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Novel Regulators of oocyte differentiation and meiosis

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Authorship Statement

I hereby declare myself as the author of this original and unpublished work. All consulted previously published literature and data is cited throughout the text and listed in the included Bibliography, giving credit to the original authors.

“He who has a why to live can bear with almost any how”.

- Friedrich Nietzsche

Dedicatory

This master thesis work is dedicated to my best friends Ana Sofia and Karyna Chornenca: your capacity for untiring pursuing of development and conquest of success are the inspirational fuel that I feed on to carry on any project I propose myself to. I also would like to dedicate this work to my Tutor Rui Silva, whose wisdom, guidance, comprehension, patience, and support kept me inspired to continue this project.

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Abstract

Oogenesis is a complex multicell process shared between several species, by which female gametes are formed. Fundamental for this process is the timely expression of different oogenesis genes and a nuclear division called meiosis. In *Drosophila melanogaster* both processes are highly regulated, with failures in this regulation leading to fertility impairments.

Drosophila oogenesis is a continuous process that starts with an asymmetric division of the germ line stem cells and terminates with meiotic completion during oocyte activation and egg deposition. In *Drosophila* there is a prolonged arrest in diplotene stage of prophase I, with oocyte chromatin condensation into the karyosome. At this stage the oocyte is transcriptionally quiescent, being all transcription performed by aiding nurse cells.

Mutations that specifically abrogate *Drosophila* karyosome formation led to meiotic chromosome segregation defects, clearly suggesting that formation of this structure is important for meiosis. Also, right before this meiotic progression, the oocyte's nucleus shows a brief transcription activity for which epigenome regulation is essential. The genes expressed during this transient reactivation are unknown. Regulation of karyosome formation, maintenance and reactivation is poorly known, our aim is to identify different proteins that regulate some or several of these processes.

We theorize that at least some of the proteins required for karyosome formation, maintenance and reactivation are likely to co-localize with the karyosome at some stages of oocyte development. The specific aim of this master thesis work was to find if different proteins from a list of candidates were present in the oocyte karyosome. The selection of the candidate proteins was based on the cumulative fulfilment of the following criteria: ii) gene ontology (GO) term that suggest interaction with DNA; ii) high expression during oogenesis measured by mass spectroscopy analysis, and iii) available GFP-tagged version of the protein. The presence of the candidate proteins in the karyosome was analyzed by confocal microscopy in which we search for colocalization of the GFP-tagged versions of the candidate proteins with the oocyte DNA stained with DAPI.

For this screen results we observed karyosome co-localization with the proteins SSRP, PIT and CG14230; interesting GFP signal was observed in other structures, namely in the germarium subareas and nucleoplasm and RNA splicing structures in latter stages.

Key Words: Oogenesis; Meiosis; Oocyte; karyosome; *Drosophila*.

Resumo

A oogénese é processo multicelular complexo partilhado por várias espécies, através do qual os gametas femininos são formados. Fundamental para este processo é a expressão temporal de diferentes genes participantes na oogénese e a divisão nuclear conhecida por meiose. Em *Drosófila melanogaster*, ambos estes processos são altamente regulados, com falhas nesta regulação resultando em danos na fertilidade.

A oogénese é um processo continuo que tem início com uma divisão assimétrica das células da linha germinativa e termina com a conclusão meiótica durante a ativação do oócito e deposição do ovo. Em *Drosófila* ocorre um sequestro prolongado na etapa diploteno da prófase I, ocorrendo a condensação da cromatina e formando o cariossoma. Durante esta fase o oócito encontra-se transcricionalmente quiescente, sendo toda a transcrição feita por células auxiliares.

Mutações que afetam especificamente a formação do Cariossoma da drosófila levam a defeitos na segregação cromossomal meiótica, sugerindo claramente que a formação desta estrutura é importante para a meiose. Adicionalmente, momentos antes da progressão meiótica, o núcleo do oócito demonstra ter uma ativação transiente da atividade transcricional, na qual a regulação do epigenoma é essencial, sendo que os genes expressos durante esta reativação transiente são desconhecidos. A regulação da formação do cariossoma, a sua manutenção e reativação, são eventos sobre os quais se sabe pouco. O nosso objetivo incide em identificar diferentes proteínas que possam regular alguns ou vários destes processos.

A nossa teoria incide no facto de que pelo menos algumas proteínas que poderão estar envolvidas na formação, manutenção e reativação do cariossoma poderão co-localizar com o mesmo em diferentes etapas do desenvolvimento do oócito. O objetivo em específico deste trabalho de tese de mestrado foi tentar determinar se diferentes proteínas, a partir de uma lista de candidatos, estavam presentes no cariossoma do oócito. A seleção destes candidatos foi feita com base nos seguintes critérios: 1) Ontologia genética (OG) que sugerisse interação com DNA; 2) altos níveis de expressão durante a oogénese medidos através de analise de espetroscopia de massa e 3) versões destas proteínas marcadas com GFP disponíveis. A presença das proteínas candidatas no cariossoma foi analisada usando microscopia confocal onde se procurou por co-localização das versões marcadas com GFP com o DNA do oócito marcado com DAPI.

Como resultados foi observada co-localização das proteínas SSRP, PIT e CG14230 com o cariossoma; padrões de sinal GFP interessantes foram também observados noutras estruturas, nomeadamente nas subáreas do germário e nucleoplasma e estruturas relacionadas com processamento de ARN em etapas mais tardias.

Termos chave: Oogénese; Meiose; Oócito; Cariossoma; *Drosófila*.

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Acronyms and Abbreviations

BAC - Bacterial Artificial Chromosome

Baf - Barrier to Autointegration Factor

Bam - bag of marbles

BMP - Bone Morphogenic Protein

CO - Crossing Over

CR - Central Region

DAPI - 4',6'-diamino-2-fenil-indol

DSB - Double Strand Breaks

Endos - Alpha-endosulfine

FSH Follicle-Stimulating Hormone

GFP - Green Fluorescence Protein

GO - Gene ontology

GRK - Gurken

GSC - germ stem cell

GVBD - Germinal Vesicle Breakdown

IFITM1 - interferon-induced transmembrane protein 1

LE - Lateral Elements

Mei-MCM - Mei Mini-Chromosome Maintenance

Mtrm - Matrimony

Ncd - Nonclaret Disjunctional

NCO - Non-Crossing Over

NHK-1 - Nucleosomal Histonal Kinase 1

OR - Oregon R.

PBS - Phosphate Buffered Saline

Pc - Polycomb

PF - Primordial Follicle

PFC - posterior follicle cell

PGC - Primordial germ cell

Pit - Pitchoune

RA - Retinoic Acid

SC - Synaptonemal Complex

SSRP - Structure Specific Recognition Protein

VDRC - Vienna *Drosophila* Resource Center

CHAPTER I

1 Introduction

Sexual reproduction in metazoans is preceded by the formation of highly specialized cells known as gametes. There are two types of gametes, the egg, and the sperm, that are form from germ cells, through a highly regulated process, the oogenesis (Porras-Gómez & Moreno-Mendoza, 2017) The goal of those gametes is to unite and form the zygote. The present work focuses on its regulation, particularly in the regulation of the chromatin of the oocyte, the karyosome. In this section we will discuss the mechanisms of oogenesis' regulation in both humans and *Drosophila melanogaster*.

1.1 Human Oogenesis

Human oogenesis starts early in embryonic development (Feher, 2017) and starts in the 24-day embryo with the formation of primordial germ cells from pluripotent epiblast cells and are initially located in a region of the dorsal endoderm of the yolk sack (Gilbert, 2016)

The Primordial germ cells (PGCs), are bipotential cells, meaning they can form either sperms or oocytes, depending on what gonad they will end up, formed in the posterior portion of the embryo. PGCs do not hold the power of deciding what kind of gamete they will give rise to; rather, this transformation will be determined by specific factors produced by each gonad (Gilbert, 2016).

Around a month and a half (6 weeks) of gestation (Feher, 2017), PGCs will migrate from their birth site in the ectoderm to the gonads in development, the genital ridges, as seen in figure 1. Different signaling molecules, receptors, and extracellular matrix proteins regulate this migration such as KIT, the KIT ligand KITLG (Farini et al., 2007), β1-integrin, E-cadherin (Bendel-Stenzel et al. 1999), interferon-induced transmembrane protein 1 (IFITM1), and IFITM3. Hindgut expansion may also regulate or facilitate this process. Gonadal colonization is mediated by CXCL12 (previously called SDF1) and its receptor CXCR4 and influenced by CXCR7.

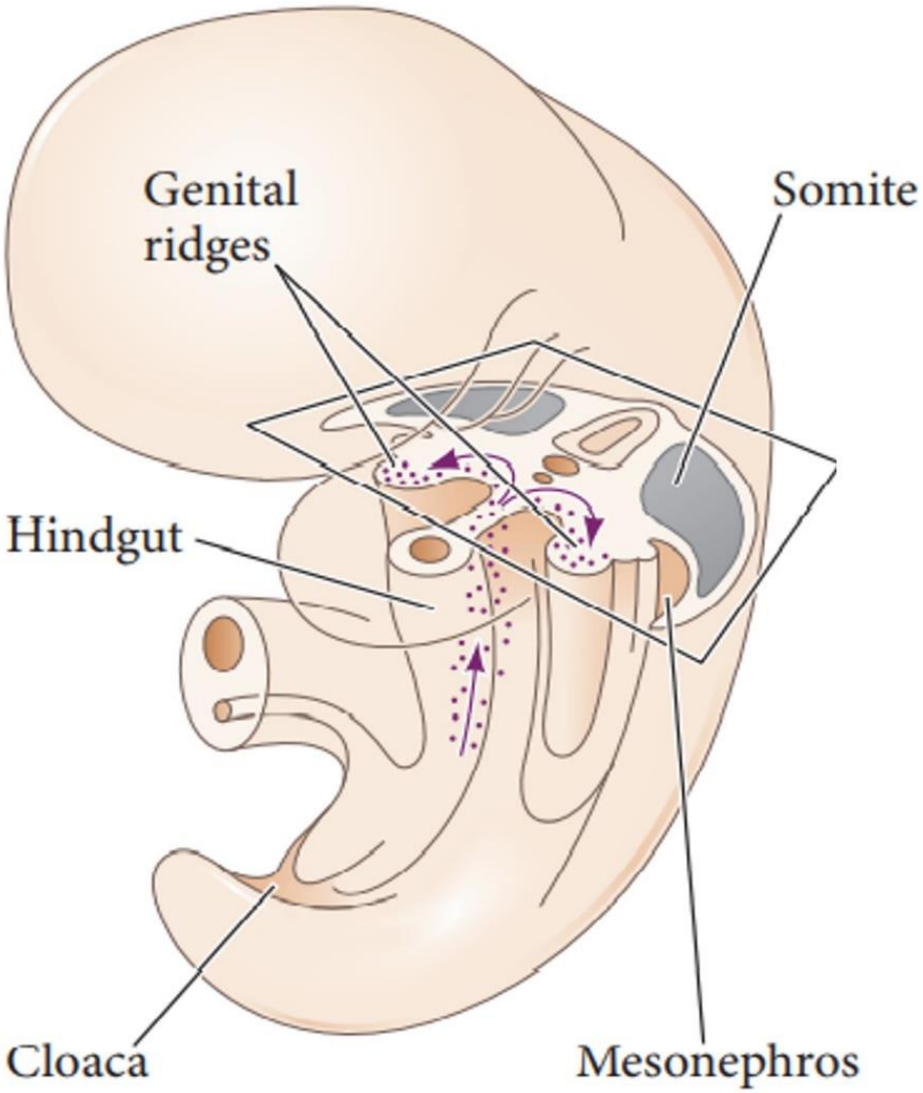

Figure 1 - **Migration of PGC's into the gonads (genital ridges)**. (adapted from (Gilbert, 2016)) PCGs (purple dots) will leave their birth site in the ectoderm, migrating through the hindgut into the genital ridge which are the precursor structures that give rise to the ovaries

During this migration, transcription and translation processes are highly repressed in these cells, assuring they migrate to the gonads and maintain their PGC identity (Richardson & Lehmann, 2010). Although the mechanics by which different species operate to maintain PGCs identity during migration to the gonads throughout development vary, four protein factors, mostly repressive in nature, seem to have roles in this task, being highly conserved between many species, being these: Vasa, Nanos, Tudor and Piwi (Ewen-Campen et al., 2010).

When PGCs reach the developing ovary, meiosis will start taking place for some of these cells, which is marked by a new cycle of DNA synthesis, promoted by Strat8 which starts to be expressed in the ovaries, up regulated by Wnt4 (Naillat et al., 2010) and Retinoic Acid (RA) (figure 2) (Baltus et al., 2006), provided by the embryo´s kidneys (Bowles et al., 2006). By the fourth month of pregnancy, around five million PGCs, now called oogonia which are the precursor cells that give rise to oocytes, already exist in the ovary (Seeley, 2017).

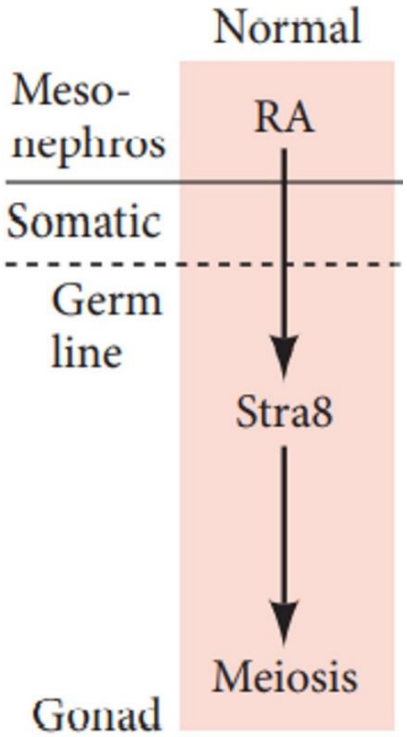

Figure 2 - **Initiation of meiosis in oogenesis** (From: (Gilbert, 2016)). Retinoic Acid from the kidneys will promote the expression of Stra8, leading to meiosis initiation.

The oogonia which enter meiosis, will progress through the first division, stopping in prophase I (Gilbert, 2016) as seen in figure 3. This meiotic arrest will endure until before the time of the ovulation. At this point, the cell is called a primary oocyte. The primary oocyte is covered with a paved epithelium layer called Granulosa layer. The structure formed by this layer and the oocyte is named Primordial follicle (PF) (Seeley, 2017) starting the folliclegenesis process at

the fourth month of gestation (Baerwald et al., 2012). Most steps of folliclegenesis take place simultaneously as seen in figure 3.

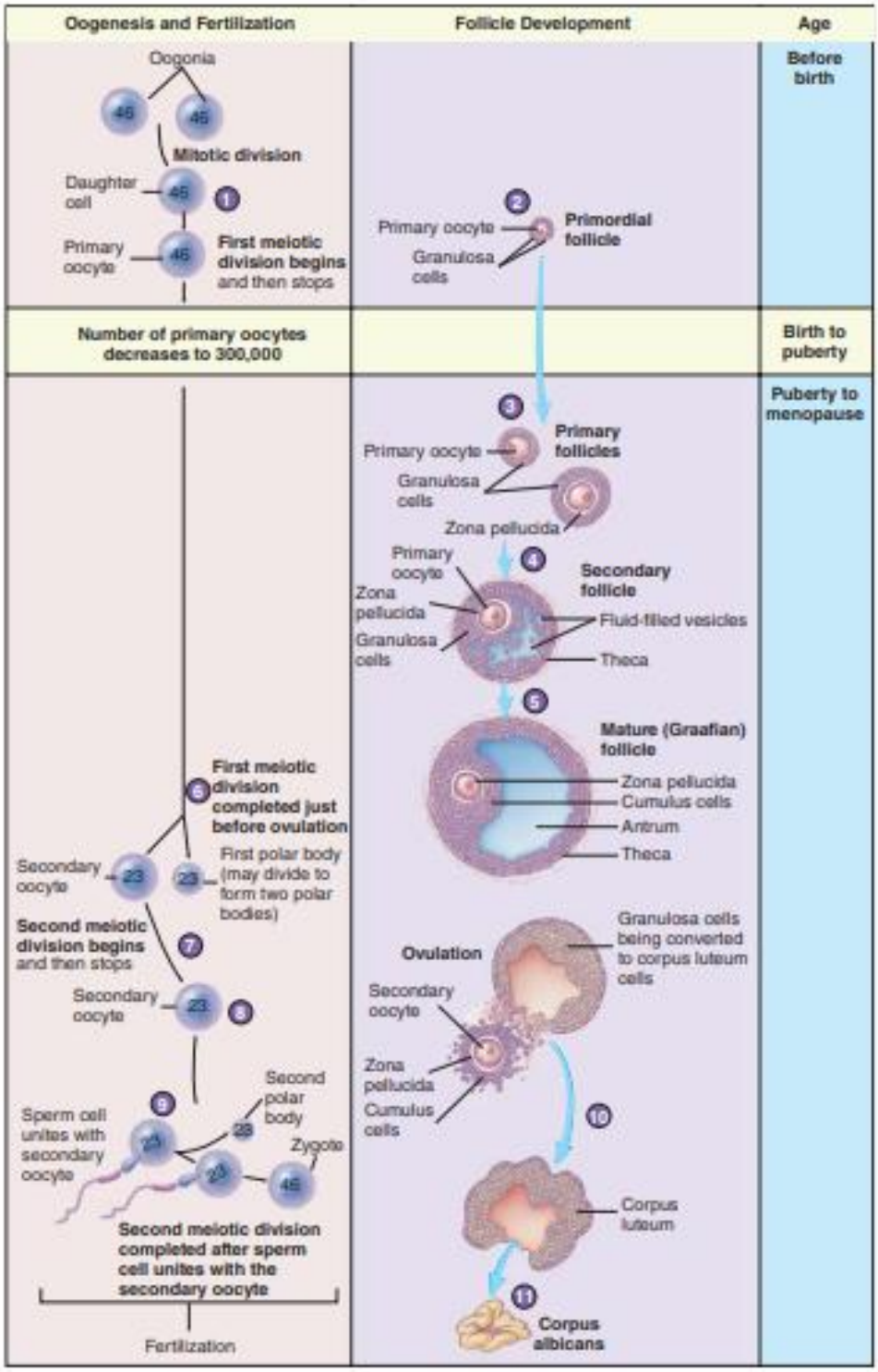

Figure 3 - **Meiosis vs oogenesis** (From:(Seeley, 2017)). Meiosis and folliclegenesis occur in the same space, the ovary, with both processes evolving synchronously. After ovulation, the oocyte leaves to the fallopian trump, while the follicular structure remains in the ovary.

From the moment of birth till female puberty, an initial number of around two million functional primordial follicles will drop to four hundred thousand (Feher, 2017; Seeley, 2017). These remaining PF's will, by the time of puberty's initiation, be stimulated by the cyclic secretions of the Follicle-Stimulating Hormone (FSH) (Messinis, 2006), and evolve to Primary Follicles, being the physiological hallmarks of this, the transition of the follicle granulosa cells from paved to cubic and the thickening of the layer, and the formation of the pellucid zone around the oocyte (Seeley, 2017).

As the granulosa layers continue to thicken, vesicle structures filled with follicle liquid begin to form among the follicle cells, marking the transition from a Primary follicle into a Secondary Follicle (Seeley, 2017).With the maturation of secondary follicles, the peripheric granulosa cells will give rise to a theca structure that subdivides into two layers: (1) the outer layer, or external theca, Fibrous in nature, and (2) the inner layer, or internal theca, vascular in nature. (Seeley, 2017).

The continuous growth of the secondary follicles and increasing of the vesicles volume will result with the fusion of these structures into one space filled with follicular liquid called Antrum (Figure 3), the secondary follicle will mature into a Graaf follicle, named after the physician Reinier Graaf, who first described these entities (Seeley, 2017). As the antrum grows in volume, the primary oocyte is pushed to periphery of the follicle, resting in a specific structure composed of follicle cells called Cumulus Oophorus. At this stage, some of the central follicle cells will gather around the oocyte to form Radiata Crown structure (Seeley, 2017)

Although during one ovarian feminine cycle several primordial follicles start folliclegenesis only one matures further to the point of suffering ovulation (Ginther et al., 2000). All the steps related to the development of the follicle are illustrated in figure 4. Right before this event, the oocyte re-enters meiosis I, finishing the first meiotic division, from which results a polar body and a second order oocyte, being the division of the cytoplasm between these cells asymmetric, with the Oocyte receiving most of the cytoplasmatic content (Bolcun-Filas & Handel, 2018). The second order oocyte immediately starts Meiosis II, being this process arrested, again, but at the stage of metaphase II. (Gilbert, 2016; Seeley, 2017)

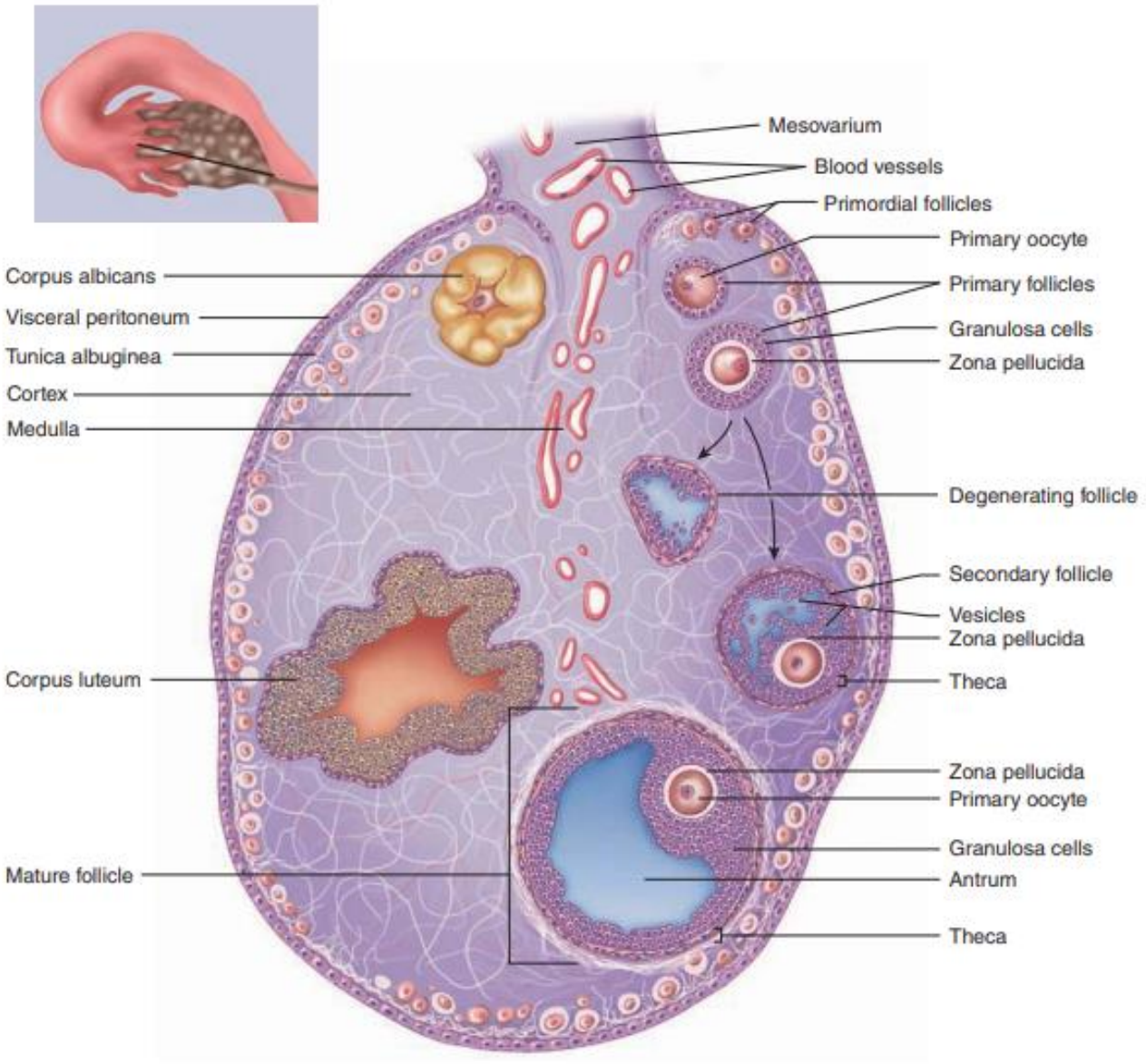

Figure 4 - **Human oogenesis** (From: (Seeley, 2017)). Depiction of the human processes that happen during the human oogenesis, starting with the formation of primary follicles and progressing through all folliculogenesis and meiosis steps that happen inside the ovary; it also depicts corpus luteum, which is maintained in the eminence of pregnancy, and corpus albicans, which is the degenerating final stage of the once follicle structure.

As ovulation occurs, the second order oocyte breaks out of the follicular structure and out of the ovary, and falls into the fallopian trump; From this point on, two possible destinies await the oocyte: (1) It crosses paths with a sperm in the fallopian trump, from where results fecundation that will trigger the reactivation of the second meiotic division; fecundation will also result in the fusion of oocyte's and sperm's nucleus, forming the zygote. (2) Fecundation does not occur and the oocyte degenerates (Seeley, 2017).

The follicle reminiscence left in the ovary becomes a glandular structure called corpus luteum, whose cells will be responsible to produce progesterone and oestrogens necessary to sustain all the physiological conditions demanded by pregnancy and efficient embryonic development (Seeley, 2017).

In the absence of pregnancy in a period of ten to twelve days after ovulation, the Corpus luteum will turn into a Corpus albicans and in time completely degenerate (Seeley, 2017). For a better understanding, Figure 4 summarizes graphically the entire human oogenesis, showcasing all processes and structures described in this section.

1.2 *Drosophila melanogaster* oogenesis

This master thesis is focused on *Drosophila melanogaster*'s oogenesis, as it presents a model with many advantages to study subject which is developmental biology.

Drosophila melanogaster is a holometabolous insect, which during its development goes through metamorphosis; its lifecycle is comprised of 4 stages: egg - larvae – pupae – fly (McLaughlin & Bratu, 2015), and a representation of this lifecycle can be observed in figure 5.

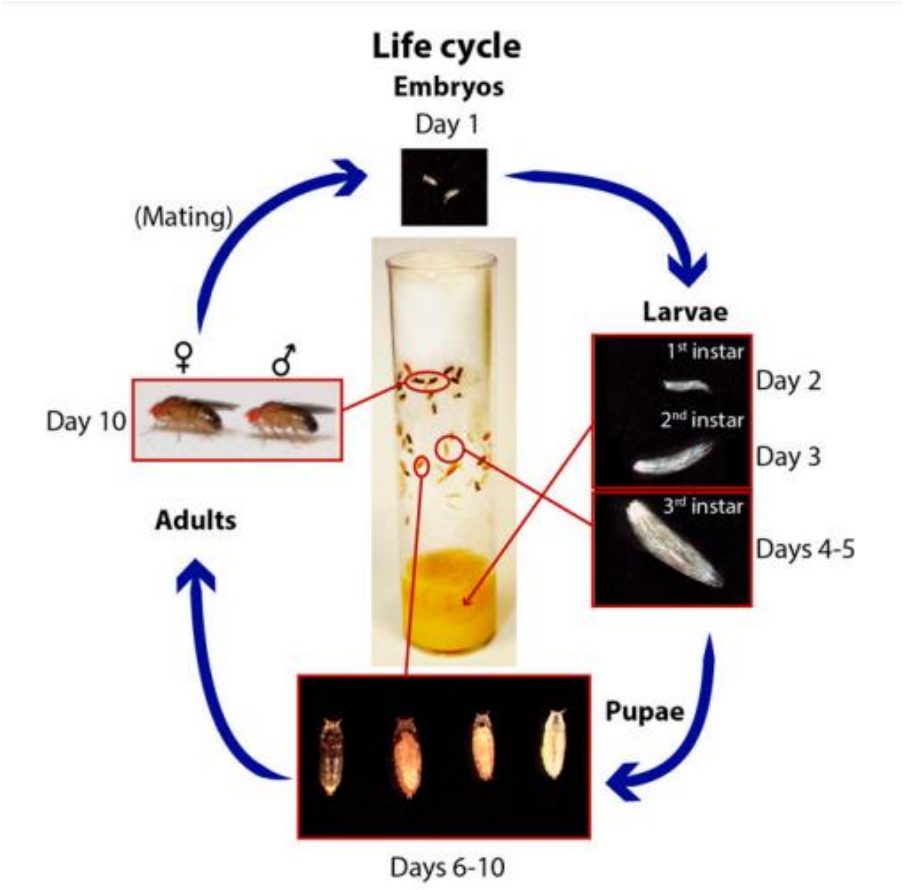

Figure 5 - ***Drosophila melanogaster*'s Life cycle** (Hales et al., 2015). Depiction of the various stages in a life cycle of a drosophila individual; fertilised eggs are laid from where larvae will hatch. Each larva will remain in this state for 4 to 5 days, transitioning to a pupae stage, which take up to 4 days; around the tenth day, adult drosophila are released from the pupae's mould and within a few maturation hours will start to mate

Drosophila's genetic composition is very simple, since the genome is all represented in four chromosomes. Due to this simplicity, it becomes easy to manipulate and study the genetic control of different processes.

Oogenesis in *Drosophila* requires all cellular processes important for the development of other tissues, such as differentiation or tissue patterning, making it useful to understand more complex processes as the ones associated with development of vertebrates and disease origin/progression. Other morphological/physiological advantages of this process include the size of the ovary and oocyte, being both, respectively, the biggest organ and cell in female *Drosophilae*. This, combined with the fact that, one ovariole from the ovary contains both germline and somatic cells as well all the stages from the differentiation of germ line stem cells

to the laying of the egg, and the fact that the ovaries are not an essential tissue, makes a diverse and extensive manipulation possible (Bastock & St Johnston, 2008).

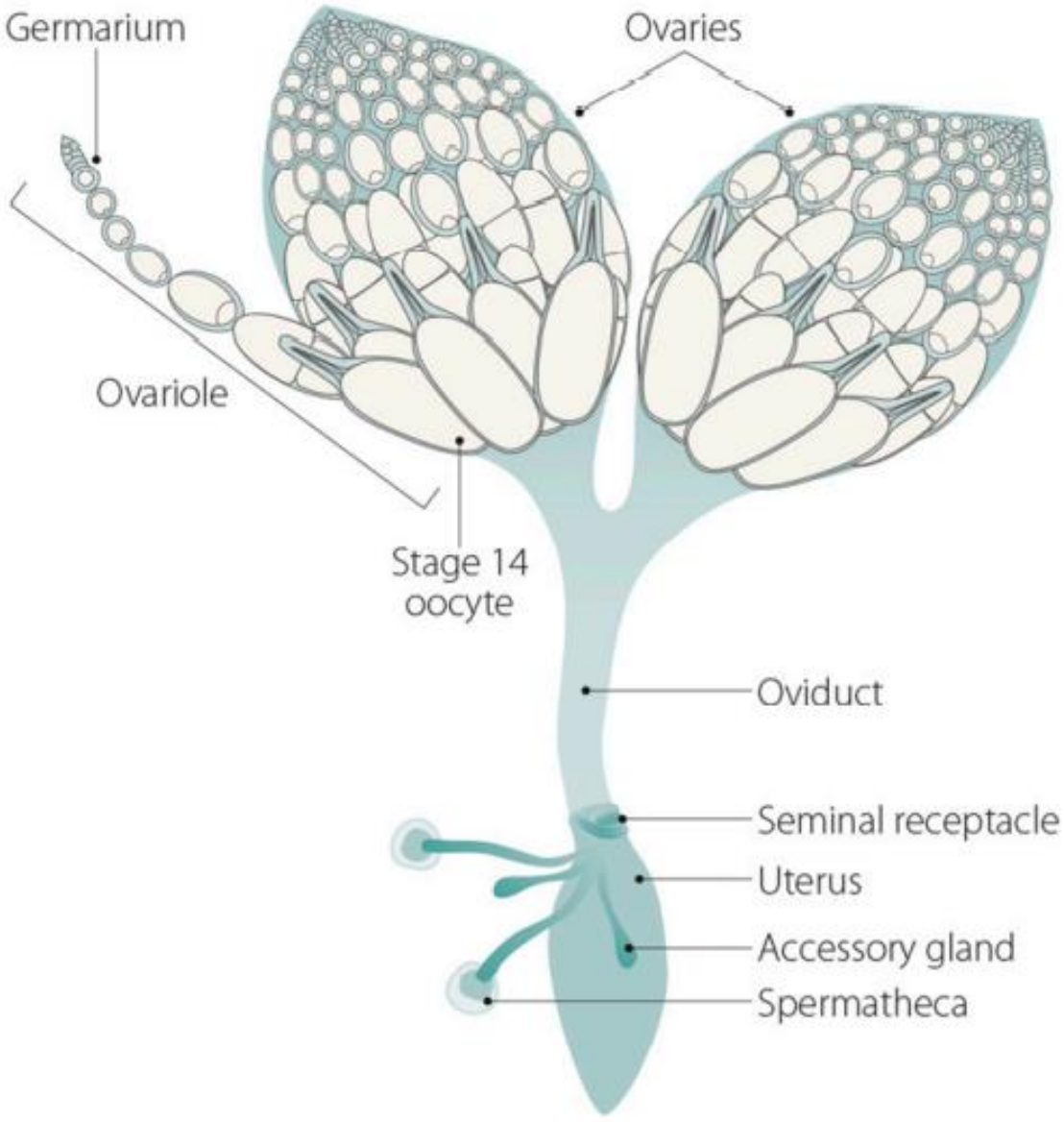

Figure 6 - **Anatomy of the *Drosophila* reproductive system** (From: (Hughes et al., 2018)). The reproductive system of *Drosophila* females is composed of a pair of ovaries containing around eighteen ovarioles each; each ovariole can generate egg chambers that will give rise to eggs which are laid through the oviduct, and, in case of fertilization, the eggs will generate new individuals.

Drosophila females possess a pair of ovaries with a set of eighteen ovarioles each (Figure 6). Each ovariole can be divided into three different structure antero-posteriorly: Terminal filament – germarium – vitellarium (McLaughlin & Bratu, 2015). *Drosophila* oogenesis starts in the

germarium, from where egg chambers bud off, going through 14 stages of development until the egg is laid, taking around one week to be completed (Bastock & St Johnston, 2008). A representation of this process can be seen in figure 7.

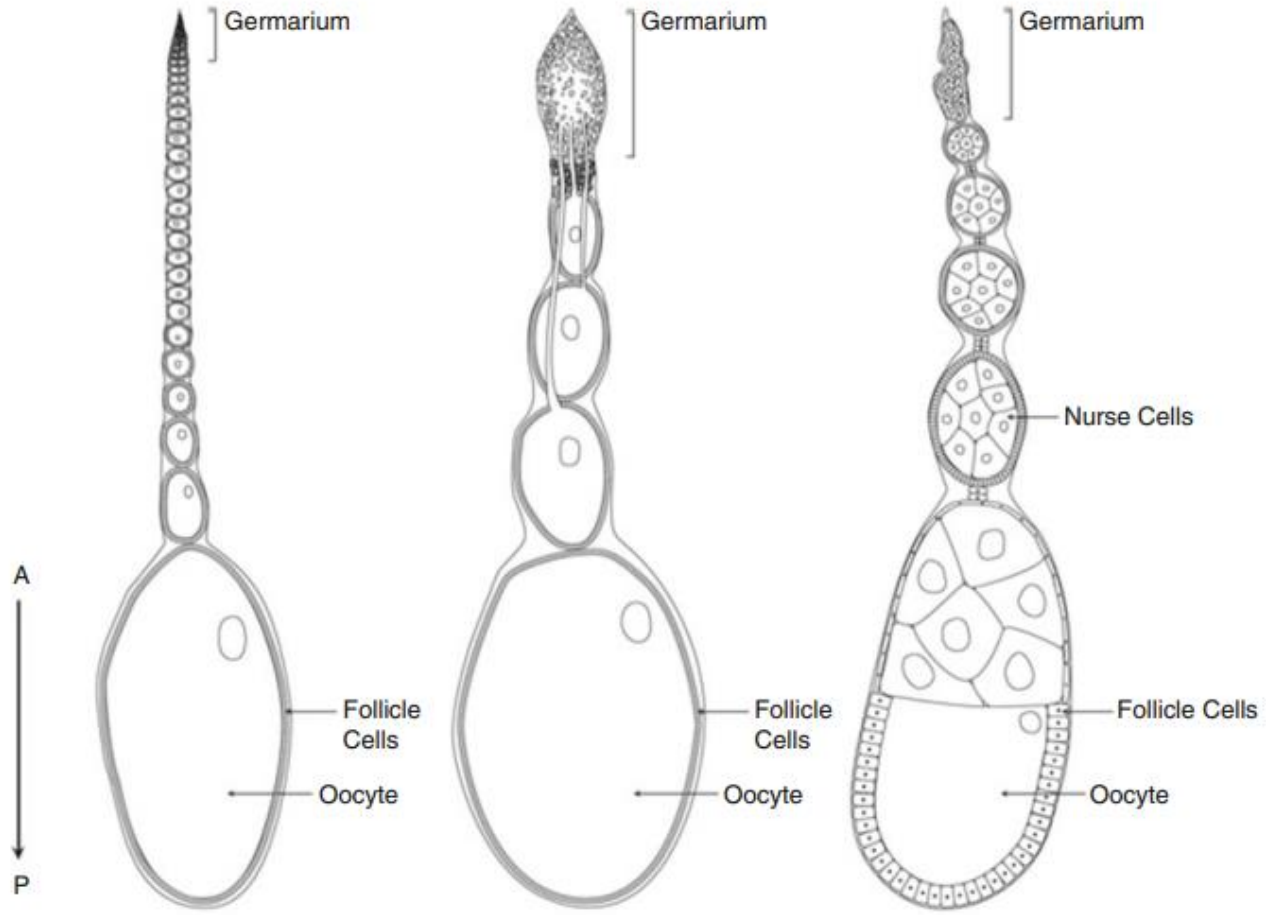

Figure 7 - Drosophila oogenesis' biology (McLaughlin & Bratu, 2015)The oogenesis starts in the most anterior part of the ovarioles, the germarium, progressing through fourteen stages. From the germarium buds one egg chamber at a time, which has the potential to generate a fertile egg. The egg chambers contain different cell composed entities such as the outer layer of follicle cells, nurse cells and the oocyte.

The germarium is subdivided in four regions: region 1, 2A, 2B and 3. Region 1 comprises the events of the formation of the cyst, 2A the determination of pro-oocytes, 2B specification of the oocyte and in the third region, the egg chamber is formed, by encapsulation of the cyst with follicle cells and budding from the germarium (McLaughlin & Bratu, 2015).

In the most anterior part of the germarium, rest the germinal stem cells, connected to the terminal filament, connected to a structure called stromal niche (Bastock & St Johnston, 2008).

These cells will divide asymmetrically, in which one will continue as a germ line stem cell and the other will suffer differentiation and form a cystoblast to form a 16-cell cyst (McLaughlin & Bratu, 2015).

The most anterior daughter cell will be exposed to BMP (Bone Morphogenic Protein) signals produced by cap cells, also resting at the anterior end of the germarium; this signaling will inhibit the expression of Bam (bag of marbles), which will enable this cell to remain with germ stem identity. The furthest posteriorly located daughter cell will not be exposed to the same signaling, leading to the expression of Bam, which triggers the differentiation into the cystoblast (precursor to the 16-cell cyst). Previous studies showed that flies that show mutations in the bam gene will not differentiate, continuing to divide, forming ovarian tumors. The stromal niche is highly dynamic as if for some reason a stem cell is lost, an adjacent cell may adjust its position, so it becomes in contact with cap cells to maintain stem identity (Bastock & St Johnston, 2008).

The Cystoblast will then suffer four consecutive, incomplete mitotic divisions, giving rise to a cyst of sixteen cells connected by ring canals. Among these cells, one will become the oocyte while others will be nurse cells, functioning as a source of nutrients and cytoplasm components to aid the development of the oocyte (Spradling, 1993).

As in most species, homolog chromosomes synapses that enables genetic recombination to occur during meiosis. In the beginning, this event is visible in several cells from the cyst, but it becomes restricted to the only two cells who are surrounded by four ring canals, and eventually, only to one which will become the oocyte. This cell will proceed with meiosis, becoming arrested in prophase I (Bastock & St Johnston, 2008).

As the cyst progresses through the germarium, it enters a region called region II; here, a structure called Balbiani body will form in the anterior of the oocyte, composed by the centrosomes, RNA, proteins and mitochondria; the follicle cells begin to surround the cyst, forcing the nucleus to join the Balbiani body; by this time, the DNA of the oocyte compacts to form the Karyosome (Bastock & St Johnston, 2008).

1.3 Patterning of the *Drosophila* oocyte

Contrary of what is known in human, *Drosophila* patterning starts during oogenesis with the establishment of an anterior-posterior and dorso-ventral patterning. Defining the polarization of the oocyte in the cyst and its maintenance is highly important because these allow the determination of the axis of the embryo while development; Patterning of the follicle cells is also important to allow a correct formation of the extra embryo structures (Bastock & St Johnston, 2008).

Patterning of the egg chambers in *Drosophila* begins with the emission of a delta signal when it's time for the cyst to leave the germarium. This Delta signal will act on the anterior follicle cells, which will become polar cells, being the hallmark of this transition the expression of JAK/STAT. This change will induce the anterior to the polar cells to become stalk cells, leading to the separation of the egg chamber from the germarium. After the bud off, a period of growth in size follows, where the nurse cells produce the components necessary to the oocyte development and the follicle cells Proliferate, until around one thousand cells are generated; this multiplication is then terminated by a new delta signaling pulse from the germline cells, promoting proper follicular maturation (Poulton & Deng, 2007).

The nucleus, mitotic organizing center, GRK (gurken) mRNA and protein are allocated to the posterior part of the oocyte (Poulton & Deng, 2007). The GRK protein contacts with posterior follicle cells, giving them posterior identity in stages between stages two and six (Steinhauer & Kalderon, 2006). These targeted cells will emit an unknown signal in response, leading to a change in the current orientation of the microtubules minus and plus ends, during stages six to 10; this modification results in the allocation of Bicoid mRNA to the anterior portion of the oocyte and oskar to the posterior – the expression of these in opposite location in the oocyte will determine the antero-posterior axis of the egg and embryo (Steinhauer & Kalderon, 2006); A graphic depiction representing this patterning can be seen in figure 8.

The dorso-ventral axis will also depend on GRK signaling, since its mRNA together with the nucleus will be allocated to the anterior portion of the oocyte and here, GRK is expressed and acts as a signal to determine this axis (Steinhauer & Kalderon, 2006).

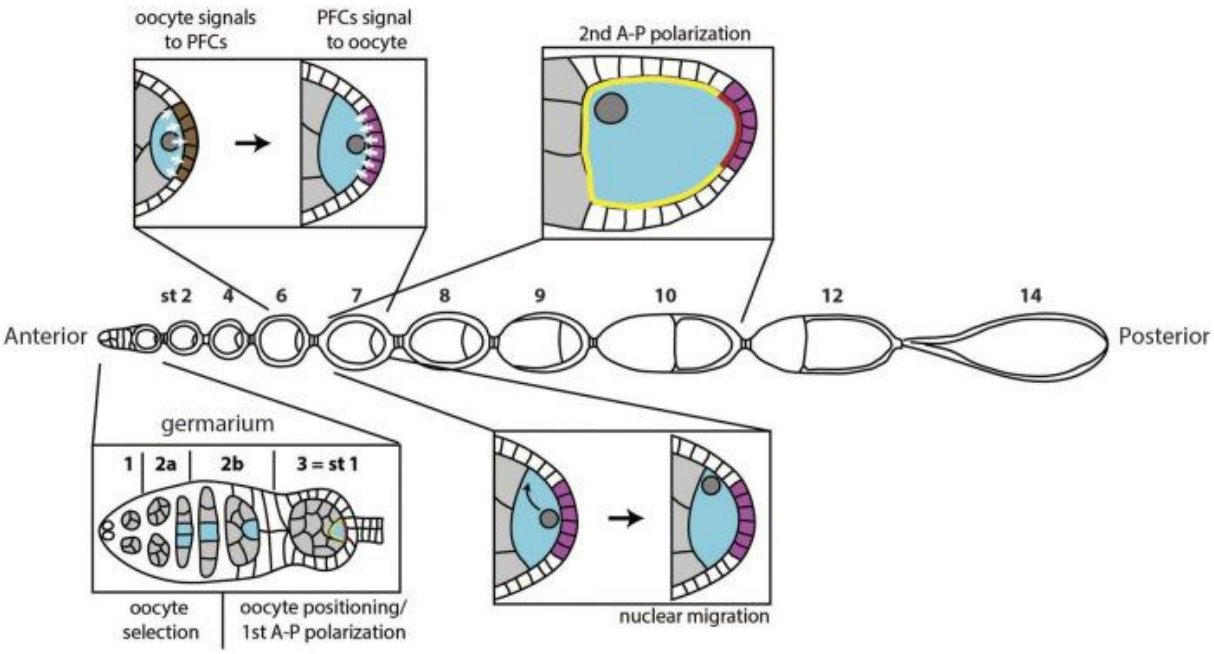

Figure 8 - **Defining the Anterior-posterior axis of the oocyte** (Ana Milas, 2022). Early Oocyte axial patterning starts in germarium region 3/stg1, with this being cell being the most posterior positioned in this structure; around stages 6/7, the oocyte will exchange signals with the PFCs (posterior follicle cells), resulting in a repositioning of its nucleus.

1.4 Meiosis: a closer look

As stated before, Oogenesis has an underlying cellular division mechanism called meiosis, which allows the formation of gametes (Bolcun-Filas & Handel, 2018). It is a double division process, form which arise four descendent cells with half of the genetic material from the ascendence; each gamete spawned possesses only one pair from each homolog chromosome ceded by its mother cell. (Gilbert, 2016; Seeley, 2017); This process enables the genetic transmission between generations and the recombination of genes from sperms and eggs, promoting allele combination diversity (Gilbert, 2016)

Starting off this topic of meiosis, a quick overview of the different phases of this process is descripted ahead.

Before the division process begins, a DNA replication takes place. (Bolcun-Filas & Handel, 2018)

The first cell division process in meiosis can be divided into four different phases each (Seeley, 2017). The first division (Meiosis I) starts with Prophase I, and subdivides into four sub-phases, which are characterized based on the chromatin/chromosomes state, as depicted in figure 9:

1. Leptotene – Chromatin density is low, making it hard to identify chromosomes, and each chromosome has one copy; both copies have two chromatids each, and homologs are unpaired (John, 1976).

2. Zygotene – chromosomes suffer synapsis, pairing together with their respective homolog; this process is aided by a protein complex called Synaptonemal complex (SC). The Structures formed by the complexation of two homologs, four chromatids and the SC are called Tetrads.

3. Pachytene – In this phase the Chromatin density increases, making the chromosome pairs now identifiable. During this stage, crossing over can occur, meaning that a chromatid from a homolog may interchange genetic material with its pair.

4. Diplotene – A phase characterized by an increased transcriptional activity and where crossing over still occurs. SC is disassembled, although the Homologs are still connected by chiasmata regions(Bolcun-Filas & Handel, 2018).

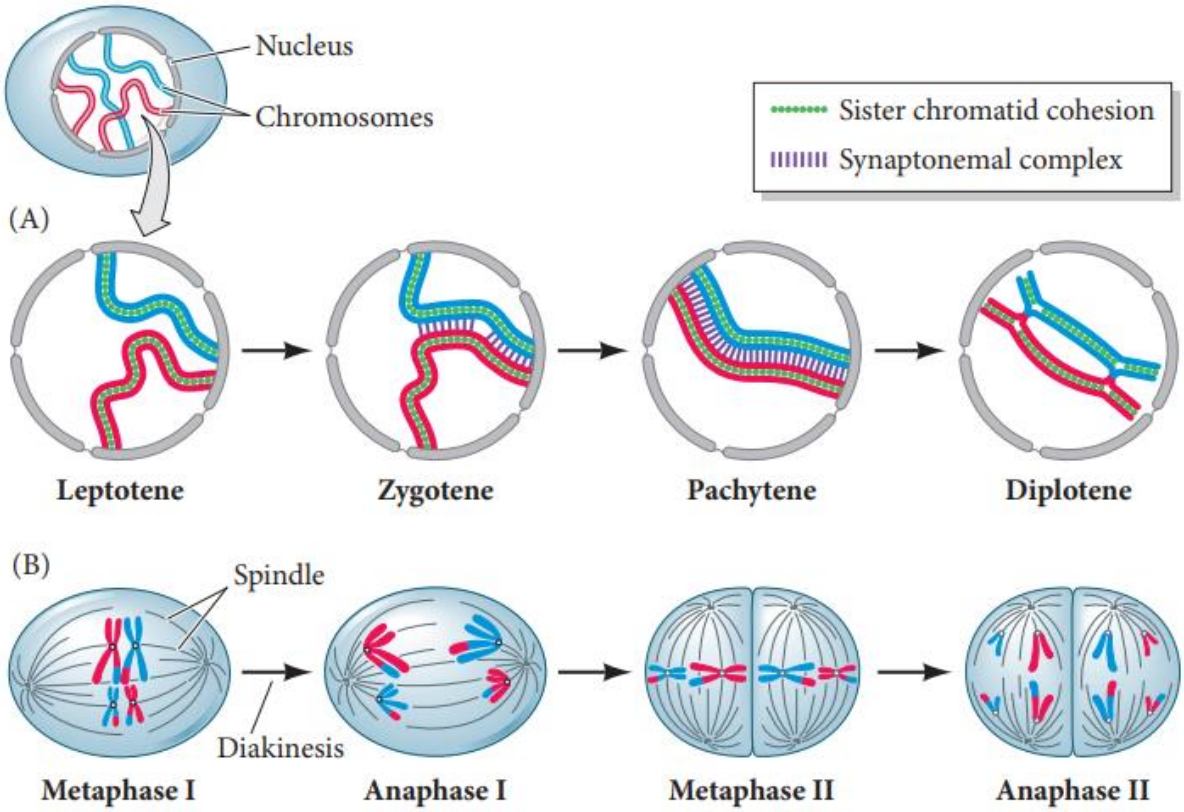

Figure 9 - **Phases of Meiosis** (From: (Gilbert, 2016))– Meiosis's prophase is divided into four subphases (A), leptotene, zygotene pachytene and diplotene, where the chromosomal synapsis

and chiasmata formation takes place; (B) shows the subsequential phases of meiosis metaphase and Anaphase I and II that culminate in the separation of homologs and chromatids respectively.

Following, comes the metaphase I in which all the chromosomes' tetrads become equatorially aligned, phenomena known as diakinesis, breakage of the nuclear envelope, and attachment of the meiotic spindle to the chromosomes (Gilbert, 2016); the homolog pairs are then separated in anaphase I, leading to the last phase, telophase I, where two descendent cells with twenty-three chromosomes each, whose number of chromatids is two, i.e., each cell receives one homolog from the original pair (Seeley, 2017).

A period of interkinesis happens between meiosis I and meiosis II. In the second division (Meiosis II), the phases are similar, borrowing the same names – Prophase II, Metaphase II, Anaphase II, and Telophase II. The same events of meiosis I occur for each of the phases in meiosis II, the difference being that he cells now dividing have only one pair of each chromosome with two chromatids; being so, the separation occurring in anaphase will be between chromatids, generating, in the end, two new haploid cells (twenty-three chromosomes per cell) with only one chromatid per chromosome (Seeley, 2017). In humans when ovulation happens, the second order oocyte will start the second meiotic division. This second meiotic division will also be arrested but this time in metaphase II and will only finish this division if fecundation by a sperm happens, fusing both nuclei.

Meiosis in *Drosophila* is also part of the oogenesis process and follows the stages of development of the ovarioles in the ovaries; it has its start in germarium which, as stated before, is subdivided into regions, showing different stages of development (Hughes et al., 2018). Figure 10 shows the chronologically paralleled events of meiosis throughout the different stages of oogenesis in *Drosophila melanogaster.*

One of the first signs of initiation of meiosis is the pairing of the homologs in the nucleus; before the formation of the cystoblast, in the germ stem cells (GSC), the chromatin of the homolog chromosomes is not paired (Christophorou et al., 2013; Joyce et al., 2013; Rubin et al., 2016). This event starts in region one of germarium, where the mitotic divisions to form the 16-cell cyst are still occurring and continues through eighth mitotic divisions (Christophorou et al., 2013; Joyce et al., 2013)

When the cystoblast comes to existence in region one of the germarium, euchromatic regions of the homologs begin pairing, while the heterochromatic ones remain unpaired (Joyce et al.,

2013). As the mitotic cyst progresses, homologs will pair and remain paired till the dismantling of the synaptonemal complex (SC) (Sherizen et al., 2005); this unpairing starts at the end of the Pachytene phase between euchromatic regions, while heterochromatic ones will remain paired (Dernburg & Sedat, 1996). Although this holds true to the somatic homologs, the X's Euchromatic regions seem to be paired by the beginning of the Pachytene phase and remain paired in the zygotene phase (Gong et al., 2005), while heterochromatic regions will be paired throughout the entire prophase I (Joyce et al., 2013).

To start up the pairing process (and thus, meiosis), we firstly witness the assembly of Synaptonemal complex along the arms of the homolog chromosomes in four nuclei of the cyst (Hughes et al., 2018), which is the protein complex which holds homologs together during meiosis (Takeo et al., 2011).

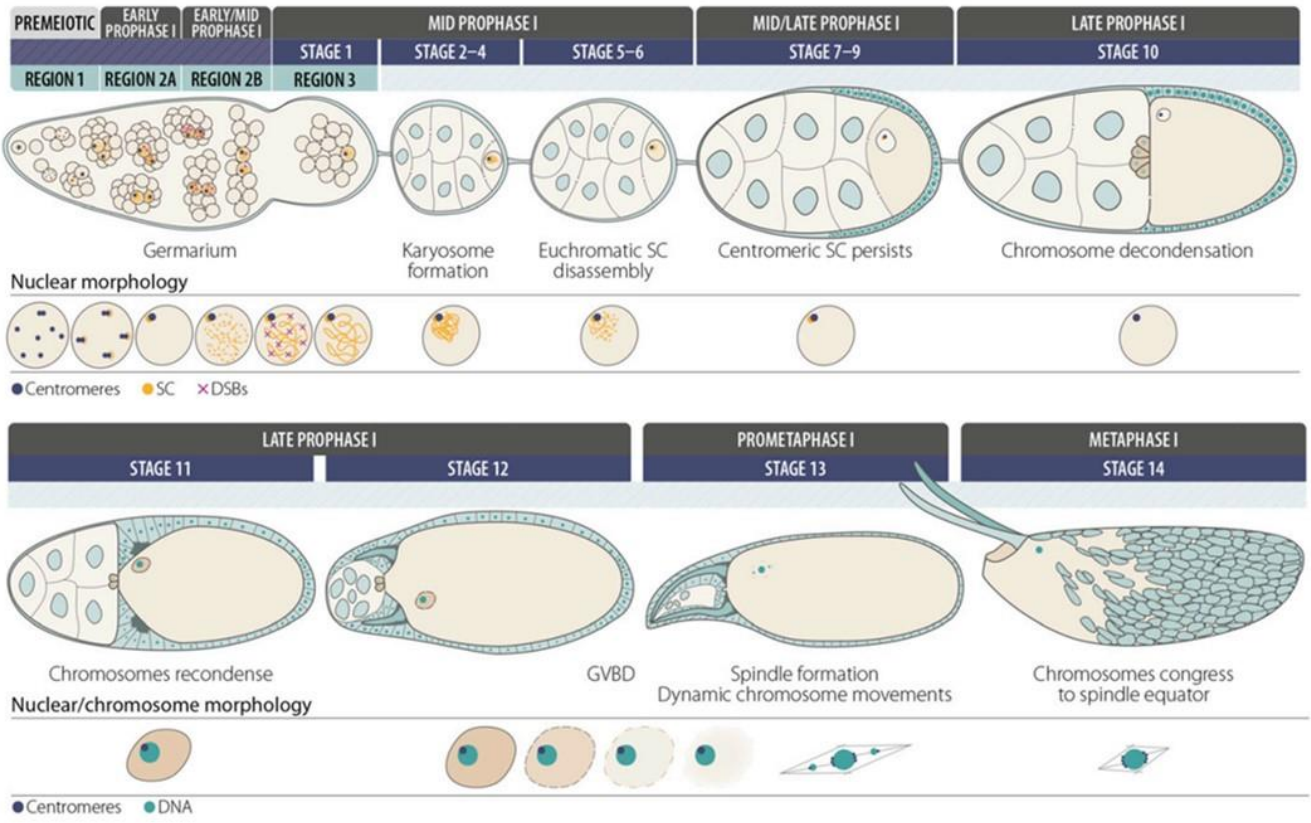

Figure 10 - **Oogenesis and meiosis** (From: (Hughes et al., 2018)). Inter and intracellular events of respectively oogenesis and meiosis occur simultaneously; the premeiotic events take place in region 1 of the germarium, affecting the GSC; prophase I events will start early in region 2A, when the Oocyte is yet to be defined. Prophase I events will then occur only on the oocyte, and last until around stage 13 of oogenesis. Metaphase one will take place at stage 14. The remainder of meiosis I and all events of meiosis II will be finalized upon egg laying.

The SC extends itself along the homolog pairs, showing a triparted structural composition: two Lateral elements (LE), which connect the chromosome cores to the Central region's elements, and is manly compose by choesin proteins; a Central Region (CR), structurally connecting the LE, composed by Transverse Filament's proteaceous components (Hughes et al., 2018), namely C(3)G, that forms a zipper type structure that links the LES (Page & Scott Hawley, 2001), Corolla, running through de CR, and Corona (Collins et al., 2014; Page et al., 2008) and finally a Central Element located in the middle of the CR (Hughes et al., 2018) (Figure 11).

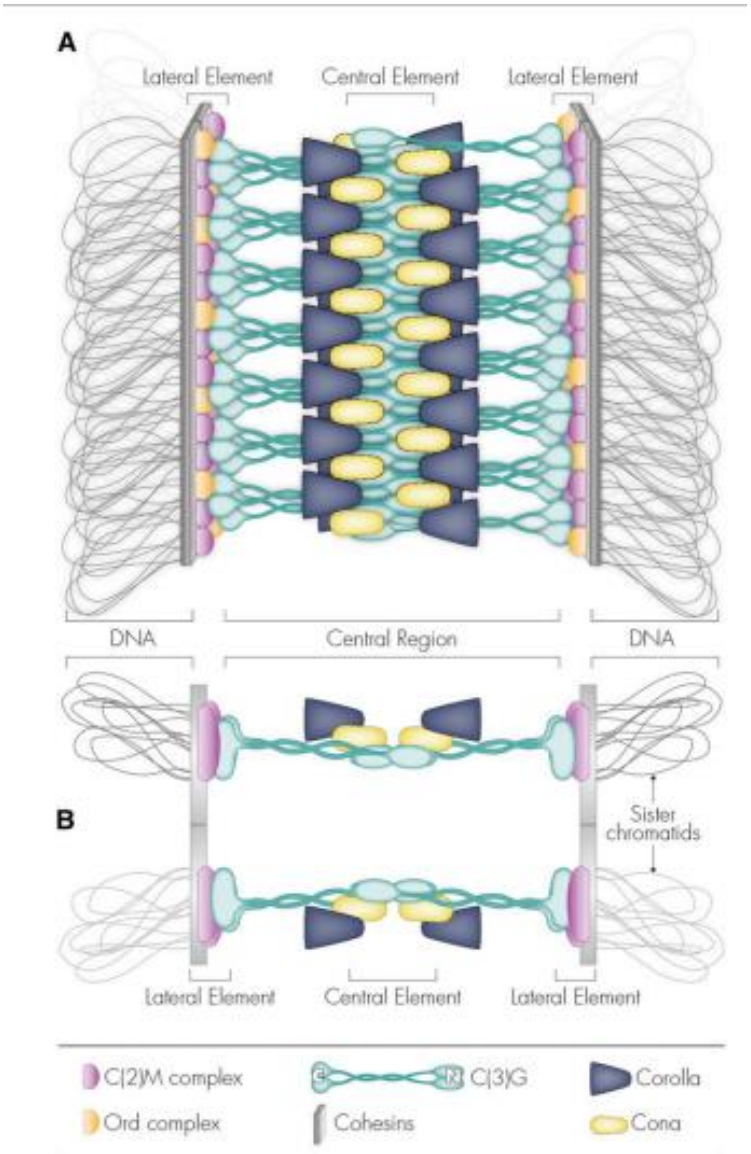

Figure 11 - **Composition of the Synaptonemal Complex** (From: (Hughes et al., 2018)) The Synaptonemal complex is a proteaceous structure that joins homologs in early meiosis, composed by different proteins; the different composing proteins make the 2 sections of this structure: Lateral elements and Central Region.

The building of the SC is a highly regulated process (Hughes et al., 2018), which starts off with the allocation of choesin Smc1/3 and Ord complex (members of the LE) to all sixteen nuclei of the cyst during the pre-meiotic mitotic divisions in 2A of the germarium (Khetani & Bickel, 2007; Webber et al., 2004). Right before the complete formation of the sixteen-cell cyst, CR components C(3)G and Corolla are assembled next to the centromeres (Christophorou et al., 2013). As the cyst reaches region 2A of the germarium, four nuclei will start to build the SC along the homolog chromosome arms, and, in this same region, two of them will now disassemble it, leaving two nuclei with incomplete chromosomal arms' SC; when region 2B is

reached, one more nucleus will disassemble this part of the SC, leaving only one of the sixteen cells with synapsed homologs by a completely assembled SC by region 3 – the Pro-oocyte. The fifteen cells left, will become nurse cells, as stated before.

By stage two of oogenesis, after the cyst left the germarium, the oocyte nucleus is reorganized giving rise to the karyosome, where the SC will remain intact. When stage five is reached, euchromatic synapsed regions of the homologs' arms will lose the SC and by stage six/eight it will be completely removed from the chromosomes' arms (Hughes et al., 2018), remaining only the centromere associated components for a few more stages (Takeo et al., 2011).

Knock out of the genes composing of the SC lead homolog recombination suppression (Collins et al., 2014; Page et al., 2008; Page & Scott Hawley, 2001); Mutants holders of these mutations form DSBs (Double Strand Breaks), although in decreased numbers, which suggests that SC is important to the maturation of DSBs into COs (Collins et al., 2014; Mehrotra & McKim, 2005). An example of this are the mutants for Ord, in which recombination at distal chromatin regions of the chromosomes is decreased (Mason et al., 1976.; Webber et al., 2004)

Programmed DSBs occur in the chromosome Homologs, after the formation of the SC (Hughes et al., 2018). In *Drosophila melanogaster*, hallmarks of these events are the presence of γH2AV, a modification to the H2A histone, after the assembly of the SC in 2A (Lake et al., 2013; Mehrotra & McKim, 2005), which can be cytologically localized with specific antibody that recognizes this modification (Lake et al., 2013; Mehrotra & McKim, 2005), and the activation of P53 in 2A and 2B (Lu et al., 2010).

Before the SC assembly, the γH2AV variant is very low, increasing its levels after its assembly (Mehrotra & McKim, 2005), and returning to residual levels when the cyst reaches region 3 of the germarium (Hughes et al., 2018).

Some of these DSBs will form up to six crossing over (CO) events in the meiosis process (Lindsley & Sandler, 1977). CO can be defined as the event that marks the location of exchange genetic material between two non-sister chromatids (Hughes et al., 2018). COs are not randomly distributed; It tends to occur frequently at euchromatic medial and distal regions, in relation to the centromere, of the chromosomes' arms, since in the proximal portions, COs are highly inhibited by polar forces coming from the centromere – the centromere effect (Miller et al., 2016). Besides this, COs are also absent from Heterochromatin, maybe because for CO to occur, DSB are necessary, which are not typical in this type of chromatin; COs are also not seen in chromosome four (Miller et al., 2016).

DSBs are shown to occur two to three more times than crossing over events also NCO (non-crossing over) events happen in an average of 11,2 per meiosis (Miller et al., 2016), which suggests the resolution of DSBs which do not form COs as NCOs as seen in figure 12 (Hughes et al., 2018).

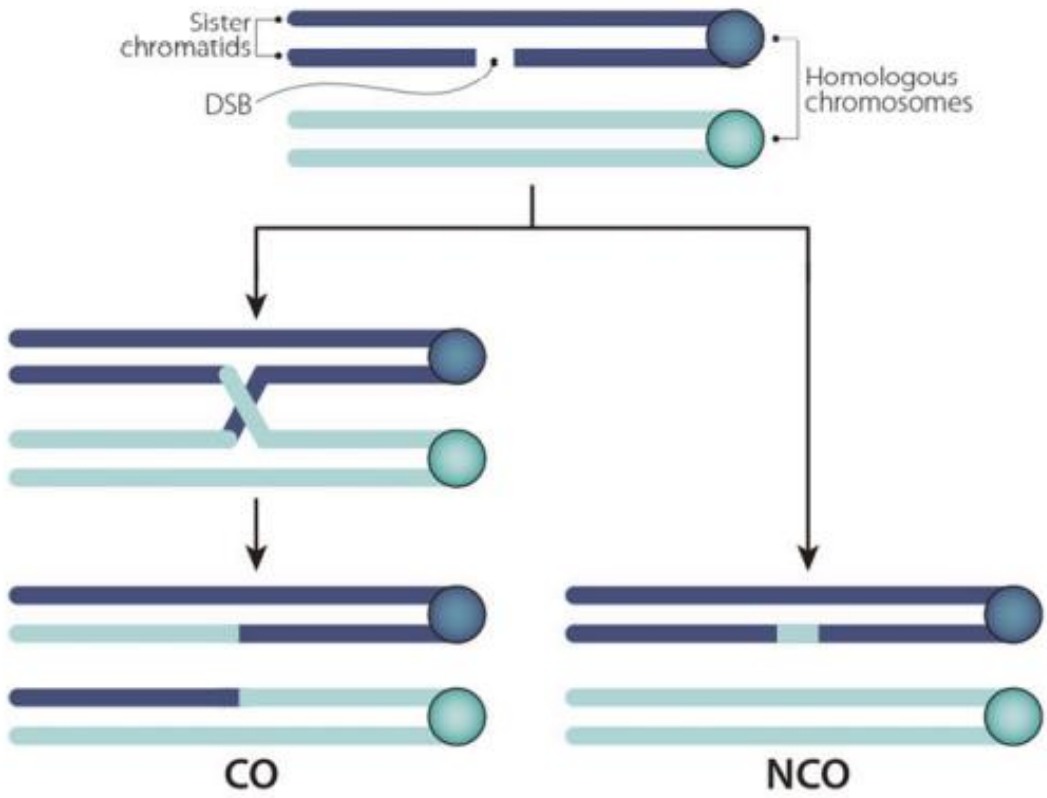

Figure 12 - **Crossing over and non-crossing over events between homologs** (Hughes et al., 2018). Double strand breaks are events that take place during meiosis; with these, breaks in the DNA chain of the homologs occur that need to be fixed for a correct progression of cell division. These breaks can be either fixed by a Crossing Over events, where genetic material is exchanged between the homolog chromosomes, or as Non-crossing Over events, where this exchange does not occur, being the fixing done by standard DNA repair methods.

Four genes seem to be necessary for the induction of DSBs: Mei-w68 (Mckim & Hayashi-Hagihara, 1998), Mei-P22 (Liu et al.,2002), Trem, whose mutations lead to a decrease in DSB's levels and abnormal chromosomal segregation (Lake et al., 2011) and Villya, whose mutants for this protein show absence of recombination, reduction in the levels of γH2A and non-segregated chromosomes (Hughes et al., 2018).

A phenomenon called Interference occurs and assures that if more than one CO happens in a homolog, these are well spaced (Berchowitz & Copenhaver, 2010).

Crossing over in many species may happen via two different pathways, class I or II, which depend on the type of enzymes used and its sensibility to Interference, being Class I COs very sensible to Interference and Class II not affected by the proximity of other crossing over events (Hughes et al., 2018). In *Drosophila*, most COs are generated by the class I pathway, but a few class two events may also occur (Miller et al., 2016).

Three main players seem to be involved in the regulation of the resolution of DSBs as COs, via Class I, which is the most common for this organism as stated above: Mei Mini-Chromosome maintenance (Mei-MCM) proteins, promote this pathway, leading to the maturation of intermediates necessary for recombination; Mei-9 complex, which facilitate the processing of said intermediates (Hughes et al., 2018) and Blm Helicase, whose function is to work as an anti-Crossing Over, by inhibiting the formation of COs via the class II pathway which is insensible to Interference (Hatkevich et al., 2017).

As meiosis progresses, COs mature into Chiasmata which will allow the physical connection between homologs, and correct biorientation in the meiotic spindle; these will ensure the correct segregation of the homolog chromosome in meiosis I (Hughes et al., 2018).

If DSB which do not generate COs are not repaired by stage 2 will lead to the activation of a check point (Hughes et al., 2018), which depends on Mei-41and Loki (Sekelsky, 2017); the activation of the checkpoint, will result in a cascade that results in defects in chromosomal condensation, abnormal development of the egg chamber and, therefore, the development of an sterile egg (Hughes et al., 2018).

All the events until now descripted regarding meiosis take place whilst the cyst is still in the germarium. After the resolution of DSBs either as COs or NCOs, and, chronologically collocating the events with oogenesis, after the budding of the cyst from region 3 of the germarium, by stage two/three, the formation of the karyosome occurs, where a reorganization of the oocyte´s nucleus takes place (Hughes et al., 2018).

This event is mediated by a kinase of the H2A histone, the nucleosomal histonal kinase 1 (NHK-1), through the phosphorylation of the Barrier to autointegration factor (Baf) Protein, whose function is to connect the nuclear envelope to the chromatin (Fiona Cullen et al., 2005). Another kinase protein the SRPK is necessary for forming and maintaining the Karyosome (Loh et al.,

2012). Mutants for these proteins fail to compact the chromatin correctly into one dense structure, leading to multiple aggregates coming to existence in the nucleus instead of just one (Fiona Cullen et al., 2005; Ivanovska et al., 2005; Loh et al., 2012). Kdm5/lid protein, also seem to be important for the formation of the karyosome, as its loss generates defects in this process (Hughes et al., 2018).

After the SC's disintegration from the chromosomal arms in stage six-eight (Hughes et al., 2018), chromosome remain connected between heterochromatic regions (Dernburg & Sedat, 1996), connection which are independent of chiasmata events (Hughes et al., 2018). These associations present an explanation to why non-recombinant homologs can segregate correctly in anaphase I.

As oogenesis reaches stage nine-stage ten, a Chromatin temporarily decondense to give rise to a brief transcriptional activity (Mahowald & Tiefert, 1970), although it is still not known what role this event has, or its relevance to meiotic progression (Hughes et al., 2018), being this one of the main motivations that led to the realization of this project as will be made clear ahead.

After the brief decondensing event, the chromatin recondenses in stage ten. This prepares the nucleus to germinal vesicle breakdown (GVBD) which happens by stage twelve and refers to the dismantling of the nuclear envelope (Hughes et al., 2018). Polo protein which has a role in initiating the mitotic process (Archambault & Glover, 2009), might have a role as well in this process, as the decreasing of the levels of its inhibitor Mtrm (Matrimony) causes early GVBD, which is suppressed by low levels of polo, and is also accelerated when polo is overexpressed (Xiang et al., 2007). On the other hand, nullification of polo's upregulator Endos (Alpha-endosulfine), delays GVBD (von Stetina et al., 2008).

Right after GVBD, a recruiting of tubulin to the chromosomes takes place (Matthies et al., 1996; Sköld et al., 2005). This process marks the beginning of the meiotic spindle assembly, which is acentriolar (no centrioles) in meiosis, since Chromosomes orientate and organize the spindle's direction (Matthies et al., 1996; Theurkauf & Hawley, 1992).

The meiotic spindle composition can be separated in three fractions: Antiparallel Microtubules, that Interact with the chromosome in the central part of the spindle; kinetochore microtubules, connecting the kinetochores to the spindle and Interpolar Microtubules, overlaying the central region of the spindle (Hughes et al., 2018).

Essential to the assembly of the complex meiotic spindle are three kinesin proteins. Nonclaret disjunctional (Ncd) is a minus terminus-oriented kinesin protein that helps not only in this construction but also aid chromosomal movement (Sköld et al., 2005). Mutations in Ncd lead to misaligned chromosomes in the spindle's center, loss of achiasmated chromosome from the spindle, abnormalities, or absence of spindle poles, and missegregation of the homologs in anaphase I (Kimble and Church,1983; Hatsumi and Endow 1992). Another motor protein which aids the spindle's building is depolymerizing Kinesin 13, KLP10A (Radford et al., 2012; Zou et al., 2008). Oocytes holders of mutations in this protein present structurally elongated spindle (Do et al., 2014; Radford et al., 2012), as well as failed biorientation and correct attachment of homologs to the microtubules and short spindles (Zou et al., 2008). At last, Kinesin 5 KLP61F has been shown to have a role in maintenance of the spindle and centromeres' symmetry (Radford et al., 2017).

For a correct alignment and Orientation of chromosomes in the spindle, it is required that the Kinetochores are well connected to the microtubules; Misconnection between these two elements must be taken down for a healthy meiotic progression (Hughes et al., 2018). Sentin protein seems to play an important role in this event, since mutations in it led to a decrease in chromosomal movements typical in prometaphase I as well as Missegregation of homologs and failure in chromosomal biorientation in the spindle (Głuszek et al., 2015). Adding to this, deficient components of the kinetochores also cause misorientation and affect the movement of chromosomes (Radford et al., 2017). During prometaphase I, as the spindle elongates, chiasmatic chromosomes are kept in the center of the spindle while the non-recombinant ones tend to move to the poles; this behavior is counterbalanced by the polar ejection force which repels these chromosomes back to the center of the spindle (Hguhes 2018).

As referred above, achiasmatic chromosomes have dynamic movement between the poles and center, during spindle assembly (Hughes et al., 2018). These movement pattern will continue until the correct alignment with the rest of the chromosomes at the center of the spindle is reached (Gilliland et al., 2009; Hughes et al., 2009).

When meiosis reaches anaphase I, homologs will segregate to opposite poles of the spindle (Hughes et al., 2009). As it's known, meiosis suffers an arrest in metaphase I in *Drosophila* females, which can only happen if at least one pair of homologs undergoes chiasmata (Jang JK, 1995). In absence of chiasmata in every chromosome, Anaphase will happen too early. This

suggests that, although recombination generates genetic diversity, its main function is to ensure a correct homolog segregation (Hughes et al., 2018).

Though recombination ensures correct segregation of recombinant homologs, a different process known as distributive system (Hughes et al., 2018), ensures that chromosomes X, which fail recombination eight to ten percent of the times, and chromosomes four, that never undergoes recombination, both segregate (Hawley et al., 1993).

The oocytes of *Drosophila* will stay arrested in metaphase for a maximum of two days (Hughes et al., 2018) and upon the passage through the oviduct, reactivation occurs instead of the fecundation being the promotor as seen in mammals (Doane ,1960; Horner & Wolfner, 2008)

Upon reactivation, meiosis one progresses with segregation of homologs in anaphase I and into meiosis two, completing the rest of the meiotic process in approximately twenty minutes (Hughes et al., 2018). A hallmark of this reactivation is a wave of calcium (Ca^{2+}), suggesting the importance of this signal to promote this event (Horner & Wolfner, 2008; Kaneuchi et al., 2015). Supporting this idea is the fact that the transition from Anaphase I to meiosis II requires the activation of Calcineurin pathway (Horner et al., 2006; Takeo et al., 2006, 2010, 2012).

As reactivation proceeds, the meiotic spindle reorients, rotating to align with the antero-posterior axis of the oocyte, elongating its central structure at the same time. Next, during Anaphase I, Chromosomes are pulled to the poles, and a structure made of microtubules is generated and passes between the chromosomes, separating the homologs (Hughes et al., 2018).

Meiosis II presents many similarities to meiosis one, with the same events taking place, but acting upon a pair of sister chromatids instead of a pair of homologs. Reaching metaphase two, these sister chromatids will segregate apart from each other, giving rise to haploid descendent cells (Hughes et al., 2018). A schematic representation can of chromosomal reduction and equation can be seen in figure 13.

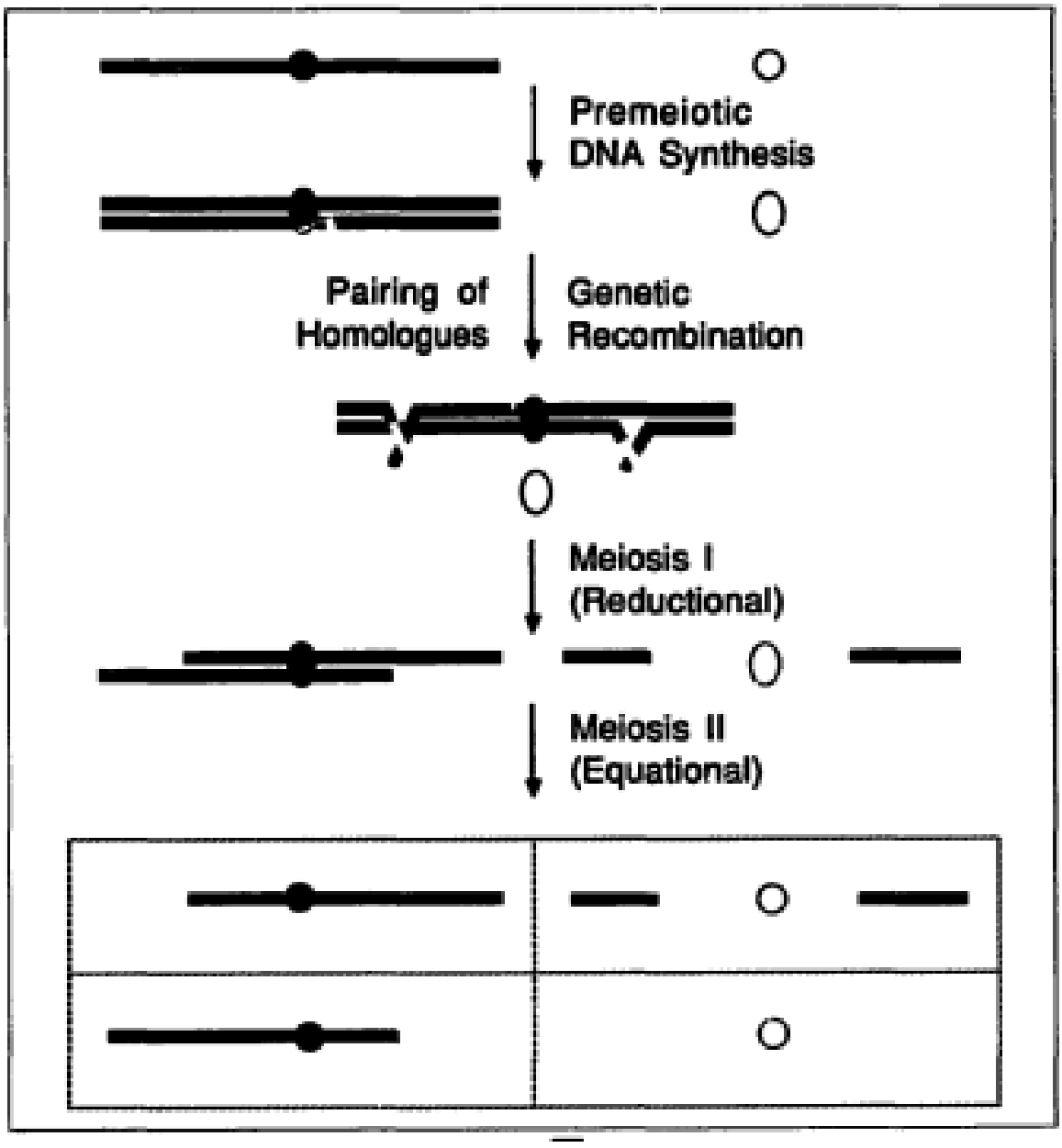

Figure 13 - **Chromosomal reduction and equation** (Roeder, 1990). At the end of the first meiotic division, the homologs will segregate, with each daughter cell keeping one of the homologs, reducing the number of chromosomes to One; at the end of meiosis II, sister chromatids of the chromosome will also segregate, equating the genetic material of the four newly formed cells.

As final note, it is important to say that meiosis two and its fast completion pace makes it a very hard process to study, as many aspects of the second meiotic division are still unknown (Hughes et al., 2018).

1.5 Masters' Thesis work objective

As the brief introductory section above shows, Oogenesis and meiosis are tightly connected biological cellular complex processes that, if correctly carried out, ensure the development of healthy and functional oocytes, both in *Drosophila melanogaster's* and human females. Said section also shows, though the description of these processes, that they are highly regulated on a subcellular level.

In *Drosophila*, the oocyte's nucleus will at some point give rise to the karyosome, a compact, chromatin composed structure, that stays transcriptionally quiescent throughout most of meiosis except for a short period of time where it briefly reactivates, as stated before.

With very little being known about the regulation processes that allow the formation, maintenance, and reactivation of the Karyosome, we theorize that some yet unidentified proteins that might have a role in this regulation might co-localize to the karyosome, interacting with it in some molecular sense, during oogenesis.

Taken this into consideration the following chapters describe a master thesis' work, carried out with the main objective of finding if previously selected candidate proteins are present in the Karyosome.

Criteria for these candidates' selection was based on their Gene Onthology suggesting their interaction with the DNA as well as high levels of gene expression throughout oogenesis, measured using mass spectroscopy analysis, and the market availability of GFP tagged versions of these proteins.

For the "How", we carried a genetic screen, where egg chambers of transgenic female *Drosophila* containing the GFP tagged versions of our candidates were observed via confocal microscopy, with the intent of searching for co-localization of these with the oocyte's DNA, previously stained with DAPI (DAPI, ou 4',6'-diamino-2-fenil-indol).

CHAPTER II

2 Material and methods:

2.1 *Drosophila* husbandry

Drosophila melanogaster was used throughout this study. *Drosophila* flies were raised using standard techniques. Fly stocks were maintained at 18 ºC in temperature-controlled incubators in polypropylene vials (23.5mm or 51mm diameter) containing enriched medium (corn flour, yeast extract, Agar-Agar, white sugar, boiled water, malt extract, niapagin) and renewed every two to three weeks, by changing the new coming generations of flies to new tubes with fresh food. For ovary dissection and staining flies are raised at 25ºC.

2.2 *Drosophila* stocks used in this study

For the conducted experiments in this project, we used a collection of stocks of transgenic flies from the Tagged FlyFos TransgeneOme Library available Vienna *Drosophila* Resource Center (VDRC) and listed in table 1.

These transgenic flies were generated by (Sarov et al., 2016) using the procedure briefly summarize here. The transgenic flies contain proteins of interest tagged with GFP (Green fluorescence protein), were produced via BAC (Bacterial artificial chromosome) engineering techniques by (Sarov et al., 2016). In short terms, a sequence of nucleotides of interest is inserted into a fosmid, along with all the elements (enhancers, promoters, for example) necessary for its expression, the created fosmid is then inserted in Bacteria for cloning (Narayanan & Chen, 2011). To develop the fosmid containing the GFP tagged proteins, (Sarov et al., 2016) used a pre-tagging non-functional cassette, consisting of an initial bacterial marker flanked by linker sequences which allow the replacement of this pre-tag in the bacteria by a tag of interest through bacterial cassette exchange via homologous recombination. Once produced, the fosmids with the GFP-tagged genes are then inserted into the flies, creating the transgenic models (Sarov et al., 2016).

Table 1 - **List of stocks used in this study**

Stocks VDRC	**Gene name**	**Genotype**
318033	Irpb	Pbac{fTRG00107.sfGFP-TVPTBF}VK00033
318048	Esc	Pbac{fTRG00140.sfGFP-TVPTBF}VK00033
318091	Chd3	Pbac{fTRG00431.sfGFP-TVPTBF}VK00033
318101	Pea	Pbac{fTRG00451.sfGFP-TVPTBF}VK00033
318107	Prp5	Pbac{fTRG00461.sfGFP-TVPTBF}VK00033
318134	CG12288	Pbac{fTRG00503.sfGFP-TVPTBF}VK00033
318138	indra	Pbac{fTRG00508.sfGFP-TVPTBF}VK00033
318168	Sin3A	Pbac{fTRG00596.sfGFP-TVPTBF}VK00033
318192	KH1	Pbac{fTRG00697.sfGFP-TVPTBF}VK00033
318211	CG10445	Pbac{fTRG00723.sfGFP-TVPTBF}VK00033
918232	Tbp	Pbac{fTRG00870.sfGFP-TVPTBF}VK00033
318239	CG9323	Pbac{fTRG00878.sfGFP-TVPTBF}VK00033
318266	Hel89B	Pbac{fTRG00912.sfGFP-TVPTBF}VK00033
318267	Ssrp	Pbac{fTRG00913.sfGFP-TVPTBF}VK00033
318268	Top3alpha	Pbac{fTRG00914.sfGFP-TVPTBF}VK00033

318270	CG5316	Pbac{fTRG00916.sfGFP-TVPTBF}VK00033
318274	Mtr4	Pbac{fTRG00921.sfGFP-TVPTBF}VK00033
318288	CG10333	Pbac{fTRG00936.sfGFP-TVPTBF}VK00033
318894	CG4612	Pbac{fTRG00942.sfGFP-TVPTBF}VK00033
318299	CG3335	Pbac{fTRG00947.sfGFP-TVPTBF}VK00033
318314	Tango6	Pbac{fTRG00963.sfGFP-TVPTBF}VK00033
318367	CG12391	Pbac{fTRG10036.sfGFP-FT}VK00033
318437	Ebi	Pbac{fTRG00006.sfGFP-TVPTBF}VK00033
318527	HP1b	Pbac{fTRG00313.sfGFP-TVPTBF}VK00033
318546	Neb-cGP	Pbac{fTRG00355.sfGFP-TVPTBF}VK00033
318560	CG30020	Pbac{fTRG00382.sfGFP-TVPTBF}VK00033
318616	CG10802	Pbac{fTRG00616.sfGFP-TVPTBF}VK00033
318660	CG11403	Pbac{fTRG00783.sfGFP-TVPTBF}VK00002
318674	spn-E	Pbac{fTRG00814.sfGFP-TVPTBF}VK00002
318678	Ball	Pbac{fTRG00823.sfGFP-TVPTBF}VK00002
318716	obe	Pbac{fTRG01000.sfGFP-TVPTBF}VK00002
318728	CG6418	Pbac{fTRG01025.sfGFP-TVPTBF}VK00002

318732	Pit	Pbac{fTRG01035.sfGFP-TVPTBF}VK00002
318747	CG8915	Pbac{fTRG01085.sfGFP-TVPTBF}VK00002
318751	Rtel1	Pbac{fTRG01096.sfGFP-TVPTBF}VK00002
318765	CG1316	Pbac{fTRG01132.sfGFP-TVPTBF}VK00002
318768	Ddx1	Pbac{fTRG01140.sfGFP-TVPTBF}VK00002
318791	CG14230	Pbac{fTRG01187.sfGFP-TVPTBF}VK00002
318808	CG2931	Pbac{fTRG01227.sfGFP-TVPTBF}VK00002
318811	ath	Pbac{fTRG01233.sfGFP-TVPTBF}VK00033
318816	Rhp	Pbac{fTRG01244.sfGFP-TVPTBF}VK00033
318858	Xpd	Pbac{fTRG01353.sfGFP-TVPTBF}VK00033
318862	mei-41	Pbac{fTRG01361.sfGFP-TVPTBF}VK00002
318865	CG11444	Pbac{fTRG01366.sfGFP-TVPTBF}VK00002

2.3 Ovaries dissection and DAPI staining

2.3.1 Ovaries dissection

Flies from the different stocks with zero to two days were transferred to new food vials supplemented with baking yeast. For each tube twelve to twenty female flies and three to six male flies were used. Flies were the incubated at 25 ºC for approximately twenty-four hours to promote oogenesis. Before dissection, flies were subjected to cold anaesthesia. Males were then discarded, and the females decapitated. Approximately sixteen to twenty (eight to ten pairs) ovaries per condition were removed through microsurgery and kept in PBS (sodium chloride 137 mM+ potassium chloride 2.7 mM+ sodium phosphate dibasic 10 mM + potassium phosphate monobasic1.8 mM).

2.3.2 DAPI staining

Dissected ovaries were fixed in 200µL of fixation solution (PBS1x, NP-40 0.5%, Formaldehyde 4%) plus 600µL heptane for ten minutes and washed three times with PBST (PBS with 0.2% tween), for ten minutes each wash. Following the last wash, ovaries were teased apart, and rinsed once in PBST.

The now separated egg chambers were subjected to an incubation at room temperature with rotation in a 4',6'-diamino-2-fenil-indol (DAPI) staining solution (DAPI 0.5µg/ml) for thirty minutes. Samples were, from this point on, shielded from light exposure.

Following the staining step, samples were washed two consecutive times for five minutes in PBST, followed by a one-minute wash in PBS (Phosphate Buffered Saline).

PBS was then replaced by mounting medium Vectashield (Vector laboratories). Egg chambers were resuspended and loaded into slides. Once finished, the slides were stored at 20ºC, protected from light exposure for later observation and imaging acquisition.

2.4 Microscope, image acquisition post-acquisition treatment

The slides were observed with a Zeiss LSM710 confocal microscope, and the acquisition of images shown in the results section was made via (ZEN2.5 Zeiss), in which specific settings were applied to the two channels used: GFP and DAPI. Following acquisition, the images were manipulated and treated using Fiji. The panels showing the results, were produced using the PowerPoint image editing tools.

CHAPTER III

3 Results

3.1 Introduction

Oogenesis is complex cellular Division process, which comprises two cellular divisions; this process allows the formation of the Haploid gametes by separating homolog chromosomes in the first meiotic incomplete division (Chromosomal reduction) and the sister chromatids in the second (Chromosomal equation). Such complex events usually are highly regulated at a genetic level; in the case of *Drosophila* melanogaster, we know that the progression of the meiosis in the oocyte is highly dependent on factors like nutrients and different biochemical signals given by the nurse cells; this way the oocyte focusses only on the progression of the meiotic process (Hughes et al., 2018).

The Meiosis I is arrested for a period during prophase I, and by this time, Chromatin is highly condensed, which makes transcriptional activity hard to accomplish, and thus the importance of Nurse cells' providing. Although this meiotic transcription quiescence prolong itself for almost all prophase I (Navarro-Costa et al., 2016) a reactivation of transcriptional activity in the oocyte seems to take place, before its progression (Mahowald & Tiefert, 1970). (Navarro-Costa et al., 2016) recently showed that Histone demethylase dkDM5 is important for temporal regulation of the meiotic transcription, with alteration to this protein leading to fertility impairments.

Taken that this and other proteins have been identified as important for meiotic progression, our working theory is that other proteins may have a role in the regulation of meiotic events, specifically in chromatin remodelling of the oocyte.

Based on go terms analysis and data presently available, our group generated a list of protein candidates with potential interactivity with the oocyte's chromatin. A subset of proteins from this list was picked, and a collection of VDRC *Drosophila* stocks GFP-tagged versions for these candidates was ordered; Our aim was to screen, mainly, for the presence of said proteins in the oocyte's chromatin in different stages of oogenesis, also reporting any other chromatin interactions that may seem relevant.

3.2 Selection of candidate proteins

The aim of this master thesis was, as previously stated, to identify possible regulators of the processes occurring during oogenesis, specifically acting on the oocyte's karyosome. To select the candidate proteins tested throughout this imaging screen, the genes had to obey the following criteria: 1) possessing Gene ontology (GO) term suggesting a role in DNA metabolism, 2) high levels of expression during oogenesis using mass spectroscopy analysis (Casas-Vila et al. 2017) and 3) the availability of GFP-tagged versions of selected proteins.

To narrow the candidate proteins into a definitive list, the five thousand genes whose proteins were more abundantly expressed in developed oocytes were picked, being retrieved from (Casas-Vila et al., 2017); from this final list, genes likely to regulate chromatin were selected, through having one or more GO terms listed in table 2.

Table 2 - **Go terms used as criteria to select candidates**

GO terms
Helicase activity
Histone binding
DNA dependent ATPase activity
chromosome condensation
chromatin remodelling
catalytic activity acting on DNA
chromatin binding
topoisomerase activity
satellite binding
regulatory DNA binding
mitotic sister chromatid separation
mitotic chromosome condensation
DNA binding
annealing activity

Finally, the list was completed by looking for GFP-tagged versions of these proteins available in the TransgeneOme collection (Sarov et al., 2016). The completed list allowed us to obtain a collection of 45 *Drosophila* stocks, each containing one GFP-tagged version of the candidate proteins listed in table 2.

For all the GFP-tagged proteins tested, their expression was under regulation of the endogenous promotor.

3.3 GFP-tagged Corola and Polycomb can be found in the oocyte chromatin with our staining protocol

Firstly, we decided to test if we could locate GFP tagged proteins via the staining and posterior confocal microscopy observation protocol determined for this work. We picked two GFP-tagged protein known to be present in the oocyte's chromatin: Corolla and PC. Polycomb (Pc) is a chromatin binding protein with genetic repressor functions, required for stable and heritable maintenance of repressed gene expression states, and acts by inducing epigenetic modifications to the targeted loci; it is also involved in the regulation of non-coding RNAs' expression that intervene in development, growth, and apoptosis processes (Enderle et al., 2011). Corolla is a structural protein component of the central region (CR) of the SC, located between the homologous cromossomes at the early steps of prophase In several cytoblast cells in region 1 of the germarium, while they are still undergoing mitotic divisions (Hughes et al., 2018).

When analysing the results, we found that corolla colocalizes with the chromatin of several cytobast in the germarium (see figure 14), becoming exclusively colocalised with the oocyte's chromatin in mid and later stages (stages three to nine) (see figures 15 and 16). This confirms previous literature since it is known that the SC is dismantled in all cells except for the one that will become the oocyte and proceed with homolog synapsis (Hughes et al., 2018). As for PC, we spotted its absence in the germarium (figure 14), and colocalization with the DNA of the oocytes (figures 15 and 16) in the later stages (three to nine). This also corroborates already existent knowledge about this protein, since it acts as a repressor for gene expression and, as celss in a differentiation process, cystoblasts have most of its genes being expressed (Deluca et al., 2020), opposite to the differentiated oocyte.

The above-described results, graphically represented by figures 14, 15 and 16, show that our staining protocol allows the detection of GFP-tagged proteins in the oocyte, thus being used as positive controls throughout the preformed imaging screen.

Table 3 - **Positive controls: corolla and polycomb. Negative control: Oregon R**. GFP-tagged Corola and Polycomb can be found in the in the oocyte chromatin showing that our staining protocol is adequate for the detection of GFP-tagged proteins in the oocyte chromatin. Results correspond to 259 biological replicas for corolla and 229 for polycomb. The total number of egg chamber and the percentage detected with GFP signal in the oocyte is indicated in this table.

	Germarium	**Stages 3-5**	**Stages 7-9**
OR	0/84 0%	0/99 0%	0/114 0%
corolla	82/82 100%	91/91 100%	86/86 100%
polycomb	54/54 100%	94/94 100%	81/81 100%

3.4 An imaging screen found that SSRP colocalizes with oocyte chromatin at different stages of oogenesis and Pit, and CG14230 are present in the oocyte's nucleus

The aim of this master thesis was to find proteins that could interact with oocyte chromatin by performing an imaging screen in which ovaries from *Drosophila* stocks expressing candidate proteins were analyzed by confocal microscopy. Co-localization of the GFP-tagged versions of the candidate proteins with the oocyte DNA stained with DAPI was investigated. For the staining process, the candidate stocks were ordered by stock numbers and divided in four groups of ten. For each group of candidate stocks tested, one of each control tagged proteins were tested in parallel. After testing all the candidate stocks, five of them were shown to be positive. A second round of staining's were performed (secondary screen) in which these five stocks

were tested again. We were able confirm the karyosome localization in three of those stocks. A third round of staining (Tertiary screen) was lastly performed upon these three stocks, confirming the results' reproductivity.

Screen results are shown in table 4; forty-five different stocks with different GFP-tagged candidates were tested, with some showing fluorescence in different structures during the oogenesis stages such as the nurse cells. Matching the original aim of this work we found three proteins that are present in the nucleus of the oocyte as shown in figures 14, 15 and 16; these refer to the proteins SSRP (structure specific recognition protein), Pit (pitchoune) and CG14230, with SSRP being shown to colocalize with the karyosome.

Taken the results obtained the positive hit rate is approximately 7% (0.068).

Ahead, the results will be described in more detail, with the representative images acquired for each control and positive results.

Table 4 -**List of results from the candidate protein screened with description**. *(*data from http://flybase.org/, version FB2022_04, released august 8, 2022)*

Protein	Germarium		Stage 3-5		Stages 7-9		Molecular Function/Go terms*	Comments
	Total Observed	GFP signal	Total observed	Karyosome' DNA GFP signal	Total observed	Karyosome' DNA GFP signal		
Irbp (Inverted repeat-binding protein)	4	0	8	0	4	0	DNA-binding;	GFP signal was detected in the DNA of the nurse cells;
Esc (extra sexcombs)	8	0	13	0	19	0	Nucleosome binding.	No alterations observed.
Chd3	4	0	10	0	5	0	ATP-dependent remodeller activity.	Weak GFP signal detected, associated to the Nurse Cells' DNA, at stages 7-9.
Pea (Peanuts)	7	0	10	0	5	0	RNA Helicase activity.	GFP signal detected, associated to the Nurse Cells' DNA, at stages 7-9.

Protein	Germarium		Stage 3-5		Stages 7-9		Molecular Function/Go terms*	Comments
	Total Observed	GFP signal	Total observed	Karyosome' DNA GFP signal	Total observed	Karyosome' DNA GFP signal		
Prp5 (pre-mRNA processing factor 5)	10	0	20	0	21	0	RNA Helicase activity	No alterations observed.
CG12288	8	0	14	0	14	0	mRNA binding	GFP signal detected, associated to the Nurse Cells' DNA, at stages 3-5
Indra	8	0	10	0	11	0	RNA polymerase II cis-regulatory region Sequence specific DNA binding.	GFP signal detected, associated to the Nurse Cells' DNA in all stages 3-9.
Sin3A	12	0	13	0	18	0	Chromatin binding.	No alterations observed.
KH1	12	0	14	0	14	0	Enables helicase activity.	No alterations observed.

Protein	Germarium		Stage 3-5		Stages 7-9		Molecular Function/Go terms*	Comments
	Total Observed	GFP signal	Total observed	Karyosome' DNA GFP signal	Total observed	Karyosome' DNA GFP signal		
							Nucleic acid binding.	
CG10445	9	0	14	0	15	0	ATP-depended activity on DNA. ATP-depended chromatin remodeller activity.	No alterations observed.
Tbp (TATA binding protein)	14	0	20	0	17	0	General transcription initiation factor binding. RNA polymerase II cis-regulatory region Sequence specific DNA binding; Transcription cis-regulatory region binding. RNA polymerase II general transcription initiation factor activity.	No alterations observed.
RHAU helicase	16	0	17	0	18	0	DNA helicase activity.	No alterations observed.

Protein	Germarium		Stage 3-5		Stages 7-9		Molecular Function/Go terms*	Comments
	Total Observed	GFP signal	Total observed	Karyosome' DNA GFP signal	Total observed	Karyosome' DNA GFP signal		
Hel89B (Helicase 89B)	6	0	11	0	11	0	ATP dependent activity over DNA: DNA binding. ATP dependent chromatin remodeler activity.	No alterations observed.
Ssrp (Structure specific recognition protein)	25	+/(25)	53	+/ (53)	51	+/ (51)	Nucleosomal DNA binding. Nucleosome binding.	Weak, but present, GFP signal detected in the karyosome DNA. Other structures showing GFP signal: NCs' DNA, ribosomal aggregates in the oocyte nucleus, nucleoplasm and possibly associated to the nuclear envelop.
Top3alpha(Topoisomerase 3α)	17	0	13	0	18	0	DNA topoisomerase activity.	No alterations observed.

Protein	Germarium		Stage 3-5		Stages 7-9		Molecular Function/Go terms*	Comments
	Total Observed	GFP signal	Total observed	Karyosome' DNA GFP signal	Total observed	Karyosome' DNA GFP signal		
CG5316	10	0	13	0	15	0	Damaged DNA Binding: DNA 5'-adenosine monophosphate hydrolase activity; Mismatched DNA binding; Single strand break-containing DNA binding; Single stranded DNA binding.	No alterations observed.
Mtr4 (Mtr4 helicase)	12	0	14	0	16	0	RNA helicase activity	No alterations observed.
CG10333	12	0	15	0	17	0	RNA helicase activity	No alterations observed.
CG4612	10	0	12	0	20	0	mRNA binding poly-A binding	No alterations observed.

Protein	Germarium		Stage 3-5		Stages 7-9		Molecular Function/Go terms*	Comments
	Total Observed	GFP signal	Total observed	Karyosome' DNA GFP signal	Total observed	Karyosome' DNA GFP signal		
CG3335	12	0	11	0	11	0	mRNA binding RNA_binding	No alterations observed.
Tango6 (Transport and Golgi organization 6)	8	0	15	0	21	0	No Go terms associated to molecular function.	No alterations observed.
CG12391	10	0	12	0	21	0	DNA binding;	No alterations observed.
Ebi	15	0	25	0	25	0	Chromatin binding. DNA-binding transcription factor binding.	No alterations observed.

Protein	Germarium		Stage 3-5		Stages 7-9		Molecular Function/Go terms*	Comments
	Total Observed	GFP signal	Total observed	Karyosome' DNA GFP signal	Total observed	Karyosome' DNA GFP signal		
HP1b (Heterochromatin Protein 1b)	11	0	22	0	19	0	Chromatin binding.	No alterations observed.
Neb-cGP	7	0	7	0	7	0	Protein phosphatase binding; chromatin Binding;	No alterations observed.
CG30020	11	0	7	0	6	0	DNA binding;	No alterations observed.
CG10802	6	0	8	0	8	0	Nucleic acid binding	No alterations observed.
CG11403	6	0	9	0	10	0	DNA binding. DNA helicase activity;	No alterations observed.
							Heterochromatin assembly.	

Protein	Germarium		Stage 3-5		Stages 7-9		Molecular Function/Go terms*	Comments
	Total Observed	GFP signal	Total observed	Karyosome' DNA GFP signal	Total observed	Karyosome' DNA GFP signal		
Spn-E (spindle E)	7	0	8	0	9	0	Mitotic chromosome condensation; Negative regulation of transposition, DNA mediated; Negative regulation of DNA-templated transcription. Oocyte karyosome formation.	No alterations observed.
Ball	7	0	8	0	10	0	Histone Threonine Kinase activity	No alterations observed.
Obe (obelus)	5	0	8	0	9	0	3'-5' DNA helicase activity. Nucleic Acid binding;	No alterations observed.
CG6418	7	0	14	0	14	0	RNA helicase activity;	No alterations observed.

Protein	Germarium		Stage 3-5		Stages 7-9		Molecular Function/Go terms*	Comments
	Total Observed	GFP signal	Total observed	Karyosome' DNA GFP signal	Total observed	Karyosome' DNA GFP signal		
Pit (pitchoune)	16	+/ (16)	30	0	30	0	RNA binding	Structures showing GFP signal: Nucleus (Nucleoplasm) of the oocyte from stages 3-9; Germarium. Follicle cells in all stages examined; NCs' DNA and ribosomal complexes in the oocyte's nucleus.
CG8915	8	0	8	0	10	0	Helicase activity;	No alterations observed.
Rtel1 (Regulator of telomere elongation helicase 1)	9	0	12	0	9	0	Chromatin binding.; DNA binding. DNA helicase activity. DNA polymerase binding.	No alterations observed.

Protein	Germarium		Stage 3-5		Stages 7-9		Molecular Function/Go terms*	Comments
	Total Observed	GFP signal	Total observed	Karyosome' DNA GFP signal	Total observed	Karyosome' DNA GFP signal		
CG1316	18	0	25	0	22	0	RNA binding mRNA binding	No alterations observed.
Ddx1 (Dead-box-1	8	0	14	0	12	0	Chromatin binding Nuclease activity Transcription coregulator activity	No alterations observed.
CG14230	20	+ / (20)	30	0	23	0	RNA binding mRNA binding	GFP signal detected in structures: nucleoplasm, nucleosome NCs' DNA, germarium and follicle cells at all stages.
							RNA binding	

Protein	Germarium		Stage 3-5		Stages 7-9		Molecular Function/Go terms*	Comments
	Total Observed	GFP signal	Total observed	Karyosome' DNA GFP signal	Total observed	Karyosome' DNA GFP signal		
CG2931	5	0	10	0	10	0	mRNA binding	No alterations observed.
Ath (athos)	6	0	13	0	13	0	rRNA binding RNA binding RNA helicase activity	GFP signal detected in NCs' DNA
Rhp (Rhophilin)	10	0	13	0	13	0	No go terms for molecular function	No alterations observed.
Xpd (Xeroderma pigmentosum D)	3	0	10	0	10	0	5'-3' DNA helicase activity Damage DNA binding DNA helicase activity	GFP signal detected in the NCs' DNA
mei-41 (meiotic 41)	22	0	42	0	44	0	No go terms for molecular function	GFP signal detected in the NCs' DNA
CG11444	26	0	33	0	35	0	RNA binding	No alterations observed.

3.5 SSRP

The results obtained during the screen for SSRP localization also showed the presence of SSRP-GFP in all the stages of the oogenesis tested. In figure 14 we can see that SSRP-GFP is present in different regions of the germarium. It shows a wave like pattern, appearing firstly in region 1, slightly fading in region 2A and 2B and reappearing in region 3, right before the budding of the egg chamber. This pattern suggests that there-might be some colocalization with DNA of the protein in the regions of the germarium where it is present, although, when looking at the panel depicting the overlapping of GFP signal with the DAPI stained DNA for SSRP in figure 14, it is clear that not all SSRP-GFP co-localizes with nuclear DNA. This pattern was consistent in all germariums observed and all the biological replicas.

SSRP-GFP signal was also detected in stages three to five observed, obeying to a consistent pattern in all the egg chambers observed in this interval of stages (table 4). As seen in the representative figure 15 SSRP-GFP shows a scattered strong signal around the karyosome, in the nucleus, namely the nucleoplasm. Also, aggregate like structures of this protein were also detected in the nucleoplasm. Analysis the panels in figure 15 showing the DNA and the marking of SSRP, it seems to be co-localisation with the karyosome's DNA, which becomes more evident when looking at the panel showing the DNA/SSRP overlapping images; this result is the only one fulfilling completely the aim of this work, since it confirms the SSRP presence in the karyosome and its colocalization with the karyosome's DNA.

For the last set of stages – seven to nine – of the oogenesis analysed, a similar pattern to the one observed in stages three to five: 1) scattered presence of SSRP around the karyosome in the nucleoplasm and 2) the colocalization of SSRP with the karyosome's DNA, which can both be seen in the panels regarding the SSRP results depicted in figure 16. For these stages, SSRP aggregate like structures were also present. A different and unique feature was, although, observed for all the stages in this range: two small points, likely small aggregates of SSRP that seem to flank and be in contact with the karyosome's boarders. The GFP signal for these structures showed itself very intense when visually comparing it the signals shown in the other structures where SSRP was also detected.

All the results obtained were confirmed in biological replicas for all stages tested.

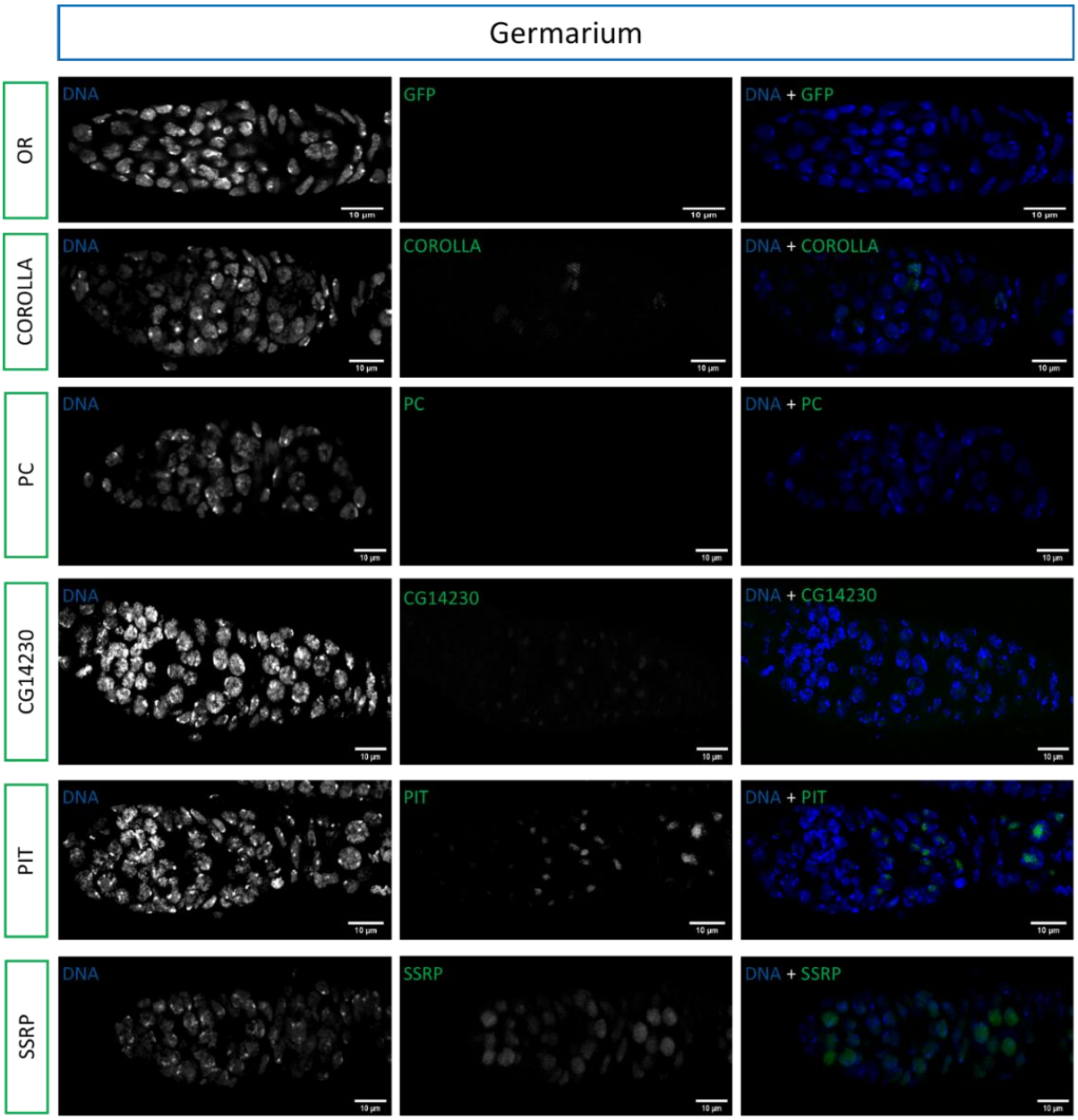

Figure 14 - **Pit, SSRP and CG14230 are present in the germarium.** GFP signal was detected in the germarium for all three positive results; OR, COROLLA and PC panel triplets, represent the negative control and both positive controls used respectively; Pit and CG14230 show a similar pattern of scattered GFP signal among the germarium cells. SSRP show round, full structures, with GFP signal, in a wave like pattern, being more visible in regions 1, 2B and 3.

3.6 Pit:

Pit-GFP was detected in all stages of the oogenesis observed during the screen, being these the germarium, stages three to five and seven to nine. As seen in figure 14, Pit-GFP signal was detected in a group of cells scattered in specific areas of the germarium, being more present in what seems to be the region 2B and 3. However, at this stage no co-localization between DNA and Pit-GFP was detected.

The Pit-GFP was also detected in stages three to five; in these stages Pit can be seen, in figure 15, around the karyosome structure, when analysed side by side with the DAPI marked DNA, in the oocyte's nucleus. It is also present in the oocyte's covering follicle cells, and similar to what is observed in germarium, it does not seem to colocalize with the DNA. Pit is also present in the nurse cells as seen in figure 15, colocalizing with their DNA. Pit can also be seen, in figure 15, around the karyosome structure, when analysed side by side with the DAPI marked DNA, in the oocyte's nucleus.

Two distinct patterns are seen in these stages: one being a scattered faded GFP signal around the nucleoplasm and the other being dense proteaceous agglomerates of Pit also in the nucleoplasm, that seem to be associated to RNA processing structures, seen in figure 15.

Similar results were observed for Pit-GFP localization in stages seven to nine, as can be seen in figure 16. This nuclear but non-DNA colocalizing patterns were also observed in the follicle cells.

The patterns described above were present in all the stages observed from all the egg chambers tested. These results were reproduced in three biological replicas.

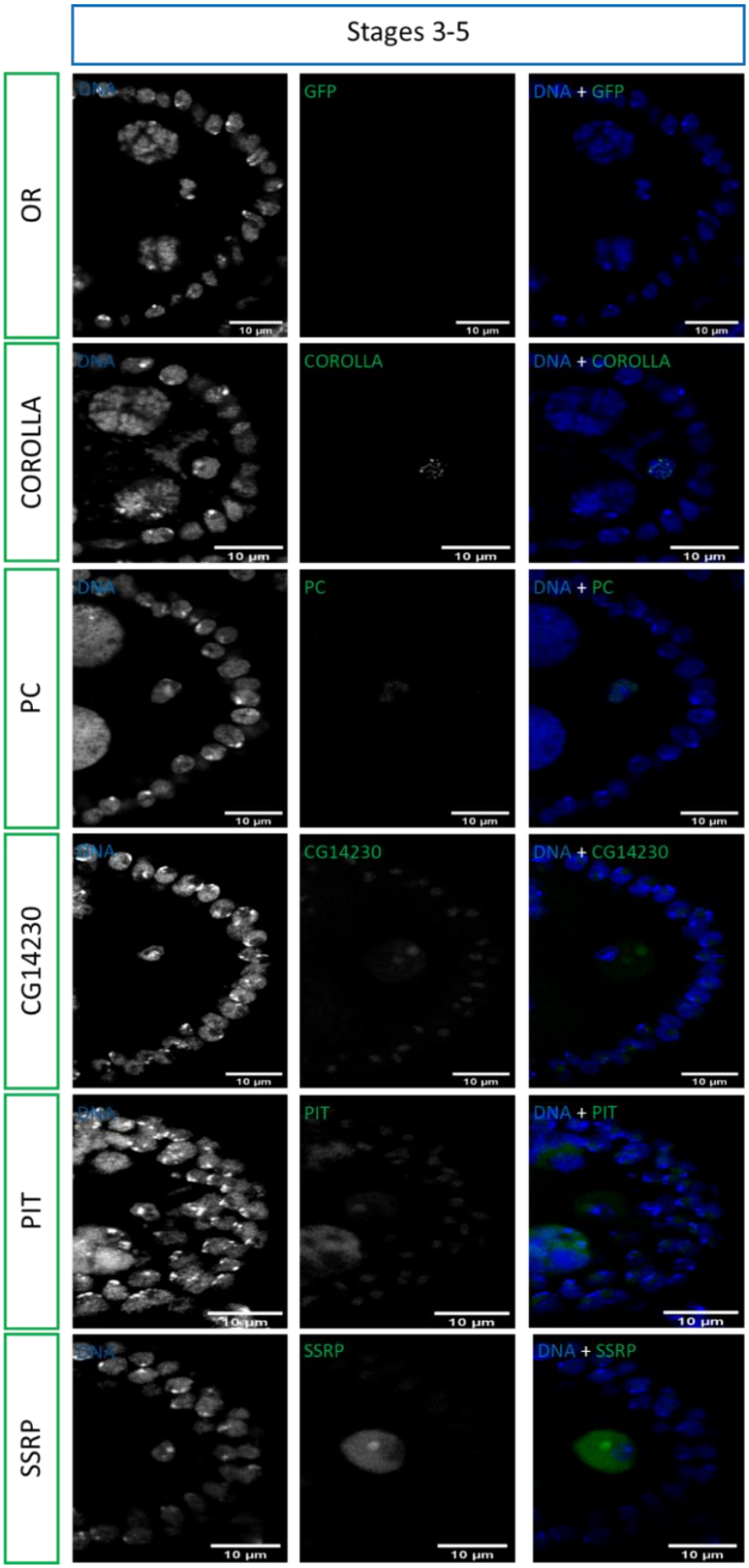
Stages 3-5
OR
COROLLA
PC
CG14230
PIT
SSRP
DNA
GFP
DNA + GFP
COROLLA
DNA + COROLLA
PC
DNA + PC
CG14230
DNA + CG14230
PIT
DNA + PIT
SSRP
DNA + SSRP
10 µm

Figure 15 - **Pit, SSRP and CG14230 are present in several subcellular structures in stages 3-5.** GFP signal was detected in stages 3-5 for all three positive results; OR, COROLLA and PC panel triplets, represent the negative control and both positive controls used respectively in this interval of stages; for both CG14230 and Pit, GFP signal was detected in the nucleoplasm, RNA splicing related structures, but not colocalizing with Karyosome's DNA; The same pattern was observed for SSRP, with this also showing colocalization with the Karyosome.

3.7 CG14230

The results for CG14230 obtained during the screen showed a weak colocalization between DNA and CG14230-GFP in the germarium. This pattern is very similar to what was observed for this stage for Pit-GFP. Both Pit and CG14230 localization in the germarium can be seen in figure 14. This pattern was seen in all he germariums observed and in all biological replicas. The GFP signal showing the presence of CG14230 seems to be very low or absent in majority of region 1, and mostly present in regions 2A, 2B and 3, in specific spots; when looking into the overlapping of DNA/CG14230 signals in figure 14, it suggests that there is no colocalization of this protein with the genetic material.

For stages three to five, all tested egg chambers and biological replicas showed a very similar pattern to what was seen for the Pit localization in these stages; CG14230 seems to be present in the follicle cells surrounding the egg chamber, but without DNA co-localization, as seen in figure 15. In the oocyte's nucleus, surrounding the karyosome, aggregates of this protein can also be seen in the nucleoplasm in the form of dense GFP-signal points, seen in figure 15, both in the panels regarding the GFP signal locating this protein. The presence of CG14230 was also detected in the nucleoplasm, like what was observed for both Pit and SSRP. As shown in the panel of figure 15 depicting the overlapping of DAPI and GFP signals, no colocalization with the karyosome's DNA was detected. These patterns in the nucleoplasm were also observed in stages seven to nine, as can be seen in figure 16, with no DNA colocalization observed in the karyosome. All results regarding these stages were confirmed by three biological replicas and were consistent in all egg chambers analysed

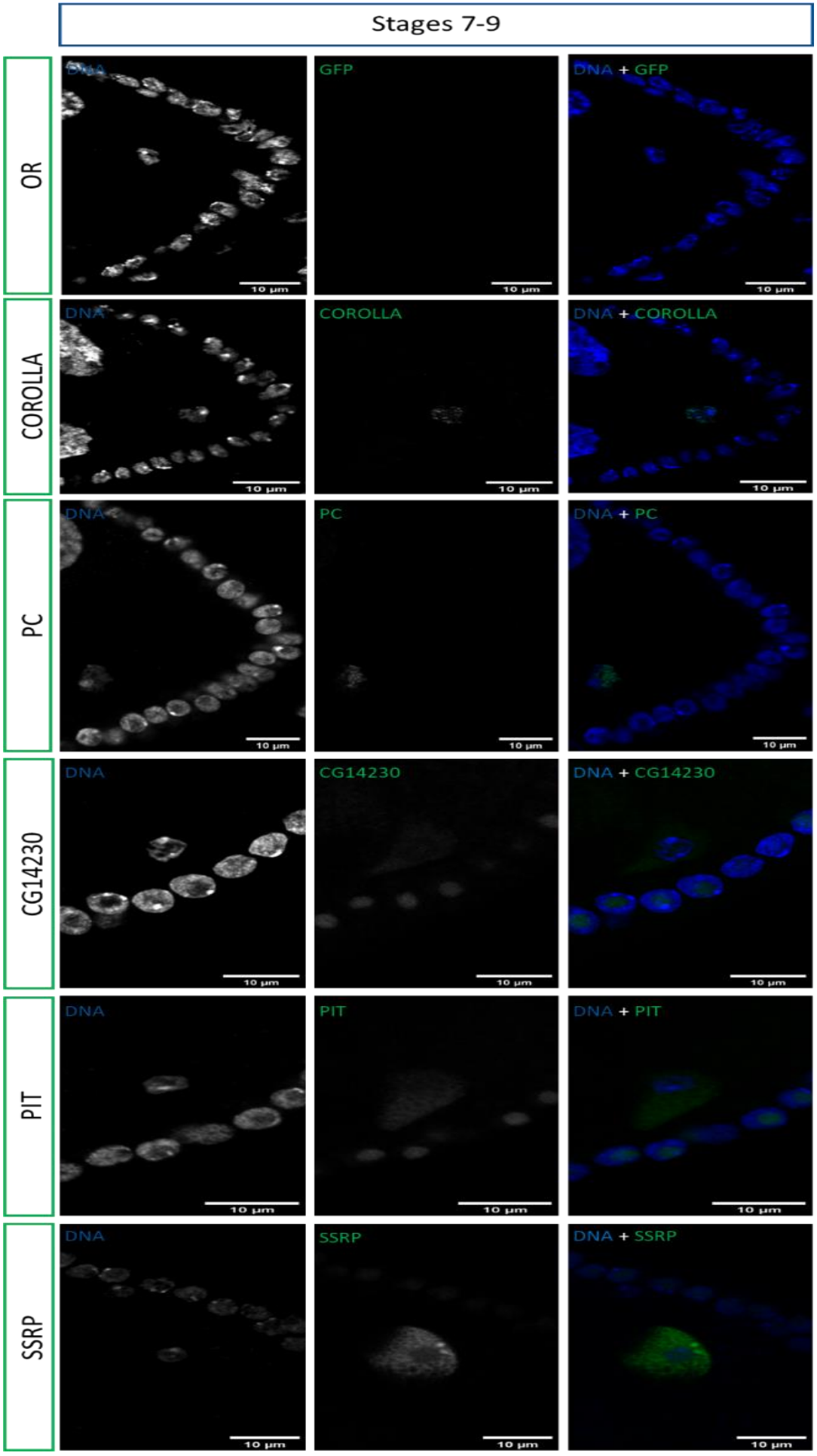
Stages 7-9
OR
DNA
GFP
DNA + GFP
COROLLA
DNA
COROLLA
DNA + COROLLA
PC
DNA
PC
DNA + PC
CG14230
DNA
CG14230
DNA + CG14230
PIT
DNA
PIT
DNA + PIT
SSRP
DNA
SSRP
DNA + SSRP
10 µm

Figure 16 - **Pit, SSRP and CG14230 are present in several subcellular structures in stages 7-9**.GFP signal was detected in stages 7-9 for all three positive results; OR, COROLLA and PC panel triplets, represent the negative control and both positive controls used respectively in this interval of stage; GFP signal was detected with an identical pattern for both PIT and CG14230, being present all around the Karyosome in the oocyte's nucleoplasm, but not colocalizing with the DNA; SSRP shows the same signal pattern in the nucleoplasm. SSRP also shows a colocalization with the karyosome's DNA and GFP signal in 2 point like structures flanking the karyosome's DNA

CHAPTER IV

4 Discussion

4.1 Imaging screen

As already stated, the genetic screening performed in this thesis' work aimed to identify potential genes involved in the regulation of key processes that allow the development of the oocyte during oogenesis. Through the use of GFP tagged versions of the candidate proteins tested, we looked for their presence in the karyosome. Through confocal microscopy we were able to visualize the localization of these candidates. We also analysed the earliest stages of oogenesis, the germarium, to test if there would be any interesting expression patterns of selected candidates.

Out of the sixty-five candidates tested, we considered three positive hits, which showed interesting GFP signal patterns in the images acquired. These, as stated in Chapter III "Results", were the proteins CG14230, SSRP and Pit.

In early planning stages for this screen, we hoped not for a big volume of positive results, which was proven to be true. However, we did hope that all positive results would fall into the main objective of this work, which was the presence of these candidates in the oocyte's nucleus, ideally in the karyosome's structure, colocalizing with its DNA; this was a weak point for our screen, since out of the three positive hits that we got, only one seems to be in fulfilment of the previously stated requirement – SSRP – since both pitchoune and CG14230 do appear to be present in the nucleus' domain of the oocyte, but not in the karyosome.

Although only one of our results is in complete alignment with the specific aim of this work, the patterns observed for SSRP are promising, since the GFP signal of this protein seems to overlap with the DAPI signal of the DNA, both in the most inner structure of the karyosome and periphery which, as will be discussed ahead, is in some extent in accordance with the literature already existent about this protein, and also supports the idea that this protein might regulate the transient transcription event that occurs in the karyosome during before meiotic progression. Other highlights of this screen include the fact that all three proteins seem to be present in the germarium phases, which opens the possibility for future studys how they might contribute to oogenesis development in that pre stage; adding to this, although not present in the karyosome, Pit and CG14230 display expression patterns inside the nucleus, which at least seem to confirm some aspects of their function already known.

In the following sections of this chapter, results obtained will be discussed and revised, for each gene in each of the stages studied.

4.2 SSRP

SSRP results show that this protein was present in all stages of oogenesis tested, depicting different patterns of expression for each stage, as seen in chapter III, "Results". The protein regarded as SSR constitutes one of the subunits of the hetrodimer FACT (Facilitates chromatin transcription); this complex protein, FACT, is capable of interaction with nucleosomes, as well as nucleosomal DNA recognition, functioning as a chromatin remodelling inducer (Tsunaka et al., 2009). FACT and consequently SSRP, are known to be involved in packing/unpacking of the nucleosomes during gene transcription, DNA replication and DNA repair, with SSRP being capable of DNA binding and histone interaction (Y. Liu et al., 2020); The DNA binding capability of SSRP was also shown by (Bruhn et al., 1992), where SSRP would bind specifically to DNA sequences modified with cisplatin. Not much is known about the regulation of FACT functions, however, according to (Y. Liu et al., 2020), the DNA binding region of the FACT complex is only revealed and accessible to DNA interaction upon the removal of the carboxy-terminal domains of SSRP and STP16 (the other subunit composing FACT), through its contact with Histone Dimer H2A-H2B; this will allow the FACT dimer to interact with DNA and Histones H3 and H4; (Tsunaka et al., 2009) also showed that SSRP can be phosphorylated/dephosphorylated as a means to regulate SSRP' DNA binding, especially in early embryogenesis stages where rapid chromatin remodelling events occur with coincident SSRP dephosphorylation.

Our results show that throughout stages seven through nine, two structures are marked with GFP signal, identifying SSRP in the periphery of the Karyosome, possibly interacting with its DNA or/and histones; we theorize that these structures might be nucleosome related, where SSRP, having relation to the FACT complex might be mediating chromatin remodelling events, like what is described above. SSRP is also shown to be seemingly colocalizing with a more intrinsic portion of the DNA of the karyosome in stages three through nine, which also reinforces the previous knowledge that SSRP is capable of DNA binding. One can theorize that this presence of the protein in earlier stages might represent an attached but inhibited form of

the protein, that might become active and ready for chromatin remodelling upon the brief transcription activity of the karyosome seen right before meiosis' progression; this inhibited state could possibly be achieved through phosphorylation of SSRP, as it is known that phosphorylation of SSRP impedes its interaction with DNA (Tsunaka et al., 2009).

Still in stages three through nine, all depicted a scattered pattern of expression for SSRP in what seems to be nucleoplasm domain. Not much information was found in the known literature that could explain this presence; nevertheless, it is possible that, since SSRP is shown to be related mostly to chromatin remodelling events, it would make sense that some quiescent inhibited forms of these protein could be stored in this space, making its recruitment to the DNA/nucleosome more immediate and faster.

In the pre-stage Germarium, SSRP was also shown to be present. As described in Chapter III "Results", its expression pattern is wavelike, showing GFP signal in region 1 of the germarium, slightly fading in regions 2A and 2B, with some subareas of this region 2B depicting complete GFP signal absence, and reappearing in region 3 right before the budding of the egg chamber. In these regions where SSRP shows to be present in the germarium, it appears that its GFP signal does overlap with some of the DNA's DAPI signal, as well occupying other cellular domains where DNA is absent.

The compact and dense structure of the germarium does difficult the distinction of cellular entities that compose the germarium, as some of the signal might be coming from form different cells such as exterior follicle cells, germ line cells (in the case of the most anterior portion of region one) and the pro-oocytes and cells that will give rise to the nurse cells. Even though this limitation is true, the presence of SSRP in this structure can still be discussed for most cells, since its function is related to chromatin remodelling, which is crucial for transcription; the fact that most of the different cells composing the germarium are transcriptionally active, reinforces the sense of these results, as only the oocyte, when defined, will enter transcription quiescence. The remaining cells will become nurse cells aiding with the production of all proteins and components necessary to the oocyte development (Hughes et al., 2018).

Taken this into consideration, the presence of SSRP in most of the cells is justified, since they are actively transcribing. As also said, some Germarium regions show a fading of the GFP signal for SSRP. This may be related to the fact that in these regions, the decision of which cell of the sixteen-cell cyst will become the oocyte was not yet taken, which would probably cause the dynamics of the chromatin in these cells to decrease, thus decreasing the expression of this

and other proteins during this time. As the cell fate decision is defined, chromatin dynamics would slowly go back in region 3, leading to the restoration of the levels of SSRP. Although plausible, more work needs to be done to test and verify these theories.

At last, a particular expression pattern was seen in stages three through five, which consisted in one or two dense points emanating a very strong GFP signal in the nucleoplasm, probably suggesting an agglomeration of SSRP in those locations. This pattern was also seen in the two other candidates considered positive in this work, as will be discussed ahead. Given the location and the display of this cluster of SSRP, one could assume that, for some reason the protein either is intervening in some DNA or RNA metabolic event that might require a great number of SSRP units, possibly acting on a substrate or that the protein is clustered, forming interactions between with more units of itself. Other theory falls upon the possibility that this protein might be involved in RNA processing, or at least is present while RNA processing is happening, since the other subunit of FACT, STP16 has a role on mRNA elongation (Tsunaka et al., 2009). On top of these theories, the most probable one might be that these might be nucleolar structures, where SSRP is known to be present (Swenson et al., 2016). Paralleling these results with the knowledge described above that SSRP is associated with chromatin remodelling and gene transcription events and with the knowledge that transcriptional quiescence of the karyosome is broken briefly during oogenesis (Navarro-Costa et al., 2016), it is fair to say that our results, copulated with further investigation, puts SSRP as a possible key player in the regulation of oocyte development and meiosis.

4.3 Pit

Pit (picthoune) belongs to the Dead-box Helicase family of protein and has as human homolog the protein MrDb (Zafran et al., 1998). Usually, this type of proteins contains ATP and RNA binding domains, behaving as helicases and are often associated with cancer and viral infections. Although not much is known about Pit itself, with no experimental evidence registered about the functions of this specific protein, the protein family that it seems to belong to, is involved in processes like ribosome biogenesis, ribonucleoprotein remodelling, export of RNA from the nucleus, regulation of transcription and RNA translation and nonsense-mediated decay (Schütz et al., 2010). Although most of the information available about these proteins

show that they are mostly related with ribosome and RNA metabolism events, with one of the most common function being double stranded RNA helicase, there is also evidence that they act as co-repressors/activators in gene transcription (Fuller-Pace, 2006; Schütz et al., 2010), which makes pitchoune a good candidate that could take part in the regulation of the development of the oocyte.

Unfortunately, no colocalization of this protein with the Karyosome's DNA was observed in all the oogenesis stages tested. Our results show that, throughout stages three to nine, Pit shows two patterns of localization, according to the GFP signal detected: 1. round agglomerates of this protein in the nucleoplasm; 2. A scattered-like pattern in all nucleoplasm.

For the first pattern we can assume that this represents the presence of this protein in the nucleolus, where it is mainly present according to (Zafran et al., 1998); its presence there also supports the fact that this protein has roles in ribosomal biogenesis and ribonucleoprotein remodelling as stated above.

Since this protein is a dead-box helicase and has roles in RNA processing and metabolism (Schütz et al., 2010), the scattered pattern across the nucleoplasm makes sense, since this is where most of these biological events take place.

For the germarium, Pit shows a consistent scattered pattern of expression in all its structure in areas around the DNA but, similar to the advanced stages, no colocalization with it as suggested by the absence overlapping signals of DNA and GFP. The fact that this protein is scattered around the DNA reinforces the already known fact Pit might be involved in RNA metabolism and Ribosome biogenesis, since all these events take place inside the nuclear space.

Although no presence in the DNA of the karyosome was detected for this protein, further investigation on its possible role in the regulation of transcription in the oocyte would be recommended, since other proteins of this family has shown to act as co-repressors and co-activators via the interaction with transcriptional machinery such as RNA polymerase II and histone deacetylases (Fuller-Pace, 2006).

4.4 CG14230

CG14230 is a *Drosophila melanogaster* gene of which very little seems to be known. It is known however that its human Ortholog is the gene Nol8 (nucleolar protein 8) (Gaudet et al.,

2011). Nol8 is a protein that is present in the nucleolus, which is the home of ribosome biogenesis and ribosomal RNA processing, as well as regulation of meiosis, mitosis and gene expression, and is known to have an RNA recognition domain (Jinawath et al., 2004)**.** Nol8 is also shown to induce exon skipping (O'Leary et al., 2009), which is a cellular mechanism of RNA splicing that allows to restore reading frames of defective genes (Aartsma-Rus et al., 2009).

The screen results obtained for this protein are very similar to the ones obtained for the Pit protein, with the advanced stages and early stages of oogenesis tested (stages three through nine) depicting a scattered GFP pattern around the karyosome and what seems to be clusters of this protein marked by round structures in the nucleoplasm (up to 1 or two observed). The pre-stage germarium also depicted some GFP signal marking the presence of this protein around DNA areas, making this pattern consistent in all stages of oogenesis examined; all stages analysed showed absence of colocalization of this protein with the DNA of the karyosome, and in the germarium no DNA colocalization was also observed.

Although this protein was not present in the karyosome, which was the ideal pattern set to be found in this work, this patterns can still indicate that this protein might be involved in the regulation of gene expression to a certain extent; assuming that CG14230 functions as its ortholog Nol8, it is expected that this protein does have the capability of recognise and interact with RNA (Jinawath et al., 2004) and act on mRNA splicing as Nol8 does. Our results do seem to be in accordance with this already published knowledge, since most of mRNA splicing is known to occur in the nuclear domain, thus justifying the abundant presence of this protein in the nucleoplasm. The round aggregates of this protein found in the nucleoplasm might also be associated with splicing activities, and, theoretically speaking, some of them might even represent spliceosome structures; Other possibility, and more probable according to the literature, is that these might be nucleolar structures where, as referred above, ortholog Nol8 is present; if CG14230 is to follow this same presence as suggested by our results, it is safe to infer that this protein might carry the similar ribosomal biogenesis and ribonuclear protein remodelling as Nol8.

In summary, the results obtained in this screen seem to be supported by the already published knowledge about these proteins or its orthologs, although their localization during oogenesis was first characterized during this master thesis All three proteins are shown to be present in nuclear space in abundance in the oocyte, which by itself is good indication of possible

regulation of gene expression to some extent; SSRP seems to be the most interesting one as the results suggest that it has direct contact with the karyosome's DNA structure, which is in accordance with its capacity for DNA binding, with the results also suggesting its interaction with histones, making a SSRP a possible gene expression regulator that acts directly at the level of the DNA and nucleosome unpacking. CG14230 and Pit, do not colocalise to the karyosome's DNA, but both are implied to be present in the nucleolus, implying their roles in ribosomal biogenesis, which seems to be confirmed in our results, assuming that the round shaped structures identified are nucleolar related. They are also inferred to have roles in mRNA processing, which would also justify their abundant presence in the nuclear space, where most of splicing activities take place, making these two proteins possible candidates for post-transcriptional gene expression regulation in the development of the oocyte.

CHAPTER V

5 Conclusions and future work

The results gained from the experiments executed during this master thesis allowed us to new putative t players in meiosis or chromatin regulation. Although this screening allowed us only to find out about the presence or absence of candidate proteins associated with the DNA of not only, but mainly, the oocyte, it may represent an opening gate for further investigation on the subject. Some of our results may corroborate, in minor scale, some of the already existent information about the candidate proteins considerate positive hits; by associating the gene ontology regarding the molecular functions of said proteins to their localization, we can now generate new hypothesis and plan functional experiments to consolidate this knowledge.

In a summary, three out of forty-four screened proteins were considerate as positive hits-Pit, CG1423 and SSRP -, with only one fulfilling the main objective, which was to find oocyte's DNA interacting proteins – the SSRP.

The highlights of our findings are: (1) all three candidates show colocalization with the DNA in the germarium, with Pit and CG14230 showing a stationary scattered pattern similar between them, and SSRP showing a wave like pattern, with some non-DNA-colocalizing GFP signal regions; (2) Pit, SSRP and CG14230 all seem to be associated to round aggregate-like structures, which are probably RNA processing related, making them potential RNA splicing players, or nucleolar structures, relating them to ribosomal biogenesis; (3) all three proteins seem to be scattered in nucleoplasm; and last, but not least, SSRP seems to be associated with DNA in stages three to five.

As for future work, functional testing for these proteins should be carried on finding more about how their disruption can affect fertility or development in the long run in *Drosophila melanogaster* females, whether by creating null mutants or inhibiting their expression via interference RNAs (RNAi).

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