation via the activation of shavenbaby and the restriction of Wingless pathway activity. Development 134, 2807–2813. doi:10.1242/dev.02878

Papatsenko, D., Levine, M., 2005. Quantitative analysis of binding motifs mediating diverse spatial readouts of the Dorsal gradient in the *Drosophila* embryo. Proc. Natl. Acad. Sci. U. S. A. 102, 4966–4971. doi:10.1073/pnas.0409414102

Pavlopoulos, A., Berghammer, A.J., Averof, M., Klingler, M., 2004. Efficient transformation of the beetle *Tribolium castaneum* using the Minos transposable element: quantitative and qualitative analysis of genomic integration events. Genetics 167, 737–746. doi:10.1534/genetics.103.023085

Payre, F., Vincent, A., Carreno, S., 1999. ovo/svb integrates Wingless and DER pathways to control epidermis differentiation. Nature 400, 271–275. doi:10.1038/22330

Phochanukul, N., Russell, S., 2010. No backbone but lots of Sox: Invertebrate Sox genes. Int. J. Biochem. Cell Biol. doi:10.1016/j.biocel.2009.06.013

Piao, S., Kim, S., Kim, J.H., Park, J.W., Lee, B.L., Ha, N.-C., 2007. Crystal structure of the serine protease domain of prophenoloxidase activating factor-I. J. Biol. Chem. 282, 10783–10791. doi:10.1074/jbc.M611556200

Ponomareff, G., Giordano, H., DeLotto, Y., DeLotto, R., 2001. Interallelic complementation at the *Drosophila melanogaster* gastrulation defective locus defines discrete functional domains of the protein. Genetics 159, 635–645.

Reach, M., Galindo, R.L., Towb, P., Allen, J.L., Karin, M., Wasserman, S.A., 1996. A gradient of cactus protein degradation establishes dorsoventral polarity in the *Drosophila* embryo. Dev. Biol. 180, 353–364. doi:10.1006/dbio.1996.0308

Remold, S.K., Lenski, R.E., 2004. Pervasive joint influence of epistasis and plasticity on mutational effects in Escherichia coli. Nat. Genet. 36, 423–426. doi:10.1038/ng1324

Richards, S., Gibbs, R.A., Weinstock, G.M., Brown, S.J., Denell, R., Beeman, R.W., Gibbs, R., Bucher, G., Friedrich, M., Grimmelikhuijzen, C.J.P., Klingler, M., Lorenzen, M., Roth, S., Schröder, R., Tautz, D., Zdobnov, E.M., Muzny, D., Attaway, T., Bell, S., Buhay, C.J., Chandrabose, M.N., Chavez, D., Clerk-Blankenburg, K.P., Cree, A., Dao, M., Davis, C., Chacko, J., Dinh, H., Dugan-Rocha, S., Fowler, G., Garner, T.T., Garnes, J., Gnirke, A., Hawes, A., Hernandez, J., Hines, S., Holder, M., Hume, J., Jhangiani, S.N., Joshi, V., Khan,

Z.M., Jackson, L., Kovar, C., Kowis, A., Lee, S., Lewis, L.R., Margolis, J., Morgan, M., Nazareth, L. V, Nguyen, N., Okwuonu, G., Parker, D., Ruiz, S.-J., Santibanez, J., Savard, J., Scherer, S.E., Schneider, B., Sodergren, E., Vattahil, S., Villasana, D., White, C.S., Wright, R., Park, Y., Lord, J., Oppert, B., Brown, S., Wang, L., Weinstock, G., Liu, Y., Worley, K., Elsik, C.G., Reese, J.T., Elhaik, E., Landan, G., Graur, D., Arensburger, P., Atkinson, P., Beidler, J., Demuth, J.P., Drury, D.W., Du, Y.-Z., Fujiwara, H., Maselli, V., Osanai, M., Robertson, H.M., Tu, Z., Wang, J., Wang, S., Song, H., Zhang, L., Werner, D., Stanke, M., Morgenstern, B., Solovyev, V., Kosarev, P., Brown, G., Chen, H.-C., Ermolaeva, O., Hlavina, W., Kapustin, Y., Kiryutin, B., Kitts, P., Maglott, D., Pruitt, K., Sapojnikov, V., Souvorov, A., Mackey, A.J., Waterhouse, R.M., Wyder, S., Kriventseva, E. V, Kadowaki, T., Bork, P., Aranda, M., Bao, R., Beermann, A., Berns, N., Bolognesi, R., Bonneton, F., Bopp, D., Butts, T., Chaumot, A., Denell, R.E., Ferrier, D.E.K., Gordon, C.M., Jindra, M., Lan, Q., Lattorff, H.M.G., Laudet, V., von Levetsow, C., Liu, Z., Lutz, R., Lynch, J.A., da Fonseca, R.N., Posnien, N., Reuter, R., Schinko, J.B., Schmitt, C., Schoppmeier, M., Shippy, T.D., Simonnet, F., Marques-Souza, H., Tomoyasu, Y., Trauner, J., Van der Zee, M., Vervoort, M., Wittkopp, N., Wimmer, E.A., Yang, X., Jones, A.K., Sattelle, D.B., Ebert, P.R., Nelson, D., Scott, J.G., Muthukrishnan, S., Kramer, K.J., Arakane, Y., Zhu, Q., Hogenkamp, D., Dixit, R., Jiang, H., Zou, Z., Marshall, J., Elpidina, E., Vinokurov, K., Oppert, C., Evans, J., Lu, Z., Zhao, P., Sumathipala, N., Altincicek, B., Vilcinskas, A., Williams, M., Hultmark, D., Hetru, C., Hauser, F., Cazzamali, G., Williamson, M., Li, B., Tanaka, Y., Predel, R., Neupert, S., Schachtner, J., Verleyen, P., Raible, F., Walden, K.K.O., Angeli, S., Forêt, S., Schuetz, S., Maleszka, R., Miller, S.C., Grossmann, D., 2008. The genome of the model beetle and pest *Tribolium castaneum*. Nature 452, 949–55. doi:10.1038/nature06784

Riechmann, V., Ephrussi, A., 2001. Axis formation during *Drosophila* oogenesis. Curr. Opin. Genet. Dev. 11, 374–383.

Rose, T., LeMosy, E.K., Cantwell, A.M., Banerjee-Roy, D., Skeath, J.B., Di Cera, E., 2003. Three-dimensional models of proteases involved in patterning of the *Drosophila* Embryo. Crucial role of predicted cation binding sites. J. Biol. Chem. 278, 11320–11330. doi:10.1074/jbc.M211820200

Roth, S., 2003. The origin of dorsoventral polarity in *Drosophila*. Philos. Trans. R. Soc. Lond. B. Biol. Sci. 358, 1317–1329; discussion 1329. doi:10.1098/rstb.2003.1325

Roth, S., Lynch, J.A., 2009. Oogenesis 1–21.

Roth, S., Schüpbach, T., 1994. The relationship between ovarian and embryonic dorsoventral patterning in *Drosophila*. Development 120, 2245–2257.

Roth, S., Stein, D., Nusslein-Volhard, C., Nüsslein-Volhard, C., 1989. A gradient of nuclear localization of the dorsal protein determines dorsoventral pattern in the *Drosophila* embryo. Cell 59, 1189–1202. doi:10.1016/0092-8674(89)90774-5

Rusch, J., Levine, M., 1996. Threshold responses to the dorsal regulatory gradient and the subdivision of primary tissue territories in the *Drosophila* embryo. Curr. Opin. Genet. Dev. 6, 416–423.

Rushlow, C.A., Han, K., Manley, J.L., Levine, M., 1989. The graded distribution of the dorsal morphogen is initiated by selective nuclear transport in *Drosophila*. Cell 59, 1165–77. doi:10.1016/0092-8674(89)90772-1

Schinko, J.B., Hillebrand, K., Bucher, G., 2012. Heat shock-mediated misexpression of genes in the beetle *Tribolium castaneum*. Dev. Genes Evol. doi:10.1007/s00427-012-0412-x

Schinko, J.B., Kreuzer, N., Offen, N., Posnien, N., Wimmer, E.A., Bucher, G., 2008. Divergent functions of orthodenticle, empty spiracles and buttonhead in early head patterning of the beetle *Tribolium castaneum* (Coleoptera). Dev. Biol. 317, 600–613. doi:10.1016/j.ydbio.2008.03.005

Schinko, J.B., Weber, M., Viktorinova, I., Kiupakis, A., Averof, M., Klingler, M., Wimmer, E. a, Bucher, G., 2010. Functionality of the GAL4/UAS system in *Tribolium* requires the use of endogenous core promoters. BMC Dev. Biol. 10, 53. doi:10.1186/1471-213X-10-53

Schneider, D.S., Jin, Y., Morisato, D., Anderson, K. V, 1994. A processed form of the Spätzle protein defines dorsal-ventral polarity in the *Drosophila* embryo. Development 120, 1243–1250.

Schüpbach, T., Roth, S., 1994. Dorsoventral patterning in *Drosophila* oogenesis. Curr. Opin. Genet. Dev. doi:10.1016/0959-437X(94)90064-A

Sen, J., Goltz, J.S., Stevens, L., Stein, D., 1998. Spatially restricted expression of pipe in the *Drosophila* egg chamber defines embryonic dorsal-ventral polarity. Cell 95, 471–481. doi:10.1016/S0092-8674(00)81615-3

Shelton, C.A., Wasserman, S.A., 1993. pelle Encodes a protein kinase required to establish dorsoventral polarity in the *Drosophila* embryo. Cell 72, 515–525. doi:10.1016/0092-8674(93)90071-W

St Johnston, D., Nüsslein-Volhard, C., 1992. The origin of pattern and polarity in the *Drosophila* embryo. Cell 68, 201–19. doi:10.1016/0092-8674(85)90275-2

Stathopoulos, A., Levine, M., 2002. Linear signaling in the Toll-Dorsal pathway of *Drosophila*: activated Pelle kinase specifies all threshold outputs of gene expression while the bHLH protein Twist specifies a subset. Development 129, 3411–3419.

Stathopoulos, A., Levine, M., 2004. Whole-genome analysis of *Drosophila* gastrulation. Curr. Opin. Genet. Dev. 14, 477–484. doi:10.1016/j.gde.2004.07.004

Stathopoulos, A., Levine, M., 2005. Localized repressors delineate the neurogenic ectoderm in the early *Drosophila* embryo. Dev. Biol. 280, 482–493. doi:10.1016/j.ydbio.2005.02.003

Stathopoulos, A., Van Drenth, M., Erives, A., Markstein, M., Levine, M., 2002. Whole-genome analysis of Dorsal-ventral patterning in the *Drosophila* embryo. Cell 111, 687–701. doi:10.1016/S0092-8674(02)01087-5

Stein, D., Roth, S., Vogelsang, E., Nüsslein-Volhard, C., 1991. The polarity of the dorsoventral axis in the *Drosophila* embryo is defined by an extracellular signal. Cell 65, 725–735. doi:10.1016/0092-8674(91)90381-8

Steward, R., 1989. Relocalization of the dorsal protein from the cytoplasm to the nucleus correlates with its function. Cell 59, 1179–1188. doi:10.1016/0092-8674(89)90773-3

Sun, H., Bristow, B.N., Qu, G., Wasserman, S.A., 2002. A heterotrimeric death domain complex in Toll signaling. Proc. Natl. Acad. Sci. U. S. A. 99, 12871–12876. doi:10.1073/pnas.202396399

Sun, H., Towb, P., Chiem, D.N., Foster, B.A., Wasserman, S.A., 2004. Regulated assembly of the Toll signaling complex drives *Drosophila* dorsoventral patterning. EMBO J. 23, 100–110. doi:10.1038/sj.emboj.7600033

Suzuki, N., Suzuki, S., Yeh, W.C., 2002. IRAK-4 as the central TIR signaling mediator in innate immunity. Trends Immunol. doi:10.1016/S1471-4906(02)02298-6

Threadgill, D.W., Dlugosz, A.A., Hansen, L.A., Tennenbaum, T., Lichti, U., Yee, D., LaMantia, C., Mourton, T., Herrup, K., Harris, R.C., 1995. Targeted disruption of mouse EGF receptor: effect of genetic background on mutant phenotype. Science 269, 230–234. doi:10.1126/science.7618084

Tomoyasu, Y., Denell, R.E., 2004. Larval RNAi in *Tribolium* (Coleoptera) for analyzing adult development. Dev. Genes Evol. 214, 575–578. doi:10.1007/s00427-004-0434-0

Tomoyasu, Y., Miller, S.C., Tomita, S., Schoppmeier, M., Grossmann, D., Bucher, G., 2008. Exploring systemic RNA interference in insects: a genome-wide survey for RNAi genes in *Tribolium*. Genome Biol. 9, R10. doi:10.1186/gb-2008-9-1-r10

Towb, P., Sun, H., Wasserman, S. a, 2009. Tube Is an IRAK-4 homolog in a Toll pathway adapted for development and immunity. J. Innate Immun. 1, 309–321. doi:10.1159/000200773

Uv, A.E., Roth, P., Xylourgidis, N., Wickberg, A., Cantera, R., Samakovlis, C., 2000. members only encodes a *Drosophila* nucleoporin required for rel protein import

and immune response activation. Genes Dev. 14, 1945–1957. doi:10.1101/gad.14.15.1945-1957.2000

Van der Zee, M., Berns, N., Roth, S., 2005. Distinct functions of the *Tribolium* zerknüllt genes in serosa specification and dorsal closure. Curr. Biol. 15, 624–636. doi:10.1016/j.cub.2005.02.057

Van der Zee, M., da Fonseca, R.N., Roth, S., 2008. TGFbeta signaling in *Tribolium*: vertebrate-like components in a beetle. Dev. Genes Evol. 218, 203–213. doi:10.1007/s00427-007-0179-7

Van der Zee, M., Stockhammer, O., von Levetzow, C., Nunes da Fonseca, R., Roth, S., 2006. Sog/Chordin is required for ventral-to-dorsal Dpp/BMP transport and head formation in a short germ insect. Proc. Natl. Acad. Sci. U. S. A. 103, 16307–12. doi:10.1073/pnas.0605154103

Van Eeden, F., St Johnston, D., 1999. The polarisation of the anterior-posterior and dorsal-ventral axes during *Drosophila* oogenesis. Curr. Opin. Genet. Dev. doi:10.1016/S0959-437X(99)80060-4

Xiao, T., Towb, P., Wasserman, S.A., Sprang, S.R., 1999. Three-dimensional structure of a complex between the death domains of Pelle and Tube. Cell 99, 545–555. doi:10.1016/S0092-8674(00)81542-1

Zee, M. Van Der, Stockhammer, O., Levetzow, C. Von, Nunes, R., Roth, S., 2006. Sog ¯ Chordin is required for ventral-to-dorsal Dpp ¯BMP transport and head formation in a short germ insect 103.

Zhang, Z., Zhu, X., Stevens, L.M., Stein, D., 2009. Distinct functional specificities are associated with protein isoforms encoded by the *Drosophila* dorsal-ventral patterning gene pipe. Development 136, 2779–2789. doi:10.1242/dev.034413

Zhu, X., Sen, J., Stevens, L., Goltz, J.S., Stein, D., 2005. *Drosophila* pipe protein activity in the ovary and the embryonic salivary gland does not require heparan sulfate glycosaminoglycans. Development 132, 3813–3822. doi:10.1242/dev.01962

Zou, Z., Evans, J.D., Lu, Z., Zhao, P., Williams, M., Sumathipala, N., Hetru, C., Hultmark, D., Jiang, H., 2007. Comparative genomic analysis of the *Tribolium* immune system. Genome Biol. 8, R177. doi:10.1186/gb-2007-8-8-r177

Appendix

A – Positive controls used in the iBeetle screen

Gene	iB-#	TC-#
achaete-scute	iB-04489	TC008433
axin	iB-04108	TC006314
broad-complex	iB-03960	TC005474
cactus	iB-00322	TC002003
CG16778	iB-04153	TC006481
Delta	iB-03691	TC004114
depapentaplegic	iB-04497	TC008466
dorsocross	iB-05219	TC012346
EGF-Receptor	iB-00647	TC003986
folded-gastrulation	iB-04203	TC006722
Frizzled-1	iB-02240	TC014055
glass-bottom-boat	iB-05543	TC014017
hairy	iB-05339	TC012851
hunchback	iB-05451	TC013553
knirps	iB-03553	TC003413
lame-duck	iB-06061	TC030749
lim1	iB-05727	TC014939
methoprene-tolerant	iB-03648	TC003908
mirror/irx	iB-03595	TC003634
odd-skipped	iB-04013	TC005785
org1	iB-05796	TC015327
patched	iB-03831	TC004745
pelle	iB-02469	TC015365
porcupine	iB-03822	TC004714
pumilio	iB-03898	TC005073
saxophone	iB-02534	TC015948
sex-combs-reduced	iB-00186	TC000917
sloppy-paired	iB-04421	TC008062
TGF-alpha	iB-03555	TC003429
tolloid	iB-01822	TC011197
Torso	iB-04720	TC009906
torso-like	iB-04423	TC008090
twisted-gastrulation	iB-00592	TC003620
wingless	iB-05552	TC014084
wnt-less	iB-00832	TC005345

yb	iB-02707	TC000053
zfh2	iB-03646	TC003891

B – List of 68 empty egg phenotypes

iB_#	TC#	iB_#	TC#
iB_00012	TC000070	iB_04022	TC005849
iB_00045	TC000220	iB_06057	TC030733
iB_00048	TC000228	iB_06067	TC030776
iB_00077	TC000228	iB_02419	TC015175
iB_00078	TC000357	iB_02467	TC015347
iB_00204	TC000994	iB_05999	TC016320
iB_00303	TC001771	iB_05837	TC015544
iB_00456	TC002852	iB_00543	TC003263
iB_00541	TC003254	iB_00708	TC003263
iB_00567	TC003425	iB_01023	TC006261
iB_00596	TC003642	iB_01161	TC007295
iB_00614	TC003787	iB_01625	TC009901
iB_00628	TC003841	iB_01775	TC011003
iB_00963	TC005928	iB_01908	TC011953
iB_01087	TC006755	iB_02419	TC015175
iB_01638	TC009977	iB_02434	TC015222
iB_01695	TC010452	iB_02501	TC015651
iB_01748	TC010852	iB_03396	TC002538
iB_02707	TC000053	iB_03756	TC004433
iB_03370	TC002405	iB_03838	TC004762
iB_03405	TC002556	iB_03911	TC005134
iB_03421	TC002620	iB_04007	TC005754
iB_03450	TC002783	iB_04191	TC006676
iB_03611	TC003753	iB_05837	TC015544
iB_03621	TC003792	iB_05999	TC016320
iB_03649	TC003909	iB_06067	TC030776
iB_03667	TC003998	iB_02429	TC015211
iB_03669	TC004005	iB_02453	TC015317

iB_03675	TC004030	iB_03515	TC003183
iB_03683	TC004064	iB_03604	TC003707
iB_03900	TC005083	iB_03605	TC003709
iB_03910	TC005133	iB_05768	TC015172
iB_03932	TC005274	iB_05791	TC015292
iB_04001	TC005715	iB_06023	TC030196

C – List of 46 cuticle phenotypes

	iB#	TC#		iB#	TC#
1	iB_00064	TC000302	24	iB_05272	TC012571
2	iB_00155	TC000689	25	iB_05342	TC012857
3	iB_00641	TC003942	26	iB_06043	TC030643
4	iB_00661	TC004088	27	iB_06060	TC030748
5	iB_01094	TC006785	28	iB_02543	TC016002
6	iB_01360	TC008511	29	iB_05219	TC012346
7	iB_01507	TC009227	30	iB_02467	TC015347
8	iB_03762	TC004453	31	iB_02707	TC000053
9	iB_03957	TC005461	32	iB_03762	TC004453
10	iB_03968	TC005500	33	iB_05623	TC014396
11	iB_05397	TC013170	34	iB_05718	TC014886
12	iB_05535	TC013978	35	iB_00676	TC004293
13	iB_05549	TC014065	36	iB_00947	TC005861
14	iB_05566	TC014135	37	iB_02370	TC014984
15	iB_00018	TC014135	38	iB_06071	TC030785
16	iB_00081	TC000379	39	iB_03810	TC004673
17	iB_00674	TC004260	40	iB_00917	TC005741
18	iB_01185	TC007423	41	iB_02340	TC014861
19	iB_01379	TC008646	42	iB_02419	TC015175
20	iB_01982	TC012466	43	iB_02466	TC015344
21	iB_03641	TC003872	44	iB_00095	TC000444
22	iB_03992	TC005625	45	iB_01901	TC011895
23	iB_04165	TC006533	46	iB_02469	TC015365

D – List of primer sequences

	Forward	Reverse
Snk1	ggccgcggATCAATATACGCCGCCACTC	cccggggcTCAAGGTCTCCCAATCGAAC
Snk2	ggccgcggATCAATATACGCCGCCACTC	cccggggcACCCCTGCACTGTTTTCAAC
Snk3	ggccgcggGCCGCTCATTGTCTCTTCTC	cccggggcCACCAACCACAACAAAATGC
Snk4	ggccgcggCGACGGTCCTAACCCTAACA	cccggggcATCAATATACGCCGCCACTC
Snk5	ggccgcggACAACAGAAGATGCCGATCC	cccggggcATCTTCAACGGGATCAGTGG
Snk6	ggccgcggGACTTTAAACCTCCTGCCAGA	cccggggcTGTTTTTCGGGCCATACAAT
Snk7	ggccgcggTCACATGGCTGTGATTGGAT	cccggggcTTTGCCTCCTTCCAAATGTC
Snk8	ggccgcggCGGCGAAAAATCCTTATCAA	cccggggcATGCCAAACGACGTAACTCC
Ea1	ggccgcggAAGGGTGAACAGGGTGACTG	cccggggcTCCCAACAGCATACCAATGA
Ea2	ggccgcggAGGATCTACGGAGGGGAGAA	cccggggcATCAGCGGACCACCACTATC
Ea3	ggccgcggGCATTTCCCACGTTTCAGTT	cccggggcGACCACCTGAATCTCCTCCA
Ea4	ggccgcggGCTGGCGTTGTTGAAGTACA	cccggggcCCCACATTCTTTCCCAAATG
Ea5	ggccgcggAGTAACCGCAGCTCATTGCT	cccggggcGTGCTTGGCATTGGACTTTT
Ea6	ggccgcggACCCAGTCGTGCGAAAATAC	cccggggcTACGACCCCAGCCACTTATC
Ea7	ggccgcggTTTTCGGATGTGGATGACAA	cccggggcACTGTCTCCGACGCAAGAAT
Ea8	ggccgcggTTGACAATTGTGCACCACCT	cccggggcAAACCCCTGGTTGATTTTCC
Ea9	ggccgcggTCGGGGTAGTGAAGATCTGG	cccggggcTGTCATTTCATGCTCCTCCA
Ea10	ggccgcggCCCATAGCACGCGAATAACT	cccggggcACATAGCGTTGGGTGTTTCC
Ndl	ggccgcggTTCGCATTGCAGAAGTTGAC	cccggggcGACTGACAATGCCAGCAAGA
Gd1	ggccgcggTTTTGCTGTTACCACCACCG	cccggggcATGTCGCACTGTTGGCAAAT
Gd2	ggccgcggATCCGACATTCTCCTCGTCC	cccggggcCTCCCTCAACTTCCCCACAT
Gd3	ggccgcggGCAGCAGTTCGGGAAATAGG	cccggggcATGTCAGTGCCATCTTCCCA
Gd4	ggccgcggTTAACCGTAGCCCACTGTGT	cccggggcGCAAGGCCACACTTATCGAG
Gd5	ggccgcggGCGATAAACACAGTAGGGCG	cccggggcTCGAGGGCTCATCGTTTCTT
Gd6	ggccgcggCGTCTGGGCTCGACTTATCT	cccggggcCGAACGATACCCCGGATACA
Gd7	ggccgcggCGATGCTGTCACAACTCGAG	cccggggcTGGTATCTGTCCGCACTGTT
Gd8	ggccgcggGCTGGCGTTGTTGAAGTACA	cccggggcTTTGTGTAAACGGCAGACCC

Gd9	ggccgcggGGAAGCTCAGATAGGCGAGT	cccggggcAACGGTCCTCCAGAATCTCC
Gd10	ggccgcggCACTACAGGCTGCGGAATTC	cccggggcGCTACCTTTATTCCCGCAGC
Gd11	ggccgcggAGCAACTACACCAGAACCGA	cccggggcTCCACACTCACTTGCGTACT

Zusammenfassung

iBeetle ist ein genomweiter RNAi-Screen zur systematischen Identifizierung von Genen, die bei der Oogenese, Embryogenese und Larvalentwicklung des roten Mehlkäfers *Tribolium castaneum* eine Rolle spielen. Im Rahmen dieser Doktorarbeit wurde nach der allgemeinen Screeningphase eine detaillierte Untersuchung der Phänotypen vorgenommen, die Defekte bei der Bildung der dorsoventralen Achse zeigten.

Bei dieser Detailanalyse wurden *Tc-nudel*, eine Komponente stromaufwärts vom Tollrezeptor, und *Tc-pelle* sowie *Tc-tube-like-kinase*, zwei Komponenten stromabwärts vom Tollrezeptor, identifiziert. *Tc-tube-like-kinase* ist von besonderem Interesse, da das Protein Homologie zu IRAK 4 von Vertebraten aufzeigt. Die Kinasedomäne von Tc-tube-like-kinase ist wahrscheinlich während der Evolution in der Abstammungslinie, die zu *Drosophila* führt, verloren gegangen. Ob die Kinasedomäne eine essentielle Funktion für die dorsoventrale Musterbildung in *Tribolium* hat, ist noch nicht geklärt.

Der Verlust von *Tc-SoxNeuro* führt zu einem Phänotyp, der dem Verlust von *Tc-sog* ähnelt, was zur Annahme führt, dass *Tc-SoxNeuro* eine Rolle im Netzwerk des BMP-Signalwegs spielt. *Drosophila SoxNeuro* ist an der frühen Neurogenese des CNS beteiligt, hat aber keine Funktion bei der Etablierung ektodermaler Zellschicksale durch BMP. In Vertebraten gibt es jedoch Hinweise, dass SoxNeuro-Homologe die BMP vermittelte dorsoventrale Musterbildung beeinflussen. Tc-SoxNeuro erfüllt somit möglicherweise eine Vertebraten-ähnliche Funktion in *Tribolium*. Diese Hypothese wird durch frühere Arbeiten gestützt, in denen gezeigt wurde, dass das BMP-Signalnetzwerk von *Tribolium* Ähnlichkeiten zu dem der Wirbeltiere hat.

Neben der Analyse von Kandidaten aus dem iBeetle Screen wurde auf unabhängige Weise versucht, die noch unbekannten Komponenten der Serinproteasekaskade des Perivitellinspalts zu identifizieren, die den Liganden

des Tollrezeptors aktivieren und somit die Bildung der embryonalen Dorsoventralachse induzieren. TC015295 ist ein mögliches Ortholog zu Gastrulation defective von *Drosophila*. Das *snake*-ähnliche Gen TC004169 könnte die Rolle von Persephone in der Immunantwort übernehmen. TC002112 korrespondiert vermutlich sowohl zu *Drosophila easter* als auch zu *Drosophila spätzle processing enzyme*, einer Komponente des Immunsystems. Diese Untersuchungen führen zur Annahme, dass die stromaufwärts agierenden Komponenten des Toll-Signalwegs in *Tribolium*, dem phylogenetisch ursprünglicheren Insekt, einfacher organisiert sind als in *Drosophila*, und, dass die Spezialisierung in immunspezifische und musterbildungsspezifische Komponenten in *Drosophila* ein abgeleitetes Merkmal darstellt.

Danksagung

First of all I would like to thank Prof. Dr. Siegfried Roth for offering me the opportunity to pursue my PhD. I am glad to work with this interesting topic under the supervision of Prof. Roth. I am very grateful for his support and the constructive discussion.

I thank the members of my thesis committee, P.D. Michael Kroiher and Prof. Dr. Henrike Scholz.

I would like to thank the iBeetle team for the incorporation during the screen. My special thanks go to Prof. Dr. Gregor Bucher and Dr. Christian Schmitt-Engel for introducing me to the iBeetle screen and for the nice cooperation.

I would like to thank all members of the AG Roth, old and new, for the nice working atmosphere. Some have become my friends; I am very happy and thankful about it.

Last but not least, I would like to thank my family and my friends for the endless support. This PhD would not have been possible without the home base and the friend circle by my side.

Erklärung

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

Lebenslauf

Persönliche Daten

Name	Van Anh Dao
Anschrift	Münchenerstr. 24
	51103 Köln
Geburtsdatum/ -ort	12.06.1986 Hanoi, Vietnam

Schulische Ausbildung

1992 – 1997	Elementary School Mai Dich
1997 – 2001	Secondary School Nguyen Tat Thanh
2001 – 2004	Highschool Nguyen Tat Thanh

Studium

2004 – 2008	Hanoi University of Science Bachelor bei Prof. Dr. Vo Thi Thuong Lan am Biomedical Laboratory Titel: „Improving the method for detecting *CYP21* gene mutations in the patients with congenital hyperplasia syndrome”
2008 – 2010	Ehime University Master bei Prof. Dr. Hisato Iwata am CMES Titel: „Assessment of metabolic potentials of polychlorinated biphenyls (PCBs) by in vitro expressed cytochrome P450 1A enzymes of the common cormorant (*Phalacrocorax carbo*)“
seit 2010	Doktorarbeit bei Prof Dr. Siegfried Roth am Institut für Entwicklungsbiologie der Universität zu Köln Titel: „ Genome-wide RNAi screening and the analysis of candidate genes for dorsoventral patterning in *Tribolium castaneum*”