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

Vazquez Faci, T.

CHAPTER 5

General conclusions and discussion

In this chapter I present the conclusions of the work of this thesis, discuss possible applications and future prospects for follow-up work. First, I will give a small introduction of the processes that we studied. Then I will give a summary of the conclusions of each chapter.

In humans after fertilization the cells start to divide. In *Tribolium castaneum*, like all insects and some other invertebrates, the nuclei and not the cells divide. The nuclei are enclosed in a single membrane, and this whole single cell is called a syncytium. Until the 8th nuclear division, the nuclei are inside of the egg. After the 8th nuclear division, they migrate to the surface of the egg and form a single layer of nuclei, called the syncytial blastoderm. Once the nuclei are at the periphery of the egg, after the 12th nuclear division, the plasma membrane surrounding the entire egg moves in between the nuclei (invagination) to separate them. Finally, the plasma membrane closes around the protocells forming a ring (1). To close the membrane to form a cell, a clamping protein creates junctions between the developing basal membrane at the bottom of the furrows and the yolk plasmalemma underneath the protocells. These junctions act as patch-clamps, allowing the basal membrane to spread until it closes off the protocell (2), creating actual cells. This process, known as cellularization, turns the syncytial blastoderm into a proper epithelium tissue, known as the cellular blastoderm.

During gastrulation the embryo starts to form more epithelia. *T. castaneum* has two extraembryonic epithelia, the amnion and the serosa. Most of the insects have these two epithelia. The blastoderm differentiates into serosa cells and the embryo (the germ anlage) (3). In most hemimetabolous and holometabolous insects, the embryo invaginates into the yolk leaving the serosa to fully cover the yolk (anatrepsis) (4). After anatrepsis serosal window closure occurs. Finally, the amnion and serosa fuse during katatrepsis. (5).

In this thesis we study these processes of cellularization and serosa window closure using different approaches. In chapter 2, we study the cellularization from a mechanical point of view. In contrast, in chapter 3, we also study the cellularization using molecular biology and genetics. Finally, in chapter 4, we study serosal window closure, which is the next major change process after gastrulation in the insect development. In general, the results of this study are important to start to understand the

developmental processes of *T. castaneum*. The availability of this data is relevant as a new biological model besides *Drosophila melanogaster* because *T. castaneum* has a more similar development compared to the majority of the insects than *D. melanogaster*. The two processes studied in this thesis are complex and we had to use different approaches from physics and biology to understand them.

Mechanics of cellular blastoderm arrangement and cell shape in insect embryos

In chapter 2, we observed that a Voronoi tessellation using the nuclei as points is similar to the arrangement of the actual protocells. In Figure 1 A, a Voronoi tessellation is shown, the plane is divided in regions such that the points in each region are closest to a given center (red dots). The boundaries between two regions (black lines) are equidistant from the corresponding centers, and perpendicular to the line connecting these centers. Although Voronoi tessellations have occasionally been used to describe cellular patterns in epithelial tissues (6–11), to the best of our knowledge, the fact that the nuclei are located at the centers of the corresponding Voronoi cells has not been shown previously. The Voronoi tessellation and the actual assembly of the cells have the same area, form and their arrangement is such that the resulting tissue is just on the liquid side of the jamming transition (12) (Figure 1 B). The jamming transition is a type of phase transition between liquid and solid state by increasing the density of the material (13). We can understand the formation of a Voronoi tessellation pattern from mechanical interactions between the cells. When the protocells grow and duplicate, they have contact with their neighbors increasing the density of protocells in the egg resulting in a mechanical reaction that causes them to stop growing towards the neighbors. These contacts translate back to a mechanical force on the nuclei of the cells, which causes them to re-position and eventually form the observed Voronoi tessellation. Therefore, mechanical interactions are a factor which determine cell arrangement and shape in the blastodermal epithelium.

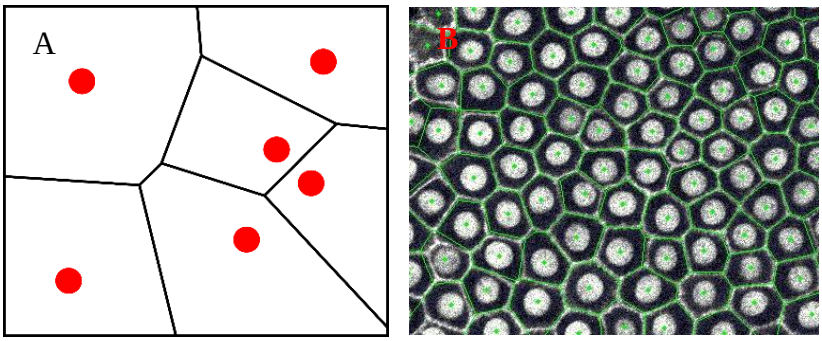

Figure 1. *A) Voronoi tessellation. Red dots are the seeds to create the tiling in the space (centers). The boundaries (black lines) between two centers are equidistant from the corresponding centers, and perpendicular to the line connecting these centers. B) In white, Image of the epithelium of the egg after cellularization with inserted Green Fluorescent Protein (GFP) in the membrane (life-actin) and the nuclei* (14, 15)*. In green, Voronoi tessellation using as centers the nuclei of the cells. The shape of the cells is similar to the shape of the Voronoi tessellation. Figure adapted from Van Drongelen et al 2017* (14)*.*

Role of Innexin 7 during cellularization in T. castaneum

In chapter 3, we also study the cellularization but using a molecular biology and genetics approach. First, we performed a screen for functions of junction proteins, then we performed an RNA sequencing experiment to study the effect on the transcriptome after parental RNAi (pRNAi) silencing of innexin 7a. Innexin 7a is a paralog of a set of 3 highly similar genes which we call further on the innexin 7 group because probably RNAi targeting cannot distinguish their function.

When we performed the screen for functions of junction proteins, we discovered that the Innexin 7 group has an important role in cellularization. When innexin 7a is silenced, the basal cell membranes are not formed and the ingressed plasma membrane retracts to the apical surface when the basal cell closure starts. Therefore, the Innexin7 group is a component of the newly identified junctions that stabilize the ingressed membrane. Furthermore, using immunolocalization, we showed that Innexin 7a forms junctions between the starting basal membrane and the yolk membrane in two ways. First, these junctions split the furrow canals immediately after the 12th division. In absence of proper splitting, membrane of a protocell can become skewed towards neighboring nuclei, as happens during

protocell delamination. Second, these junctions stabilize the furrow canals during the phase of actual basal cell closure. Absence of this stabilization leads to the retraction of the ingressed membrane to the apical surface, causing a complete reversal of the cellularization process.

We performed RNAseq of RNA isolated from eggs of which the parents were injected with dsRNA targeting innexin 7a (pRNAi) to study the effect of the silencing on the transcription of all genes. The results from the differential expression of genes after pRNAi as compared to the control show a large set of genes that are different expressed. It is important to mention that based on the close homology of the three identified innexin 7 paralogs we assume that the effects of the pRNAi approach could be the result of silencing of all three paralogs and therefore we use the generic name innexin 7 to indicate the entire gene group. Additionally, we performed gene enrichment analysis to describe the effects of the pRNAi on the transcriptome. Functional annotation charts obtained from DAVID (Data base for Annotation, Visualization and Integrated Discovery) showed a strong enrichment with very low P-values of many genes that are linked to development, particularly linked to insect cuticle development. Considering that the RNAi targeting inx7a resulted in severe developmental differences, it is very likely that many effects at the RNA level are indirectly caused by differences in developmental timing. In addition, our RNAseq results indicate a possible connection of inx7 with DNA replication. In this context, the publication of Doble et al (2004) that shows that phosphorylation of serine 262 in the gap junction protein connexin-43 regulates DNA synthesis in human cardiomyocytes supports our findings. It is therefore interesting to further investigate the role of gap junction regulation in the DNA replication process.

It might seem surprising that the sole depletion of Inx7a shows a strong cellularization phenotype, while the other innexins Tc-inx1, inx2, inx3, inx456 are also expressed during cellularization. However, specific protein properties might distinguish the Innexin7 paralogs from the other expressed Innexins. Since in other organisms, paralogs often develop to have a specialized function in heteromeric complexes (16) it wouldn't be surprising if the paralogs of the innexin 7 group are forming heteromeric gap junctions and therefore have an equally important function.

The proposed mechanism of basal cell closure involving Inx7 could be unique to *Tribolium*. However, since the *Drosophila* mode of

cellularization involving columnar cells is evolutionary derived, it seems more likely that the Inx7-mediated process is ancestral.

Role of the trimer Laminin α1,2, β and γ during serosal window closure in T. castaneum

In chapter 4, we studied the role of Laminin during serosal window closure. The protein of Laminin is in the basement membrane of animals (17–20). It is made by three subunits: Laminin α, β and γ. Depending on the organism there are different heterotrimers which they are made by different chains of α, β or γ. *T. castaneum* has four laminin chains: two α chains (α1,2 and α3,5), one β chain and one γ chain. We noted that the trimer Laminin α1,2, β and γ (Laminin 1) is one of the key cell adhesion molecules and the first cellular component involved in serosal window closure in *Tribolium castaneum*. Furthermore, we show the enrichment and potentially extracellular localization of Laminin γ protein around the necklace cells of the serosal window. Finally, we show that closure of the serosal window is delayed in laminin deficient embryos.

The phenotype we observe after laminin RNAi is comparable to the one reported for dorsocross RNAi (21). Not only the delay of closure of the serosal window is similar, but also the curved germ band that is positioned more towards the posterior (21). In absence of proper separation of the amnion and the serosa, the amnion connects the germ band to the serosa which is anchored to the outer vitelline membrane, preventing anterior movement of the germ band (21, 22). This probably happens in both *dorsocross* and laminin RNAi. In addition, the expression pattern of laminin γ resembles the reported expression pattern of *dorsocross* (21). This makes it highly likely that laminins are important target genes of the Dorsocross transcription factor during closure of the serosal window in *Tribolium*.

In conclusion, we have shown that Laminin is required for closure of the serosal window, a crucial step in the development of the serosa. As more genetic tools such as RNAi and CRISPR-Cas are now available for non-model organisms, more genes will probably soon be described to be involved in morphogenesis of this crucial extraembryonic epithelium in insects.

Applications

The applications of studying the early development of insects range from agriculture to material science. In agriculture, the study of the early development of insects can be applied to create new kinds of pesticides, less harmful to human health. For example, carbofuran is an insecticide that stops pests by disturbing the reproduction and development of insects. But, this insecticide does not just kill insects, it also has toxic effects in humans and other mammals (23). Understanding the early development of insects, can help in identifying a hormone or gene that has the same effect in insect development as carbofuran (El-Sheikh, Kamita, & Hammock, 2016; "Handbook of Hormones" 2016). In this way, insect pests can be eliminated with less environmental contamination. This kind of pesticides are called third-generation pesticides (26). There are already hormones on the market which can be used instead of pesticides (https://www.koppert.nl/). One example is Vidi Terrum, that is used to stimulate the defense and stress resistance of crops.

There are also applications for studies of the serosa that are not directly obvious, like the process of cryopreservation. Cryopreservation is a freezing process used to preserve the eggs of the insects for a long period and to hatch them after years of been fertilized (27–29). The possible use of this process is to make a bank of fertilized embryos similar to the seed bank in Norway (30). Unfortunately, not all insects are easily cryopreserved, such as the bee (31). The bee is a very important insect for human survival since it is the main pollinator on the planet (32). In the University of Humboldt in Germany, Dr. Jakob Wegener aims to preserve bee eggs by cryopreservation (33). He found that the permeability of the bee egg to the cryopreservation chemicals is the limiting factor. The serosa is one of the epithelia that is responsible of the permeability of the egg (34). Therefore, knowledge of the composition and formation of serosa is important to be able to cryopreserve bee and other insect eggs.

Other interesting applications could be in material science, by creating new materials that avoid desiccation. According to Jacobs et al 2013, the serosa protects the eggs from desiccation. The study of serosa components can help us to understand the biological principles of protection against desiccation. This can lead to the creation of new

biomimetic materials which are resistant to dry environments. These new materials can be used in deserts, in the Antarctic, by astronauts or for food preservation.

In conclusion the study of the development of *T. castaneum* has a lot of possible applications. Some of the applications are already on the market and some of them need more study and comprehension. Therefore, it is important to keep studying the development of *T. castaneum* specially from a multidisciplinary perspective.

Future work

In chapter 3, the next step for future investigations is to further study the role of innexins in signaling pathways. One of the reasons for this are the results of the study by Lechner, et al. 2007 (34) They observed that innexin 2 is part of the hedgehog pathway. The hedgehog signaling pathway is essential for the expression of wingless and Delta/Notch. Hedgehog and wingless regulate gap junction communication by transcriptionally activating the innexin 2 gene. In a feedback loop, innexin 2 is needed for the transcriptional activation of hedgehog, wingless and Delta/Notch (34). Our transcriptome study of chapter 3 also indicates a link of innexin 7 function with Notch signaling. To further study this in *Tribolium* is relevant because to the best of our knowledge there is not a link between innexin function and signaling pathways known in any species.

Following up from chapter 2, future work could address the physical properties of the egg. The egg can be seen as a liquid, and we can study properties such as the viscosity or diffusion constant. They can be measured by observing how a particle moves inside of the egg. To reach the aim we could study the movement of fluorescent beads through the egg of *D. melanogaster* and *T. castaneum* obtaining the diffusion constant by tracking the beads. The viscosity can be calculated by using the Stroke-Einstein equation (35):

$$\eta = \frac{kT}{6\pi Dr} \qquad \text{Eq 1}$$

Where D is the diffusion constant, η is the viscosity, k is the Boltzmann constant, T is the temperature of the medium and r is the radio of a spherical particle.

In chapter 4, the role of the second trimer of Laminin (α3,5, β and γ) could be studied. Although, it is possible that Laminin 2 has not any role in serosal window closure because in *D. melanogaster* null mutations of α3,5 produces embryonic lethality with defects in somatic muscles, dorsal vessel and endoderm (36, 37). It is known that the α3,5 chain is also required for localization of anterio-posterior markers in the oocyte and to bind pioneer axons in the brain (38, 39). Therefore, it is highly possible that in the case of *T. castaneum* the silencing of Laminin α3,5, β and γ can produce different effects than Laminin α1,2, β and γ. Using electron microscopy or observing Collagen IV or Perlecan proteins we could know if the basal membrane of the serosal epithelium is disturbed by knocking down Laminin α3,5, β and γ.

References

1. Handel, K., C.G. Grünfelder, S. Roth, and K. Sander. 2000. Tribolium embryogenesis: a SEM study of cell shapes and movements from blastoderm to serosal closure. Dev. Genes Evol. 210: 167–179.
2. van der Zee, M., M.A. Benton, T. Vazquez-Faci, G.E.M. Lamers, C.G.C. Jacobs, and C. Rabouille. 2015. Innexin7a forms junctions that stabilize the basal membrane during cellularization of the blastoderm in Tribolium castaneum. Development. 142: 2173–2183.
3. Roth, S. 2004. Gastrulation in other insects. In: Gastrulations: from cells to embryo. .
4. Panfilio, K.A. 2008. Extraembryonic development in insects and the acrobatics of blastokinesis. Dev. Biol. 313: 471–491.
5. Panfilio, K.A. 2009. Late extraembryonic morphogenesis and its zenRNAi-induced failure in the milkweed bug Oncopeltus fasciatus. Dev. Biol. .
6. Honda, H. 1978. Description of cellular patterns by Dirichlet domains: the two-dimensional case. J. Theor. Biol. 72: 523–543.

7. Sulsky, D., S. Childress, and J.K. Percus. 1984. A model of cell sorting. J. Theor. Biol. 106: 275–301.
8. Weliky, M., and G. Oster. 1990. The mechanical basis of cell rearrangement. I. Epithelial morphogenesis during Fundulus epiboly. Development. 109: 373–386.
9. Sharma, V., M. Crne, J.O. Park, and M. Srinivasarao. 2009. Structural origin of circularly polarized iridescence in jeweled beetles. Science (80-.). 325: 449–451.
10. Bock, M., A.K. Tyagi, J.-U. Kreft, and W. Alt. 2010. Generalized Voronoi tessellation as a model of two-dimensional cell tissue dynamics. Bull. Math. Biol. 72: 1696–1731.
11. Yu, W., H.K. Lee, S. Hariharan, W. Bu, and S. Ahmed. 2010. Evolving generalized voronoi diagrams for accurate cellular image segmentation. Cytom. Part A. 77: 379–386.
12. Bi, D., X. Yang, M.C. Marchetti, and M.L. Manning. 2016. Motility-driven glass and jamming transitions in biological tissues. Phys. Rev. X. 6: 021011.
13. Biroli, G., C.E.A. Saclay, and O. Merisiers. 2007. JAMMING A new kind of phase transition ? 3: 222–223.
14. Drongelen, R. Van, T. Vazquez-Faci, M. Van Der Zee, and T. Idema. 2017. Mechanics of epithelial tissue formation. : 1–11.
15. Sarrazin, a. F., a. D. Peel, and M. Averof. 2012. A Segmentation Clock with Two-Segment Periodicity in Insects. Science (80-.). 336: 338–341.
16. Kramer, J.M. 2005. Basement membranes. WormBook. 27: 1–15.
17. Engel, J., E. Odermatt, A. Engel, J.A. Madri, H. Furthmayr, H. Rohde, and R. Timpl. 1981. Shapes, domain organizations and flexibility of laminin and fibronectin, two multifunctional proteins of the extracellular matrix. J. Mol. Biol. 150: 97–120.
18. Kusche-Gullberg, M., K. Garrison, A.J. MacKrell, L.I. Fessler, and J.H. Fessler. 1992. Laminin A chain: expression during Drosophila development and genomic sequence. EMBO J. 11: 4519–27.
19. Fessler, J.H., and L.I. Fessler. 1989. DROSOPHILA EXTRACELLULAR MATRIX. Annu. Rev. Cell Biol. 5: 309–39.
20. Horn, T., and K.A. Panfilio. 2016. Novel functions for Dorsocross in epithelial morphogenesis in the beetle Tribolium castaneum. Dev. .

21. Münster, S., A. Jain, A. Mietke, A. Pavlopoulos, S.W. Grill, and P. Tomancak. 2019. Attachment of the blastoderm to the vitelline envelope affects gastrulation of insects. Nature. .
22. Gupta, R.C. 1994. Carbofuran toxicity. J. Toxicol. Environ. Health. .
23. El-Sheikh, E.S.A., S.G. Kamita, and B.D. Hammock. 2016. Effects of juvenile hormone (JH) analog insecticides on larval development and JH esterase activity in two spodopterans. Pestic. Biochem. Physiol. 128: 30–36.
24. 2016. Handbook of Hormones. Edited by: Yoshio Takei, Hironori Ando and Kazuyoshi Tsutsui. Second edition. Elsevier Inc.
25. Omkar. 2016. Ecofriendly Pest Management for Food Security. .
26. Leopold, R.A. 2012. Cryopreservation of Insect Germplasm: Cells, Tissues and Organisms. In: Insects at Low Temperature. .
27. Štětina, T., P. Hůla, M. Moos, P. Šimek, P. Šmilauer, and V. Koštál. 2018. Recovery from supercooling, freezing, and cryopreservation stress in larvae of the drosophilid fly, Chymomyza costata. Sci. Rep. 8: 1–13.
28. Vyletova, L., L.V.P. Rennalls, K.J.L. Wood, and V.M. Good. 2016. Long-term, large scale cryopreservation of insect cells at −80 °C. Cytotechnology. 68: 303–311.
29. Charles, D. 2006. SPECIES CONSERVATION: A “Forever” Seed Bank Takes Root in the Arctic. Science (80-.). .
30. Collins, A.M. 2002. Collection of honey bee eggs for cryopreservation. J. Apic. Res. 41: 89–95.
31. Klein, A.M., B.E. Vaissière, J.H. Cane, I. Steffan-Dewenter, S.A. Cunningham, C. Kremen, and T. Tscharntke. 2007. Importance of pollinators in changing landscapes for world crops. Proc. R. Soc. B Biol. Sci. 274: 303–313.
32. Jakob Wegener, S.A.-K.& K.B. 2009. Collection of viable honey bee (Apis mellifera) larvae after hatching in vitro. J. Apic. Res. 48.
33. Jacobs, C.G.C., G.L. Rezende, G.E.M. Lamers, and M. van der Zee. 2013. The extraembryonic serosa protects the insect egg against desiccation. Proc. R. Soc. B Biol. Sci. 280.
34. Lechner, H., F. Josten, B. Fuss, R. Bauer, and M. Hoch. 2007. Cross regulation of intercellular gap junction communication and paracrine signaling pathways during organogenesis in Drosophila. Dev. Biol. 310: 23–34.

35. Hoffmann, M.M., M.D. Too, M. Vogel, T. Gutmann, and G. Buntkowsky. 2020. Breakdown of the Stokes-Einstein Equation for Solutions of Water in Oil Reverse Micelles. J. Phys. Chem. B. 124(41):9115-9125.
36. Henchcliffe, C., L. Garcia-Alonso, J. Tang, and C.S. Goodman. 1993. Genetic analysis of laminin A reveals diverse functions during morphogenesis in Drosophila. Development. 18(2):325-37.
37. Yarnitzky, T., and T. Volk. 1995. Laminin is required for heart, somatic muscles, and gut development in the Drosophila embryo. Dev. Biol. 169(2):609-18.
38. Deng, W.M., and H. Ruohola-Baker. 2000. Laminin A is required for follicle cell-oocyte signaling that leads to establishment of the anterior-posterior axis in Drosophila. Curr. Biol. 10(11):683-6.
39. García-Alonso, L., R.D. Fetter, and C.S. Goodman. 1996. Genetic analysis of Laminin A in Drosophila: Extracellular matrix containing laminin A is required for ocellar axon pathfinding. Development. 122(9):2611-21.