Mechanical and genetics basis of cellularization and serosal window closure in Tribolium castaneum

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CHAPTER 1

General introduction

Biological processes are complex and we need a multidisciplinary perspective to understand them. However, sometimes different fields take different perspectives to study the same process. One example is the development of organisms. In the 1930s it was studied by two separate fields: genetics and developmental embryology. Geneticists thought that it could be explained as result of a heritable function of the organism. On the other hand, embryologists thought that development was not necessarily related to a gene because it was not known yet that a gene was linked to the delicate timing of all events occurring during development (1).

The separation between developmental embryology and genetics lasted for more than 60 years until all the concepts of developmental embryology and genetics were understood. In 1942, Glueksohn discovered a mutation in the T-locus genes of mice that causes an aberration in the dorsal axis of the embryo. She traced the effects through the development and demonstrated that the defect in the embryo started during the induction of the dorsal axis in gastrulation (2). In 1953, the DNA structure was discovered by Watson and Crick (3). Later, in 1961, the gene concept was introduced by Jacob and Monod (4). The union between developmental embryology and genetics started in 1970 when Waddington found genes responsible for wing malformations in *Drosophila melanogaster*. Waddington showed that the malformations start in the early developmental stages of *D. melanogaster* (5). Another important step in understanding the connection between genes and development was made by Nüsslein-Volhard and Wieschaus which was awarded with the Nobel prize in 1995 for physiology or medicine. For instance in 1980, their groups published their work about 15 loci from *D. melanogaster*. When these luci mutate the segmental pattern of the larva is altered (6). Nowadays, we have a better understanding of the general principles of genetics and developmental embryology and we cannot study the development of an organism without combining the two fields.

In this thesis we combine the concepts and methods of genetics and developmental embryology to study the early development of *Tribolium castaneum*. For example, we use the RNA interference method to study the role of genes during the early development of *T. castaneum* (Figure 1). This method interferes in the translation of a specific gene by introducing a double-strand of RNA (dsRNA) into the cells (7). A dsRNA with the same

sequence as the target mRNA is injected into the cell. This dsRNA binds to a dicer protein, which is an endonuclease protein. Then, the dicer protein cuts the dsRNA into segments. After that, the argonaut protein attaches to the dicer protein and the dsRNA selecting one strain of the dsRNA. This complex of proteins and RNA is called RISC (RNA-induced silencing complex). Later, the full complex binds to a specific target of the mRNA by base paring. At the end, the Argonaut protein catalyzes the mRNA, which will be later degraded. If the mRNA is degraded, the translation of the protein does not occur and the gene is silenced (8). This method is commonly used for screening gene function (9, 10), to study the role of genes in a particular stage during development (11–13) and even for therapeutic applications (14).

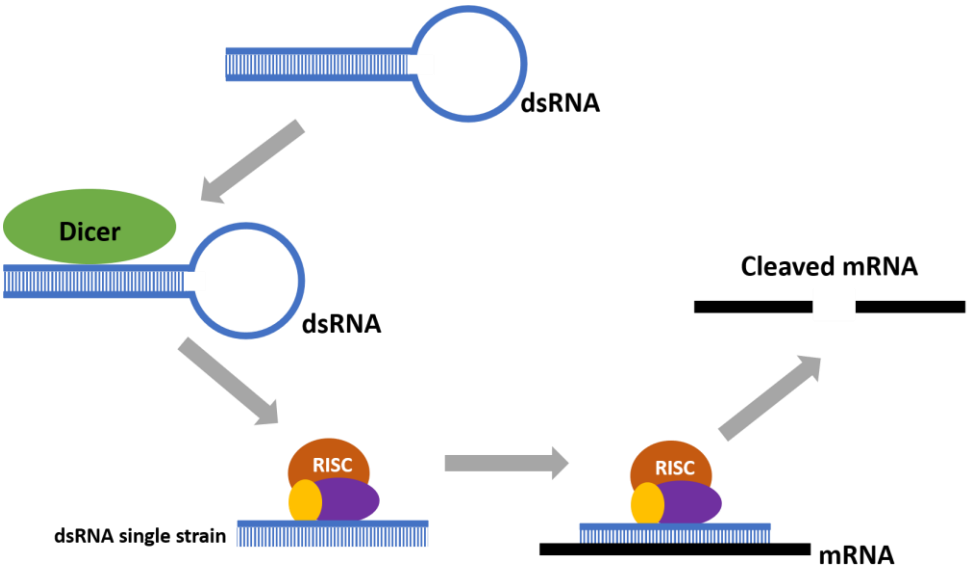

Figure 1. *The mechanism of RNAi interference. First the dsRNA binds to the dicer (endonuclease protein) cutting the dsRNA into segments. Then the dsRNA attaches to the RNA induced silence complex (RISC). At the end, the complex binds to the target mRNA producing its degradation.*

Early development of *Tribolium castaneum*

In this thesis we study the development of the red flour beetle, *Tribolium castaneum.* We follow processes from the early embryonic nuclear divisions until the formation of the larva and the extraembryonic

epithelium (serosal window closure) as explained below. Although *D. melanogaster* is the most used insect model in Biology, the utilization of *T. castaneum* to study insect development is becoming more common because the development of *T. castaneum* is closer to most of the insects (15).

Nuclear divisions and cellularization

In Biology, fertilization is the first step to start reproduction in diploids organisms. It is well-known that in humans the cellular division starts right after fertilization (1). In the case of insects and some vertebrates, in the first steps of embryogenesis, nuclei divide instead of cells (16–18). All the nuclei are enclosed in a single membrane, this arrangement is called syncytium (Figure 2). In *T. castaneum* the nuclei divide 12 times (14) (Figure 2). After the 12^{th} nuclear division the membrane starts to invaginate between the nuclei. In other words, the membrane starts to surrounds individual nuclei. This part of the membrane is called furrow canals. Cells are forming and connect through proteins called basal junctions, while the membrane grows to separate the nuclei. At this point the forming cells are called protocells. In the last step, the protein Innexin 7, that we describe further in this introduction, attaches to the sides of the furrow canals enclosing the nuclei (19). At this moment discrete cellular structures are formed. The process when the membrane starts to envelop the nuclei until the creation of cells is called cellularization.

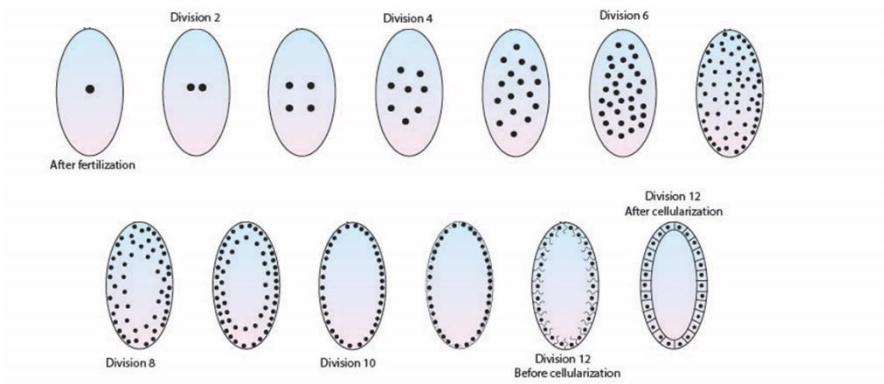

Figure 2 *Development of a T. castaneum embryo, from fertilization until cellularization. After fertilization, the nuclei divide surrounded by the same membrane, leading to a structure called syncytium. The cellularization starts after the 12th nuclear division, when all the nuclei are at the surface of the egg. At this stage, membrane material will separate each nucleus. At the end of cellularization the membrane closes around each nuclei leading to the formation of cells. Figure adapted from Gilbert et al, 2000 (1)*

Serosal window closure

After cellularization, the formation of a serosa starts. A serosa is an extraembryonic epithelium which surrounds the embryonic rudiment. *D. melanogaster*, has amnioserosa and it does not surrounds the entire embryo (Figure 3).

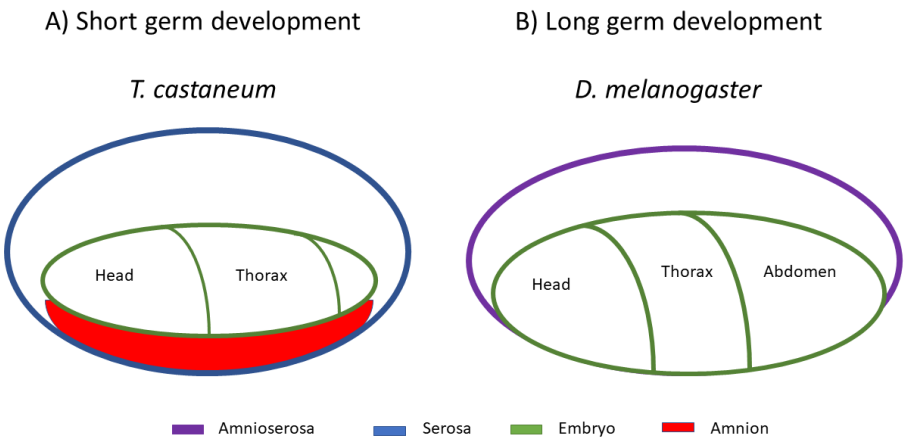

Figure 3. *The two types of development in insects. A) One example of the short germ development is shown for T. castaneum where the blastoderm only has the head and the thorax after gastrulation. Serosa and amnion are two different epithelia. B) D. melanogaster development is an example of the long germ development where all the segments are presents in the blastoderm after gastrulation. Serosa and amnion are combined in one epithelium called amnioserosa.*

The use of *T. castaneum* to study serosa has increased because the development of *T. castaneum* is more similar to the development of most of the insects than *D. melanogaster* (15). However, *D. melanogaster* is the most studied insect in Biology and therefore there is most information available.

D. melanogaster has a long germ development which means that after gastrulation all the segments develop at the same time. Additionally, the two extraembryonic epithelia have been strongly reduced to a single small amnioserosa that covers the yolk dorsally (Figure 3B) (19). In contrast, *T. castaneum* has a short germ development, as is the case for most of the insects. In this kind of development just the head and the thorax are present in the blastoderm . After gastrulation, the rest of the segments are developed. Additionally, *T. castaneum* has no amnioserosa, but two separate epithelia, amnion and serosa (Figure 3A). Most of the insects have these two epithelia during their embryonic stage. The serosa differentiates from the germ anlage at the blastoderm stage during development (21). In most hemimetabolous insects, the embryo invaginates into the yolk leaving the serosa to fully cover the yolk (katatrepsis) (Figure 4A) (22). Finally, the serosa retracts and the embryo emerges from the yolk (Figure 4B) (12).

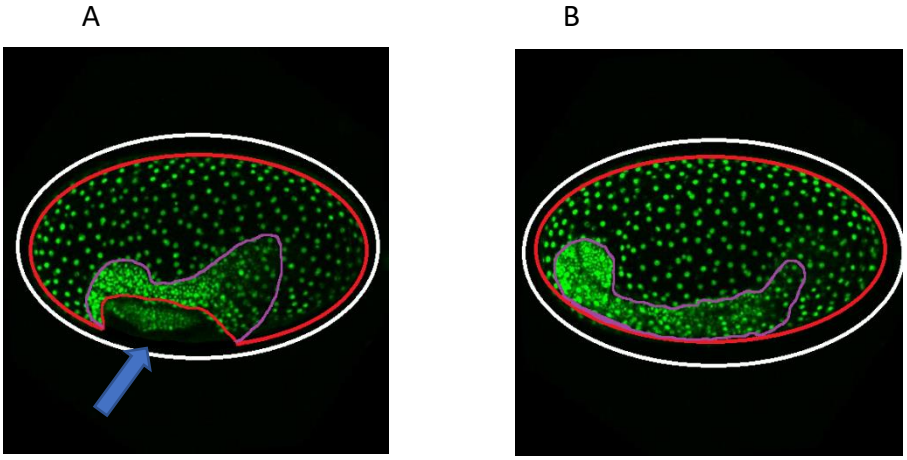

Figure 4 *T. castaneum embryo during the formation of serosa. In white is the chorion, in red the serosa and in purple the embryonic rudiment. The arrow points at the serosal window. : A) The serosa is folding over the germ rudiment and the serosal window (arrow) is still open. B) Germ band growing and the serosal window has closed*

Role of Innexins and Laminins during the early development

In this thesis we study the role of Laminin and Innexin in the development of *T. castaneum*. Laminin is an extracellular matrix protein and an essential component of the basal lamina. Innexin is an integral membrane protein. Integral membrane proteins are proteins which are attached to the cellular membrane. They are important because they are part of several cell functions and structures such as cell communication, channels, linkers, structural membrane-anchoring, cell adhesion, among others (23–26).

Gap junctions and Innexins

Gap junctions are channels that connect neighboring cells and enable cells to transport small molecules up to 1-2 kDa between cells. The gap junctions are important because they open and closed controlling various cell signals. For example, the transport of calcium during apoptosis (26).

There are three protein families that make gap-junction channels, Connexins, Pannexins and Innexins. Connexins and Pannexins are found

only in vertebrates while Innexins are in invertebrates (23, 28) (Figure 5). Until now 3 pannexins (29), 20 connexins (28) and 8 innexins (30) have been discovered. The first Innexins were identified in *D. melanogaster* and *C. elegans* (24, 31, 32). In particular, *D. melanogaster* has 8 different innexins in its genome. They play a role in the adult visual system, embryonic epithelia organization, morphogenesis, germ cell differentiation processes (33), and in neuronal connections (34).

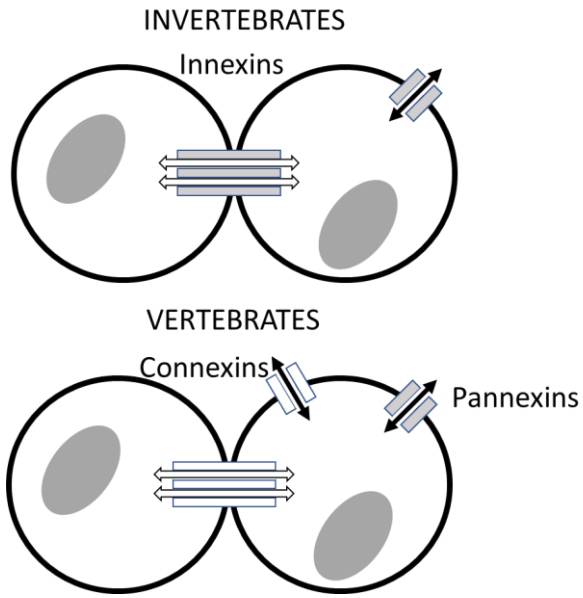

Figure 5. *The cell communication is mediated by gap junctions. In invertebrates are called innexins. In vertebrates there are two types of gap junctions pannexins and connexins. Figure adapted from Scemes et al 2008 (27)*

Innexins are important for cellular communication, which has an important role in several cellular functions. For example, in the immune system, cell communication is needed to react to external pathogens, and in the muscular system to stretch or retract the muscles. Blocking innexin function can lead to prevention of some parasitic diseases by blocking the communication between cells (23).

In this thesis we focus on Innexin 7 paralogs based on a small genetic knockdown screen that identified this gene group as important for development. These results showed that in *T. castaneum* Innexin 7

stabilizes the invagination of the furrow canals after the last nuclear division. Just before finishing the cellularization, Innexin 7 induces the cell closure by attaching the ends of the furrow canals. After the closure, neither the membrane or the nuclei elongate, leading to a thin layer of cells with cuboidal form (19). In comparison, Innexin 7 does not have any role in *D. melanogaster* cellularization. In this case, Actin protein plays a crucial role in the process of cellularization (16).

Laminins

Laminin is found in the basement membrane of animals and it plays a role in cell adhesion (35–38) (Figure 6). It is made by three subunits α, β and γ. The α subunit is the only subunit which is secreted independently of the other two. The secretion of β and γ subunits depends on the secretion of the α subunit (39).

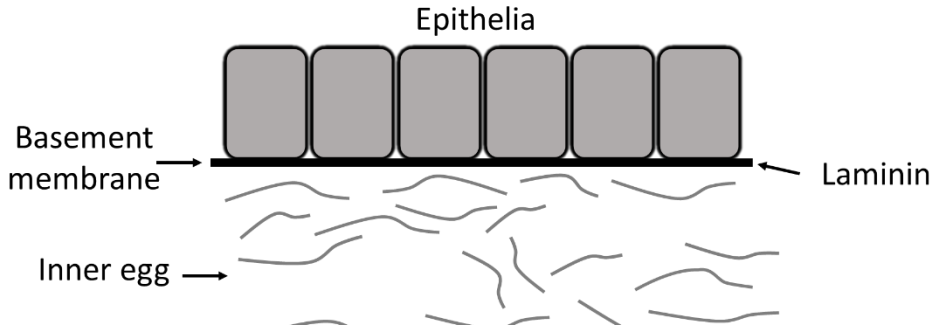

Figure 6. *The basement membrane is a thin, dense sheet matrix that surround most animal tissues. Laminin is a component of the basement membrane. Figure adapted from Jayadev et al, 2017 (39)*

There are different variants of Laminin subunits depending on the species. The mouse was the first animal where the variants were discovered, it has 15 variants of Laminin α (39). The genetics and biochemistry of Laminin in *T. castaneum* is similar to Laminin in *D. melanogaster*. Laminin in *T. castaneum* has four laminin chains: two α chains (α1,2 and α3,5), one β chain and one γ chain. In comparison, *D. melanogaster* has also four laminin chains: two α chains (α1,2 and α3,5), one β chain and one γ chain. They are arranged in two different trimers: the trimer formed by α3,5; β1; α1 is called laminin A and the trimer formed by α1,2; β1; γ1 is called laminin W. The Laminin α1,2 and α3,5 of *T.*

castaneum are orthologues of *D. melanogaster* Laminin α1,2 and α 3,5, respectively.

When Laminin is silenced this leads to different effects in animals. For example, in mammals, particularly in mice, it inhibits murine lung morphogenesis (41). Another example is seen in nematodes, specifically embryos of *C. elegans* suffer an arrest when they elongate and the organs are formed (19). Kao et al (42) observed a detachment of cells from tissues giving rise to cell disorganization. In the case of insects, the silencing of Laminin β produces blisters in the wings of adult silk worms (25); additionally Laminin has been found in the limb bud of grasshoppers where the tibial pioneer neurons grow (43). . In tissues of *D. melanogaster* there is an abnormal accumulation of major basement membrane components, such as Collagen IV and Perlecan. Additionally, silencing prevents the normal morphogenesis of most organs and tissues, including the gut, trachea, muscles and nervous system (45). In *D. melanogaster* adults when Laminin is silenced it produces wing blisters (46). In the case of *T. castaneum*, the knock down of Laminin induces a decrement of the circumference of the abdomen, generates problems with the development of muscles and results in bending of legs and antennas. Sometimes even the pupae do not hatch, hatching is delayed or their wings show blisters (https://ibeetle-base.uni-goettingen.de).

Outline

The main goal of this work is to understand the process of cellularization and serosal window closure in tribolium. This thesis is divided in five chapters. Chapter 1 describes in a broad sense cellularization and serosal window closure and gives an outline of the thesis.

In chapter 2, we study the cell shape during cellularization in *T. castaneum* and *D. melanogaster*. We created a new transgenic line of *T. castaneum*. We inserted the green fluorescent protein (GFP) gene of a jellyfish into the genome to create a gene fusion with the gene encoding the cytoskeletal actin protein of *T. castaneum* in order to visualize the membrane before and during cellularization (47). As a result the visualized membranes allowed us to make videos of the eggs to recognize the shape of the (proto)cells. In order to do this, we used a pattern recognition program. In this thesis we used the program of Aigouy et al., 2010. We

observed that the membranes of the cells are arranged in a pattern known as a Voronoi tessellation. To understand this natural pattern-forming process, we simulated the growth of the cells using a mechanical model comprising the nuclei, radial microtubules and actin cortex of the cells. The result of the simulation and the experiments are consistent.

In Chapter 3 we study the role of the gene Innexin 7a and its paralogs in *T. castaneum* development. We used parental RNA of interference (pRNAi) for a screen of junction proteins and to silence the gene Innexin 7a (8, 49, 50) . We found that knocking down innexin7a leads to failure of cellularization. We used RNA deep sequencing (RNAseq) to study the effect on transcriptome expression of signal transduction pathways when Innexin 7a is silenced (51). RNAseq analysis shows that the pRNAi targeting of Innexin 7a had a very strong effect on the transcription of a large group of genes including several genes of the innexin family during the early development.

In Chapter 4, we study the role of Laminin α1,2, β and γ (Laminin 1) in the serosal window closure of *T. castaneum* using pRNAi. We focused on the trimer Laminin 1 because the knock down of Laminin α1,2 induces wing blisters. This phenotype is indicative of cell adhesion defects which lead to morphogenetical mutations such as defects in serosal window closure. We observed that the trimer Laminin 1 is one of the key cell adhesion molecules during serosal window closure. To the best of our knowledge, Laminin 1 is the first discovered cellular component in the process of serosal window closure in *T. castaneum*.

In Chapter 5, we present the general conclusions and possibilities for future work and applications for the study of cellularization and serosal window closure.

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