Molecular keys in Structural Evolution of Insect Ovaries

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Abstract

The present work aims at establishing the first bases for studying the insect oocyte polarity in a phylogenetically basal species, the cockroach *Blattella germanica*. Our approach was to choose some genes that have been described as key in the establishment of oocyte polarity in *Drosophila melanogaster*. Among them we studied, Capicua, a HMG box transcription factor repressor in *B. germanica*, which is required for the establishment of dorsal-ventral patterning of eggshell and embryo. The epidermal growth factor receptor (EGFR) that is also essential for the polarity establishment in the oocyte. We study the function of Notch pathway and how the components of this pathway participate in the proper development of the oocyte. Otherwise, trying to study the relationships between Capicua and EGFR, we assess the function of Pipe in *B. germanica* ovary, and embryo.

Resumen

El presente trabajo quiere asentar las bases para estudiar la polaridad en el oocito en una especie filogenéticamente basal como la cucaracha *Blattella germanica.* Para ello, y basándonos en la información disponible sobre genes implicados en el establecimiento de la polaridad en *Drosophila melanogaster*, escogimos algunos genes clave. Hemos estudiado el factor de transcripción Capicua, que en *B. germanica* actúa como represor de la transcripción, y es imprescindible para la formación del eje dorso-ventral del huevo y del embrión. EGFR (epidermal growth factor receptor) que participa también en el establecimiento de la polaridad en el folículo ovárico. Estudiamos la función de la vía Notch, y como los componentes de esta vía afectan el correcto desarrollo del oocito. Además, tratando de establecer una relación entre Capicua y EGFR estudiamos la función de Pipe en el ovario y el embrión de *B. germanica*.

Preface

The aim of the present thesis was to participate in the understanding of insect reproduction, and how this process has been evolved. When this work starts most of the information available in physiology of the reproduction was from hemimetabolan insect species, as most of the laboratories that were working in insect endocrinology, used species of this group as models. However, most of the work done to understand the gene function, by means of molecular tools, was done using *Drosophila melanogaster* as a model, principally due to the great possibilities that this species offers to the researchers (genome availability, transgenesis…). With the development of RNAi methodologies these kinds of researches were extended to other insect species, and between them we can include the cockroach *Blattella germanica*.

In fact, during the last 25 years, the group has been working on a number of features related to development and reproduction of this model. First was the physiology and endocrinology of the reproduction, studying the regulation and the levels of the main hormones involved in the reproduction, and how these hormones affects vitellogenesis and choriogenesis in these species. Later on, studying those genes directly involved in the oocyte growth and maturation.

To understand how evolved the function of these genes structurally conserved, is one of the main objectives in the laboratory and in which this thesis is involved.

In the present thesis we studied in *Blattella germanica*, a cockroach with panoistic ovaries, different genes that regulates the oocyte polarity, trying to provide a bit of information on this model. We focused on the transcription factor Capicua (Cic) required for the establishment of dorsal-ventral axis in *D. melanogaster*. However, Cic in *B. germanica* is involved in the establishment of anterior-posterior axis of the ovarian follicles. Then, we studied epidermal growth factor receptor (EGFR) which is essential in *D. melanogaster* for the further establishment of both anterior-posterior and dorsal-ventral polarity in the oocyte. EGFR in *B. germanica* plays a role in the control of cell proliferation, through interaction between Hippo and Notch pathways. Related with EGFR signaling, we studied Notch pathway that regulates the egg elongation through the follicular cell polarization. Finally, we studied Pipe, a gene that is an important target for EGFR. Pipe in *B. germanica* is essential for the correct development of the embryo.

We know that with this work we laid the keys to open more doors in this particular subject. But, we expect to be helping to unveil one of the most interesting questions in developmental biology. How an egg is organized to give an organism.

Abbreviations

BLAST   Basic Local Alignment Search Tool

cDNA   Complementary DNA

Cic   Capicua

DAPI   4',6-diamidino-2-phenylindole

DEPC   Diethylpyrocarbonate

DNase   Deoxyribonuclease

dsRNA   double strand RNA

DV   dorsal-ventral

EGFR   Epidermal Growth Factor Receptor

FE   Follicular epithelium

HMG-box   High Mobility Group box protein

JH   Juvenile hormone

MAPK   Mitogen-activated protein kinases

miRNA   MicroRNA

N   Notch

NICD   Notch Intercellular Domain

ORF   Open reading frame

PBS   Phosphate buffer saline

pH3   Phospho-Histone H3

qRT-PCR   Quantitative real time PCR

REST	Relative Expression Software Tool
RNAi	RNA interference
s.e.m	Standard error of the mean
SEM	Scanning electron microscopy
TRICT	Tetramethylrhodamine isothiocyanate

Content

1. INTRODUCTION AND OBJECTIVES

1.1. Introduction

The fundamental goal of developmental biology is to understand the molecular processes by a single cell gives rise to an adult body complex. It has been long documented that insects are the most diverse group of organisms, in terms of the number of species. Their capacity to generate large number of progeny is one reason of their evolutionary success. Most insects reproduce sexually requiring efficient formation from the specialized gametes, a process that in females called oogenesis.

a) The insect ovary types

The reproductive organs of insects are similar in structure and function to those of vertebrates. The female's reproductive system consists of two pairs of ovaries which are subdivided into a number of egg tubes called ovarioles. The functional units of insect ovaries are the ovarioles, where the eggs are actually produced. Between the insects the ovaries are distinct in morphology and they are classified in two main types Panoistic and Meroistic (Figure 1.1).

The panoistic ovary (Figure 1.1A), the ancestral type, is the simplest in morphology. In this model all stem germ cell develop into oocytes that will be surrounded by monolayer of follicular cells, and they do not have any specialized nutritive cells accompanying the oocytes during the maturation. The oocyte nucleus is who synthesize all the RNAs that will need the oocyte to mature and growth (Mahowald, 1972). The meroistic ovariole has more complicated cytological derivations and physiologies. The
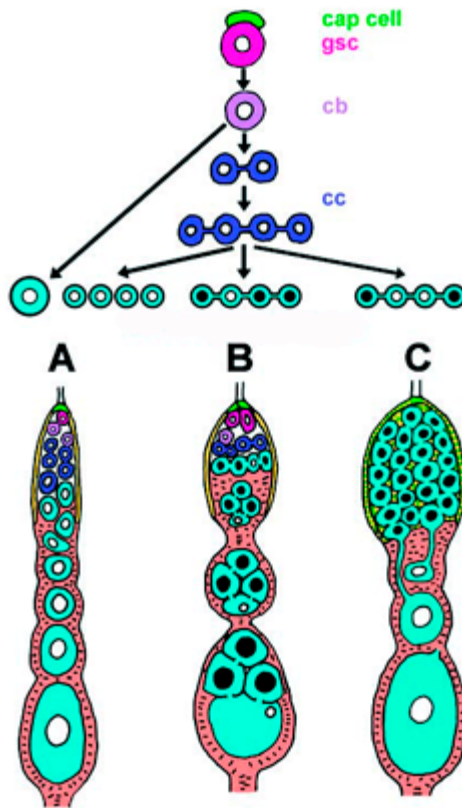
Figure 1.1: The ovary types in insects. A: panoistic, B-C: meroistic (B: polytrophic, C: telotrophic). The schema of the ovarioles is shown, together with cluster genesis, adopted from *D. melanogaster*. In panoistic ovarioles all germinal cells develop into oocytes. In meroistic ovarioles, only one cell from the cluster of cystocytes can develop as an oocyte, while all other cystocytes differentiate as nurse cells. In polytrophic ovarioles the nurse cells escort the oocyte. In Telotrophic ovarioles the nurse cells are retained in the germarium, connecting with the oocytes through cords.

stem germ cells divide to form a cluster of sister cells that remain connected by cytoplasmic bridges. One of them will become the oocyte and the remainder becomes nurse cells (Büning, 1994a). These nurse cells will provide to the oocyte all the mRNA and proteins necessaries for the oocyte maturation and growth.

Depending on the position of these nurse cells within the ovariole, the meroistic ovaries can be subdivided into two types: the meroistic polytrophic (Figure 1.1B), in which the nurse cells are grouped together close to the oocyte in the same egg chamber, and meroistic telotrophic (Figure 1.1C), in which the nurse cells are localized in the germarium and are connected with oocytes by cytoplasmic chords (nutritive chord) (King and Büning, 1985), (Štys and Bilinski, 1990).

Büning (Büning, 1993; 1994a) studied the evolutionary changes in the reproductive organs of the insects describing the distribution of the different ovary types in relation with the phylogeny of the insect orders (Figure 1.2). At the base of the tree, is placed the panoistic ovary, the ancestral ovary type, mostly present in the basal insect orders. In the other side of the tree (Figure 1.2) are placed those insect orders that have meroistic ovaries. Between these orders are placed the Dermaptera that have an ovary type which was considered the point of transition from panoistics to meroistics (Büning, 1993). Büning (1993) considered that this ovary had a development parallel to the others since the germ stem cell divides once given one oocyte and one nurse cell.

But the classification of insect ovaries is not so simple. In some orders there are species that have ovaries were secondarily modified (Büning, 1994a). For instance, in Plecoptera, a basal insect order, the cystocytes remain connected by intercellular bridges until complete their maturation as happens in meroistic ovaries (Gottanka and Büning, 1990). Also, among the orders that were expected to find meroistic ovaries appeared some groups with the panoistic
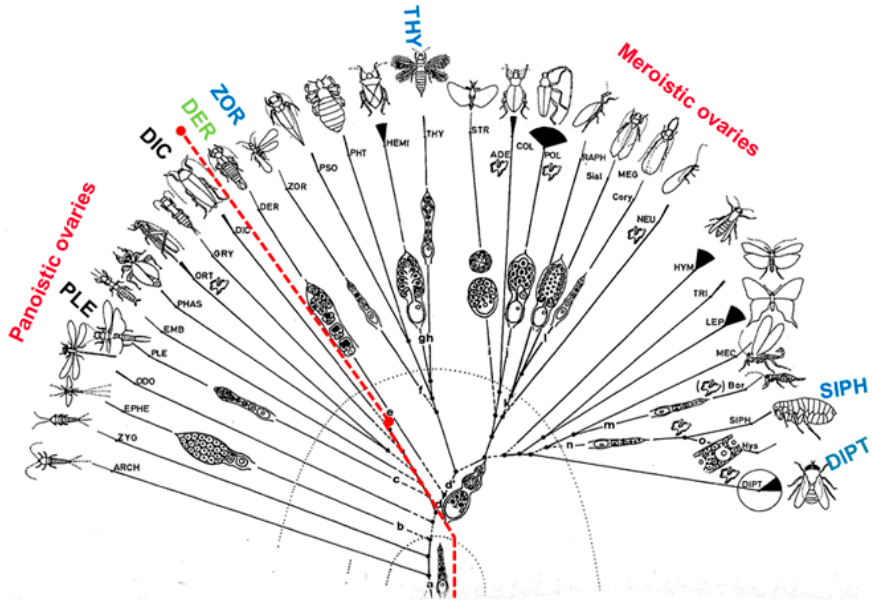
Figure 1.2: Hypothetical dendrogram of insect ovariole types. At the base of insects, cluster formation in female gonads was repressed, resulting in panoistic ovary. (PLE) Plecoptera: clusters are built but all cystocytes remain oocytes, which detach later on. (DIC) (Dictyoptera): the order where the cockroach species were represented, *Blattella germanica* and where the great division between panoistic and meroistic ovaries occurs. (DER) Dermaptera: first order which develops a polytrophic meroistic ovary with stem cell clusters and regular split of cystocyte clusters. (ZOR) Zoraptera, (THY) Thysanoptera and (SIPH) Siphonaptera are the orders that have neopanoistic ovaries evolved to meroistic ovaries. (DIPT) order Diptera where the flies' species were represented, *Drosophila melanogaster* the most modified species. Modified from Büning 1993.

type, named secondary panoistic or neopanoistic ovaries (Štys and Bilinski, 1990), and they include the Zoraptera (Büning, 1993), Thysanoptera (Pritsch and Büning, 1989) and Siphonaptera among others. The fundamental objective of the laboratory is to understand how the transition from panoistic to meroistic ovaries occurred during evolution. The morphological differences are evident and have been extensively studied, but still much to know about the

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functional differences which should be carried out in the view of molecular mechanism.

b) *Blattella germanica* as a model

As stated above, a good model for studying oogenesis is the insect with the panoistic ovary, especially, characterized for lacking nurse cells escorting the germinal cell or oocyte. Instead of this, each ovarian follicle only consists of an oocyte surrounded by the follicular epithelium. Panoistic ovary is characteristic from phylogenetically basal insects as the hemimetabolan species, the cockroach *Blattella germanica*, which is the experimental model insect in this work, and is the most common ovary type in invertebrates and vertebrates (Tworzydlo et al 2014. Zoology).

There are some differences between the models *B. germanica* and *D. melanogaster*, the ovary in *B. germanica* is panoistic, and the ovarian follicle do not have nurse cells, whereas in *D. melanogaster* the ovary is meroistic polytrophic, and the oocyte is accompanied by nurse cells that provide it with the necessary macromolecules. The follicular cells surrounding the oocyte in *B. germanica* are binucleated when the oocyte reaches the maturity, while in *D. melanogaster* is mononucleated. The oocyte nucleus of *B. germanica* is transcriptionally active and moves to the ventral central part of the oocyte; however, in *D. melanogaster* is inactive and moves to the dorsal anterior part.

We can distinguish three different regions along the ovariole (Figure 1.3), vitellarium, which holds the developing ovarian

follicles; a germarium, where the germ and somatic stem cells are placed, and a terminal filament that is an apical extension from the protein layer that covers the ovariole. All the filaments combined together to fix the ovary to the body wall. Each ovariole has a pedicel that connects all the ovarioles in a lateral oviduct and lead into the oviduct, which connects both ovaries (Klowden, 2008).
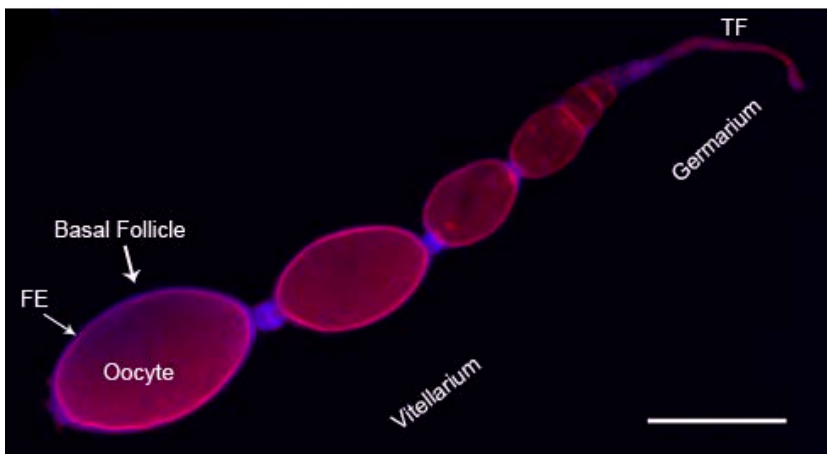
Figure 1.3: *B. germanica* **ovariole.** In the picture are shown the vitellarium holding the developing follicles, the germarium, and the terminal filament (TF). Each follicle is consisted of an oocyte surrounded by a follicular epithelium (FE). Scale Bar: 1000 µm.

The cockroach *B. germanica* has around 20 ovarioles per ovary and only the basal oocyte of each ovariole develops, is fertilized and is oviposited at the end of the corresponding gonadotrophic cycle, while the subsequent oocytes are held, arresting or reducing their development until the onset of the next cycle.

The first gonadotrophic cycle starts on the last (sixth) nymph instar and culminates with the oviposition, eight days after adult emergence. This period can be divided into three stages, pre-vitellogenesis,

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vitellogenesis and choriogenesis. The pre-vitellogenic period includes the last nymph instar and the first three days of adult life, in which the cells of the follicular epithelium divide mitotically and increase its number up to 10 fold. During vitellogenesis, the follicular cells arrest cytokinesis and become binucleated (Ciudad et al., 2007; Irles et al., 2009a). The basal oocytes grow exponentially due to the incorporation of vitellogenin, which is synthesized in the fat body in response to the presence of juvenile hormone in the hemolymph. Thereafter, the vitellogenin will be internalized in the oocyte by receptor-mediated endocytosis (Comas et al., 2001; Ciudad et al., 2006). During this period, the follicular cells enter in the endocycle program, beginning polyploidy (Irles et al., 2009a; b). The internalization of the storage proteins is possible because follicular cells shrink and leave intracellular spaces among them, a phenomenon known as *patency*, which is also under juvenile hormone (JH) control (Davey and Huebner, 1974; Pascual et al., 1992). Choriogenesis is the last stage of gonadotrophic cycle, it starts later in 5-day-old females, when the follicular cells, under the action of the hormone 20-hydroxyecdysone, synthesize the chorion proteins (Belles et al., 1993; Irles et al., 2009b; Irles and Piulachs, 2011). The resulting chorion is a multilayered envelope for the egg (the eggshell) that sustains and protects it. Once the chorion is formed, the egg is fertilized egg and oviposited. In the case of our model, the eggs are oviposited and are packaged in an ootheca, which is carried by the female during the embryo development until hatching. Those embryos are short germ band located in the ventral side of the egg, which differs from *D. melanogaster* embryos that are long germ band and are located in the

dorsal side. After the embryos hatch and once is release from the ootheca, a new gonadotrophic cycle starts.

The whole process of growth and maturation of the ovarian follicle is controlled by a series of signals, mainly hormones. In adult female, the JH is released from *corpora allata* into the hemolymph reaching high levels in the hemolymph (Treiblmayr et al., 2006), where acts in the fat body activating the expression and synthesis of vitellogenin (Martin et al., 1996; Comas et al., 2001) that will be uptake by the growing oocyte (Ciudad et al., 2006). The JH acts in the follicular cells determining the patency and facilitating the passage of the vitellogenin through the epithelium.

Also we cannot forget to mention the extensive knowledge about the physiology of the reproduction of this species that has the laboratory, which outcome of years of use *B. germanica* as our research model.

However, despite the different ovary types, the final function of the oogenesis is to obtain a correct embryo development. Reaching to this final goal, series of genes should be transcribed and translated onsite in the ovarian follicle at the precise time and place. We hypothesized that some genes involved in oogenesis are specific for panoistic ovaries and other specific for meroistic ovary whereas some are conserved in both structure and function.

After more studies done in our laboratory and analyzing the sequenced genome, now available, we found that may be more genes are structurally conserved between the different ovary types. But, is the function of these genes, the same?

To contribute in answering this question, we established this work

focused on studying genes that were necessary for oocyte polarity and the establishment of axes during the oogenesis in basal insects. We studied in *B. germanica* the genes involved in oocyte polarity in *D. melanogaster* as Capicua (Cic) gene, which plays a role in DV patterning (Goff et al., 2001), the epidermal growth factor receptor (EGFR) signaling which essential participating in the DV patterning of the egg and embryo (Gonzalez-Reyes et al., 1995; Roth et al., 1995), the Pipe gene, which is the target of EGFR signaling and is relevant to DV axis patterning (Sen et al., 1998) and Notch pathway which contribute to regulate the oocyte polarity formation by controlling the temporal and spatial pattern of follicle cell differentiation and proliferation (Yu et al., 2008).

1.2 Objectives of the Thesis

The main objective of the present thesis was to put the basis in the study of the establishment of the oocyte polarity in panoistic ovaries using the German cockroach *Blattella germanica* as a model. To achieve this main objective, we followed these specific objectives:

1. To identify and characterize *B. germanica* Capicua (BgCic) and describe its function in panoistic ovaries.

2. To study *B. germanica* EGFR (BgEGFR) in the panoistic ovary and characterize it functionally and its interaction with other signaling pathways.

3. To study Notch pathway in *B. germanica* ovary and unveil its function in oocyte polarity.

4. To analyze *B. germanica* Pipe (BgPipe), describing its function in the oocyte and in the embryo, and correlate BgPipe with BgCic and BgEGFR signaling.

1.3. Reference

Belles, X., Cassier, P., Cerda, X., Pascual, N., Andre, M., Rosso, Y., Piulachs, M.D., 1993. Induction of choriogenesis by 20-hydroxyecdysone in the German cockroach. Tissue & cell 25, 195-204.

Büning, J., 1993. Germ cell cluster formation in insect ovaries. Int. J. Insect Morphology & Embryology 22, 237-253.

Büning, J., 1994. The Insect Ovary. Chapman & Hall, London, UK.

Ciudad, L., Belles, X., Piulachs, M.D., 2007. Structural and RNAi characterization of the German cockroach lipophorin receptor, and the evolutionary relationships of lipoprotein receptors. BMC molecular biology 8, 53.

Ciudad, L., Piulachs, M.D., Belles, X., 2006. Systemic RNAi of the cockroach vitellogenin receptor results in a phenotype similar to that of the *Drosophila* yolkless mutant. The FEBS journal 273, 325-335.

Comas, D., Piulachs, M.D., Belles, X., 2001. Induction of vitellogenin gene transcription in vitro by juvenile hormone in *Blattella germanica*. Molecular and cellular endocrinology 183, 93-100.

Davey, K.G., Huebner, E., 1974. The response of the follicle cells of Rhodnius prolixus to juvenile hormone and antigonadotropin in vitro. Canadian journal of zoology 52, 1407-1412.

Goff, D.J., Nilson, L.A., Morisato, D., 2001. Establishment of dorsal-ventral polarity of the *Drosophila* egg requires capicua action in ovarian follicle cells. Development 128, 4553-4562.

Gonzalez-Reyes, A., Elliott, H., St Johnston, D., 1995. Polarization of both major body axes in *Drosophila* by gurken-torpedo signaling. Nature 375, 654-658.

Gottanka, J., Büning, J., 1990. Oocytes develop from interconnected cystocytes in the panoistic ovary of Nemoura sp. (Pictet) (Plecoptera: Nemouridae). Int J Insect Morphology & Embryology 19, 219- 225.

Irles, P., Belles, X., Piulachs, M.D., 2009a. Brownie, a gene involved in building complex respiratory devices in insect eggshells. PloS one 4, e8353.

Irles, P., Belles, X., Piulachs, M.D., 2009b. Identifying genes related to choriogenesis in insect panoistic ovaries by Suppression Subtractive Hybridization. BMC genomics 10, 206.

Irles, P., Piulachs, M.D., 2011. Citrus, a key insect eggshell protein. Insect biochemistry and molecular biology 41, 101-108.

Irles, P., Piulachs, M.D., 2014. Unlike in *Drosophila* meroistic ovaries, Hippo represses Notch in *Blattella germanica* panoistic ovaries, triggering the mitosis-endocycle switch in the follicular cells.

King, R.C., Büning, J., 1985. The origin and functioning of insect oocytes and nurse cells. Comprehensive Insect Physiology, Biochemistry and Pharmacology 1 (ed. G. A. Kerkut and L. I. Gilbert), 37-82.

Klowden, M.J., 2008. Reproductive Systems. In Physiological Systems in Insects. San Diego: Elsevier-Academic Press, 198-255.

Mahowald, A.P., 1972. oogenesis. Developmental systems: insects 1 (Counce, S J; Waddington, CH, eds, Academic Press; London, UK), 1- 48.

Martin, D., Piulachs, M.D., Belles, X., 1996. Inhibition of vitellogenin production by allatostatin in the German cockroach. Molecular and cellular endocrinology 121, 191-196.

Pascual, N., Cerdá, X., Benito, B., Tomás, J., Piulachs, M.D., Belles, X., 1992. Ovarian ecdysteroid levels and basal oöcyte development during maturation in the cockroach *Blattella germanica* (L.). Insect Physiol 38, 339-348.

Pritsch, M., Büning, J., 1989. Germ cell cluster in the panoistic ovary of Thysanoptera (Insecta). Zoomorphology 108, 309- 313.

Roth, S., Neuman-Silberberg, F.S., Barcelo, G., Schupbach, T., 1995. cornichon and the EGF receptor signaling process are necessary for both anterior-posterior and dorsal-ventral pattern formation in *Drosophila*. Cell 81, 967-978.

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.

Štys, P., Bilinski, S., 1990. Ovariole types and the phylogeny of hexapods. Biological Reviews 65, 401 - 429.

Treiblmayr, K., Pascual, N., Piulachs, M.D., Keller, T., Belles, X., 2006. Juvenile hormone titer versus juvenile hormone synthesis in female nymphs and adults of the German cockroach, *Blattella germanica*. J Insect Sci 6, 1-7.

Yu, J., Poulton, J., Huang, Y.C., Deng, W.M., 2008. The hippo pathway promotes Notch signaling in regulation of cell differentiation, proliferation, and oocyte polarity. PloS one 3, e1761.

2. CAPICUA IN PANOISTIC OVARIES MAINTAINS THE ANTERIOR-POSTERIOR AXIS

Capicua in panoistic ovaries maintains the anterior-posterior axis

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2.1. Abstract

The establishment of the symmetry axis is crucial for the development of all organisms. In insects, this begins to happen early in the oogenesis with the correct distribution of the mRNAs and proteins in the oocyte, and Capicua (cic) is a protein that participates organizing this distribution. The function of cic has been studied in *Drosophila melanogaster* in relation to oogenesis and embryo development, it is maternally expressed and is required for the establishment of dorsal-ventral axis, acting as a repressor of terminal genes along the dorsal-ventral axis. Although the sequence of Cic is highly conserved, its function in other insect ovary types is unknown. We wondered if the function of Cic, in insects, has been maintained through evolution in spite of the ovary type, or if it has been modified in a parallel to the ovary evolution. To fill this gap we studied the Cic function in a phylogenetically basal insect, the cockroach *Blattella germanica*, a species with panoistic ovaries. The Cic sequence of *B. germanica* has an 80% of identity compared to *D. melanogaster* Cic, it is essential for the oocyte development as it participates in the establishment of the anterior-posterior axis, and a depletion of this protein results female sterility.

2.2. Introduction

Oogenesis is a crucial process in the animal kingdom, as it ensures the continuity of the species, that it is not surprising that it is a finely regulated process. A good model for studying oogenesis is

the insect ovary and, especially, the panoistic ovary which is characterized by the absence of nurse cells escorting the germinal cell or oocyte. Instead of this organization, each ovarian follicle only consists in an oocyte surrounded by a monolayered follicular epithelium (Büning, 1994b). The panoistic ovary is characteristic of phylogenetically basal insects like the cockroach *Blattella germanica*, ours insect model, and it is the most common ovarian type in invertebrates and vertebrates (Telfer, 1975). In addition, during *B. germanica* oogenesis, the basal follicle is the only one that matures in each reproductive cycle, while the other ovarian follicles remain in the vitellarium, waiting to occupy the basal position in subsequent cycles (Tanaka and Piulachs, 2012; Irles and Piulachs, 2014). The ovarian follicles in meroistic ovaries, like those of *Drosophila melanogaster* ovary, are clearly asymmetric, as they are formed by a cluster of nurse cells and the oocyte, both surrounded by a monolayer of follicular cells (Büning, 1994b). The dorsal-ventral (DV) asymmetry is established in the oocyte at mid oogenesis, when the nucleus takes a dorsal-anterior position (Roth and Lynch, 2009). In contrast, in panoistic ovaries, as those of *B. germanica*, the ovarian follicles are more symmetric due to the absence of nurse cells and to the short migration of oocyte nucleus which moves late in the oogenesis from the center to a mid-ventral position of the oocyte (Tanaka, 1973). This discrete change in the nucleus position and the oval shape of young panoistic ovarian follicles makes difficult to distinguish the DV axis in them. In *B. germanica*, however, the asymmetric distribution of F-actins in the oocyte membrane, and the maintenance of patency in the ventral

follicular cells, facilitates the identification of the ventral side of the basal ovarian follicle (Zhang and Kunkel, 1992).

These asymmetries in the oocyte morphology have a correspondence in the asymmetric distribution of the mRNAs encoding for patterning factors (Medioni et al., 2012). The distribution of these patterning factors have been thoroughly studied in holometabolan insects (Fonseca et al., 2009; Wilson et al., 2011; Wilson and Dearden, 2013), especially in *D. melanogaster* (Roth and Lynch, 2012; Shin and Hong, 2014), in relation to embryogenesis, where minor modifications in mRNA or protein localization in the oocyte may be lethal for the embryo. We have adopted the panoistic ovary of *B. germanica* as model with the aim of unveiling key factors involved in establishing and maintaining the distribution of the mRNAs in the oocyte, and we have started by studying the function of the transcription factor capicua (cic) in this basal ovary type. Cic encodes for an HMG-box protein that acts as a transcriptional factor repressor, and is structurally conserved from cnidarians to vertebrates (Jimenez et al., 2000; Lam et al., 2006). In *D. melanogaster, cic* has been reported as a maternally expressed gene that is required for the establishment of DV patterning of the eggshell and the embryonic termini during embryogenesis (Jimenez et al., 2000; Goff et al., 2001). In humans and mice, cic is involved in the development of the nervous system (Lee et al., 2002; Lee et al., 2011), and in humans, cic inactivation has been related with different cancer processes (Alentorn et al., 2012; Chan et al., 2014) . Our results show that Cic in the panoistic ovary of *B. germanica* is necessary to maintain the anterior posterior axis in the ovarian

follicles as is regulating the stabilization of the F-actins in the oocyte, a function that is exerted early during the oocyte development.

2.3. Material and Methods

a) Insects

Blattella germanica (L.) specimens were obtained from a colony reared in the dark at 29 ± 1 °C and 60–70% r.h. (Belles et al., 1987a). Adult females were maintained with males during all the first gonadotrophic cycle, and mating was confirmed at the end of the experiments by assessing the presence of spermatozoa in the spermatheca. All dissections and tissue sampling were carried out on carbon dioxide-anesthetized specimens. Tissues were frozen on liquid nitrogen and stored at -80º until use.

b) Cloning and Sequencing

A fragment of *B. germanica* Capicua (BgCic) sequence (493 bp) was obtained from an mRNA library available in the laboratory. The sequence of BgCic was completed by 3´- and 5´- rapid amplification of cDNA ends (RACE), according to the manufacturer protocol (Ambion, Huntingdon, Cambridgeshire, UK). The amplified fragments were cloned into the pSTBlue-1 vector (Novagen, Madison, WI, USA) and sequenced.

c) Sequence comparisons and phylogenetic analysis

Sequences of Cic protein from insects and the respective orthologues in other invertebrates and vertebrates were obtained from GenBank. The search was enlarged by Blast using the BgCic (Accession number LN623700) and *D. melanogaster* Cic as queries. The sequences used in the phylogenetical analysis were: *D. melanogaster* (NP_001247203.1), *Culex quinquefasciatus* (XP_001862155.1), *Aedes aegypti* (XP_001649010.1), *Danaus plexippus* (EHJ68194.1), *Bombyx mori* (XP_004931421.1), *Tribolium castaneum* (XP_968497.2), *Dendroctonus ponderosae* (ERL94807.1), *Acromyrmex echinatior* (EGI66064.1), *Camponotus floridanus* (EFN68385.1), *Harpegnathos saltator* (EFN88589.1), *Bombus terrestris* (XP_003393598.1), *Apis mellifera* (XP_003249448.1), *Nasonia vitripennis* (XP_001607793.2), *Pediculus humanus corporis* XP_002423691.1, *Acyrthosiphon pisum* XP_003241821.1, *Ciona intestinalis* XP_002120586.1, *Danio rerio* XP_005173570.1, *Takifugu rubripes* XP_003966398.1, *Sarcophilus harrisii* XP_003766506.1, *Mus musculus* NP_082158.2, *Rattus norvegicus* NP_001100960.2, *Pan troglodytes* JAA29672.1, *Papio anubis* XP_003915670.1, and *Homo sapiens* NP_055940.3.

Protein sequences were aligned with BgCic using clustalX (Larkin et al., 2007), and poorly aligned positions and divergent regions were eliminated by Gblocks 0.91b (http://molevol.ibmb.csic.es/Gblocks_server/) (Castresana, 2000). The resulting alignment was analyzed by the PHYML 3.0 program (Guindon and Gascuel, 2003) based on the maximum-likelihood

principle with the amino acid substitution model. Four substitution rate categories with a gamma shape parameter of 1.444 were used. The data was bootstrapped for 100 replicates using PHYML.

d) RNA extraction and expression studies

Total RNA was isolated using GenElute Mammalian Total RNA kit (Sigma, Madrid, Spain). An amount of 400 ng from each RNA extraction was DNAse treated (Promega, Madison, WI, USA) and reverse transcribed with Superscript II reverse transcriptase (Invitrogen, Carlsbad CA, USA) and random hexamers (Promega). RNA quantity and quality was estimated by spectrophotometric absorption at 260/280 nm in a Nanodrop Spectrophotometer ND-1000® (NanoDrop Technologies, Wilmington, DE, USA).

Expression of BgCic was determined by quantitative real-time PCR (qRT-PCR) in the last nymphal instar and in the adult stage during the first gonadotrophic cycle. PCR primers used in qRT-PCR expression studies were designed using the Primer Express 2.0 software (Applied Biosystems, Foster City, CA, USA), and are detailed in Table 2.S1. The Actin-5c gene of *B. germanica* (Accession number AJ862721) was used as a reference. qRT-PCR reactions were performed using the SYBR Green Supermix (BioRad) containing 200 nM of each specific primer, and were run in triplicate. Amplification reactions were carried out at 95ºC for 2 min, and 40 cycles of 95ºC for 15 s and 60ºC for 30s, using MyIQ Single Color RTPCR Detection System (BioRad). After the

amplification phase, a dissociation curve was carried out to ensure that there was only one product (Irles et al., 2009a).

e) RNA interference

To knockdown BgCic and asses the specificity of the phenotype, 2 different dsRNAs were designed and synthesized as previously described (Ciudad et al., 2006). The dsBgCic-1 was 324 bp in length (from nucleotides 5874 to 6197) and the second one, dsBgCic-2 392 bp (from nucleotides 4366 to 4757). As dsRNA control (dsMock) a fragment (300bp) of the sequence of *Autographa californica nucleopolyhedrovirus* (GenBank: K01149) was used. The corresponding cDNAs were amplified by PCR and cloned into pSTBlue-1 vector. Newly emerged sixth nymphal instar females or newly emerged adult females were independently treated with 1 μg of dsBgCic-1 or 1 μg dsBgCic-2. As the same ovary phenotype was found using both dsRNA, we will refer to RNAi treatments as dsBgCic. The same dose and conditions used in dsBgCic treatments were applied to dsMock-treated females.

f) Immunohistochemistry

After dissection, ovaries were fixed immediately for 2 h with 4% paraformaldehyde in PBS, washed in PBT (PBS; 0.3% Triton-X100), treated with 50 μg/ml proteinase K for 2 min, washed for 2 min in 2 mg/ml glycine in PBT, washed for 10 min in PBT and fixed again for 20 min in the same solution. Then the ovaries were

washed three times for 10 min with PBT (PBS; 0.1% Triton-X100, 0.1% BSA). After three washes with PBT the ovaries were saturated for 1 h at room temperature in PBTBN (PBS; 0.1% Triton-X100, 0.5% BSA and 5% normal goat serum), and incubated overnight at 4º C with the primary antibody, The primary antibodies used were rat anti-Cic (1:200, generated against the C-terminal half of *D. melanogaster* Cic protein, kindly provided by Jordi Casanova), mouse anti-EGFR (15µg/ml) and mouse anti-Eya (1:50) (Developmental Studies Hybridoma Bank, University of Iowa, Department of Biology, Iowa City, IA, USA). Tissues were washed with PBTBN three times and incubated for 2 h with Alexa-Fluor 647 goat anti- mouse or anti-rat IgG (Molecular Probes, Carlsbad, CA, USA) diluted 1:400 in PBTBN. For F-actin visualization, ovaries were incubated for 20 min in 1µg/ml phalloidin-TRITC (Sigma), and after washing they were mounted in UltraCruz™ Mounting Medium (Santa Cruz Biotechnology®, inc., Delaware CA, USA), which contains DAPI for DNA staining. Samples were observed by epifluorescence microscopy using a Zeiss AxioImager.Z1 microscope (ApoTome), using the software Zen 2012, blue edition (Carl Zeiss MicroImaging).

g) Statistics

Data are expressed as mean ± standard error of the mean (s.e.m). Statistical analysis of gene expression values was carried out using the REST 2008 program (Relative Expression Software Tool V 2.0.7; Corbett Research) (Pfaffl et al., 2002). This program makes

no assumptions about the distributions, evaluating the significance of the derived results by Pair-Wise Fixed Reallocation Randomization Test tool in REST (Pfaffl et al., 2002).

2.4. Result

a) *Blattella germanica* has a structurally conserved Capicua ortholog

Capicua from *B. germanica* (BgCic), was amplified, cloned and sequenced from ovarian tissues, which only expressed one isoform of BgCic. The complete sequence of BgCic has 7,631bp with an ORF encoding for a protein of 2,279 amino acids (nucleotide positions 553-7,392). BgCic possesses a HMG-box domain (from amino acid 1,206 to 1,274) characteristic of cic proteins that is highly conserved when compared among insects, from 98% of identity with *Acromyrmex echinator* Cic, to 80% with *D. melanogaster* Cic.

A maximum-likelihood analysis of Cic sequences available in the literature and databases, including insects and chordates, gave the tree shown in Figure 2.1. The tree topology clearly separates two clades, one is including the insect sequences and that cluster the vertebrate and the urochordate *Ciona intestinalis* sequences. The topology of Cic in the insect cluster matches the currently accepted phylogeny of the included species. Sequences of cic from Diptera and Lepidoptera form a group with longer branches that indicates a faster rate of divergence than in the other insect groups. Another

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cluster joined the cic sequences from hemimetabolan insects, those

of *A. pisum*, *P. humanus* and *B. germanica*.
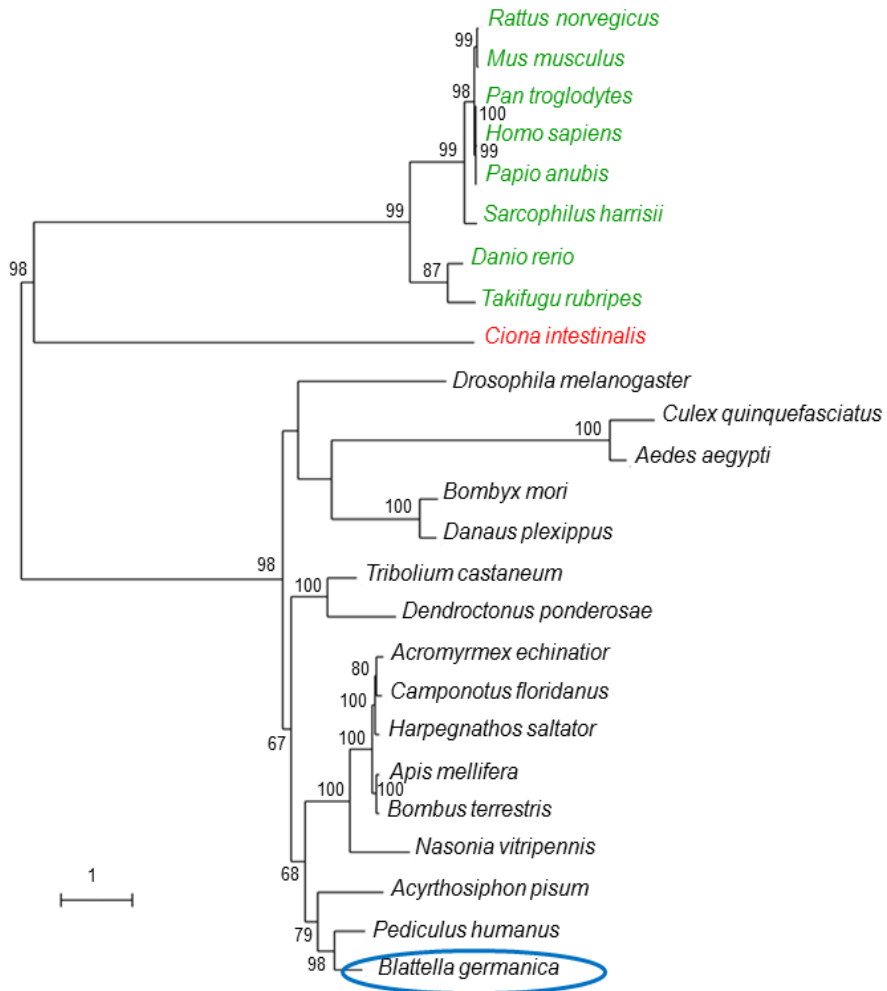
Figure 2.1. Phylogenetic analysis of the transcription factor Capicua in insects and chordates. Insect Capicua sequences are depicted in black. That of the tunicate *Ciona intestinalis*, (in red) clusters as sister group of the vertebrate node (in green). The tree is based in the maximum-likelihood principle with the amino acid substitution model was built with the sequences publicly available. Only bootstrap values higher than 60 are shown in the main basal nodes.

b) BgCic is highly expressed in the ovary during the last nymphal instar

The expression of BgCic mRNA was studied in different tissues from 3-day-old adult females, namely ovary, thoracic ganglia, colleterial glands, fat body, brain and midgut (Figure 2.2A). BgCic is expressed in all tissues analyzed, showing the highest levels in brain and ovary. In general, in ovaries from sixth instar nymphs, the levels of BgCic mRNA are fluctuated, but higher than its expression in the adult and in the last days of the fifth nymphal instar (Figure 2.2B). After the imaginal moult the expression of BgCic shows a tendency to decrease, reaching the lowest levels just before oviposition (Figure 2.2B). These expression patterns suggest that in the ovary BgCic plays its main function during the last nymphal instar.

c) BgCic protein localizes in somatic and germ cells

A heterologous antibody against Cic protein of *D. melanogaster* was used to localize BgCic in ovaries. BgCic labeling was detected in all ovarian follicles through the ovariole, and in both germinal and somatic cells (Figure 2.2C). However, there is a clear differential distribution of BgCic in the growing oocyte. In the basal follicle of 0-day-old sixth instar nymphs, BgCic labeling appears as a thin layer close to the oocyte membrane (Figure 2.2D), being less

intense in the anterior and posterior oocyte poles. Later, in 6-day-old (Figure 2.2E) and 8-day-old sixth instar nymphs (Figure 2.2F), BgCic labeling spreads through the ooplasm and becomes more intense, even accumulating as a thick layer in the boundary of the oocyte. This labeling is displayed in all ovarian follicles, from the basal to the most distal, as well as in the germarium (Figure 2.2C).

In 8-day-old sixth instar nymphs, as the female approaches the adult moult, BgCic labeling appears in the oocyte nucleus, spreads with a faint signal throughout the oocyte cytoplasm, although the signal increase in the oocyte membrane (Figure 2.2F'). After the imaginal moult, BgCic labeling in the oocyte becomes very faint (Figure 2.2G).

Figure 2.2. **Expression and localization of Capicua (BgCic) in the ovaries of** *B. germanica*. (A) Expression of BgCic was quantified in ovary (Ov), thoracic ganglia (TG), colleterial glands (CG), fat body (FB), brain (Br) and midgut (MG) from adult females. (B) Expression of BgCic in the ovary during sixth instar nymph and in the adult; the expression in ovaries in the last day of fifth nymphal instar is also showed. Data in A and B are expressed as copies of BgCic per 1000 copies of actin-5C (relative expression) and are expressed as the mean ± s.e.m. (C) Immunolocalization of BgCic in an ovariole from a 6-day-old sixth instar nymph showing the asymmetric distribution of actins in the basal follicle. (D-F) Immunolocalization of BgCic in basal follicles from 0-day-old (D), 6-day-old (E), and 8-day-old (F and F') sixth instar nymphs, showing the changes of distribution related with the age of the female, from an accumulation in the oocyte membrane to an extensive spread through the cytoplasm and into the nucleus (F'). (G) Basal ovarian follicle from a 0-day-old adult female showing a weak BgCic labelling. (H-I) Optical section of basal follicles from 8-day-old sixth instar nymph (H) and 0-day-old adult (I), showing the distribution and the different levels of BgCic labelling in the ooplasm and in the follicular cells. The arrows indicate the layer of endosymbiont bacteriocytes. Oo: ooplasm. The anterior pole in C and G is towards the right, in D-F is to the top of the image. Cic: green, F-actins: red, DAPI: blue.

DAPI
TRICT
Capicua
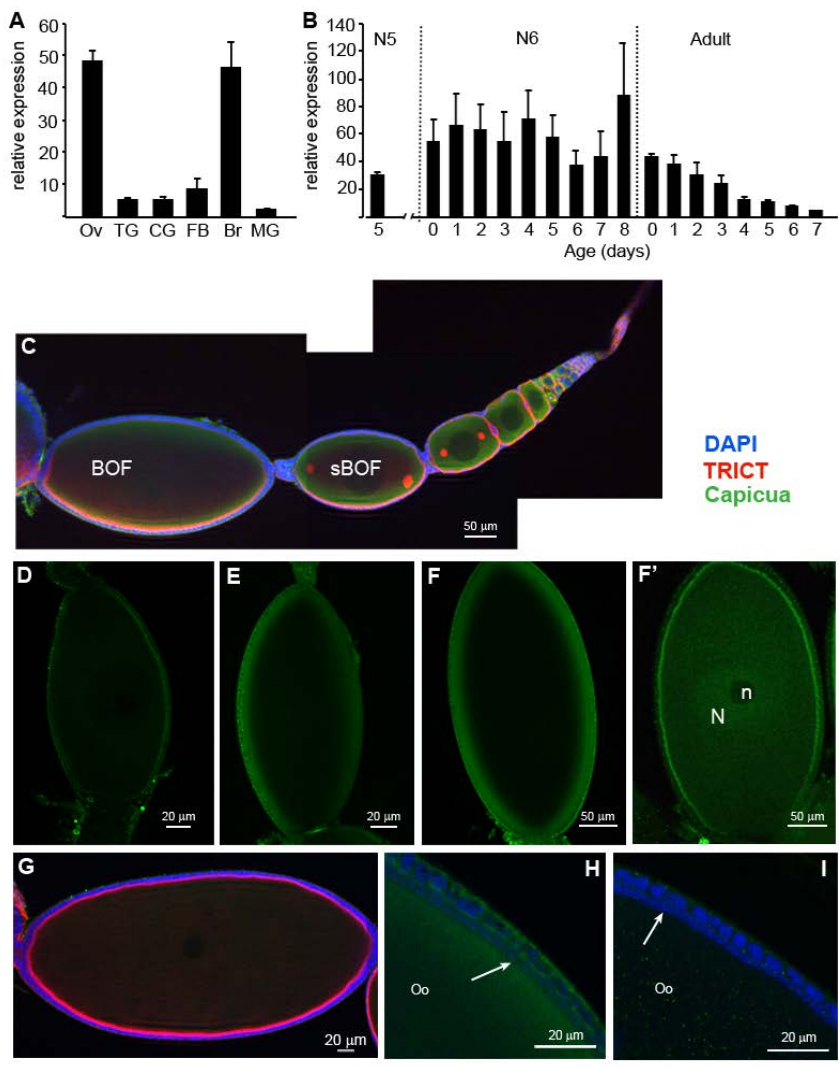
Concerning follicular cells, in 8-day-old sixth instar nymphs, BgCic appears abundant in the cytoplasm (Figure 2.2H), whereas in 0-day-old adults, labeling in the follicular cells became fainter and concentrated in the basal pole (Figure 2.2I). The whole results indicate that the BgCic localization in the ovary experience sequential and relevant changes, which suggests that it plays different roles during oocyte maturation.

d) BgCic is involved in oogenesis of *B. germanica*

To unveil the functions of BgCic in the panoistic ovaries of *B. germanica*, 1µg of dsBgCic was injected to two different experimental models: 0-day-old adult females and 0-day-old sixth instar female nymphs. In both experiments, the females were maintained with untreated males until oviposition. In the group of females that were treated in the adult stage, the mRNA levels for BgCic in ovary were measured 48 after the treatment with the dsBgCic, and they show only a 27% of reduction. A depletion that was enough to perturb the development in the embryos from these treated females. From these females treated as adult (n= 45) 78% oviposited and formed the ootheca correctly. However, 60% of these dropped the ootheca between 48-72 h after its formation, while the remaining females maintained the ootheca attached to the genital pouch, which gave rise to nymphs that emerged normally without delays. The rest of females that were treated as adults either did not oviposit (18%) or died before ovipositing (4%).
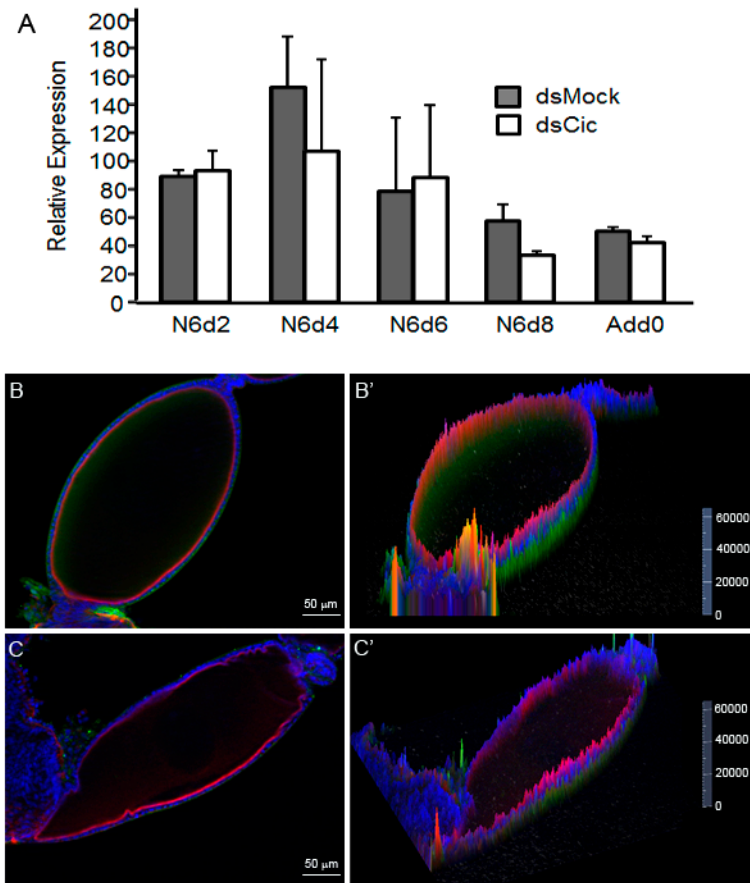
Figure 2.3. BgCic in ovaries of dsBgCic treated females. (A) Expression of BgCic mRNA in ovaries of dsBgCic-treated sixth instar nymphs and adults. Nymphs were treated just after the moult to the last nymphal instar. Data are expressed as copies of BgCic per 1000 copies of Actin-5C. (B, B') Cic protein in basal follicles of 8-day-old dsMock and (C, C') and dsBgCic-treated sixth instar nymph. The signal intensity of the different fluorophores is show (B' and C') using the option 2.5D of the Zen 2012 blue edition software from Zeiss. Anterior pole of the basal follicle is towards the right. Cic: green, F-actins: red, DAPI: blue.

To analyze this phenotype due to the BgCic depletion, a new batch of dsBgCic-treated (n=20) and dsMock-treated (n=20) 0-day-old sixth instar nymphs was prepared to examine the ovarian follicle at different ages, in the last nymphal instar and in the adult. The basal

ovarian follicles from 8-day-old dsBgCic-treated sixth instar nymphs appeared elongated (Figure 2.3C and 4), and the anterior pole of the oocyte became narrow and penetrates into the lumen of the stalk (Figure 2.4A, B). Conversely, the posterior pole showed "fishtail-like" morphology (Figure 2.4C, D). Later, in 3-day-old adults, the basal ovarian follicle of dsBgcic-treated females appeared still more elongated (Figure 2.4E) and the apical pole of the oocyte that penetrates in the lumen of the stalk appeared engulfed by those follicular cells that localize close to the apical pole of the ovarian follicle (Figure 2.4 F, G).

In general, in both poles of the younger oocytes in the vitellarium of dsMock-treated females, it is appeared an accumulation of cytoplasmic F-actins (microfilaments). From these concentration of F-actins is established an extensive network that extends through the ooplasm connecting both poles (Figure 2.4H). This accumulation of cytoplasmic F-actins usually disappears in the basal oocytes. However, in the basal oocyte of 8-day-old dsBgCic-treated sixth instar nymphs the cytoplasmic F-actins is still present and strongly labeled (Figure 2.4D, I, J), and it is maintained and even bigger in the basal ovarian follicle of 3-day-old adults from the dsBgCic-treated group (Figure 2.4K).

In ovaries of 7-day-old adults, when the chorion synthesis begins in dsMock-treated females, all the ovarioles of the dsBgCic-treated specimens lost the basal and the sub-basal oocytes, remaining only the sheaths of follicular epithelium that were covering the oocytes (Figure 2.4L). Moreover, the more distal oocytes which still remain in the vitellarium exhibit an unusual hourglass morphology; possibly due to a generalized enrichment of F-actins in the ooplasm (Figure 2.4M).

e) Depletion of BgCic affects the younger ovarian follicles

Observing in detail all ovarian follicles in the ovariole, of these 8-day-old dsBgCic-treated sixth instar nymphs (Figure 2.5), we observed that the nucleus in the oocytes is not centered, as occurs in controls (Figure 2.5A). Rather, it takes a posterior position, in the basal oocyte (Figure 2.5B) and in the immature oocytes within the ovariole as well (Figure 2.5C, D and E). It appears as if the nucleus in this position were pushing the oocyte, towards the posterior part of the ovarian follicle, and as a consequence more than one oocyte can be encapsulated in one ovarian follicle (Figure 2.5C, D). This is facilitated by the absence of follicular cells in the poles and by the absence of stalk cells separating young ovarian follicles, which determines the fusion of the oocyte membranes, thus giving the aspect of a single oocyte with more than one nucleus (Figure 2.5C, D and E, arrowhead).
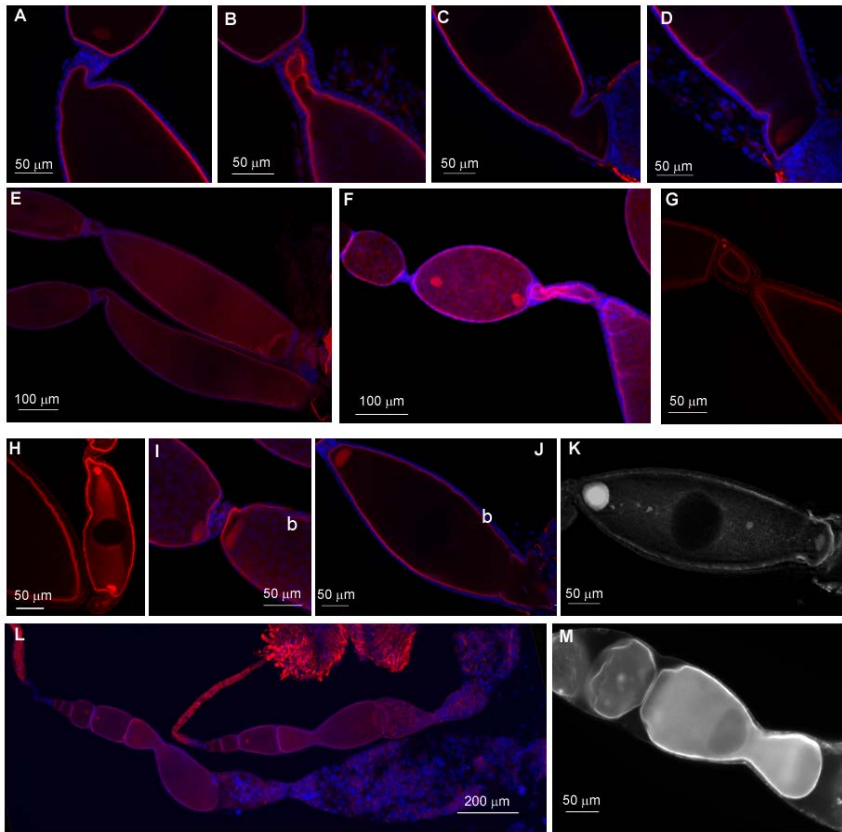
Figure 2.4. Ovarian follicles from dsBgCic-treated last instar nymphs. Basal
ovarian follicles from 8-day-old dsBgCic-treated sixth instar nymphs, showing a
different degree of narrowing in the anterior pole (A, B), and the "fishtail-like"
phenotype, with a high concentration of F-actins in the posterior pole (C, D). (E-
G) Ovarioles from 3-day-old adults from the dsBgCic-treated group, showing the
elongated shape of the basal follicle (E), and the engulfment of the anterior pole
of the basal oocyte (F, G) by the follicular epithelia. (H) Sub-basal ovarian
follicle from 8-day-old sixth instar nymphs showing the network of F-actins
connecting the accumulation of F-actins placed in both poles of the oocyte. (I, J)
Basal oocyte (b) from an 8-day-old sixth instar nymph treated with dsBgCic,
showing the F-actins concentration in the anterior pole. (K) Basal ovarian follicle
from a 3-day-old adult that had been treated with dsBgCic, showing the network
of F-actins, with a big F-actins concentration in the anterior pole of the oocyte,
when, usually at this age the F-actins concentration and the network are not
detected. (L) Ovarioles from a 7-day-old dsBgCic-treated adult, the basal and
sub-basal oocytes were lost (fallen in the oviduct) before they complete their
development, the ovarioles keep the follicular epithelium that was surrounding
these oocytes; in these ovarioles, the first oocyte that remains shows an hourglass
shape (M), and the stalk separating the ovarian follicles is absent. Anterior pole is
towards the left. F-actins: red, DAPI: blue.

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Moreover, the absence of the stalk connecting successive ovarian follicles leads to a general disorganization of the ovariole that especially affects those ovarian follicles that just have been formed and are ready to leave the germarium.

As a consequence of these dysfunctions, in older adult females (7-day-old), it can be observed that the malformed basal follicle has fallen prematurely and is lost into the oviduct, without reaching complete maturation.

f) Depletion of Cic affects other pathways involved in insect oogenesis

The complex phenotype produced by the depletion of BgCic suggests that this protein could interact with other pathways related with cell proliferation and stalk formation (Irles and Piulachs, 2014). Even with the EGFR signaling, as the anterior-posterior polarity in the ovarian follicles is disrupted in EGFR-depleted nymphs, which provokes the disorganization of the ovariole (Elshaer and Piulachs, in press).

The expression of BgEGFR in ovaries from 8-day-old sixth instar nymphs that had been treated with dsBgCic tended to decrease (Figure 2.S1). However, the localization of BgEGFR protein in this treated nymphs appeared modified (Figure 2.5G), as BgEGFR labeling is restricted to the mid region of the basal oocyte and around the nucleus but, in dsMock-treated nymphs it spreads over the cytoplasm and concentrates around and within the nucleus (Figure 2.5F).
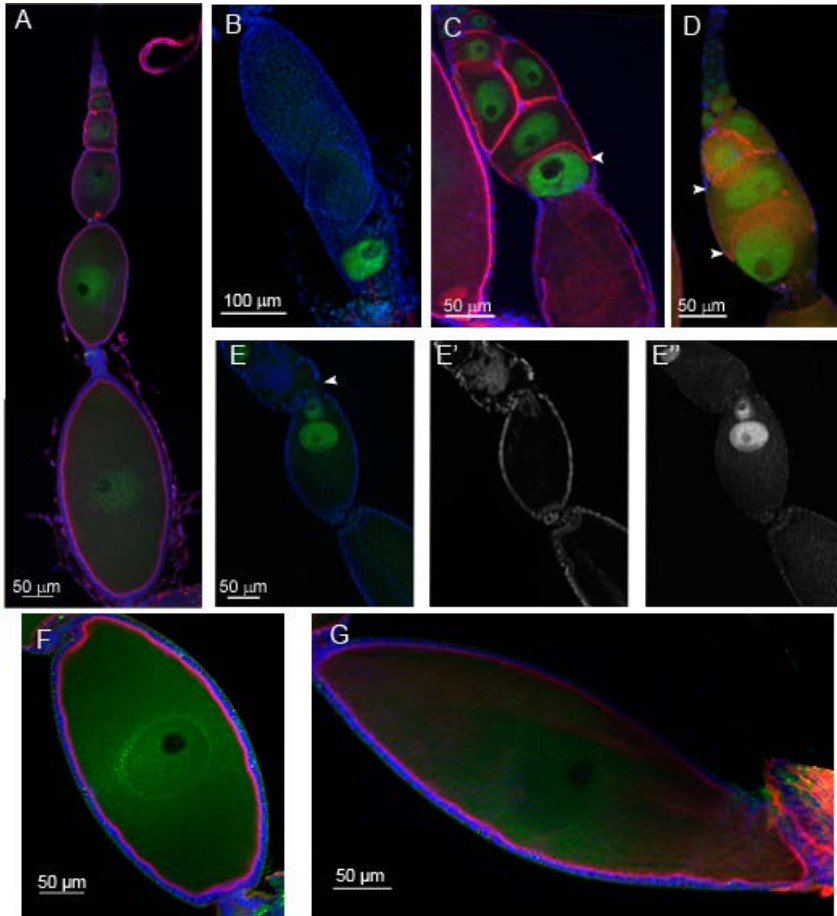
Figure 2.5. Localization of the oocyte nucleus in dsBgCic-treated sixth instar nymphs. (A) Ovariole from 8-day-old sixth instar nymphs that were treated with dsMock, showing the localization of oocyte nucleus in the ovarian follicles along the ovariole; the image results from the composition of three pictures. (B) Basal ovarian follicle from 8-day-old female that had been treated with dsBgCic, showing the posterior position of the oocyte nucleus. (C, D) The loss of the stalks determines the disorganization of the ovarian follicles in the vitellarium which show the oocyte nuclei mis-localized (arrowhead). (E- E'') Two fused oocytes resulting in a single ovarian follicle with two nuclei. (F) Basal ovarian follicle from 8-day-old dsMock-treated sixth instar nymph, showing the EGFR protein diffused through the ooplasm, showing a high labelling around and inside the oocyte nucleus. (G) Basal ovarian follicle of dsBgCic-treated nymph, the EGFR localization appeared restricted to the central region of the oocyte around the nucleus and towards the oocyte ventral side. DAPI: blue, Phaloidin-TRICT: red, and the oocyte nucleus in (A- E) labelled by anti-Eya antibody (green) while in (F, G), the green shows the labelling of EGFR protein. The arrowhead in the images indicates the place where it should be placed the stalk. The anterior pole is up towards the left.

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In relation to proliferation, we measured the expression of Hippo (BgHpo), which significantly down-regulated (0.351 fold change) in BgCic knockdowns (Figure 2.S1), although we did not detect higher levels of proliferation in the follicular epithelium of these specimens. As regards the Hippo pathway, and related to the loss of stalk formation, we analyzed the expression of Notch (BgN) and Delta (BgDl), from Notch pathway (Irles and Piulachs, 2014) (Irles, Elshaer and Piulachs submitted). As expected, the expression of BgN has a tendency to decrease, while its ligand BgDl is significantly up-regulated (1.161 fold change) in dsBgCic-treated last instar nymphs (Figure 2.S1).

Finally, the modifications observed in the anterior and posterior poles in ovarian follicles from dsBgCic-treated sixth instar nymphs, suggested us looking for genes that contribute to drive the terminal position in *D. melanogaster* egg chambers and in embryos. Therefore, we examined the response of Oskar (BgOsk), Staufen (BgStau), Bruno (BgBru) and Tailless (Bgtll) (Figure 2.S1). Results show that in dsBgCic knockdowns, the expression of BgOsk tends to decrease, whereas BgStau, BgBru and Bgtll mRNA levels are significantly down-regulated (0.712, 0.491 and 0.355 of fold change, respectively). We additionally measured the expression of the transcription factor Dorsal (BgDL), which is required for the determination of DV polarity, observing that it decreased significantly (0.408 fold change) (Figure 2.S1).

2.5. Discussion

Cic sequence is highly conserved among animals, from cnidarians to vertebrates (Lee et al., 2002; Lam et al., 2006) and in most insects, including *B. germanica,* it is expressed as a single isoform, while in *D. melanogaster* and in mammals at least two isoforms are expressed (Jimenez et al., 2012). We have shown that in *B. germanica* Cic is particularly well expressed in the brain and in the ovaries. The high levels of BgCic expression in *B. germanica* brain suggest that BgCic might have an important role in the development of the central nervous system (CNS), a function described for Cic in vertebrates, which show a high expression in immature granule cells in the cerebellum, in the hippocampus and in the olfactory bulb (Lee et al., 2002), while mutations in the Cic gene that determine an absence of the protein are detected in oligodendroglial tumors (Alentorn et al., 2012; Chan et al., 2014). In the present study; however, we have focused on the expression and function of Cic in the ovaries. Indeed, the role of Cic in insects has been thoroughly documented in *D. melanogaster* in relation to oogenesis and to the specification of terminal regions in the embryo (Jimenez et al., 2000; Goff et al., 2001; Atkey et al., 2006), as the loss of Cic function results in a dorsalization of the eggshell and the embryo (Goff et al., 2001) and affects the anterior-posterior embryonic axis (Andreu et al., 2012).

In the panoistic ovary of *B. germanica*, BgCic protein accumulates in the basal oocyte of sixth instar nymphs as they progress in the instar. However, after the imaginal moult, labeling for this protein in the basal oocyte becomes very weakly. This confirms that in the

panoistic ovary, the most important steps for oogenesis and further oocyte maturation occur during the last nymphal instar, thus when the female reach the adult stage, the basal follicles are ready to incorporate storage proteins into the oocyte and to complete growth (Ciudad et al., 2006; Tanaka and Piulachs, 2012; Herraiz et al., 2014; Irles and Piulachs, 2014). Our results show that the function of BgCic is essential during oogenesis, and it takes place before the ovarian follicle reach the maturity, and its depletion results in female sterility. Equivalent observations have been reported for *D. melanogaster* (Jimenez et al., 2000), where Cic has a fundamental function in oocyte development and its depletion determines female sterility as well. However, the mechanisms underlying these phenotypes are different in both species and in both ovaries types.

Females of *D. melanogaster* mutants for Cic are able to oviposit, but the eggs have defects in the eggshell and the embryos start developing although are not able to complete it and hatch. Conversely, *B. germanica* Cic-depleted females do not oviposit correctly as the oocyte is pushed to the oviduct before to complete its development, and these females never formed an ootheca.

In the panoistic ovaries of *B. germanica*, BgCic is required to maintain the anterior-posterior axis, as a depletion of BgCic triggers morphological modifications in both poles of the basal ovarian follicles as a result of important changes in the distribution of F-actins microfilaments, in both the oocyte and in the follicular cells. Actins are the main component of the cytoskeleton and are crucial in different cellular processes, like cell motility and shaping (Riparbelli and Callaini, 1995; Sun and Schatten, 2006). Related to

the change in the F-actins distribution in the dsBgCic-treated females, cytoplasmic F-actins is maintained in each pole of the basal oocyte in the transition from nymph to adult, and a network of F-actins can be detected connecting both poles. Usually the cytoplasmic F-actins disappeared from the basal oocytes in the last days of the sixth nymphal instar, and they are never detected in the basal oocytes from an adult female. Indeed, the microtubule organizing center (MTOC) is a significant organizer of the microtubule network, which is important for the distribution of mRNAs and proteins which is necessary in the *D. melanogaster* oocyte growth (Cooley and Theurkauf, 1994; Roth and Lynch, 2012), the actin cytoskeleton also would provide the oocyte growth and differentiation (Riparbelli and Callaini, 1995) and polarity establishment (Sun and Schatten, 2006), but the information about the role of the MTOC or the microfilaments in the distribution of proteins or mRNAs in a panoistic ovary is practically none. The maintenance of this network of F-actins in dsBgCic-treated females probably affects the distribution of BgEGFR protein that appears modified in basal oocytes, as the protein appears restricted to the middle region of the oocyte, and is not spread by all the cytoplasm as happens in control basal oocytes.

The results obtained by the depletion of BgCic affecting the stalk formation, the polarity of the oocyte or cell proliferation, suggested that BgCic might be interacting with different pathways contributing to oocyte development. For example, it has been reported that Notch pathway is involved in the formation of the stalk (Irles and Piulachs, 2014), and in *D. melanogaster*, EGFR

appears to indirectly promote cell proliferation by inhibiting Cic and thus de-repressing genes acting on cell proliferation, like Hippo (Herranz et al., 2012). In contrast, depletion of BgCic in *B. germanica* results in BgHpo down-regulation although the follicular epithelium continues proliferating, suggesting that the level of BgN in the ovarian follicle is sufficient for keeping the proliferation in the adequate level as it is described in *B. germanica* (Irles and Piulachs, 2014) which in the absence of BgHpo, the follicular epithelium continues proliferating because of the BgN is maintained in the follicular cells.

Moreover, the depletion of BgCic seems to modify the expression of those genes that in *D. melanogaster* are required to establish the anterior-posterior polarity or the DV axis (like oskar, tailless, staufen, bruno and dorsal) (Roth and Lynch, 2009; Lynch and Roth, 2011), as they were down-regulated. It will be necessary to study the function of all these genes in a panoistic ovary, were the polarity of the oocyte differs greatly of that of a meroistic oocyte, and to unveil how its function has been modified through the evolution.

The main function described for Cic was to be a transcriptional repressor, in *D. melanogaster* and other species (Jimenez et al., 2000). However, our results suggest that Cic in the panoistic ovary, the ancestral ovary type, might act as inducer of the transcriptional activity. To unveil how was produced the transition from a transcriptional inductor to a transcriptional repressor, will be necessary to study the Cic function in different insect models and in relation with the other pathways that controls oocyte organization and maturation.

2.6. Acknowledgments

2.7. References

Alentorn, A., Sanson, M., Idbaih, A., 2012. Oligodendrogliomas: new insights from the genetics and perspectives. Current opinion in oncology 24, 687-693.

Andreu, M.J., Gonzalez-Perez, E., Ajuria, L., Samper, N., Gonzalez-Crespo, S., Campuzano, S., Jimenez, G., 2012. Mirror represses pipe expression in follicle cells to initiate dorsoventral axis formation in *Drosophila*. Development 139, 1110-1114.

Atkey, M.R., Lachance, J.F., Walczak, M., Rebello, T., Nilson, L.A., 2006. Capicua regulates follicle cell fate in the *Drosophila* ovary through repression of mirror. Development 133, 2115-2123.

Belles, X., Casas, J., Messeguer, A., Piulachs, M.D., 1987. In vitro biosynthesis of JH III by the corpora allata of adult females of *Blattella germanica* (L). Insect Biochemistry 17, 1007-1010.

Büning, J., 1994. The Insect Ovary. Chapman & Hall, London, UK.

Castresana, J., 2000. Selection of conserved blocks from multiple alignments for their use in phylogenetic analysis. Molecular biology and evolution 17, 540-552.

Ciudad, L., Piulachs, M.D., Belles, X., 2006. Systemic RNAi of the cockroach vitellogenin receptor results in a phenotype similar to that of the *Drosophila* yolkless mutant. The FEBS journal 273, 325-335.

Cooley, L., Theurkauf, W.E., 1994. Cytoskeletal functions during *Drosophila* oogenesis. Science 266, 590-596.

Chan, A.K., Pang, J.C., Chung, N.Y., Li, K.K., Poon, W.S., Chan, D.T., Shi, Z., Chen, L., Zhou, L., Ng, H.K., 2014. Loss of CIC and FUBP1 expressions are potential markers of shorter time to recurrence in oligodendroglial tumors. Modern pathology : an official journal of the United States and Canadian Academy of Pathology, Inc 27, 332-342.

Elshaer, N., Piulachs, M.D., 2015. Crosstalk of EGFR signaling with Notch and Hippo pathways to regulate cell specification, migration and proliferation in cockroach panoistic ovaries. Biology of the Cell, in press.

Fonseca, R.N., Lynch, J.A., Roth, S., 2009. Evolution of axis formation: mRNA localization, regulatory circuits and posterior specification in non-model arthropods. Current opinion in genetics & development 19, 404-411.

Goff, D.J., Nilson, L.A., Morisato, D., 2001. Establishment of dorsal-ventral polarity of the *Drosophila* egg requires capicua action in ovarian follicle cells. Development 128, 4553-4562.

Guindon, S., Gascuel, O., 2003. A simple, fast, and accurate algorithm to estimate large phylogenies by maximum likelihood. Syst Biol 52, 696-704.

Herraiz, A., Belles, X., Piulachs, M.D., 2014. Chorion formation in panoistic ovaries requires windei and trimethylation of histone 3 lysine 9. Experimental cell research 320, 46-53.

Herranz, H., Hong, X., Cohen, S.M., 2012. Mutual repression by bantam miRNA and Capicua links the EGFR/MAPK and Hippo pathways in growth control. Current biology : CB 22, 651-657.

Irles, P., Belles, X., Piulachs, M.D., 2009. Identifying genes related to choriogenesis in insect panoistic ovaries by Suppression Subtractive Hybridization. BMC genomics 10, 206.

Irles, P., Piulachs, M.D., 2014. Unlike in *Drosophila* Meroistic Ovaries, Hippo Represses Notch in *Blattella germanica* Panoistic Ovaries, Triggering the Mitosis-Endocycle Switch in the Follicular Cells. PloS one 9, e113850.

Jimenez, G., Guichet, A., Ephrussi, A., Casanova, J., 2000. Relief of gene repression by torso RTK signaling: role of capicua in *Drosophila* terminal and dorsoventral patterning. Genes & development 14, 224-231.

Jimenez, G., Shvartsman, S.Y., Paroush, Z., 2012. The Capicua repressor--a general sensor of RTK signaling in development and disease. Journal of cell science 125, 1383-1391.

Lam, Y.C., Bowman, A.B., Jafar-Nejad, P., Lim, J., Richman, R., Fryer, J.D., Hyun, E.D., Duvick, L.A., Orr, H.T., Botas, J., Zoghbi, H.Y., 2006. ATAXIN-1 interacts with the repressor Capicua in its native complex to cause SCA1 neuropathology. Cell 127, 1335-1347.

Larkin, M.A., Blackshields, G., Brown, N.P., Chenna, R., McGettigan, P.A., McWilliam, H., Valentin, F., Wallace, I.M., Wilm, A., Lopez, R., Thompson, J.D., Gibson, T.J., Higgins, D.G., 2007. Clustal W and Clustal X version 2.0. Bioinformatics 23, 2947-2948.

Lee, C.J., Chan, W.I., Cheung, M., Cheng, Y.C., Appleby, V.J., Orme, A.T., Scotting, P.J., 2002. CIC, a member of a novel subfamily of the HMG-box superfamily, is transiently expressed in developing granule neurons. Brain research. Molecular brain research 106, 151-156.

Lee, Y., Fryer, J.D., Kang, H., Crespo-Barreto, J., Bowman, A.B., Gao, Y., Kahle, J.J., Hong, J.S., Kheradmand, F., Orr, H.T., Finegold, M.J., Zoghbi, H.Y., 2011. ATXN1 protein family and CIC regulate extracellular matrix remodeling and lung alveolarization. Developmental cell 21, 746-757.

Lynch, J.A., Roth, S., 2011. The evolution of dorsal-ventral patterning mechanisms in insects. Genes & development 25, 107-118.

Medioni, C., Mowry, K., Besse, F., 2012. Principles and roles of mRNA localization in animal development. Development 139, 3263-3276.

Pfaffl, M.W., Horgan, G.W., Dempfle, L., 2002. Relative expression software tool (REST) for group-wise comparison and statistical analysis of relative expression results in real-time PCR. Nucleic acids research 30, e36.

Riparbelli, M.G., Callaini, G., 1995. Cytoskeleton of the *Drosophila* egg chamber: new observations on microfilament distribution during oocyte growth. Cell motility and the cytoskeleton 31, 298-306.

Roth, S., Lynch, J., 2012. Axis formation: microtubules push in the right direction. Current biology : CB 22, R537-539.

Roth, S., Lynch, J.A., 2009. Symmetry breaking during *Drosophila* oogenesis. Cold Spring Harbor perspectives in biology 1, a001891.

Shin, D.H., Hong, J.W., 2014. Capicua is involved in Dorsal-mediated repression of zerknullt expression in *Drosophila* embryo. BMB reports 47, 518-523.

Sun, Q.Y., Schatten, H., 2006. Regulation of dynamic events by microfilaments during oocyte maturation and fertilization. Reproduction 131, 193-205.

Tanaka, A., 1973. General account on the oocyte growth and the idintification of vitellogenin by means of immunospecificity in the cockroach, *Blattella germanica* (L.). Development, Growth and Differentiation 15, 153-168.

Tanaka, E.D., Piulachs, M.D., 2012. Dicer-1 is a key enzyme in the regulation of oogenesis in panoistic ovaries. Biology of the cell / under the auspices of the European Cell Biology Organization 104, 452-461.

Telfer, W.H., 1975. Development and Physiology of the Oöcyte-Nurse Cell Syncytium. Advances in Insect Physiology, 223- 320.

Wilson, M.J., Abbott, H., Dearden, P.K., 2011. The evolution of oocyte patterning in insects: multiple cell-signaling pathways are active during honeybee oogenesis and are likely to play a role in axis patterning. Evolution & development 13, 127-137.

Wilson, M.J., Dearden, P.K., 2013. RNA localization in the honeybee (*Apis mellifera*) oocyte reveals insights about the evolution of RNA localization mechanisms. Developmental biology 375, 193-201.

Zhang, Y., Kunkel, J.G., 1992. Program of F.actin in the follicular epithelium during oogenesis of the german cockroach, *Blattella germanica*. Tissue and Cell 24, 905-917.

2.8. Supplementary material
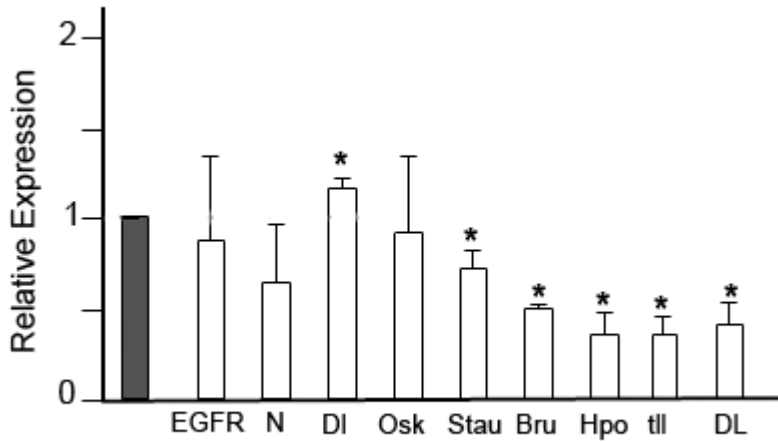
Figure 2.S1: Genes expression in 8-day-old sixth nymph instar treated with dsBgCic. mRNA levels of EGFR, Notch, Delta, Oskar, Staufen, Bruno, Hippo, Tailless and Dorsal in ovaries of 8-day-old dsMock and dsBgCic-treated instar nymphs. All of the studied genes are down-regulated but only Delta (the ligand of Notch) is significantly increased. qRT-PCR data represent three biological replicates and are normalized against control ovaries (reference value=1); expression of actin-5c was used as a reference. (*) indicates statistically significant change.

Supplementary Table 2.S1: **Primer sequence used for qRT-PCR and RNAi experiments.** The accession numbers of studied sequences are indicated. F: Primer forward. R: Primer reverse. BgActin-5c was used as housekeeping gene in the expression studies.

	Accession number	Primer name		Primer sequence
1	LN623700	BgCic-RT	F R	5' AACCCGCAAGGTTGTCAGT 3' 5' ACTCTGCTGCTCAAGCACAA 3'
2	LN623700	BgCic-dsRNA1	F R	5' ACTTGAGAGGAGAACCAGA 3' 5' ATGGACAGGGTTCTCGAAAC 3'
3	LN623700	BgCic-dsRNA2	F R	5' CCAGCAAGGTTAACATCTTGCAA 3' 5' CAACTCGACCGAAGTCAACAG 3'
4	LN623701	BgEGFR-RT	F R	5' GAGTACAAAGCAGCAGGAG 3' 5' CCAACATCGGATATTCACTCAC 3'
5	LN623703	BgDL-RT	F R	5' GGTTTCTCTCATCGCAGTCA 3' 5' CAGTGGTGTCTGAACCCATC 3'
6	LN623702	BgTll	F R	5' GACAGCGTCAGTACGTTTGC 3' 5' ATGAACAAGGATGCGGTACA 3'
7		BgOsk	F R	5' AGATTCAATCAAGATGTCTGC 3' 5' GCAGACATCTTGATTGAATCT 3'
8		BgBru	F R	5' GGTCATTAACGGGCCAGAC 3' 5' ACAATGGCACTGGGCTTG 3'
9		BgStau	F R	5' ACGAAATGTTGCATCACCAA 3' 5' TTTGGTGTCTATGCCAACCA 3'
10	HF969255	BgN-RT	F R	5' GCTAAGAGGCTGTTGGATGC 3' 5' TGCCAGTGTTGTCCTGAGAG 3'
11	HF969256	BgDl-RT	F R	5' CCACTACAAGTGTTCGCCAA 3' 5' TACCTCTCGCATTCGTCACA 3'
12	HF969251	BgHpo-RT	F R	5' GACATTTGGAGCCTTGGCAT 3' 5' AGGTTTCCCTTCAGCCATTTC 3'
13	AJ862721	BgActin-5c	F R	5' AGCTTCCTGATGGTCAGGTGA 3' 5' ACCATGTACCCTGGAATTGCCGACA 3'

3. CROSSTALK OF EGFR SIGNALING WITH NOTCH AND HIPPO PATHWAYS TO REGULATE CELL SPECIFICATION, MIGRATION AND PROLIFERATION IN COCKROACH PANOISTIC OVARIES

Crosstalk of EGFR signaling with Notch and Hippo pathways to regulate cell specification, migration and proliferation in cockroach panoistic ovaries

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Elshaer N, Piulachs MD. Crosstalk of EGFR signalling with Notch and Hippo pathways to regulate cell specification, migration and proliferation in cockroach panoistic ovaries. Biol Cell. 2015 Aug;107(8):273-85. doi: 10.1111/boc.201500003

4. THE NOTCH PATHWAY KEEPS THE FOLLICULAR CELLS IN A PROLIFERATIVE AND ANTI-APOPTOTIC STATE

The Notch Pathway keeps the follicular cells in a proliferative and anti-apoptotic state

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Irles P, Elshaer N, Piulachs MD. The Notch pathway regulates both the proliferation and differentiation of follicular cells in the panoistic ovary of Blattella germanica. Open Biol. 2016 Jan;6(1):150197. doi: 10.1098/rsob.150197.

5. THE EXPRESSION OF PIPE IN *BLATTELLA GERMANICA* OVARIES IS ESSENTIAL FOR EMBRYO DEVELOPMENT.

The expression of Pipe in *Blattella germanica* ovaries is essential for embryo development.

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5.1. Abstract

The Pipe gene express for a heparan sulfate 2-O-sulfotransferase, that has been well studied in the meroistic ovaries of *Drosophila melanogaster*, where it plays a pivotal role in dorsal-ventral polarity of the embryo activating the protease cascade triggering Toll pathway, and restricting the area of action. The depletion of Pipe in *Blattella germanica* adult ovaries, a species with panoistic ovaries, causes defects in the dorsal-ventral axis of the embryos affecting their development.

While the effect of BgPipe depletion on the embryos only happens by dsRNA injection in early adult stage not in nymphal stage or later of adult stage, this specifies the maternal effect of BgPipe.

5.2. Introduction

In many invertebrates and some vertebrates, the establishment of axes polarity in the embryo depends on prior establishment of polarity in the egg chamber which are determined during oogenesis by the distribution of maternal mRNAs and proteins. The formation of dorsoventral (DV) axis in *D. melanogaster* requires a localized activation of a serine protease cascade, formed by Gastrulation Defective, Snake, and Easter, in the perivitelline space surrounding the developing embryo (Morisato and Anderson, 1995; LeMosy et

al., 2001; Moussian and Roth, 2005). This activation of protease cascade causes ventral to dorsal gradient of Toll receptor activation in the embryonic membrane, which regulates the DV patterning of the embryo.

The ventral restriction activation of the protease cascade relies on cues in the vitelline membrane which is the first chorion layer secreted by the follicular cells. It has been reported that Pipe, that encodes for a sulfotransferase modifies some structural components of the vitelline membrane becoming the responsible of this restriction (Sen et al., 1998; Moussian and Roth, 2005; Zhang et al., 2009a). In *D. melanogaster* Pipe encodes for ten different isoforms (Sen et al., 1998; Sergeev et al., 2001) and only one among them, called Pipe-ST2, has been shown to be crucial for the embryonic DV axis polarity (Zhang et al., 2009b). The expression of this isoform in the ovary is restricted to the cells in ventral side of the follicular epithelium, where is required to determine the DV axis in the future embryo (Nilson and Schupbach, 1998; Sen et al., 1998; James et al., 2002; Peri et al., 2002). After fertilization and oviposition the action of Pipe sulfating some proteins in the ventral side of the vitelline membrane, initiate a localized proteolytic cascade, and thus to the initiation of embryonic DV axis formation (Dissing et al., 2001; LeMosy et al., 2001; Moussian and Roth, 2005; Cho et al., 2010). Therefore, Pipe provides a link between the ovarian and embryonic DV polarity, as Pipe mutant females produced dorsalized embryos (Sen et al., 1998).

We report the function of Pipe in hemimetabolan insect, the cockroach *Blattella germanica*, which has panoistic ovaries and an

embryo that is of short germ band type. The depletion of BgPipe expression in newly adult females leads to embryos with dorsal-ventral defects. While the depletion of Pipe, in sixth nymphal instar females or at the end of adult stage leads to oviposit normal embryos. These findings indicate that BgPipe has a maternal effect and play a pivotal role in the process that defines the embryonic dorsal-ventral axis.

5.3. Material and Methods

a) Cockroach colony and tissue sampling

Freshly ecdysed sixth instar nymphs or adult females of *B. germanica* were obtained from a colony fed on Panlab dog chow and water ad libitum, and reared in the dark at 29±1°C and 60–70% r.h. (Belles et al., 1987). In adult females the length of the basal oocyte was used to stage the age of the female. After the moult to adult, females were maintained with males during all the first gonadotrophic cycle, and mating was confirmed at the end of the experiments by assessing the presence of spermatozoa in the spermatheca. All dissections and tissue sampling were carried out on carbon dioxide-anaesthetized specimens. After the dissection, the tissues were frozen in liquid nitrogen and stored at –80°C until use.

b) RNA extraction and retrotranscription to cDNA

All RNA extractions were performed using the GenElute Mammalian Total RNA kit (Sigma, Madrid, Spain). RNA quantity and quality were estimated by spectrophotometric absorption at 260 nm/280 nm in a Nanodrop Spectrophotometer ND-1000® (Nano Drop Technologies, Wilmington, DE, USA). A sample of 400ng of total RNA from each extraction was DNase treated (Promega, Madison, WI, USA) and reverse transcribed with Transcriptor First Strand cDNA Synthesis Kit (Roche, Sant Cugat del Valles, Barcelona, Spain). In all cases we followed the manufacturer's protocols.

c) Expression studies

The expression of *B. germanica* Pipe (BgPipe) (Accession number LN623705) was determined by quantitative real-time PCR (qRT-PCR) in the ovary during the last nymphal instar and in the adult during the first gonadotrophic cycle. PCR primers used in qRT-PCR expression studies were designed using the Primer Express 2.0 software (Applied Biosystems, Foster City, CA, USA), and are indicated in Table 5.1. qRT-PCR reactions were carried in an iQ5 Real-Time PCR Detection System (Bio-Rad Laboratories, Madrid, Spain), using IQ™ SYBR Green Supermix (BioRad). The Actin-5c gene of *B. germanica* (Accession number AJ862721) was used as a reference. The efficiency of primers was first validated by constructing a standard curve through four serial dilutions of cDNA from ovaries. At least three independent qRT-PCR experiments

(biological replicates) were performed, and each measurement was done in triplicate (technical replicates). qRT-PCR reactions were performed and analyzed as previously described (Irles et al., 2009b). Fold change expression was calculated using the REST-2008 program (Relative Expression Software Tool V 2.0.7; Corbett Research) (Pfaffl et al., 2002).

Table 5.1: **Primer sequence used for RNAi experiments, qRT-PCR and In Situ-Hybridization experiments.** F: Primer forward. R: Primer reverse. BgActin-5c was used as housekeeping gene in the expression studies

Primer name	Primer sequence	Amplicon length (bp)
BgPipe-dsRNA	F: 5' TGAGGTCGTCCAGTGTT 3'	417
	R: 5' GGAAGCCAAACGTTTATGGA 3'	
BgPipe-RT	F: 5' GCAGCGTTAATTGTTGAGTCC 3'	155
	R: 5' CTCAGACACACAGTAAACCAACA 3'	
BgPipe-in Situ H.	F: 5' TCCCTTGCCAGATCCACATT 3'	265
	R: 5' TCTACCCTTACTGTACTTGAGCA 3'	
BgActin5c	F: 5' AGCTTCCTGATGGTCAGGTGA 3'	213
	R:5'ACCATGTACCCTGGAATTGCCGACA3'	

d) Pipe depletion experiments

To deplete BgPipe, a dsRNA (dsBgPipe) were prepared encompassing a 417 bp. The fragment was amplified by PCR and cloned into pSTBlue-1 vector. As dsRNA control (dsMock), a fragment (300 bp) of the sequences of *Autographa californica nucleopolyhedrovirus* (GenBank: K01149) was used. Preparation of the dsRNA was performed as previously described (Ciudad et al., 2006). Freshly emerged last (sixth) instar nymphs, freshly emerged

adults and 6-day-old adult females were treated with 1μg of dsBgPipe injected in the abdomen in a 1μL of water-DEPC. Control specimens were treated similarly with 1μg of dsMock, or injected with 1μL of DEPC water to assess the absence of possible unspecific effects from dsMock injection.

e) Whole-Mount In Situ Hybridization

Sense and anti-sense RNA probes of Pipe transcripts in the ovary labelled with digoxigenin (DIG) were generated by transcription in vitro using SP6 or T7 RNA polymerases (Fermentas) and DIG RNA labelling mix (Roche), representing a 265 bp fragment.

The ovaries for whole mount procedures were dissected in PBS 0.2 M, pH 6.8; ovaries fixation and subsequent hybridization were carried out as previously reported (Irles et al., 2009b). For alkaline phosphatase (AP) detection reaction, the ovaries were incubated with reabsorbed anti-DIG antibody conjugated with AP in blocking buffer. For Fluorescence, in situ hybridization RNA probes (sense and antisense) were detected with Alexa Fluor® 647, following the manufacturer's protocol (FISH Tag^{TM} RNA Kit, Molecular Probes).

f) Scanning electron microscopy (SEM)

Selected ovarioles from 7-day-old dsBgPipe-treated and dsMock-treated females were processed to observe the structure of the chorion layers in the basal ovarian follicles. Females were dissected late on 7-day-old adult in order to assess that the chorion was

completely formed in controls. Samples were prepared as previously described (Irles and Piulachs, 2011). After fixation with 2.5% glutaraldehyde in cacodylate buffer 0.2 M, ovarian follicles were gently ripped with a micro-forceps in order to expose the chorion layers. Samples were observed with a Hitachi S-3500N scanning electron microscope at 5 kV (Hitachi High-Technologies Corporation, Tokyo, Japan).

5.4. Result

a) BgPipe and its expression in ovaries

The complete sequence of BgPipe has 2444 bp with an ORF encoding for a heparan sulfate 2-O-sulfotransferase. Pipe has 395 amino acids, with an estimated molecular mass of 44.98 kDa and an isoelectric point of 8.8, and no isoforms were detected by BLAST analysis against the *B. germanica* genome.

The expression of BgPipe in ovaries was measured in sixth instar nymphs and in adult females during the first gonadotrophic cycle (Figure 5.1). The expression of BgPipe mRNA in ovaries was at very low levels, although in old sixth instar nymphs, just before the imaginal moult, the expression of Pipe increased. After the moult to adult, the expression levels decrease and the low levels were maintained during most of the cycle, as unexpectedly the last day of the gonadotrophic cycle, before is done the chorion synthesis, the expression BgPipe is highly increased, around a 10-fold (Figure 5.1).
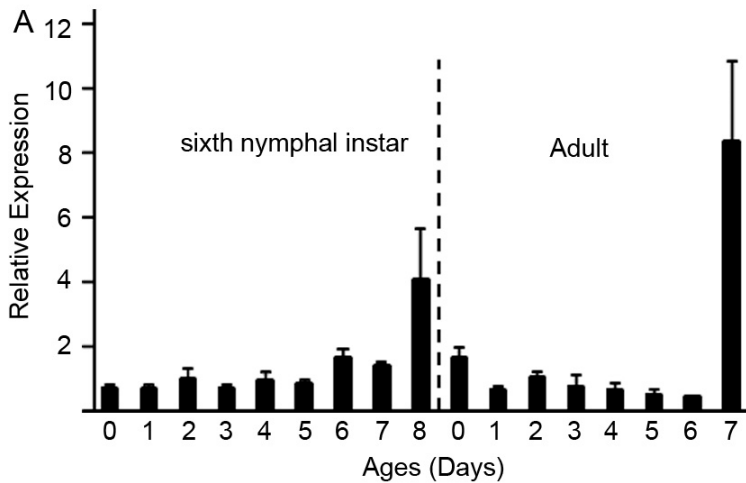
Figure 5.1: Expression of BgPipe in ovaries of *B. germanica*. The expression of BgPipe was measured by qRT-PCR in ovaries of *B. germanica* during the sixth nymphal instar and the first gonadotrophic cycle of adults. Data represent copies of BgPipe mRNA per 1,000 copies of actin-5c mRNA, and are expressed as the mean ± SEM (n = 3 in sixth instar nymph, n = 2 in adults). The dashed line indicates the imaginal moult to adult.

b) Depletion of BgPipe affects embryo viability

The function of BgPipe in the ovary was studied using the RNA interference. Therefore, we prepared a dsRNA that was injected at a dose of 1 µg in females of *B. germanica* following 2 different strategies. In the first one (Figure 5.2A), dsBgPipe was injected to 0-day-old sixth instar nymph females (n= 28), and in parallel a non-coding sequence (dsMock) was injected at the same dose (n= 25). The expression of BgPipe was measured in 8-day-old sixth instar nymphs (Figure 5.2B), showing that BgPipe mRNA levels were depleted a 60%, but no phenotype was observed in the ovaries at this age. All the treated nymphs moulted to adult at the same time than dsMock-treated nymphs, with no problems in the moult. These

adult females were maintained with males, and both dsMock-treated and dsBgPipe-treated females oviposited and formed the ootheca correctly, and the offspring hatched without any delay and any defects.
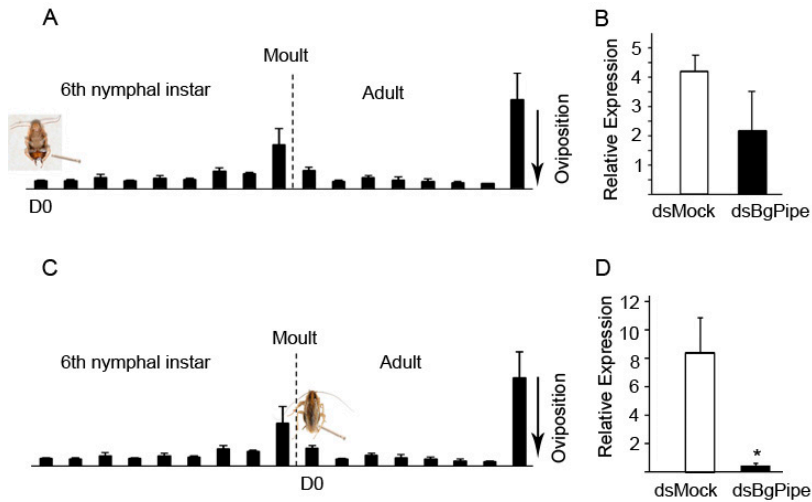
Figure 5.2: BgPipe in ovaries of *B. germanica*. (A) Strategy followed injecting dsBgPipe in 0-day old sixth instar nymphs, and measuring the expression in ovaries from 8-day-old sixth instar nymphs. (B) Expression of BgPipe after the treatment showed in A. (C) Strategy followed injecting dsBgPipe in 0-day old adult females, and measuring the expression in ovaries from 7-day-old adults. (D) Expression of BgPipe after the treatment showed in C. Data represent copies of BgPipe mRNA per 1,000 copies of actin-5c mRNA, and are expressed as the mean ± SEM (n = 3).

The second strategy (Figure 5.2C) was to inject dsBgPipe (n= 27) and dsMock (n= 28) into freshly emerged adult females, and observe if these females were fertile. We measured the levels BgPipe mRNA in ovaries of 7-day-old adults (Figure 5.2D), and we confirm that BgPipe expression was reduced a 95% in dsBgPipe-treated females respect to dsMock-treated females. All the dsBgPipe-treated and dsMock-treated females oviposited and

formed the ootheca normally, eight days after the moult to adult. However, no nymph hatches from the oothecae of dsBgPipe-treated females, while in dsMock-treated females the embryogenesis takes 18 days and after this period the nymphs hatched. The dsBgPipe-treated females maintain the ootheca attached to the genital pouch more than 20 days and at the end the ootheca drops. These dsBgPipe-treated females start immediately a new gonadotrophic cycle that was completed normally, and after oviposition they form a second ootheca where the embryos developed correctly as the nymphs hatched normally.

c) BgPipe is necessary in *B. germanica* embryogenesis

To study the function of BgPipe in the embryogenesis of *B. germanica*, we follow the second strategy described before, preparing a new batch of experiments and observing the embryos in different periods. Three-day-old oothecae from dsMock-treated (n= 5 oothecae) and dsBgPipe-treated females (n= 6 oothecae) were dissected. At this age, in the embryos from dsMock-treated females (Figure 5.3A) the segmentation is being established, the caudal end starts to fold ventrally and it is possible to distinguish the head and the primordia of the legs. However, in dsBgPipe-treated females a 40% of the eggs do not form the germ band (Figure 5.3B, arrow) although the cleaved energids are spread through the egg, indicating that the zygote was formed. The rest of the eggs (60%) showed different degrees of defects in the germ band (Figure 5.3C- G). Some eggs only have an amorphous mass of distinct tissue which in

the ventral part of the egg (Figure 5.3C), while other show a different degrees of segmentation in the germ band.

Figure 5.3: *B. germanica* **embryos from dsBgPipe-treated females.** (A) 3-day-old embryo from dsMock-treated female, the arrowhead indicates the position of the head. (B-G) 3-day-old embryo from dsBgPipe-treated females. (B) The germ band is absent, although the energids are clearly visible. The arrow indicates where should be the germ band. (C) In the place of the germ band appears an amorphous mass of distinct tissue without traces of segmentation. (D, E) Germ bands with bad segmentation, with the head and the abdomen not well differentiate. The thorax shows a tightly segmentation. (F) Germ band that appeared splitted in three parts (arrows). (G) Germ band bad segmented, the head, thorax and abdomen are differentiated, but they are poorly developed. In A and G the ventral side is towards the top, in B-F is towards the right. DAPI was used as staining. Scale bar: 200 µm.

It is possible to observe germ bands with the abdomen not segmented but the thorax partially segmented and with the head and the tail poorly developed (Figure 5.3D, E), or the germ band appeared divided in separated parts (Figure 5.3F, arrowheads), or the germ band although having the correct size shows an incomplete segmentation and poorly developed (Figure 5.3G).

We wondered whether the phenotypes observed might simply be the consequence of a slower development or if the development of the embryos was arrested at this age. Therefore, we carried out a new batch of experiments dissecting the oothecae when the embryos were 14-day-old (n= 4 oothecae per each injection dsMock and dsBgPipe-treated females). At this age, in the embryos from dsMock-treated females (Figure 5.4A) the eyes are completely formed and their color is becoming more intense. The tips of antennae and the hind legs reach the fifth abdominal segment, the dorsal closure of the body wall is complete and the mycetocytes that have been developed in the abdominal fat body are visible through the cuticle (Tanaka, 1976; Piulachs et al., 2010). Whereas in the oothecae from dsBgPipe-treated females, a 65% of the embryos have arrested their development in early steps of the embryogenesis, in agreement with the results observed in 3-day-old embryos. The rest of the embryos that have advanced in their development (35%) showed a big range of phenotypic defects, affecting both axes anterior-posterior and dorso-ventral. All the embryos had short limbs (legs and antennae) which reach only to the first abdominal segment (Figure 5.4B, C), the caudal end appeared curved towards the dorsal side (Figure 5.4B, D), and although was possible to

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distinguish the abdominal segments they were not completely differentiated. In these embryos the pleuropodia was still present when usually it starts to shrink in 13-day-old embryos (Figure 5.4C, arrow).
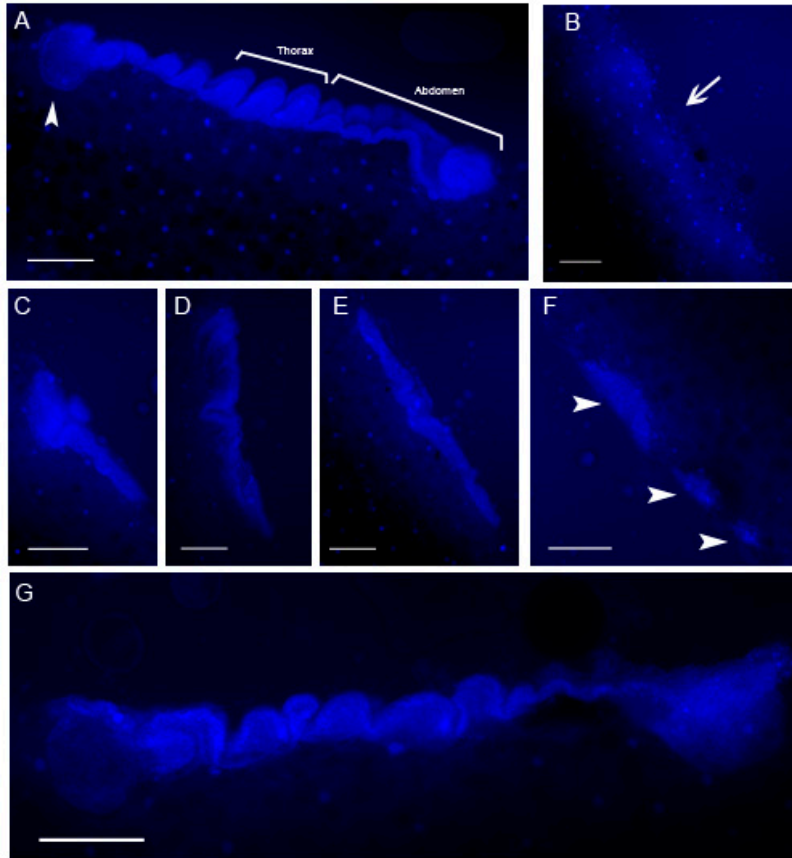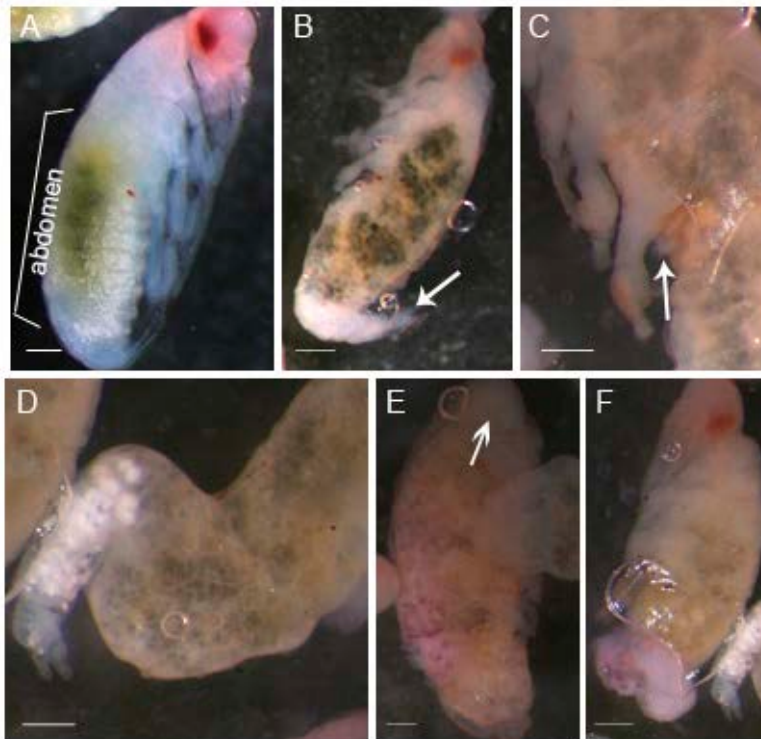

Figure 5.4: *B. germanica* **embryos from dsBgPipe-treated females.** (A) 14-day-old embryo from dsMock-treated females. At this age the tips of antennae and the hind legs almost reach between the fourth and the fifth abdominal segment and the pigment in the eyes increase. (B-F) 14-day-old embryo from dsBgPipe-treated females. (B) The embryos have short limps (antenna and legs), an abdomen larger than in controls and present the caudal end curved towards the back of the embryo (arrow). (C) Thoracic segments showing the short legs. The arrow indicates the existence of the pleuropodia. (D) Caudal end of an embryo showing the enlargement of the abdomen, the incomplete segmentation, the end of the abdomen curved presenting a concentration of the fat body in this part. (E) Embryo with the dorsal closure not completed, showing the epidermis that do not enclose all the embryo body. The arrow indicates the eye not pigmented. (F) Embryo with the abdomen poorly segmented, showing the caudal region not enclosed by the epidermis. The head is towards the right top in A, B, E and F. Scale bar: 200 µm.

In some of these embryos the eyes were not pigmented yet (Figure 5.4E, arrow), and in few of them the body wall has not been closed and the dorsal organ still arises (Figure 5.4E, F), showing the internal part of the body that it seems not developed while in this age of dsMock-treated females, the developing body wall has been completely enclosed dorsally and at the caudal end of the egg and the secondary dorsal organ has been shut by the wall.

d) BgPipe as a maternal-effector gene.

The above observations in embryos from dsBgPipe-treated females, led us to question of whether this phenotype is due to an early signal of BgPipe in the oocyte or only due to a decrease of the huge peak of expression that appeared in the ovaries from 7-day-old adults (Figure 5.1). To unveil why is necessary this punctual increase of the expression at the end of the gonadotrophic, cycle just before the synthesis of the chorion take place, we performed an in situ hybridization to localize in which ovary cells the mRNA of Pipe was expressed, at this age. Although the big size of the basal follicle at this age, and the rich content of proteins and lipids in the oocyte limits our observations to the follicular cells, we found that the mRNA of BgPipe was expressed in the cytoplasm of the follicular cells in the basal follicle (Figure 5.5A, B), whereas in the sub-basal follicle, was detected in the cytoplasm of both, the oocyte and the follicular cells (Figure 5.5C). At the beginning of choriogenesis, when those follicular cells placed in the apical pole of the basal oocyte become differentiated (Irles et al., 2009a), the mRNA of BgPipe appears localized in these cells (Figure 5.5D) that

are responsible for the formation of the sponge-like body, the structure that is essential in the ootheca contains the micropyle and allows the exchange of oxygen between the egg and the exterior. This expression of BgPipe in these anterior follicular cells could be one possibility to explain the high levels of expression observed at the end
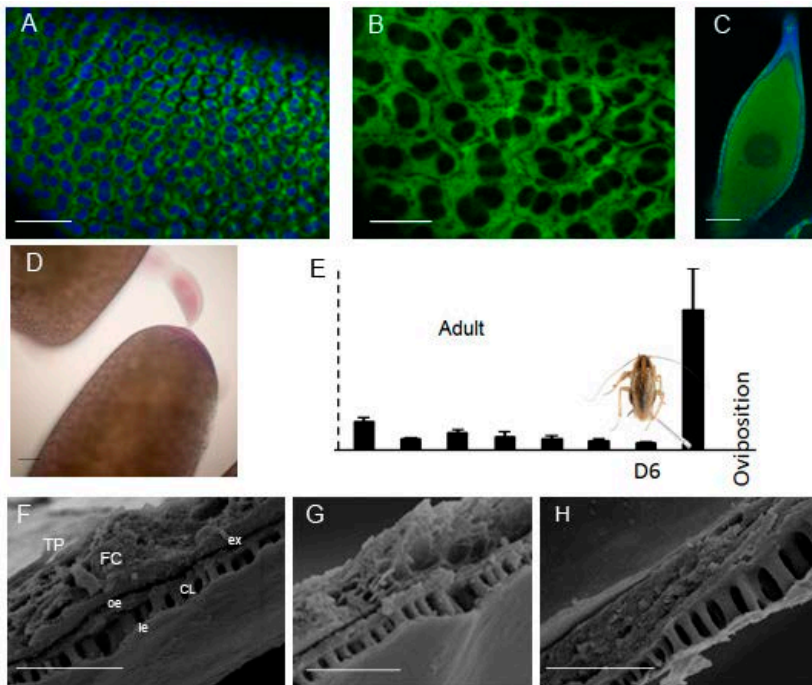

Figure 5.5: BgPipe at the end of adult cycle. (A, B) The BgPipe mRNA appeared localized of in the cytoplasm of the follicular cells from the basal ovarian follicles. (C) Sub-basal ovarian follicle showing the localization of BgPipe mRNA in the ooplasm as well as in the cytoplasm of the follicular cells. (D) Basal ovarian follicle, in the early steps of chorion synthesis, showing BgPipe mRNA localized in the cells placed in apical pole. (E) Strategy followed injecting dsBgPipe in 6-day-old adult females, 24 h before the high levels of BgPipe expression in ovaries. (F-H) Depletion of BgPipe does not affect the chorion formation. (F) 7-day-old dsMock-treated females. (G) Chorion layers from dsBgPipe injected in 6-day old adult females. (H) Chorion layers from dsBgPipe injected in 0-day-old adult females. CL: columnar layer, ex: exochorion, FC: follicular cells, ie: inner-endo chorion, oe: outer-endo chorion, TP: tunica propria. DNA stained with DAPI: blue, BgPipe mRNA labelling in green. Scale bar in C: 50 µm, in A, B and D: 100 µm, and in F- H: 5 µm.

of the gonadotrophic cycle, and suggest a function of this enzyme related with the choriogenesis.

To understand how important is the increase of BgPipe expression in ovaries from 7-day-old adult females, a new experiment was designed to deplete specifically this peak of expression. When the synthesis of the chorion layers is starting, the basal oocyte is not permeable to any molecule coming from the haemolymph (Ciudad et al., 2006), knowing that we injected dsBgPipe to 6-day-old females when the first layers of chorion have been formed, expecting that the dsRNA will affect the follicular cells but do not enter into the oocyte (Figure 5.5E). 24h later the ovaries were dissected and processed to observe the formation of the chorion layers by scanning electron microscopy (SEM). As a reference to this experiment, 0-day-old females were treated with dsBgPipe, and when they reach 7-day-old the ovaries were dissected and processed for their observation at the SEM. In all the oocytes processed and observed at the SEM, from control and treated females (from the two treatments), all the chorion layers were properly deposited and was easily to distinguish the three main layers: the endochorion, the columnar layer and the exochorion (Figure 5.5F- H), suggesting that the action of BgPipe do not seems essential for the chorion formation, and that the peak at the end has a distinct function that should be unveil.

Part of these treated females, from dsMock and from the two dsBgPipe treatments, were maintained alive to confirm the expected phenotype in the embryos. In those females treated just after the emergency to the adult, the nymphs do not hatch, accordingly with

the previous results. Those females treated when they were 6-day-old start to oviposit 24 h after the treatment as dsMock-treated females, and the nymphs hatched normally 18 days after the oviposition, suggesting that the functions of BgPipe is maternal and is stored in the oocytes the first days of the adult life.

5.5. Discussion

In *B. germanica* there is one isoform of Pipe, as in most of the insect species studied (Zhang et al., 2009a; Zhang et al., 2009b). The exception is *D. melanogaster*, which exhibit ten different Pipe isoforms with two of them expressed in the ventral follicular cells of the egg chambers (Neuman-Silberberg and Schupbach, 1993; Sen et al., 1998; Sergeev et al., 2001; Zhang et al., 2009b). The Pipe-ST2 isoform of *D. melanogaster* is the most similar to the Pipe protein present in the rest of insects, and this suggests to Zhang et al. (2009b) to propose that the function of Pipe-ST2 regulating the formation of dorsal-ventral embryonic axes is the ancestral function of Pipe in insects (Zhang et al., 2009b).

The expression of BgPipe in ovaries occurs at very low levels during the sixth nymphal instar and in the adult. However, the expression increases in two critical moments, just before the imaginal moult and in the adult, when the main chorion layers should be synthesized. We know that in *B. germanica*, others genes are increasing its expression at the days before or after the moult (Herraiz et al., 2011; Irles et al., 2013; Herraiz et al., 2014; Irles and Piulachs, 2014), and this will be related to the change of program in

the oocyte, that should start to growth and mature. On the other hand, we assumed that the peak of expression in ovaries from7-day-old adult females was related with the chorion synthesis as was described in *D. melanogaster* (Zhang et al., 2009a), as Pipe is essential to determine the ventral part of the future embryo sulfating some components of the vitelline membrane. However, in *B. germanica*, the depletion of Pipe does not affect the main chorion layers as they appeared well formed and correctly distributed when were observed at the SEM. However is not possible to dismiss any role of BgPipe in the vitelline membrane, an issue that remains to be clarified. Moreover, the experiments performed to deplete specifically the expression of Pipe at the end of the cycle, do not affect the chorion or the embryo development, suggesting again a different role for Pipe in the ovary of *B. germanica*, that could be related with the expression of BgPipe in the younger ovarian follicles.

In *B. germanica*, the expression of Pipe is localized in all the follicular cells in the basal ovarian follicle and in the germinal and follicular cells in the younger ovarian follicles. This localization of BgPipe contrast with the localization of Pipe in *D. melanogaster* eggs, where the expression of Pipe mRNA is limited to the ventral follicular cells (Sen et al., 1998), restricting the localization of other genes in the follicular epithelium (Sen et al., 1998; Moussian and Roth, 2005; Zhang et al., 2009a).

Our results suggest that BgPipe is a maternal mRNA, or protein, that is accumulated in the oocyte, early in the adult, and this accumulation is essential for the correct formation of the embryo.

Low levels of BgPipe gives rise to embryos that have defects along the anterior-posterior and DV axes, affecting the development of the eyes, the limbs (legs and antennae are shorter), the abdomen (larger and poorly segmented) and the caudal region (folded to the dorsal side). These phenotypes are in agreement with the lack of Pipe function in *D. melanogaster* females, which leads dorsalized embryos with deficiencies in elements that pattern the ventral, ventrolateral and dorsolateral parts (Anderson and Nusslein-Volhard, 1986; Schupbach and Wieschaus, 1989).

Our investigations on Pipe function in panoistic ovaries demonstrate that Pipe is a maternal expressed gene that is accumulated in the ovary of the early adult and is essential for the establishment of the axes in the embryo, as its depletion results lethal.

5.6. Acknowledgments

5.7. References

Anderson, K.V., Nusslein-Volhard, C., 1986. Dorsal-group genes of *Drosophila*. Gametogenesis and the Early Embryo, J. Gall ed. (New York: Alan R. Liss, Inc.), 177- 194.

Belles, X., Casas, J., Messeguer, A., Piulachs, M.D., 1987. In vitro biosynthesis of JH III by the corpora allata of adult females of *Blattella germanica* (L). Insect biochemistry and molecular biology 17, 1007-1010.

Ciudad, L., Piulachs, M.D., Belles, X., 2006. Systemic RNAi of the cockroach vitellogenin receptor results in a phenotype similar to that of the *Drosophila* yolkless mutant. The FEBS journal 273, 325-335.

Cho, Y.S., Stevens, L.M., Stein, D., 2010. Pipe-dependent ventral processing of Easter by Snake is the defining step in *Drosophila* embryo DV axis formation. Curr Biol 20, 1133-1137.

Dissing, M., Giordano, H., DeLotto, R., 2001. Autoproteolysis and feedback in a protease cascade directing *Drosophila* dorsal-ventral cell fate. The EMBO journal 20, 2387-2393.

Herraiz, A., Belles, X., Piulachs, M.D., 2014. Chorion formation in panoistic ovaries requires windei and trimethylation of histone 3 lysine 9. Experimental cell research 320, 46-53.

Herraiz, A., Chauvigne, F., Cerda, J., Belles, X., Piulachs, M.D., 2011. Identification and functional characterization of an ovarian aquaporin from the cockroach *Blattella germanica* L. (Dictyoptera, Blattellidae). The Journal of experimental biology 214, 3630-3638.

Irles, P., Belles, X., Piulachs, M.D., 2009a. Brownie, a gene involved in building complex respiratory devices in insect eggshells. PloS one 4, e8353.

Irles, P., Belles, X., Piulachs, M.D., 2009b. Identifying genes related to choriogenesis in insect panoistic ovaries by Suppression Subtractive Hybridization. BMC genomics 10, 206.

Irles, P., Piulachs, M.D., 2011. Citrus, a key insect eggshell protein. Insect biochemistry and molecular biology 41, 101-108.

Irles, P., Piulachs, M.D., 2014. Unlike in *Drosophila* Meroistic Ovaries, Hippo Represses Notch in *Blattella germanica* Panoistic Ovaries, Triggering the Mitosis-Endocycle Switch in the Follicular Cells. PloS one 9, e113850.

Irles, P., Silva-Torres, F.A., Piulachs, M.D., 2013. RNAi reveals the key role of Nervana 1 in cockroach oogenesis and embryo development. Insect biochemistry and molecular biology 43, 178-188.

James, K.E., Dorman, J.B., Berg, C.A., 2002. Mosaic analyses reveal the function of *Drosophila* Ras in embryonic dorsoventral patterning and dorsal follicle cell morphogenesis. Development 129, 2209-2222.

LeMosy, E.K., Tan, Y.Q., Hashimoto, C., 2001. Activation of a protease cascade involved in patterning the *Drosophila* embryo. Proceedings of the National Academy of Sciences of the United States of America 98, 5055-5060.

Morisato, D., Anderson, K.V., 1995. Signaling pathways that establish the dorsal-ventral pattern of the *Drosophila* embryo. Annual review of genetics 29, 371-399.

Moussian, B., Roth, S., 2005. Dorsoventral axis formation in the *Drosophila* embryo--shaping and transducing a morphogen gradient. Current biology : CB 15, R887-899.

Neuman-Silberberg, F.S., Schupbach, T., 1993. The *Drosophila* dorsoventral patterning gene gurken produces a dorsally localized RNA and encodes a TGF alpha-like protein. Cell 75, 165-174.

Nilson, L.A., Schupbach, T., 1998. Localized requirements for windbeutel and pipe reveal a dorsoventral prepattern within the follicular epithelium of the *Drosophila* ovary. Cell 93, 253-262.

Peri, F., Technau, M., Roth, S., 2002. Mechanisms of Gurken-dependent pipe regulation and the robustness of dorsoventral patterning in *Drosophila*. Development 129, 2965-2975.

Pfaffl, M.W., Horgan, G.W., Dempfle, L., 2002. Relative expression software tool (REST) for group-wise comparison and statistical analysis of relative expression results in real-time PCR. Nucleic acids research 30, e36.

Piulachs, M.D., Pagone, V., Belles, X., 2010. Key roles of the Broad-Complex gene in insect embryogenesis. Insect biochemistry and molecular biology 40, 468-475.

Schupbach, T., Wieschaus, E., 1989. Female sterile mutations on the second chromosome of *Drosophila* melanogaster. I. Maternal effect mutations. Genetics 121, 101-117.

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.

Sergeev, P., Streit, A., Heller, A., Steinmann-Zwicky, M., 2001. The *Drosophila* dorsoventral determinant PIPE contains ten copies of a variable domain homologous to mammalian heparan sulfate 2-sulfotransferase. Developmental dynamics : an official publication of the American Association of Anatomists 220, 122-132.

Tanaka, A., 1976. Stages in the embryonic development of the German Cockroach, *Blattella germanica Linné* (Blattaria, Blattellidae). Kontyu, Tokyo 44, 513-525.

Zhang, Z., Stevens, L.M., Stein, D., 2009a. Sulfation of eggshell components by Pipe defines dorsal-ventral polarity in the *Drosophila* embryo. Current biology : CB 19, 1200-1205.

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

6. DISCUSSION

6.1. Discussion

The proper embryo development is the final product resulting from fundamental processes that take place in the egg. The oogenesis is the essential process that produces the female gametes (the oocyte) from its differentiation in the germarium until to reach the maturation. During all this process the oocyte stores all the materials is necessary for the embryo development (Gilbert, 2000), and this storage will be very well organized, in time and space, in order to be successful in reproduction.

Our long term objective is to elucidate how is regulated the insect oogenesis taking into account the structural diversity of ovary types and their evolutionary history between insects. Thus, we have been based our project on a basal insect species with panoistic ovaries: the cockroach *Blattella germanica*.

It was hypothesized that some genes involved in oogenesis are conserved in structure and function in both panoistic and meroistic ovaries, whereas others are specific for each one. During last years, information on the oogenesis of *B. germanica* has been accumulated (Herraiz et al., 2011; Irles et al., 2013; Piulachs et al., 2010) and more other studies were accomplish helping to understand how the oogenesis is organized in panoistic ovarioles (Irles and Piulachs, 2014) (Elshaer and Piulachs, in press).

To participate in understanding the oogenesis in panoistic ovary, we built up this work which aimed to provide the grounds for further research on the mechanism that regulates oocyte polarization and the establishment of the anterior-posterior and dorsal-ventral axes,

during oogenesis in our model insect. To develop this objective, we chose some genes that were described in most modified insects, *D. melanogaster*, as determinants of oocyte polarization.

BgCic and the anterior-posterior axe regulation

Cic was identified as HMG box transcription factor which regulates multiple stages of *D. melanogaster* development (Jimenez et al., 2000). Later, it was found that Cic has important functions in vertebrates which is expressed in the developing cerebellum, controls matrix metalloproteases in the developing lung, and is implicated in different human diseases, including several cancers (Bettegowda et al., 2011; Kawamura-Saito et al., 2006; Lam et al., 2006; Lee et al., 2002; Lee et al., 2011; Lim et al., 2008).

Cic is required for the establishment of the anterior-posterior axis of the ovarian follicle in panoistic ovary. The depletion of BgCic modifies the shape of germinal and somatic cells, resulting in an elongated basal follicle with a fishtail-like aspect in the posterior pole. The anterior pole of the oocyte is also elongated entering into the lumen of the stalk. The defects in the anterior and posterior poles of younger ovarian follicles, due to depletion of BgCic, provoke the fusion of follicles as well the fusion of oocytes. The action of BgCic might be exerted regulating the stabilization of the F-actins in ovaries, modifying the distribution of other proteins in the oocyte. BgCic is also interacting with Hippo and Notch pathways to control cell proliferation and the differentiation of the stalk cells.

EGFR signaling in ovaries of *B. germanica*

We tried to demonstrate, in *B. germanica*, the same relationship between Cic and EGFR signaling as was described for *D. melanogaster* (Atkey et al., 2006; Goff et al., 2001; Roch et al., 2002). BgEGFR protein is localized in the oocyte, in both the cytoplasm and in the nucleus, and in the follicular cells. The nuclear localization of EGFR is not exclusive of the *B. germanica* oocyte, as it has been described in many cell lines and in cells from different cancer types, where it is acting as a co-transcription factor activating genes involved in cell proliferation (Brand et al., 2011; Brand et al., 2013; Lin et al., 2001). EGFR resulted very important for the proper development of the ovarian follicles, as the depletion of EGFR impedes the oviposition, probably due to the premature death of the follicular cells and to modifications in the F-actins distribution. In the young ovarian follicles, BgEGFR depletion deeply affects their structure, as they lost the ovoid morphology and can even overlap each other. These changes in the morphology might result from the concentration of F-actins that appear in the equatorial zone of the follicle, tightening this area and given these follicles an hourglass-shape, while the overlapping of the young follicles is the result of a reduction in the number of follicular cells that are not sufficient to encapsulate the oocyte and the incomplete development of the stalk. This reduction of follicular cells is also important at the level of the germarium, as they are not enough to impede germ cell proliferation, breaking the equilibrium between

the number of somatic cells and germinal cells that left the germarium (Matsuoka et al., 2013).

Notch and EGFR signaling interact in the panoistic ovary

Notch, as well as the Hippo pathway is necessary for cell proliferation in *B. germanica* ovaries in which Hippo represses Notch inducing the transition from mitosis to endocycle, in the follicular cells (Irles and Piulachs, 2014). We assess the function of *B. germanica* Notch (BgN) pathway and how it is contributing in the establishment of oocyte polarity. Depletion of BgN causes spherical ovarian follicles not allowing the ovarian follicles to elongate and have the proper elliptical shape, keeps the follicular cells immature with small nucleus and do not become binucleated (Irles and Piulachs, 2014). BgN participates in the control of the switch from mitosis to endocycle, as a depletion of BgN results in the absence of mitosis in the follicular cells, and an up-regulation of BgCyclin B, both indirect mechanisms that regulate the cell cycle transition, and suggest that these small follicular cells might be entering in the endocycle.

Pipe a target of EGFR in panoistic ovaries

As described in *D. melanogaster*, Pipe is an important target for EGFR in oogenesis (Fuchs et al., 2012). The expression of BgPipe in ovaries occurs at very low levels during the sixth nymphal instar and in the adult. However, the expression increases in two critical moments, just before the imaginal moult and in the adult, when the

main chorion layers should be synthesized. In *D. melanogaster* (Zhang et al., 2009a), Pipe is essential to determine the ventral part of the future embryo sulfating some components of the vitelline membrane. However in *B. germanica*, depletion of Pipe does not result in defects in the chorion but affects the proper development of the embryo as is essential for the establishment of the axes in the embryo. Our experiments suggest that Pipe is a maternal expressed gene that is accumulated in the ovary of the early adult and, as its depletion results lethal for the embryo.

6.2. Final Remarks

From the results obtained during the development of the present work, it is clear that although the genes are conserved in structure between the different insect species, their function has been modified. Now, it is important to know how these changes have occurred, and which factors are involved regulating these transitions.

Much work remains to be done to understand the mechanism that regulates oocyte polarization and the establishment of the anterior-posterior and dorsal-ventral axes, in insect oogenesis. And this work should be done in a great variety of insect species with different models of reproduction. When starting my job I caught the relief of other students who worked on this issue, now I am passing the torch to future students to continue with it.

6.3. References

Atkey, M.R., Lachance, J.F., Walczak, M., Rebello, T., Nilson, L.A., 2006. Capicua regulates follicle cell fate in the *Drosophila* ovary through repression of mirror. Development 133, 2115-2123.

Bettegowda, C., Agrawal, N., Jiao, Y., Sausen, M., Wood, L.D., Hruban, R.H., Rodriguez, F.J., Cahill, D.P., McLendon, R., Riggins, G., Velculescu, V.E., Oba-Shinjo, S.M., Marie, S.K., Vogelstein, B., Bigner, D., Yan, H., Papadopoulos, N., Kinzler, K.W., 2011. Mutations in CIC and FUBP1 contribute to human oligodendroglioma. Science 333, 1453-1455.

Brand, T.M., Iida, M., Li, C., Wheeler, D.L., 2011. The nuclear epidermal growth factor receptor signaling network and its role in cancer. Discovery medicine 12, 419-432.

Brand, T.M., Iida, M., Luthar, N., Starr, M.M., Huppert, E.J., Wheeler, D.L., 2013. Nuclear EGFR as a molecular target in cancer. Radiotherapy and oncology : journal of the European Society for Therapeutic Radiology and Oncology 108, 370-377.

Fuchs, A., Cheung, L.S., Charbonnier, E., Shvartsman, S.Y., Pyrowolakis, G., 2012. Transcriptional interpretation of the EGF receptor signaling gradient. Proceedings of the National Academy of Sciences of the United States of America 109, 1572-1577.

Gilbert, S.F., 2000. The saga of the germ line: oogenesis, 6th ed. Sinauer Associates.

Goff, D.J., Nilson, L.A., Morisato, D., 2001. Establishment of dorsal-ventral polarity of the *Drosophila* egg requires capicua action in ovarian follicle cells. Development 128, 4553-4562.

Herraiz, A., Chauvigne, F., Cerda, J., Belles, X., Piulachs, M.D., 2011. Identification and functional characterization of an ovarian aquaporin from the cockroach *Blattella germanica* L. (Dictyoptera, Blattellidae). The Journal of experimental biology 214, 3630-3638.

Irles, P., Piulachs, M.D., 2014. Unlike in *Drosophila* Meroistic Ovaries, hippo represses notch in *Blattella germanica* Panoistic ovaries, triggering the mitosis-endocycle switch in the follicular cells. PloS one 9, e113850.

Irles, P., Silva-Torres, F.A., Piulachs, M.D., 2013. RNAi reveals the key role of Nervana 1 in cockroach oogenesis and embryo development. Insect biochemistry and molecular biology 43, 178-188.

Jimenez, G., Guichet, A., Ephrussi, A., Casanova, J., 2000. Relief of gene repression by torso RTK signaling: role of capicua in *Drosophila* terminal and dorsoventral patterning. Genes & development 14, 224-231.

Kawamura-Saito, M., Yamazaki, Y., Kaneko, K., Kawaguchi, N., Kanda, H., Mukai, H., Gotoh, T., Motoi, T., Fukayama, M., Aburatani, H., Takizawa, T., Nakamura, T., 2006. Fusion between CIC and DUX4 up-regulates PEA3 family genes in Ewing-like sarcomas with t(4;19)(q35;q13) translocation. Human molecular genetics 15, 2125-2137.

Lam, Y.C., Bowman, A.B., Jafar-Nejad, P., Lim, J., Richman, R., Fryer, J.D., Hyun, E.D., Duvick, L.A., Orr, H.T., Botas, J., Zoghbi, H.Y., 2006. ATAXIN-1 interacts with the repressor Capicua in its native complex to cause SCA1 neuropathology. Cell 127, 1335-1347.

Lee, C.J., Chan, W.I., Cheung, M., Cheng, Y.C., Appleby, V.J., Orme, A.T., Scotting, P.J., 2002. CIC, a member of a novel subfamily of the HMG-box superfamily, is transiently expressed in developing granule neurons. Brain research. Molecular brain research 106, 151-156.

Lee, Y., Fryer, J.D., Kang, H., Crespo-Barreto, J., Bowman, A.B., Gao, Y., Kahle, J.J., Hong, J.S., Kheradmand, F., Orr, H.T., Finegold, M.J., Zoghbi, H.Y., 2011. ATXN1 protein family and CIC regulate extracellular matrix remodeling and lung alveolarization. Developmental cell 21, 746-757.

Lim, J., Crespo-Barreto, J., Jafar-Nejad, P., Bowman, A.B., Richman, R., Hill, D.E., Orr, H.T., Zoghbi, H.Y., 2008. Opposing effects of polyglutamine expansion on native protein complexes contribute to SCA1. Nature 452, 713-718.

Lin, S.Y., Makino, K., Xia, W., Matin, A., Wen, Y., Kwong, K.Y., Bourguignon, L., Hung, M.C., 2001. Nuclear localization of EGF receptor and its potential new role as a transcription factor. Nature cell biology 3, 802-808.

Matsuoka, S., Hiromi, Y., Asaoka, M., 2013. Egfr signaling controls the size of the stem cell precursor pool in the *Drosophila* ovary. Mechanisms of development 130, 241-253.

Piulachs, M.D., Pagone, V., Belles, X., 2010. Key roles of the Broad-Complex gene in insect embryogenesis. Insect biochemistry and molecular biology 40, 468-475.

Roch, F., Jimenez, G., Casanova, J., 2002. EGFR signalling inhibits Capicua-dependent repression during specification of *Drosophila* wing veins. Development 129, 993-1002.

7. CONCLUSIONS

Conclusions

From the results obtained during this present work which are disclosed here, the following conclusions can be derived and refer to the species *Blattella germanica*:

1. The function of Capicua is essential during the early steps of oogenesis in *B. germanica,* as a depletion of BgCic results in female sterility. The main function of Capicua is to establish the polarization of the ovarian follicles. A function mainly played by regulating the stabilization of the F-actins in the oocytes along the anterior-posterior axis.

2. Capicua in the panoistic ovary acts as inducer of transcriptional activity, which is opposite to the function described in the most modified meroistic ovary of *Drosophila melanogaste*r, where Capicua acts as a transcriptional repressor. Our findings suggest that the function of Capicua as inducer of transcriptional activity would be the ancestral function of this transcription factor.

3. EGFR in the panoistic ovary of *B. germanica* is necessary for the proper development of the ovarian follicles, as its depletion prevents oviposition and ootheca formation. The action of EGFR is different depending on the stage of the development of each follicle. The basal oocytes fail the oviposition, due to the premature death of the follicular cells and changes in actins distribution. In younger ovarian follicles, BgEGFR depletion deeply affects their structure, due to changes in F-actins architecture that appear concentrated in

the equatorial zone, to these follicles giving them an hourglass-shape.

4. Depletion of EGFR affects cell proliferation, cell fate specification and cell migration in the follicular epithelium in the ovary of *B. germanica*, which indicates that it contributes to these processes signaling of EGFR is mediated by the Hippo and Notch pathways.

5. In the panoistic ovary of *B. germanica* EGFR is necessary to maintain the balance between the number of follicular cells and the number of oocytes produced in the germarium. Depletion of EGFR reduces the quantity of follicular cells that are not enough to impede germ cell proliferation.

6. The role of Notch pathway is crucial during the oogenesis in *B. germanica*, since the absence of any of the components of Notch pathway leads to sterile females. Notch participates in ovarian follicle elongation through regulation of cytoskeleton network.

7. In follicular cells of *B. germanica* ovaries, Notch depletion results in the absence of mitosis in the follicular cells, and an up-regulation of Cyclin B, thus indicating that cells can enter in the endocycle.

8. *B. germanica* Pipe is accumulated in the ovary in the early adult stages and can be considered as a maternal gene. Pipe is required for the proper development of the embryo, participating in the establishment of anterior-posterior and dorsal-ventral axes.