UNIVERSIDADE DE LISBOA
FACULDADE DE CIÊNCIAS
DEPARTAMENTO DE BIOLOGIA ANIMAL

Defining a non-canonical role for Condensin II in transcription regulation during spermatogenesis

Neide Fernanda Meireles da Silva

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Dr. Paulo Navarro-Costa
Drª. Gabriela Rodrigues

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Abstract

Condensins are conserved protein complexes that are mainly recognized for their role in chromosome condensation and segregation. In Drosophila, previous studies have shown that Condensin II is essential for meiotic fidelity and male fertility. However, data from previous work in the lab suggest noncanonical roles for this protein complex in spermatogenesis, as rescuing the chromosome segregation defects of Condensin II mutants is insufficient to restore male fertility. Based on these observations, the current thesis work builds on the hypothesis that Condensin II is also required for the regulation of gene expression during male germ cell development.

Using cytological analysis, we confirmed that spermatocytes from mutants for the two non-SMC subunits of the Condensin II complex (CAP-D3 and CAP-H2) do not form the meiotic chromosome territories, despite being able to complete meiosis and initiate post-meiotic cytodifferentiation. We observed that cytodifferentiation was prematurely disrupted in these mutants, ultimately contributing to male sterility due to significantly reduced sperm counts. To directly test for a potential role in gene expression, we used RNA-seq of CAP-D3 mutant testes, revealing that the absence of Cap-D3 strongly affects the expression of 563 genes (239 up- and 324 down-regulated). 36 strongly downregulated genes were selected for functional testing using germ cell-specific RNAi (driver: bam-GAL4). This analysis revealed that the depletion of one of these downregulated genes - *Ogre* - recapitulated infertility and the testicular phenotype of the Condensin II mutants. In both cases, the silencing resulted in significant defects in the assembly and maintenance of the post-meiotic sperm individualization complexes.

Our data show that, in *Drosophila*, Condensin II is also essential for gene expression regulation during spermatogenesis. More specifically, Condensin II-mediated transcription is required for the correct differentiation of post-meiotic germ cells into mature sperm, thus uncovering a new function of this protein complex in male fertility.

Keywords: condensin II, *Drosophila*, spermatogenesis, gene regulation.

Resumo

A espermatogénese é um processo altamente especializado que engloba todas as etapas desde o início da diferenciação da célula germinal inicial até à individualização do espermatozoide. Devido à sua importância, este processo é altamente conservado entre espécies de organismos com reprodução sexuada. De um modo geral, a espermatogénese começa quando uma célula estaminal da linhagem germinativa inicia a sua diferenciação. Esta dá origem à espermatogónia que passa por uma fase de amplificação, que corresponde a várias rondas de divisão mitótica com citocinese incompleta, no fim das quais surgem os espermatócitos. Estes passam pelas duas divisões da meiose (ambas com citocinese incompleta, também) resultando nos espermatídios (células haplóides) que passam por uma fase de alongamento, seguida de individualização. Os espermatozoides resultantes deste processo são posteriormente encaminhados para fora do testículo, onde são armazenados até à cópula. Dada a natureza delicada deste processo, perturbações que alterem o seu normal decorrer podem ter repercussões significativas na fertilidade.

Durante o processo de diferenciação, as células passam por diferentes modificações conformacionais até chegarem ao espermatozoide maduro. A par destas alterações, a cromatina no interior dos seus núcleos vai também passando por uma série de alterações ao longo das diferentes etapas. Existem diversas proteínas envolvidas ao nível nuclear nestas transformações e entre elas encontram-se as condensinas.

As condensinas são complexos proteicos conservados, que são amplamente conhecidos pelo seu papel na condensação e segregação de cromossomas. Existem dois destes complexos identificados como Condensina I e Condensina II. Cada complexo é constituído por cinco subunidades das quais duas são partilhadas. Estas são ATPases e pertencem à família de moléculas responsáveis pela manutenção da estrutura dos cromossomas, sendo normalmente designadas por SMC2 e SMC4. As restantes três subunidades são específicas de cada um dos complexos: Cap-H, Cap-D2 e Cap-G constituem a condensina I; Cap-H2, Cap-D3 e Cap-G2 a condensina II. Ambos os complexos possuem funções diferentes, mas complementares. A condensina I atua ao nível da compactação lateral e a condensina II é responsável pela compactação axial. Estudos anteriores demonstram a relevância dos seus papeis nas divisões celulares, principalmente na mitose, a onde a condensina I é fundamental para o correto desenvolver do processo. No entanto, em *Drosophila,* foi demonstrado que é a condensina II o complexo essencial para a fidelidade meiótica e para a fertilidade masculina.

Nas células da linha germinal, a condensina II localiza-se no interior do núcleo, onde na interfase é responsável pelas alterações na organização da cromatina. Na ausência deste complexo funcional a prófase I dos espermatócitos é afetada sendo que deixa de ocorrer a formação dos três territórios cromossómicos. Apesar desta alteração, ao nível da organização da cromatina, as células conseguem prosseguir com a meiose e realizar a posterior citodiferenciação. No entanto, estudos realizados anteriormente reportaram que apesar da conclusão da meiose nestes machos há um aumento significativo dos erros na segregação de cromossomas como, por exemplo, pontes entre cromossomas, não disjunção e atrasos na movimentação (“lagging”), aquando da migração até aos pólos opostos da célula, na anáfase I. Estes defeitos levam ao aumento significativo de alterações na carga cromossómica de cada célula-filha que, por sua vez, culminam no aumento de casos de aneuploidia nos espermatozoides destes machos. Adicionalmente, machos que possuem este complexo com a sua funcionalidade afetada são também reportados como sendo completamente estéreis. Curiosamente, dados de trabalhos anteriores sugerem a possibilidade de existirem funções não canónicas para este complexo proteico na espermatogénese, dado que a correção destes erros de segregação nos mutantes para a condensina II é insuficiente para recuperar a fertilidade masculina perdida. Baseando-nos nestas

observações, propomos a hipótese de que a condensina II é também necessária para a regulação da expressão génica durante o desenvolvimento da célula germinal masculina.

Com o intuito de testarmos a nossa hipótese, usamos duas linhas de mutantes de drosófila, uma para cada uma das subunidades específicas da condensina II (Cap-H2 e Cap-D3). Inicialmente, procedemos com um conjunto de análises com o propósito de confirmar que estas linhas de mutantes recapitulavam os fenótipos previamente descritos. Usando a análise citológica, confirmamos que os espermatócitos dos mutantes para as duas subunidades não-SMC do complexo de condensina II não formam os territórios cromossómicos observados na meiose, apesar de serem capazes de completar a meiose e iniciar a citodiferenciação pós-meiótica. Adicionalmente, observamos que a citodiferenciação é prematuramente interrompida nestes mutantes na última etapa da espermatogénese – a individualização. A individualização, como o nome indica consiste na separação dos espermatídios-irmãos através da quebra das pontes citoplasmáticas existentes entre eles e na formação de uma membrana individualizada. São os complexos de individualização que levam a cabo esta função. Estes consistem em cones de actina que se formam em torno do DNA destas células (o que será a futura "cabeça" do espermatozoide), e se movem sincronizadamente através do seu axonema individualizando-as à medida que avançam. A interrupção desta etapa, acaba por contribuir para a esterilidade masculina dado que há uma redução significativa no número de espermatozoides produzidos por estes machos.

Para testarmos diretamente a condensina II para um potencial papel na expressão génica, realizamos uma análise da mesma recorrendo à técnica de RNAseq em testículos dos mutantes Cap-D3. Esta análise, por sua vez, revelou que a ausência de CAP-D3 afeta consideravelmente a expressão de 563 genes (239 sobre- e 324 sub-expressos). Foram selecionados para testes funcionais 36 genes, que tiveram a sua expressão mais significativamente reduzida. Usamos linhas RNAi cruzadas com um driver meiótico específico das células germinais (driver: *bam*-GAL4) e procedemos à realização de quatro rondas independentes de testes de fertilidade. Esta análise revelou que a remoção de um destes genes (*Ogre*) cuja expressão foi significativamente reduzida, recapitula a infertilidade e o fenótipo testicular observado nos mutantes para a condensina II. *Ogre* (de seu nome gânglio ótico reduzido) é um gene pertencente à família das inexinas, proteínas que estão envolvidas na formação de junções comunicantes. Na literatura existem referências a fenótipos resultantes da ausência deste gene, mas nenhum associado à fertilidade. Posteriormente, averiguamos se os machos, em que este gene se encontrava silenciado, apresentavam uma redução significa no número de espermatozoides como tinha sido anteriormente observado nas linhas mutantes. Após, percebermos que o mesmo era replicado por estes machos vimos ainda que em ambos os casos, o silenciar da expressão resulta em defeitos significativos na capacidade da célula construir e manter os complexos pós-meióticos de individualização de espermatozoides.

Quando olhamos para os nossos dados como um todo, vemos que na ausência de condensina II tanto a organização da cromatina como a expressão génica são afetadas. Esta alteração leva à diminuição da capacidade de as células conseguirem formar complexos de individualização que, por sua vez, culmina na diminuição da produção de espermatozoides. O gene *Ogre* parece ter um papel importante neste processo, já que junções comunicantes são essenciais para assegurar uma comunicação celular adequada na espermatogénese. Os nossos dados mostram assim que, em *Drosophila*, a condensina II é também essencial para a regulação da expressão génica durante a espermatogénese. Mais especificamente, a transcrição mediada pela Condensina II é necessária para a correta diferenciação das células germinais pós-meióticas em espermatozoides, demonstrando assim, uma nova função para este complexo proteico na fertilidade masculina. Em suma, propomos que a diminuição da produção de espermatozoides em conjunto com a aneuploidia anteriormente documentada contribuem ativamente para a esterilidade observada nestes mutantes.

Palavras-chave: condensina II, Drosófila, espermatogénese, regulação genética

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Abbreviations

DNA – Deoxyribonucleic Acid

GB – Gonialblast

JAK-STAT – Janus Kinase/signal transducer and activator of transcription

CT(s) – Chromosome Territory(-ies)

SMC – Structural Maintenance of Chromosomes

CAP – Chromosome Associated Protein

HEAT – Huntingtin, **E**longation factor 3 (EF3) 1, protein phosphatase 2**A** (PP2A) 2, and the yeast PI3-kinase **T**OR1

NEBD – Nuclear Envelope Breakdown

AMP – Antimicrobial peptides

SAC – Spindle Assembly Checkpoint

APC – Anaphase Promoting Complex

UAS – Upstream activation sequence

RNAi – Interference ribonucleic acid

Bam – Bag of Marbles

Rpl3 – 60S Ribosomal Protein L3

OR – Oregon-R

IGC – Instituto Gulbenkian da Ciência

CO_2 – Carbon dioxide

TB – Testes Buffer

RNA – Ribonucleic Acid

DNase – Deoxyribonuclease

RCF – Relative Centrifugal Force

RNase - Ribonuclease

RNAseq – Ribonucleic Acid Sequencing

H_2O – Water

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

WGA –Wheat germ agglutinin

GFP – Green Fluorescent Protein

B+P – Blocking and Permeabilization

BBT – PBS-T with BSA and donkey serum

PBS – Phosphate-buffered saline

PBS-T – Phosphate-buffered saline with 0.1% of Triton X (a detergent

ICs – Individualization Complexes

Ctrl – Control

Ogre – Optical ganglion reduced

Mut – Mutant

PCA – Principal Component Analysis

DGE – Differential Gene Expression

Zpg – Zero population growth

RT-qPCR – Reverse transcription-quantitative polymerase chain reaction

dH_2O – distilled water

KCl – Potassium chloride

NaCl – Sodium chloride

Tris-HCl – Tris-hydrochloride

EDTA – Ethylenediamine tetraacetic acid

PMSF – Phenylmethylsulfonyl fluoride

PFA – Paraformaldehyde

NP-40 – Nonionic polyoxyethylene surfactant

1 - Introduction

An appreciation of *Drosophila melanogaster* as a model organism

Since the beginning of the 20th century, scientists have been using *Drosophila melanogaster* to answer biological questions. Today, more than one hundred years later, *Drosophila* is one of the most used organisms in scientific research. Throughout this century of work, scientists were able to develop several methods of genetic manipulation and techniques that allowed them to maximize the understanding and increased the utility of this model.

The history of the fruit fly as a scientific model begins in the first decade of 1900 when Thomas Hunt Morgan selected these animals to perform a study on evolution. Although this choice was based on convenience (as it will be clarified later the text) it was the beginning of an incredible story of discoveries. In its initial project, Morgan tried to use different selective pressures as a means of inducing mutations. The experiment wasn't a success, but it led to Morgan discovering a white-eyed male among his wild-type (with red eyes) population. From this finding, he went on to show that genes are located in chromosomes and that chromosomes play an important role in heredity, as he reported that the white eye color was linked to X-chromosome inheritance (Morgan, 1910). One of his students, Hermann Muller, years later discovered that X-ray radiation-induced DNA mutations (Carlson, 2013). This finding at the time was not only revolutionary, but later as the atomic age began, his work was taken into consideration in terms of the genetic risks that the usage of atomic energy would pose to us, humans.

A few decades later, in the 70s, another major breakthrough happened with *Drosophila*. This time the field of developmental biology was forever changed by the discovery of what are now known as the Hox genes. Ed Lewis unveiled the role of the bithorax gene complex, revealing that this complex was able to control thoracic and abdominal development – affecting their respective segmentation patterns (Lewis, 1978). His work inspired other scientists to further explore the bithorax complex and another homeotic gene complex - the antennapedia (Kaufman et al., 1980; Garber et al., 1983; Sánchez-Herrero et al., 1985). These studies ultimately culminated in the discovery that both complexes were constituted by genes that coded for evolutionarily-conserved homeobox-containing proteins (Duboule, 2007). Later, the existence of four of these homeotic gene complexes (named Hox genes) was reported in vertebrates. Not only did these genes act similarly to their *Drosophila* homologs, but they also were proven essential for the correct development of all vertebrate species (Mallo et al., 2010). It was also thanks to the *Drosophila* model that we uncovered surprising things such as the genetic basis of behavior (Takahashi *et al.*, 2008), learning and memory (Alberini, 1999; McGuire et al., 2005).

The contribution of *Drosophila* to our actual knowledge is immense and most of it is due to the particular characteristics of this small insect. There are several aspects that make *Drosophila* one of the best and easiest organisms to work with. They are very easy and inexpensive to maintain due to their size and metabolic requirements. Additionally, as a result of its rapid life cycle, we can obtain many adult flies in a matter of days (Jennings, 2011). Although an insect species, *Drosophila* presents several similarities to mammals in different physiological processes. Organs such as the gut and the reproductive system have equivalent functions and organization compared with their mammalian equivalents (Hackstein et al., 2000; Apidianakis et al., 2011). One of the most striking qualities is the extensive homology between the Drosophila and mammalian genomes, with *Drosophila* having homologs for around 60% of all human protein-coding genes (Ugur et al., 2016).

Drosophila spermatogenesis

Spermatogenesis is a highly specialized process that includes all the steps starting from the differentiation of diploid male germ line stem cells to the individualization of haploid spermatozoa. Due to its importance, this process is well-conserved among species of sexual organisms, even at a genetic level (Correia et al., 2022). In the *Drosophila* early embryo, germ cells are located in the posterior embryonic region before initiating their migration through the hindgut primordium. After migration, they will finally settle in the embryonic gonads, alongside the somatic gonadal cells (Zhao and Garbers, 2002). These cells arrange themselves to create a spherical gonad (Le Bras and Van Doren, 2006; de Cuevas and Matunis, 2011). It is then that the *Drosophila melanogaster* testis begins its life as a disc. In this disc, there's the establishment of the germline system and the beginning of the differentiation process. In the late stages of testis development, it connects to the remaining structures of the reproductive tract that originated from the genital disc. Derivatives from this disc bind to the posterior region of the testis, leading to the migration of muscle cells that end up surrounding the testis (Kozopas et al., 1998; Whitworth Jimenez and van Doren, 2012). Together these two events lead to the elongation of the male gonad. The testis is then developed and takes the shape of a coiled tube with blunt ends where spermatogenesis occurs in a well-defined continuum throughout the fly's lifespan. Overall, there are four essential phases in spermatogenesis. Briefly, spermatogenesis begins with the commitment of the germ line stem cell to differentiation, thus originating a gonialblast (GB). This cell goes through a mitotic amplification cycle that consists of four rounds of division. The resulting cells enter meiosis (spermatocytes), and at the end of the two meiotic divisions we have the haploid spermatids. Finally, spermatids begin a process of cytodifferentiation (also known as spermiogenesis) that transforms these round post-meiotic cells into long, mature sperm cells (Fig1.1).

It is in the apical tip of the adult testis that we can find the male germ line stem cells. Like other stem cells, these male germ cell precursors require a specific cellular microenvironment (known as niche) to ensure their maintenance and function (Yamashita et al., 2005). In the *Drosophila* testes, this niche is composed of three different cell types: the germ line stem cells, the somatic hub cells, and the somatic cyst stem cells (Hardy *et al.*, 1979). Hub cells form a cluster that houses the other two cell types, ensuring proper cell signaling in the niche. The somatic cells from the hub are responsible for the maintenance of the germ cells through the activation of the JAK-STAT pathway (Tulina and Matunis, 2001; Kiger et al., 2001).

Germline stem cells divide asymmetrically, originating two different daughter cells: one that retains stemness characteristics (to maintain the stem cell population), the other acquiring a new cell fate (the GB) through the activation of a cellular differentiation program. This division is only possible due to the hub acting as a structural component, allowing an asymmetrical division by modulating the orientation of the mitotic spindle (Yamashita et al., 2003; Inaba et al., 2010). When germ cells enter mitosis, centrosomes move inside the cell so that the spindle forms in a perpendicular position in relation to the hub. With this conformation, upon division one of the daughter cells will keep in contact with hub cells (receiving its signals) and thus maintain its identity, while the cell that will be further away from the hub will enter the differentiation process (Kiger et al., 2000; Kiger et al., 2001). This way, after cell division is complete, the GB is encapsulated by two cyst cells and evicted from the hub. The cyst cells differentiate from the somatic cyst stem cells that are in contact with the hub. The communication that is then established between the somatic and the germ cell lineages is kept throughout all stages of spermatogenesis, and is essential for the correct differentiation of the male germ cell precursors into mature sperm (Yamashita et al., 2005).

Following this asymmetric division and the establishment of the germ cell–soma interactions, spermatogenesis begins with four rounds of mitosis with incomplete cytokinesis. This process is referred

to as mitotic amplification and ends with the formation of a cyst consisting of 16 spermatogonia all interconnected via cytoplasmic sharing through the ring canals. These canals form cytoplasmic bridges between neighboring cells, thus allowing the passage of products between cells and contributing to the synchrony of the processes and divisions inside the cyst (Guo and Zheng, 2004).

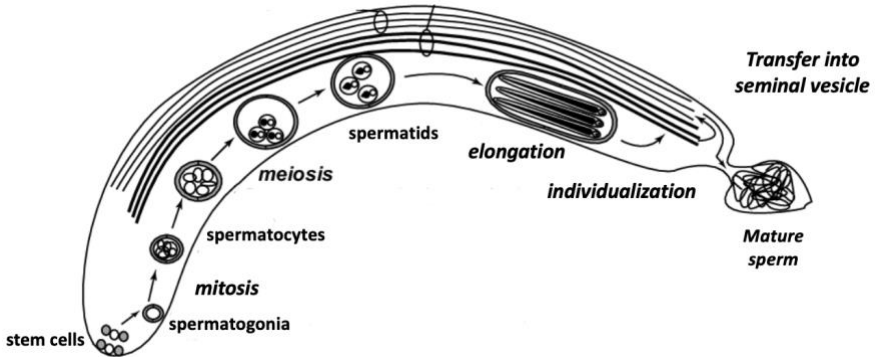

Figure 1.1 - Schematic representation of the Drosophila melanogaster spermatogenesis. *Inside the blunt testis all stages of spermatogenesis are present in a very-well defined continuum. In this image is possible to observe some of the cellular stages and all the steps that are part of this process. Adapted from Metzendorf, 2010.*

Spermatogonia undergo a premeiotic S-phase before beginning the program of cellular growth and nuclear enlargement that will prepare them to go through meiosis as spermatocytes. This requires cells to undergo several alterations and rearrangements at the nuclear level, ultimately affecting chromatin organization and transcription (Cenci *et al.*, 1994). It is at this stage that *Drosophila* male germ cells produce practically all transcripts that will be later needed to complete spermatogenesis. Therefore, by the end of this phase, the cellular volume of these cells is approximately twenty-five times bigger than that of mitotically amplifying spermatogonia, with cells becoming ready to undergo the two rounds of cell division that constitute meiosis (Cenci *et al.*, 1994). The two meiotic divisions result in a total of sixty-four haploid spermatids inside the cyst and, as in the preceding mitotic amplification, incomplete cytokinesis ensures that all daughter cells remain interconnected. The resulting spermatids will then enter a cytodifferentiation program, characterized by profound changes in cell morphology (Tokuyasu et al., 1972; Fabian and Brill, 2012). The most obvious being an elongation stage characterized by the formation of the sperm axoneme. Elongated spermatids complete spermatogenesis when they are released from the other germ cells in the cyst. This last stage – individualization - begins with the formation of actin cones around the sixty-four spermatid nuclei. The sixty-four actin cones will then move simultaneously along the spermatids' axoneme. During this movement, cytoplasm and vesicles accumulate around the actin cones, forming what is known as the cystic bulge. In this region, a new cellular membrane that will encapsulate the sperm axoneme is formed through the remodeling of the cyst cell membrane (Noguchi and Miller, 2003). When this process is finished the resulting mature sperm cells are transferred to and stored in the seminal vesicles.

Meiosis

In sexually reproducing animal species, meiosis is a key stage of gametogenesis as it produces the haploid cells required for fertilization. For this to occur, the cells replicate their DNA only once before entering two rounds of division. As in mitosis, such replication results in cells with two homologous chromosomes, each containing two sister chromatids. By the end of the two rounds of division (Meiosis I and II), each daughter cell will only have one of these chromatids. Meiosis I is referred to as a reductional division, since the homologous chromosomes segregate between the two daughter cells. On

the other hand, Meiosis II is, similarly to mitosis, an equational division as sister chromatids are segregated between the daughters (Petronczki et al., 2003). Organismal fertility largely requires that these divisions occur without errors. Meiotic defects, such as segregation errors, often result in aneuploidy, a process that can have extreme consequences for fertility and/or for the well-being of the offspring.

Meiosis is an extremely well-conserved process across eukaryotes, but in *Drosophila*, more specifically in *Drosophila* males, there are a few twists to the traditional scheme. The main one is that these males do meiosis without genetic recombination. For this reason, Drosophila male meiotic chromosomes are characterized by a lack of synapsis and crossovers, reflecting the absence of both the synaptonemal complex and chiasmata (Grishaeva and Bogdanov, 2018; McKee et al., 2012). To ensure faithful segregation, homologous chromosomes are already paired before the beginning of prophase I, and remain so until anaphase. As previously mentioned, male meiotic cells are referred to as spermatocytes, and in Drosophila these cells are located close to the apical portion of the testis, organized as 16 cell cysts kept together via cytoplasmic bridges.

After the fourth mitotic round, spermatogonia activate the premeiotic DNA synthesis phase (S phase), signaling their transition into primary spermatocytes (Cenci *et al.*, 1994). These cells progress to the largely atypical meiotic G2, characterized by a substantial increase in cell volume. At this stage, homologous chromosomes already exhibit high levels of pairing. In other species, such a high level of pairing is only observed later, in prophase I. This premature pairing is one of the biggest differences found in Drosophila spermatocytes, alongside the absence of the classic elements that characterize prophase I and the fact that paired chromosomes remain decondensed until prometaphase (Cenci *et al.*, 1994). For this reason, a specific nomenclature was defined to classify the G2 - prophase I continuum characteristic of Drosophila male meiosis. The continuum was divided into 7 phases, from S1 to S6 (with S2 divided into S2a and S2b). This categorization was established based on the distribution of chromatin inside the nucleus, as well as on nuclear size and shape (Cenci *et al.*, 1994).

The uniqueness of Drosophila male prophase I

In the first stage, S1, spermatocytes still retain a spermatogonia-like appearance, with compacted chromatin in the center of the nucleus (where the paired chromosomes are intertwined) (Fig1.2S1). Subsequent stages are characterized by substantial changes in chromatin organization as if a chromosome dance was happening inside the nucleus while the nuclear volume expands. During this dance, chromatin organizes itself into three different spatially defined units. Stage S2, which is divided into two phases, begins with the compact chromatin occupying the center of the nucleus (phase S2a) as displayed in Fig1.2S2a. During this stage, the cell increases its size, and chromatin changes. By phase S2b chromatin divides into three clumps that localize near the nuclear membrane. In this stage, it is very common to only observe two clusters of chromatin (Fig1.2S2b) that correspond to the two larger chromosome pairs – chromosomes II and III (Cenci *et al.*, 1994). However, in S3 spermatocytes the chromatin is perfectly divided into three well-defined regions, as seen in Fig1.2S3, known as chromosome territories (CTs).

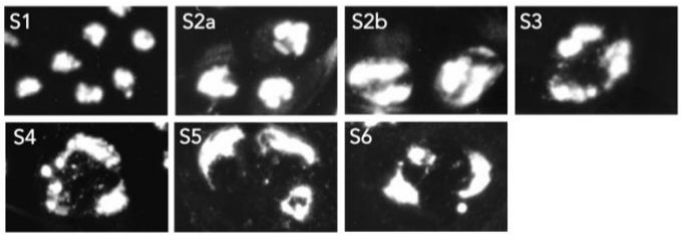

***Figure 1.2 - Drosophila male meiosis G2 to prophase I continuum**. This continuum is divided into 7 phases from S1 to S6. These phases were established based on the chromatin (seen in each image stained with Hoechst) distribution inside the spermatocyte's nucleus - as depicted in the figure. Additionally, it was also taken into consideration the nuclear size and shape for this classification (not shown). Adapted from Cenci et al., 1994.*

These CTs have moved from the center of the nucleus to the vicinity of the inner membrane of the nuclear envelope, each one opposing the other two territories. Each territory consists of the two homologs of the three major *Drosophila* chromosomes (Cenci *et al.*, 1994). The fourth chromosome pair, the smallest, is occasionally separated from the other chromosomes (not easily visualized) or tends to cluster with the sex chromosomes in their corresponding territory. It is also in S3 that the formation of Y chromosome loops starts. These loops only occur at the spermatocyte stage and correspond to fertility factors. Loop formation marks the beginning of the transcription of these fertility factors (Bonaccorsi *et al.*, 1988). In the S4 stage (Fig1.2S4), cells continue to increase their nuclear size and there's also the formation of a third and final loop. In S5, the nucleus has reached its maximum volume, with the sets of homologous chromosomes being the furthest apart from each other (Fig1.2S5). In this stage, the shape of the nucleus also presents some alterations to the classical round shape, acquiring a more uneven outline. The Y chromosome loops also reach their maximum size in these mature spermatocytes (Bonaccorsi *et al.*, 1988). Finally, in the last stage (S6) the nucleolus disintegrates, and the chromosomes finally begin to condense into compact round structures that remain at the nuclear periphery (Fig1.2S6).

Meiosis continued – the dynamics of chromosome segregation

Cells enter prometaphase after prophase I is complete. At this stage, the nucleus suffers a reduction in size, while microtubules begin to reorganize into two asters. These are star-like structures formed by centrosomes and their associated matrix and microtubules. The asters move to opposite sides of the nucleus, staying in close proximity with the nuclear envelope. Throughout these processes, the chromatin is also going through changes, with the bivalents becoming fully condensed and randomly distributed inside the nucleus. Around this time, the nuclear lamina begins to break down and chromosomes are moved into the correct metaphase orientation as the microtubules from the asters begin to reach in and bind to the bivalents.

Metaphase begins when the bivalents are finally organized in the metaphase plate. Homologous chromosomes are pulled to opposite poles in a movement that occurs very fast and marks the onset of anaphase I. The distance between homologs increases throughout the duration of anaphase, in preparation for the assembly of what will become the daughter nuclei. In telophase I, these nuclei individualize themselves from the cytoplasm and the cells acquire an hourglass shape as force is exerted on the microtubules that form the central spindle. As telophase progresses there's also an increase in the size of the nuclei and the chromatin decondenses and occupies the center of the nucleus. There's a new formation of asters that repeat their movement to the poles of the nucleus.

After this brief period, cells begin to prepare for Meiosis II. Chromosomes condense once more, and the nuclear lamina disassembles. Then microtubules are able to access and bind to the chromatids in order to complete prometaphase. After that, the chromosomes (each with its two chromatids) are moved to the metaphase II plate. Then anaphase II takes place through the swift movement of chromatids to opposite poles. During this phase, the daughter nuclei progressively increase their distance up until the onset of telophase II where the central spindle is compressed (conferring the diving cells an hourglass shape), and the two nuclei reassemble their nuclear envelopes.

In meiosis, chromatin condensation and organization are essential for division fidelity, requiring the involvement of different molecules and pathways. In this work, we will focus on the meiotic functions of key players for the structural maintenance of chromosomes.

Condensin Complexes

As previously mentioned, during spermatogenesis germ cells undergo substantial alterations in their morphology until finally becoming individualized mature sperm cells. During these changes, the chromatin inside the nucleus also experiences several conformational rearrangements. Some of the key players in this process are the multi-protein complexes known as condensins.

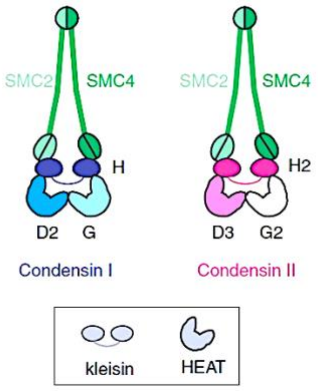

Figure 1.3 - Condensin Complexes architecture. *Conserved subunits SMC2 and SMC4 are ATPases. As indicated in the figure: Cap-H/H2 are kleisin subunits, Cap-G/G2 and Cap-D2/D3 possess HEAT repeats. Adapted from Hirano,2005*

In vertebrates, there are two different condensin complexes: Condensin I and II, both sharing the same structural maintenance of chromosomes (SMC) subunits (SMC2 and SMC4) and differing in their non-SMC subunits (Cap-H, Cap-D2, and Cap-G in Condensin I, and Cap-H2, Cap-D3, and Cap-G2 in Condensin II) (Hirano et al., 1997; Ono et al., 2003). The SMC subunits are part of a family of chromosomal ATPases; the Cap-H/H2 subunits are SMC-interacting proteins belonging to the kleisin family; and the remaining Cap-D2/D3 and Cap-G/G2 subunits possess tandem repeats known as HEAT repeats. When assembled as a complex, the SMC subunits form a V-shaped protein which is connected to the kleisin subunit (Cap-H or H2) that itself functions as a platform to facilitate the binding of the other non-SMC subunits (Fig.1). When all subunits are properly assembled, condensins acquire a ring-like structure reflecting the typical conformation of the SMC proteins.

As their name suggests, condensins are mainly known for their role in chromosome condensation and segregation during cell division. These complexes have different, yet complementary, functions; condensin I is responsible for lateral compaction while condensin II is responsible for axial compaction. Their localization dynamics inside the cell also differs: condensin I is located in the cytoplasm and is only able to interact with chromatin after nuclear envelope breakdown (NEBD) in prometaphase, whereas condensin II is found inside the nucleus in close contact with chromatin for the majority of the cell cycle (Ono *et al.*, 2003; Hirota *et al.*, 2004). This difference in localization is also related to the different roles of these complexes, as Condensin II initiates condensation during prophase, a process that is later continued by Condensin I when it gains access to chromatin (Hirano, 2005). After NEBD, both condensins act together to ensure correct chromosome assembly and the resolution of chromatids for metaphase. Later in anaphase, they are responsible for ensuring correct DNA segregation. It is proposed that these complexes achieve this through their loop extrusion activity. Chromosomes are known to be organized in chromatin loops that facilitate transcription, by simplifying enhancer-promoter interactions, for example, and even help promote segregation, among other things (Merkenschlager and Odom, 2013; Bompadre and Andrey, 2019; Yatskevich, Rhodes and Nasmyth, 2019). These loops were shown to be formed in vivo by SMC complexes like condensins (Ganji *et al.*, 2018). Condensins originate the loops by binding to chromatin pulling it and extruding it in the form of a loop.

Regardless of their well-established canonical role in chromosome compaction, over the last few years, new functions have been reported for these complexes and their subunits. Initially, condensins were only associated with mitosis but further investigation revealed that these complexes played a key role in chromosome architecture in meiosis. In particular, Condensin II is involved in homologous chromosome pairing as well as in the formation of the previously mentioned CTs. The latter are observed not only in meiotic prophase I spermatocytes but also in other cell types such as salivary glands and nurse cells (Hart et al., 2008; Bauer et al., 2012). Condensins have also been shown to be involved in the response to DNA damage, in the maintenance of the ribosomal DNA cluster, and even in the regulation of gene

expression (Kobayashi, 2006; Sakamoto *et al.*, 2011; Hirano, 2012; Lancaster *et al.*, 2021). This transcriptional role is likely associated with condensins' substantial effect at the level of chromatin organization.

The transcriptional role of Condensin II

Despite their well-established role as chromatin organizers, condensins have been implicated in several other non-canonical processes, one of them being the regulation of gene expression. This connection was first established in *Caenorhabditis elegans*, in association with a developmental process known as X-chromosome compensation (Meyer and Casson, 1986). All organisms whose sex is determined by chromosomes go through this process, despite through different mechanisms. This regulation of gene expression ensures that both males and females share equivalent levels of X-gene products despite having a different number of X chromosomes. As previously mentioned, there are different approaches to how an organism regulates these levels but in *C.elegans* the molecule responsible for this regulation is a condensin-like complex (Chuang et al., 1994). This protein complex is constituted by a homolog of the shared SMC subunits (SMC2 and SMC4) together with the condensin I non-SMC subunits Cap-H and Cap-D2 (Chuang et al., 1996). In *C.elegans*, this complex associates with the X chromosomes halving the gene expression levels of both chromosomes in XX cells (Chuang et al., 1996).

Some years later, work developed by Valerio Orlando's group reported that the *Drosophila melanogaster* protein Barren (also known as Cap-H) could promote gene silencing during embryo development (Lupo *et al.*, 2001). In *Drosophila*, the Abdominal-B gene controls the posterior body segment's identity (Sánchez-Herrero *et al.*, 1985). This is achieved by the existence of segment-specific domains. Each segment is composed by a series of silencers and enhancers that are flanked by insulators. The activity of these domains is controlled by embryonic proteins that are transiently expressed and contribute to the patterning. The inactivation of these domains is driven by the Polycomb proteins as they bind to a series of domain-specific regulatory elements (the Polycomb response elements) (Simon and Tamkun, 2002). Through chromatin immunoprecipitation assays it was observed that Barren, alongside with Topoisomerase II, was specifically binding to Polycomb sequences, hence that Cap-H2 was involved in gene silencing. Additionally, it was also observed that Barren mutants had defects in Polycomb response elements-mediated gene silencing (Lupo et al., 2001). This observation gave additional support to the idea that condensins were directly involved in gene expression regulation.

Collectively, these studies were only the beginning of a series of reports on how condensins influence gene expression. The first observations pointed to a repressive role, with condensins (mainly condensin I) promoting gene expression silencing. More recently, several studies have shown that these complexes are also able to upregulate gene expression.

Among the latter, a study performed in mouse embryonic cells reported that Condensin II interacts with the transcription factor IIIC complex and together they localize to active promoters. Such localization is facilitated by the interaction of the Cap-D3 subunit with H3K4me3 - an epigenetic modification in histone H3 that is recognized by the cell as a transcriptionally permissive mark (Beacon *et al.*, 2021). More importantly, it was shown that Condensin II is essential for the maintenance of high expression levels of histone gene clusters (containing canonical histone genes: H2A, H2B, H3, and H4), and to allow the establishment of interactions between these clusters (Yuen, Slaughter and Gerton, 2017). Also in human cancer cells, Condensin I and Condensin II are reported to have a role in the positive regulation of the estrogen ligand-dependent enhancer activation, by allowing the full enhancer RNA transcription and the formation of the enhancer-promotor looping that results in the coding gene activation (Li *et al.*, 2015). Additionally, there are also studies of Condensin II-specific subunits acting alone as transcription regulators. Such is the case of the Cap-D3 subunit, which is required for the transcriptional activation

of the antimicrobial peptide (AMP) genes in *Drosophila* fat body cells. In these cells, Cap-D3 binds directly to the diptericin locus (one of the Drosophila AMP genes) thus promoting its expression (Longworth *et al.*, 2012).

Condensin II and *Drosophila* male meiosis

In *Drosophila*, contrary to what is commonly observed in other species, Condensin II only has four subunits (Herzog *et al.*, 2013). More specifically, the sequence for the G2 subunit seems to be missing from the fruit fly genome. Despite this particularity, the *Drosophila* Condensin II complex has been shown to be essential for spermatogenesis. Condensin II localizes in the interphase nucleus and in *Drosophila* male germ cells it is not only responsible for the beginning of condensation, but also for antagonizing homologous chromosome pairing and transvection (Bauer, Hartl and Bosco, 2012; Smith *et al.*, 2013). In the absence of functional Condensin II, spermatocyte prophase I is affected: as soon as cells enter the S2 stage it is possible to detect defects in chromosome architecture, as revealed by the incapability of chromatin to separate into two distinct blobs. By S3, cells do not form the typical three well-defined CTs, as chromatin appears distended and spread throughout the nucleus (Hart *et al.*, 2008). Despite these profound changes, cells are still able to progress through and complete meiosis: previous work has shown that, by metaphase, chromosomes are able to condense. However, the separation of homologous chromosomes at the onset of anaphase occurs with a significant increase in segregation errors (Hart *et al.*, 2008). Condensin II-defective cells are characterized by extensive Meiosis I segregation errors such as chromosome lagging, bridges, and non-disjunction. These errors are propagated to Meiosis II and result in aneuploidy. Of note, Condensin II-defective males were also found to be completely sterile. Not surprisingly, meiotic defects were typically considered the cause of male sterility, given the dramatic effects of paternal aneuploidy for embryo development (Siegel and Amon, 2012).

However, previous work performed in the lab strongly suggests that other factors need to be invoked when explaining the sterility of Condensin II-defective males. The work of Cíntia Ramos and colleagues has explored the role of Condensin II in meiosis by using Cap-H2 mutants (Horta, Tavares and Oliveira, 2022). Their data have shown that meiosis occurs faster in these mutants since the lack of Condensin II impairs the spindle assembly checkpoint (SAC). Thus, these cells are not capable of delaying segregation until the correct attachment of chromosomes to the spindle microtubules is guaranteed. Interestingly, the rescue of the segregation defects was made possible by artificially extending division time. To do so, a strategy of diminishing the levels of the anaphase-promoting complex (APC) and consistently delaying meiotic progression was adopted. By performing RNAi against one of the APC subunits (the cdc23 subunit) in Cap-H2 mutants, Horta and colleagues were able to prolong the duration of Meiosis I from approximately 25 to 60 minutes. Such strategy led to a significant reduction of segregation errors to values comparable with that of controls. After this successful rescue of meiotic fidelity, it was expectable that fertility would also be rescued. Surprisingly, the rescued males remained sterile (Horta, Tavares and Oliveira, 2022). The Giovanni Bosco lab also performed an equivalent experiment, using a different Cap-H2 mutant line and another meiotic rescue strategy. In this work, they also observed several segregations defects – mainly anaphase I bridges. In order to attempt to rescue this defect, they inserted a mutation in the Teflon gene on their Cap-H2 mutant background. This gene is known for its role in promoting the maintenance of autosome pairing (Tomkiel, Wakimoto and Briscoe, 2001; Arya *et al.*, 2006). Without it, these mutants lose autosomal pairing even before anaphase I (Arya *et al.*, 2006). Thus, the Cap-H2 mutant males with this background had a significant decrease in segregation defects. As in the case of Horta and colleagues' work, the successfully rescued males remained sterile (Hart *et al.*, 2008), suggesting that there might be more to Condensin II in spermatogenesis than just chromosome segregation.

It is from these observations that the hypothesis that drives this work was born. If the aneuploidy resulting from segregation errors in Condesin II-depleted cells is not sufficient to fully explain the sterility phenotype, it follows that this complex must have other functions in spermatogenesis. Accordingly, we posit that Condensin II is also required for gene expression regulation in the male germ line. To test this hypothesis, I analyzed a possible effect of Condensin II on male germ cell transcription and its functional implications for post-meiotic development. How these analyses were performed, the corresponding results and their possible significance will be the focus of the next chapters.

2 - Materials and Methods

Stocks of *Drosophila melanogaster*

Drosophila upstream activation sequence (UAS) lines with RNAi hairpins targeting each of the Condensin II subunits (Cap-H2, Cap-D3, SMC2, and SMC4 were purchased from the Vienna Drosophila Resource Center and the Bloomington Drosophila Stock Center. Lines for the Cap-D3 and Cap-H2 mutants were ordered from the Bloomington Drosophila Stock Center (#15026 and #2608 for Cap-D3 and Cap-H2, respectively). These lines were selected based on previous reports of male sterility and meiotic chromatin organization defects(Savvidou *et al.,* 2005; Hart *et al.,* 2008). Analyses were performed on hemizygous males resulting from crosses with corresponding chromosomal deficiencies: Cap-D3^{EY00456}/Cap-D3$^{Df(2L)\ Exel7023}$ and Cap-H2^{TH1}/Cap-H2$^{Df(3R)\ Exel6159}$ (#7797 and #7638 for Cap-D3 and Cap-H2, respectively). RNAi lines for candidate effector genes for the sterility phenotype of the Condensin II subunit mutants were also purchased from the Vienna and Bloomington stock centers. Please check Table SM1 for a list of all RNAi lines used in this thesis.

For the gene silencing experiments, UAS lines were crossed with a *Bam*-GAL4 line (gift from Renate Renkawitz-Pohl, Philipps University of Marburg) with the purpose of silencing the genes at the beginning of meiosis. We had two controls for the fertility tests using these lines: a negative control line with a RNAi against the mCherry fluorophore (not present in the genome of the tested flies), and a positive control line with RNAi against Rpl3 (a ribosomal protein indispensable for cell survival) (#35785 and #36596, respectively). For the mutant crosses, we used as controls males resulting from the following crosses: Cap-D3$^{Df(2L)\ Exel7023}$/wild-type and Cap-H2$^{Df(3R)\ Exel6159}$/wild-type for the Cap-D3 and Cap-H2 mutants, respectively. Wild type corresponds to an Oregon-R (OR) stock. It is originally from the Roseburg region in Oregon (United States of America) and was first collected in the year 1925 (Zych, 2008). Since then, it has become an extremely inbreed population and it is widely used in *Drosophila melanogaster* studies as it provides a great baseline for comparison. The stock used in this work was taken from Instituto Gulbenkian da Ciência's (IGC) stock collection.

Additionally, the RNAi lines against the Condensin II subunits were also crossed with a *Nanos*-GAL4 line. We used the same negative and positive controls for this assay as for the *Bam*-GAL4 experiments but crossed with the *Nanos*-GAL4 stock.

Food media

Food media was prepared according to the Vienna recipe (ingredients and quantities in Supplementary Material – Table SM2) and was ordered from the Champalimaud Foundation's *Drosophila* platform.

Rearing Conditions

All fruit fly lines were kept in vials (27 mm diameter, from SARSTEDT), except the OR, Nanos-GAL4, and Bam-GAL4 lines that were exclusively kept in bottles (51mm diameter, from SARSTEDT). Lines were kept at 22ºC (room temperature). Moreover, the Cap-D3^{EY00356} and Cap-H2$^{Df(3R)\ Exel6159}$ lines were also kept in bottles to increase virgin acquisition. These bottles were flipped once or twice per week

depending on the number of virgin females needed for crossings. Mutant crosses were also performed in bottles due to the number of males needed for experiments. The UAS-RNAi and *Nanos*-RNAi crosses were all performed in vials.

Fly pushing

For fly selection, transfer, and overall handling, we used carbon dioxide (CO_2) to anesthetize the flies and handled them with a soft paintbrush to avoid damage. *Drosophila melanogaster* has sexual dimorphism, so there are several differences between males and females. For example, males are smaller than females, have darker posterior plates, and possess sex comb structures (Ashburner et al., 2005). This makes males and females easily distinguishable and simplifies the selection of flies for the crosses.

Virgin Collection

Drosophila females can store sperm from previous matings and use these cells to fertilize their eggs at a later time (Ashburner et al., 2005). Therefore, the only way to guarantee that the whole progeny results from our intended crossing scheme is to resort to the use of virgin females. To select them, we collected only recently eclosed females (with around 3-6 hours of age). Furthermore, these females were then kept in vials for at least 24 hours before being used, to guarantee that they had not been inseminated. This virgin collection was done two to three times per day.

Fertility Tests

The male reproductive fitness of selected genotypes was quantitated by 4 rounds of independent fertility tests. In each, 2 males of the genotype of interest were placed in a vial with 4 virgin wild-type females for 24h at 25ºC with a light cycle of 12 hours light /12 hours darkness. Afterward, flies were discarded, and the laid eggs were counted. The vials were then returned to the incubator where they were kept in the same conditions for another 24h. After this incubation, the number of eggs that had hatched were counted. The overall fertility rate was calculated as the average of the number of hatched eggs divided by the total number of eggs across the 4 independent tests. Male reproductive fitness was considered affected if the overall fertility rate was lower than 75% (Correia et al., 2022).

Testes and Seminal Vesicles dissection

Selected male flies were anesthetized with CO_2 and decapitated. Afterwards, the testes and seminal vesicles were isolated using a stereoscope (Leica MZ12.5 with objective Leica 10446230), in a drop of Testis Buffer (TB, on table SM3) placed on a dissection plate. This process consisted in the removal of the reproductive system by an incision in the anal plate performed with suitable tweezers (nr.5 – 11295-10 Dumostar-Biology).

Cytological Analysis

Phase contrast microscopy was employed to observe different male germ cell developmental stages and to qualitatively assess sperm production. Following dissection, testes and seminal vesicles were immediately transferred to a slide with TB. On average, each preparation consisted of 4 pairs of testes in 4µL of TB. After adding a cover slip, these squash preparations were immediately analyzed in a phase contrast microscope (Nikon Eclipse E400). Images were acquired with an IDS camera (UI-3370CP-C-HQ) using the uEye software. The process after testes dissection was very fast to preserve the tissue intact and the cells alive. This was particularly important when looking at sperm cells, as their movement (or lack thereof) is relevant to identify the possible cause of infertility.

RNA sequencing analysis

Total RNA was extracted from *Drosophila* testes using the PureLink RNA Mini Kit with the PureLink DNAse Solution (Invitrogen). The testes of 30 Cap-D3 mutant and 30 control males were dissected and

temporally stored in wells with 200uL of TB in ice. They were then transferred to an eppendorf with 50µL of PureLink RNA Mini kit lysis buffer supplemented with 2-mercaptoethanol. Testes were disrupted with a pestle, followed by the addition of 150µL of supplemented lysis buffer and of 200µL of 70% ethanol. From this solution, 200µL were loaded in a spin cartridge (with a collection tube) and the liquid soaked the filter for 1 minute. The spin cartridges were then centrifuged for 15 seconds at 16,000 relative centrifugal force (rcf) and the resulting flowthrough was discarded. 350µL of Wash Buffer I was added before another centrifugation round. Afterward, Wash Buffer II was added (500µL) prior to another centrifugation. The cartridge was then transferred to a new collection tube and centrifuged for 1 minute at 16,000 rcf. Finally, the cartridge was transferred to a recovery tube and 30µL of RNAse-free H_2O were added prior to a 1 min incubation. Then, a final centrifugation was performed (1 minute and 30 seconds at 16,000 rcf), with the eluate (total RNA) being collected and stored on ice for immediate quantification (Nanodrop). RNA was stored at -80ºC until analysis. This process was done in parallel for mutant and control males and repeated 3 times (temporally independent replicates). Males with 3 to 9 days of age were used for all extractions.

The RNA samples obtained were submitted to IGC's genomic unit where they were processed for both quality and sequencing. The quality of the RNA samples was analyzed using the Agilent Method DNF-472T22 - HS Total R and was performed in the 5200 Fragment Analyzer (software version: 1.1.0.11). cDNA libraries were prepared using the Zymo-Seq RiboFree Total RNA library kit. Finally, the sequencing was performed in a Ilumina nextseq. Gene expression analysis was performed by Daniel Sobral (Instituto Nacional de Saúde Dr. Ricardo Jorge), with differentially expressed genes fulfilling the following criteria: having a detectable expression in both mutant and control groups (log2CPM>1), the difference in expression between controls and mutants should be of at least two-fold [-1<log2(FC)>1], and that the difference observed needed to be statistically significant (FDR <0.05).

Immunofluorescence and Confocal Imaging

To confirm the previously reported chromatin phenotype in spermatocytes from Condensin II subunit mutants, the chromatin (DAPI; that was present in the Vectashield mounting medium solution (H-1200)) and the nuclear envelope (WGA or mouse anti-Dm0 Lamin) of these cells were analyzed. A mouse anti-Dm0 Lamin (Adl 84.12 c; Hybridoma Bank) antibody was used (1:100 dilution), with detection being performed by an anti-mouse secondary antibody conjugated with the AF488 fluorophore (1:1000). Alternatively, WGA was used in a 1:500 concentration. For the observation and scoring of sperm Individualization Complexes, DAPI was used to stain the sperm heads while phalloidin conjugated with Tetramethylrhodamine B (Phalloidin-TRITC, Mfcd00278840) allowed the visualization of the actin cones.

For the immunofluorescence staining, males were dissected as previously indicated and testes were collected in a glass plate well with 200µL of TB. When all testes had been collected, TB was removed from the wells and immediately replaced with 200µL of freshly prepared pre-fix solution (Table SM4). Testes were then transferred to a protein low-binding 1.5mL tube containing heptane (600µL) and fix-solution (200µL). Fixation was performed by incubating for 20 minutes in a rotating platform. Afterwards, testes were washed 3 times in PBS 1x before being transferred to a standard tube with 1mL of Blocking and Permeabilization solution (B+P) (Table SM8) and incubated with rotation for 1 hour. Next, this solution was substituted by 500µL of BBT (Table SM9), in preparation for a 5-minute incubation. Subsequently, the tissue was incubated for 1 hour in rotation and protected from the light, and in a BBT solution containing AF647-conjugated WGA (1:500). Then the tissue was washed 3 times (five minutes each) in PBS-T. Whenever phalloidin staining was also performed, it would be added in the second wash in PBS-T at a dilution of 1:200, for 10 minutes with rotation. At the end of the washes, the tissue was rinsed in 500µL of PBS 1x, prior to the addition of 2 drops of DAPI-supplemented

Vectashield mounting medium (H-1200). Finally, the testes in the mounting medium were transferred to a slide (VWR Microscope slide Ground Edges), covered with a 20x20mm coverslip, and the preparations were sealed with nail polish for storage at 4ºC.

In the following days, we would take these preparations to the Confocal Microscope and we would acquire images.

Image Acquisition and Analysis

Images were acquired with a Leica Stellaris5 Confocal Microscope, with the objective HCX PL APO CS 63.0x1.4 oil, and under the following settings: format – 512x512, speed – 400 Hertz, zoom factor was either 0.75 (for the distal portion of the testes) or 10 for S3 spermatocytes, the pinhole – 1 Airy Unit, the smart intensity was kept around 4%, the smart gain was adjusted whether we were observing testes or spermatocytes staying around 40-60%. The acquisition software was the Leica Application Suit X, version 4.5.0.25531. ImageJ (Fiji for Mac OS X version 1.53v) was used to process confocal and phase contrast images.

When assessing S3 spermatocytes, we acquired 20 images from different testes for both mutants and controls. In the controls, this phenotype is very clear due to the chromatin organization and size of the nuclear envelope. On the other hand, in the mutant lines, we identified the S3 spermatocytes due to the size of the nuclear envelope and the position of these cells in the testis. Spermatocytes were classified into three groups based on chromatin condensation and organization. We defined them as compact territories – with chromatin divided into three compact territories; diffuse territories – chromatin appeared distended, but it was possible to visualize a certain organization into what appeared to be the chromosome territories; and no territories – when chromatin appeared not only distended but also completely dispersed.

The assessment of sperm production was indirectly assessed by the quantity of sperm visible inside of the seminal vesicles. In this assay, we scored 20 seminal vesicles from both mutant lines males, *ogre*-silenced males, and their respective controls. The seminal vesicles were then distributed into three groups. These were: full – these seminal vesicles were large and completely full sperm cells; empty – in these seminal vesicles no sperm cells could be detected (and they also were smaller than the full ones); and residual – in these seminal vesicles we were able to observe only a few sperm cells, despite being quite similar to the empty seminal vesicles.

To analyze the integrity of the individualization complexes, we collected images from 20 of these complexes for each of the mutant lines, *ogre* RNAi, and controls. Despite observing different types of defects with different severity levels, we decided to simplify this quantitation and divide the ICs into two categories: regular IC – with the actin cones arranged in an almost perfect line parallel to the sperm DNA; and irregular IC – where we grouped all defects ranging from the asynchronous positioning of the actin cones to the more severe cases with scattered cones.

Statistical Analysis

Statical analysis was performed using GraphPad Prism version 6.01. The reported P-values correspond to two-tailed unpaired tests.

3 - Results and Discussion

Selection of an experimental strategy to interfere with Condensin II in spermatogenesis

Initially, we intended to use in this work RNAi-silenced males for Condensin II-exclusive subunits (i.e., of the non-SMC type). This would give us a more adequate genetic background control for the subsequent gene expression analysis we were planning to perform. Therefore, we began this project by testing all commercially available RNAi lines targeting the Condensin II subunits (both exclusive to this complex and shared with Condensin I). Previous studies had reported that Condensin II mutant males were sterile (Hart *et al.,* 2008), so our expectation was to recapitulate this phenotype using the RNAi lines.

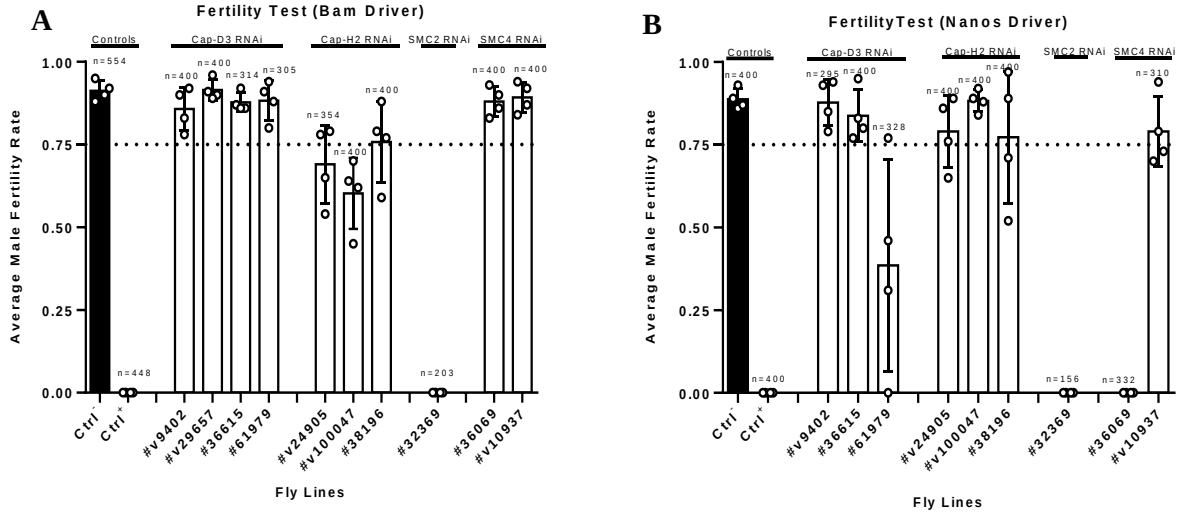

***Figure 3.1 - Fertility tests with RNAi lines for Condensin II subunits**. **(A)** RNAi lines crossed with meiotic driver Bam had their fertility accessed. The same RNAi lines were crossed with Nanos driver **(B)** expressed in germ-line stem cells. Neither of the crossings repeated the sterility phenotype previously reported for Cap-D3 and Cap-H2 subunits. The empty dots represent the results of each individual fertility assay. The dotted line represents the arbitrary cutoff of 0.75 below which we consider fertility affected. The negative control (mCherry) is shown with a black bar. n – represents the total number of eggs counted.*

All lines were crossed with flies carrying the *Bam*-GAL4 driver, a classic spermatogenesis driver (White-Cooper, 2012) that induces the silencing of the targeted genes in the late spermatogonia stage prior to meiosis. The reproductive fitness of the RNAi-silenced males was tested in four independent fertility assays, by mating tested males with wild-type virgin females and quantitating the resulting egg-hatching rate (fertility rate). In Fig.3.1A, we present the individual results of these four tests (each result represented by a circle) and the overall average fertility rate of each tested line (represented by a vertical bar). Based on our previous results using a similar set-up to identify evolutionarily conserved spermatogenesis genes (Correia et al., 2022), fertility rates below 0.75 were considered indicative of impaired male reproductive fitness.

Quite unexpectedly, all tested Cap-D3 lines had high average fertility (>0.80) and while two of the lines for the Cap-H2 subunit (#v24905 and #v100047) had decreased fertility, they were far from being sterile as expected (fertility rates of approximately: 0,69 and 0.60, respectively). Only the line for the shared SMC2 subunit had a substantial impact on fertility, with the silenced males being completely sterile. However, we could not use this line for further analyses as it would not allow the distinction of Condensin II-specific defects from those originating from the disruption of Condensin I.

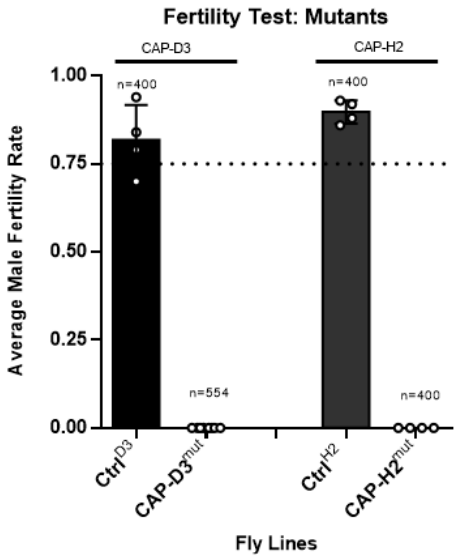

Figure 3.2 - Fertility tests of the mutant lines confirmed the sterility previously reported. *The controls for the respective mutant lines are shown with dark and gray bars. The empty dots represent the results of each individual fertility assay. The dotted line represents the arbitrary cutoff of 0.75 below which we consider fertility affected. n – represents the total number of eggs counted.*

Based on these results, we hypothesized that the lack of particularly noticeable defects in fertility was due to the silencing being induced at a sub-optimal developmental stage. Since we had used a driver that induces silencing upon meiotic entry (*Bam*-GAL4), we envisaged that Condensin II could have already exerted its functional role on the germ cell chromosomes by that point. In order to test if that was the case, we crossed the same RNAi lines this time with a *Nanos*-GAL4 line (*Nos*-GAL4; Fig.3.1B). Since *Nanos* is expressed at the very initial stages of germ cell development (primordial germ cells) and also in male germline stem cells (Doren, Williamson and Lehmann, 1998), it would allow us to identify a possible earlier role (prior to the mitotic to meiotic transition in the adult gonad) of Condensin II in the male germ line. Several differences were observed when comparing the *nos*-driven silencing with the *bam* one. For starters, one of the Cap-D3 lines (#61979) had a steep decline of its male fertility rate (from 0.88 to 0.38), although still far from the sterility phenotype we were expecting to recapitulate. In addition, one of the SMC4 lines (#36069) now presented a male sterility phenotype, alongside the SMC2 line which was already sterile with *bam*-GAL4. For the reasons previously stated for the SMC2 line (the impossibility to distinguish the effects of Condensin II from those of Condensin I), we also could not use the SMC4 line for further work. Collectively, these experiments indicated that the RNAi approach was not suitable for our needs. Despite trying different promotors, the RNAi lines against the Condensin II-specific subunits did not recapitulate the sterility phenotype of the previously reported mutants. Different reasons could explain this result. Since Condensin II protein levels are already so low under normal conditions (Herzog *et al.*, 2013), an RNAi approach might not be the best option to interfere with the gene (it is possible that the transcripts that escape silencing are still enough to ensure Condensin II function). The fact that Condensin II has a very fast half-life, hence it is being consistently produced and degraded, can also explain why we could not recapitulate this phenotype. Or (quite unlikely), Condensin II acts via a non-germ cell-specific mechanism (i.e., silencing the gene in germ cells only has a partial effect). The efficacy of the RNAi itself could be an issue. With our positive control, we can establish that our GAL4 driver is working, but we would only be able to confirm that the RNAi line is actually silencing the targeted gene if we analyzed the transcript's expression level.

Based on the results of the RNAi assay, we went back to the literature and selected previously reported Cap-H2 and Cap-D3 mutant lines that had been associated with male reproductive impairment. These selected mutants (hemizygotes) are the product of crossing a loss-of-function mutation with the respective deficiency line: Cap-D3^{EY00456}/Cap-D3$^{Df(2L)\ Exel7023}$ and Cap-H2^{TH1}/Cap-H2$^{Df(3R)\ Exel6159)}$.

To confirm that the resulting male progeny of these crosses was sterile and therefore matched what had been previously reported, we quantitated the reproductive fitness of these males as before, with four independent fertility tests for each condition. As controls, we used the male progeny resulting from the cross of each deficiency line with a wild-type stock (referred to as $Ctrl^{D3}$ and $Ctrl^{H2}$). We observed that the Condensin II mutants were completely sterile (Fig.3.2), thus confirming the reported phenotype.

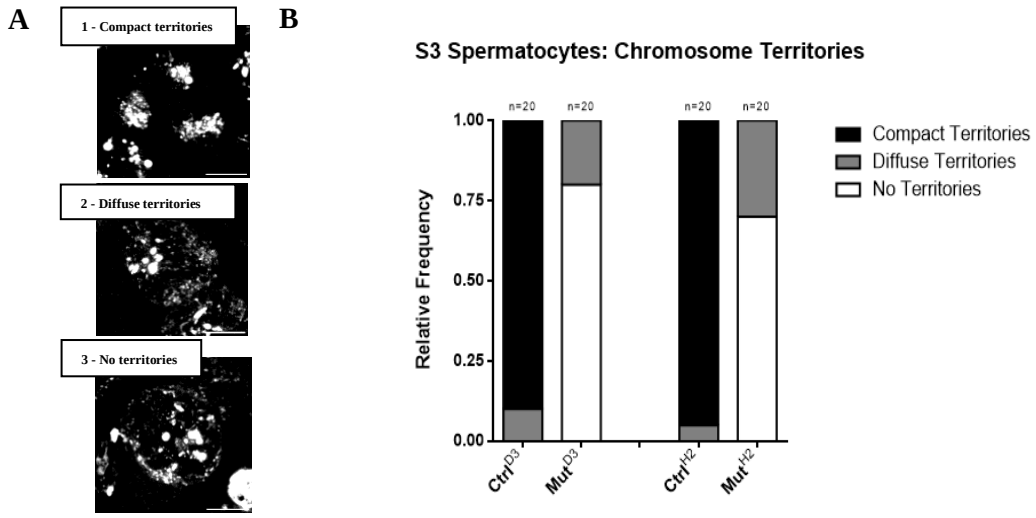

Figure 3.3 – Condensin II mutants do not form chromosome territories. (A) *Cytology of the chromosome territories in control and mutant males representing the categories used to classify the chromatin organization and condensation state. Chromatin was stained with DAPI. Scale bar corresponds to 5μM.* ***(B)*** *Quantification of the phenotypic classification of the 20 spermatocytes observed in the three defined categories. The majority of the mutants do not form territories and in some cases is possible to observe what appears to be an attempt of assembly despite the distended chromatin.*

Despite the sterility phenotype, we still needed to confirm if these males also had defects in meiotic chromatin, as previously reported. For this, we dissected male gonads and performed an immunofluorescence staining for DNA (DAPI) and the nuclear envelope (WGA or anti-Dm0 Lamin antibody). By imaging the apical portion of the testes where the early stages of spermatogenesis reside, we observed that the chromatin organization in spermatocytes appeared to be affected as early as in the S2 stage, with the phenotype becoming clear by S3. To quantitate this phenotype, we acquired images of twenty different S3 spermatocytes from three independent experiments and allocated them to one of 3 classes based on the degree of chromatin organization and condensation. These categories were: compact chromosome territories (CTs), no territories, and an intermediate category defined by the presence of diffuse, albeit still separate territories. In both controls, we could observe that most cells (>90%) had well-defined CTs (Figure 3.3 – A1). Yet, the majority of mutant spermatocytes were not able to form the CTs (approximately 70% and 80% (for Cap-H2 and Cap-D3 mutants, respectively) with no territories; Figure 3.3 – A3). Interestingly, we could also observe mutant cells that despite having irregularly distended chromatin were still able to arrange their chromosome pairs into three distinct clusters. We considered this conformation as a failed attempt of forming fully functional CTs (Figure 3.3 – A2). We observed that around 25% and 30% of all spermatocytes had this chromatin conformation in the Cap-D3 and Cap-H2 mutants. This phenotype was also recorded in the control lines, but at a much lower frequency (<1%).

Based on these data, we could successfully confirm, under our experimental conditions, the sterility and meiotic chromatin phenotype of the previously reported Cap-D3 and Cap-H2 mutants. These conditions were therefore selected for all subsequent experiments in the project. Additionally, we could further refine previous observations by noting that the lack of functional Condensin II in these mutants can, in

some cases, still be compatible with some level of chromatin arrangement inside the nucleus (via the formation of incipient CTs).

Gene expression defects in the male germ line of Condensin II mutants

In this portion of the work, I counted with the help of Joana Almeida and Alexandra Tavares, who kindly performed the dissections of the male testes with me.

After confirming that the lines we were working with presented the previously reported phenotype, it was time to put our hypothesis to the test. In order to confirm or refute our hypothesis, we needed to determine if gene expression was altered in Condensin II mutants. To do so, we performed a whole testis RNAseq analysis to assess if there were significant differences between the mutant and control transcriptomes.

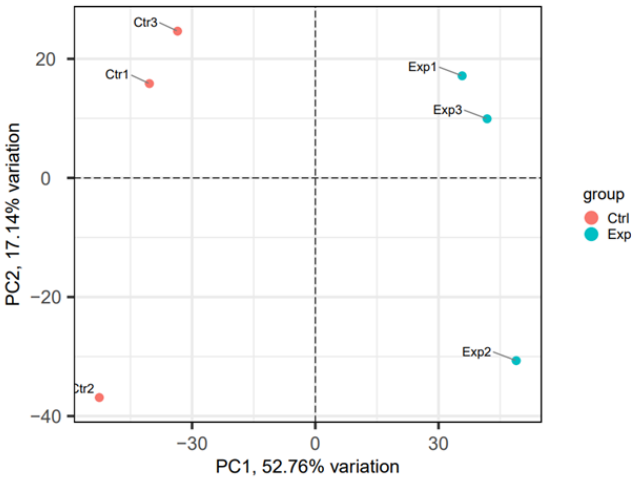

Figure 3.4 – Control and mutant males are clearly separated. *PCA analysis revealed that Cap-D3 mutants (Exp) have a different expression from their control (Ctrl).*

For this assay, we selected the Cap-D3 mutant (and its respective control) to perform a transcriptomic analysis (bulk RNAseq) in male gametogenic tissue. This choice was purely based on the practicality of the crossing scheme, as the cross between Cap-D3^{EY00456}/ Cap-D3$^{Df(2L)\ Exel7023}$ and the Cap-D3$^{Df(2L)\ Exel7023}$/OR lines consistently resulted in a larger number of males with the desired genetic constitution than its Cap-H2 counterpart.

Three independent RNA extractions from the testes of mutants and controls were performed. For each extraction, 30 males from each experimental group were used. The total RNA that was extracted from the testes was of high quality (RNA quality number of the samples between 8.5 and 10), as assessed by capillary gel electrophoresis (Fragment Analyzer). RNAseq libraries were prepared and sequenced at the IGC gene expression unit, and the expression data were analyzed by Daniel Sobral at the National Institute of Health Dr. Ricardo Jorge. A principal component analysis (PCA) plot revealed a clear separation between mutants and controls (Fig.3.4). It should be noted that in one of the extractions (extraction 2), both the control and mutant are further apart from their respective clusters (but still clearly different from each other). We tried to pinpoint a possible influencing factor during the extraction of these two samples, but we could not find a definitive explanation for this outcome.

A differential gene expression (DGE) analysis revealed that, as we had hypothesized, the testicular transcriptome of Cap-D3 mutants differed from that of controls (Fig. 3.5). From a total of 15,172 expressed genes, 563 (approximately 4%) were differentially expressed in the mutant, with 324 genes being downregulated in the mutant and the remaining 239 upregulated. We were quite strict when defining the parameters to consider a gene as differently expressed to minimize artifacts introduced by the high transcriptional noise characteristic of spermatogenesis (Correia et al., 2022). Accordingly, we defined that all differentially expressed genes must have a detectable expression in the mutant and control groups (log2CPM>1), that the difference in expression should be at least two-fold [-1<log2(FC)>1], and the difference observed was statistically significant (FDR <0.05).

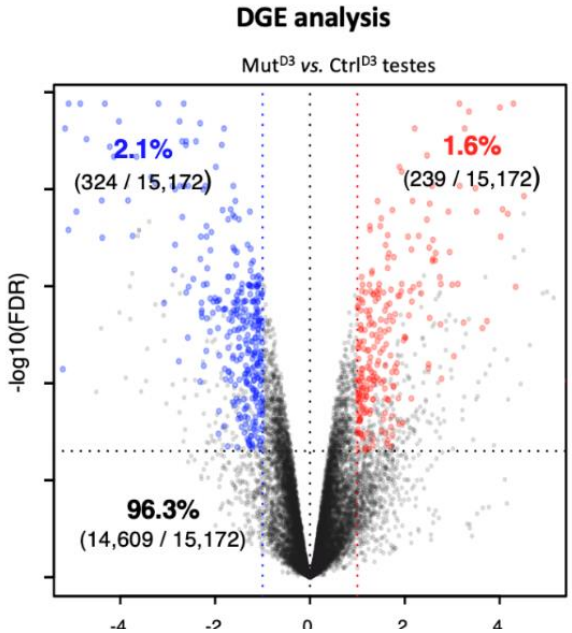

Figure 3.5 – Volcano Plot of the gene expression assay (RNAseq). *Log2(FC) indicates the magnitude of change in the expression level of the genes, in this case, a two-fold change; -log10(FDR) indicates the significance. Each dot corresponds to one gene. Black dots represent no significant differentially expressed genes. Blue dots represent significantly downregulated genes. Red dots represent significantly upregulated genes.*

We next focused on the downregulated genes, since we expected that the decrease in the transcript levels of these genes would likely have a negative functional impact in a process as delicate as spermatogenesis. Out of these 324 genes, we selected for functional testing those that were at least four-fold less expressed in the mutant (log2FC <-2). This led to a list of 71 severely downregulated genes that also included non-coding genes. After filtering these out from the list and selecting only those for which there were RNAi lines available for testing, we ended up with a final selection of 36 testable severely downregulated protein-coding genes (Fig. 3.6). Supplementary Figure SM1 lists the expression levels of all top 50 up and downregulated genes.

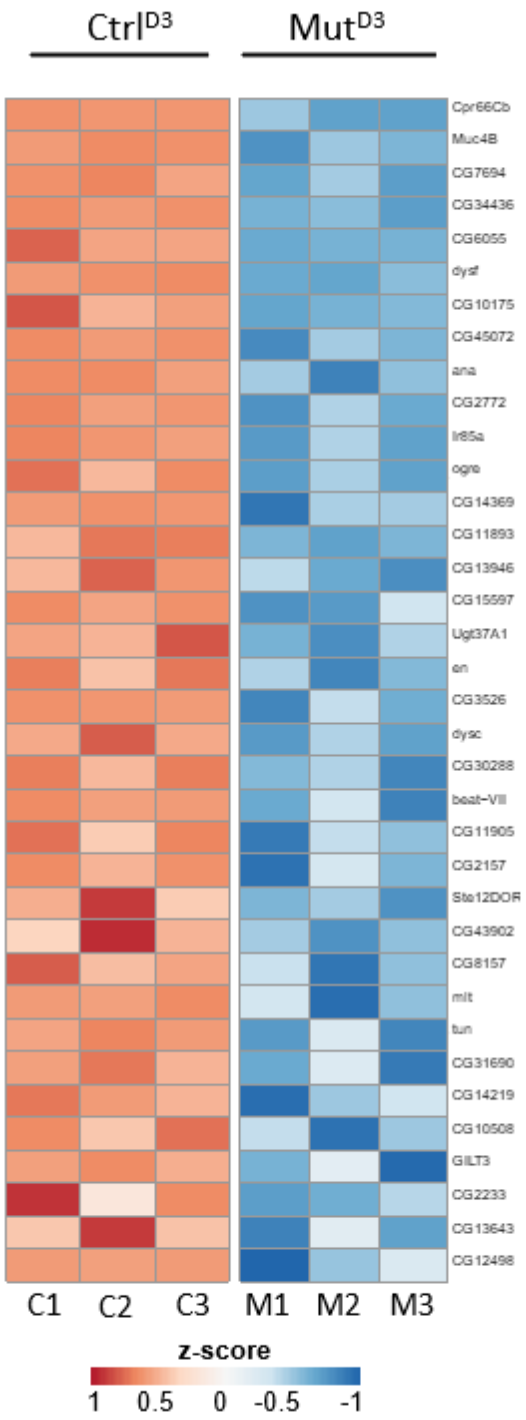

Figure 3.6 – Final list of the 36 genes selected for test and their expression levels for controls and mutants in each extraction.

Identifying a Condensin II-dependent new spermatogenesis gene

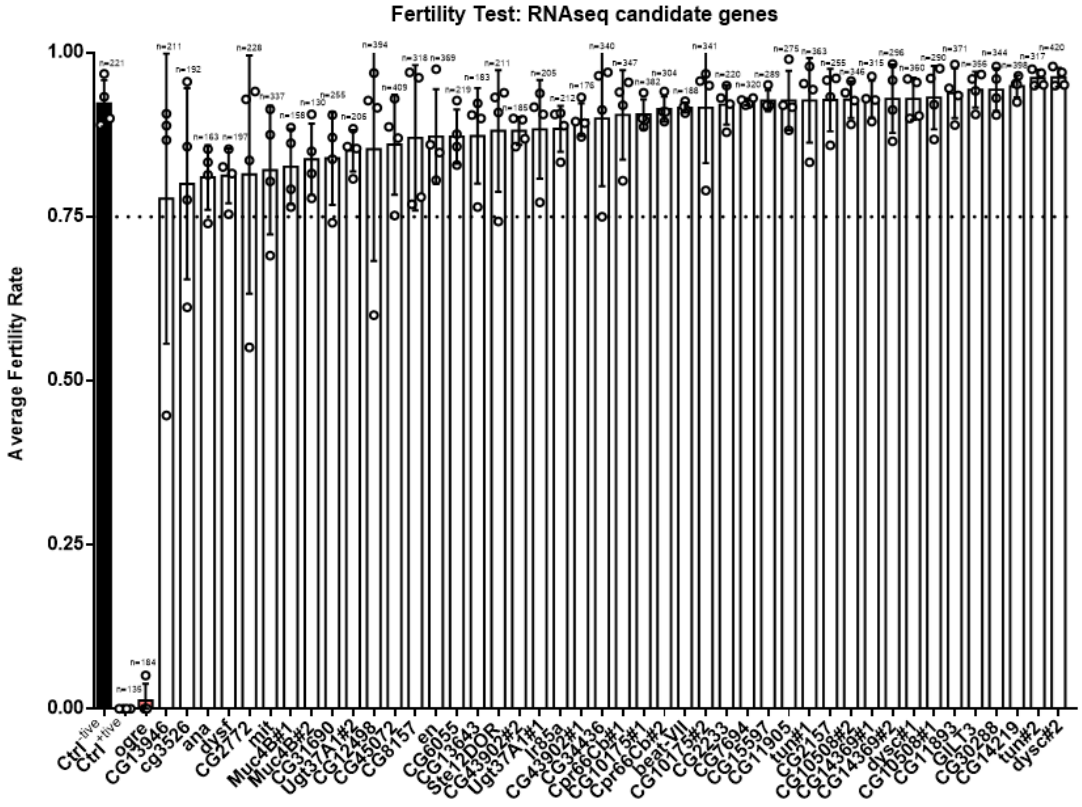

Figure 3.7 – Testing the downregulated genes' impact on fertility. *The silencing of these genes was induced upon meiotic entry using the Bam-GAL4 driver. Each dot corresponds to the result of an independent test. The negative control (mCherry) is represented by a black bar. The dotted line represents the cutoff of 0.75 below which we consider fertility affected. Ogre (red bar) was the only gene whose silencing severely affected fertility.*

In order to attempt to recapitulate the male sterility phenotype of the Condesin II mutants via the silencing of a downregulated gene, we ordered a total of 42 RNAi lines that corresponded to the 36 previously selected candidate genes (see previous section for selection strategy). This means that for some of the genes we ended up testing two RNAi lines – this was not an intentional decision for specific genes, it simply reflected the number of available lines for each candidate.

As before, silencing was induced upon meiotic entry using the *Bam*-GAL4 driver, and the reproductive fitness of the silenced males was assessed by four independent fertility tests (Fig.3.7). Out of the 36 tested candidates (corresponding to 42 RNAi lines), only one had severely impaired fertility (average fertility: 0.01): the optical ganglion-reduced gene, commonly referred to as *ogre* (Lipshitz and Kankel, 1985a).

Ogre encodes one of the eight innexin genes found in *Drosophila* (more specifically, it encodes for innexin 1). Together with two other innexin subunits (innexin 2 and 3), ogre forms the homo- or hetero-oligomers that define gap junctions in invertebrates (Phelan et al., 2001). Gap junctions consist of intercellular channels that connect the cytoplasm of neighboring cells, allowing cellular communication through cell-to-cell transfer of ions and small molecules (Phelan et al., 2001). Based on the literature, ogre is known for its role in postembryonic neurogenesis, with *ogre* mutants being described as having a small nervous system, particularly due to a significant decrease in optic lobe size (phenotype by which this gene is named), and by having defects in neural architecture organization (Lipshitz et al., 1985;

Watanabe et al., 1992; Holcroft et al., 2013). From all the phenotypes described, we could not find any indication of a previously reported role of ogre in spermatogenesis.

Sperm production is affected both in the Condensin II mutants and in the *ogre* RNAi

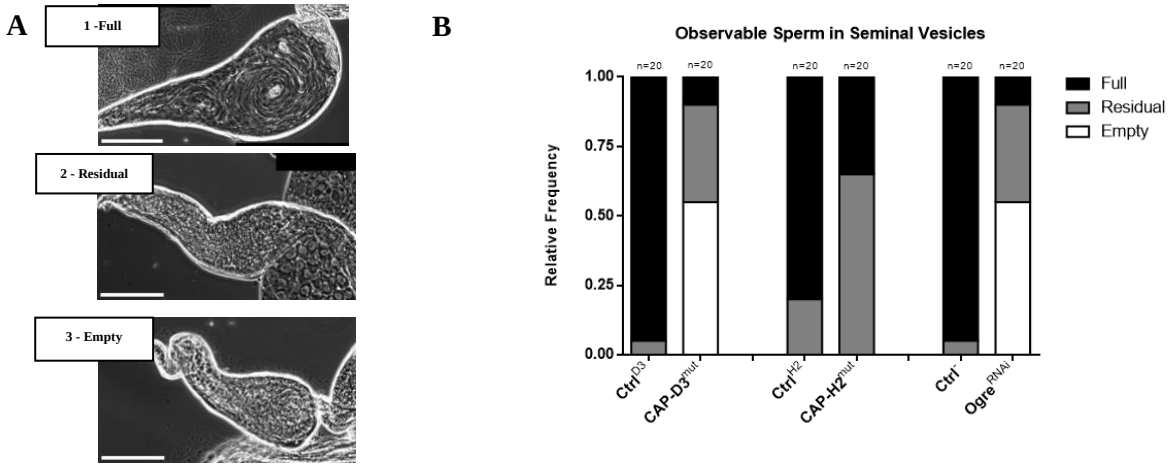

Figure 3.8 – Sperm production is affected in condensin II mutants and ogre-silenced males. (A) *Seminal vesicles from controls, mutant, and ogre-silenced males were analyzed in order to assess sperm production. The scale bar corresponds to 10μM.* ***(B)*** *Quantification of the phenotypic classification of the 20 seminal vesicles observed in the three defined categories.*

When starting this project, we knew that Condensin II mutants were able to finish meiosis despite their segregation defects (Horta et al., 2022) and that Cap-H2 mutants were sterile due to a defect in gamete production (Hart *et al.*, 2008). Accordingly, we decided to explore in greater detail the post-meiotic stages of spermatogenesis to better understand the functional requirements of Condensin II to produce male gametes. For this, we assessed the impact of Condensin II mutations, and of the silencing of the Condensin II-regulated gene *ogre*, on sperm production. To do so we isolated males for a period of 48 hours to maximize sperm accumulation in the seminal vesicles. In our assay, seminal vesicles were scored, using a phase contrast microscope, based on the amount of sperm cells they contained. Three categories were defined: full seminal vesicles, empty seminal vesicles (no observable sperm cells), and an intermediate phenotype that we classified as containing residual sperm. In this last category, we could observe a considerably lower number of sperm cells compared with a full seminal vesicle.

Our data (Fig.3.8) revealed that in Cap-D3 mutants the majority of the males do not produce sperm cells (55% of the scored vesicles were empty), but that there was still a fraction of males capable of completing spermatogenesis, albeit at very low levels (35% of the vesicles with residual sperm). We found evidence of normal sperm production (full seminal vesicle) in only two out of the twenty seminal vesicles analyzed. *Ogre*-silenced males perfectly recapitulated the phenotype of Cap-D3 mutants. Quite unexpectedly, Cap-H2 mutants appeared to have a less severe spermatogenic impairment phenotype. Contrary to what was previously reported for these mutants (Hart *et al.*, 2008), we always observed sperm cells in the seminal vesicles of these males (either at levels indistinguishable from controls or severely reduced). It is important to point out that the finding that these mutants are still able to produce sperm does not contradict their sterility phenotype, as these sperm cells can have either pre or post-fertilization defects – the aneuploidy reported in these males can contribute to the latter.

Nevertheless, collectively, all Condensin II mutants and *ogre*-silenced males had sperm production affected to some degree. With this in mind, we decided to look at the last stage of spermatogenesis – the individualization of the fully differentiated sperm cells.

Sperm individualization is impaired both in Condensin II mutants and in *ogre*-silenced males

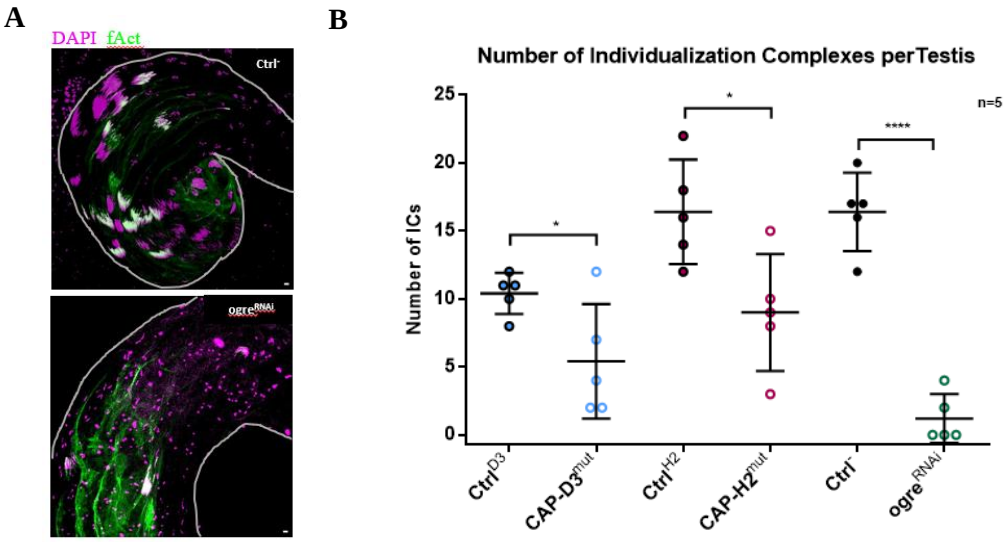

Figure 3.9 - Condensin II mutants and ogre-silenced males are less capable of assembling Individualization Complexes. ***(A)*** *Example of how the distal portion of the testis of control (top image) and Ogre-silenced males (bottom image) – where it is appreciable a clear difference in the number of ICs.* ***(B)*** *Quantification of the observed individualization complexes inside five different testes revealed that in the absence of condensin II and ogre, the males can't assemble ICs. Each dot represents one testis. *, p-value<0.1; ****, p-value<0.0001.*

Sperm individualization is the process through which elongated spermatids lose the bridges that unite them to their sister cells inside the cyst, thus becoming fully separate male gametes. As previously mentioned, sperm individualization occurs when actin cones form around the nucleus of the germ cells and migrate alongside the axoneme thus assembling, as they progress, the individual plasma membrane of each of the 64 gametes within the cyst. Disturbances in this process can critically affect sperm production (Yuan *et al.*, 2019). To observe if sperm individualization was somehow affected in our lines of interest, we decided to look at the distal portion of the testis where this process takes place and analyze the structure of the individualization complexes (ICs).

To analyze these complexes, we stained the actin cones using phalloidin and the germ cell DNA using DAPI. For each tested line, we counted the total number of individualization complexes that could be identified in the full volume of the distal portion of 5 different testes. Overall, we observed a significant reduction in the number of ICs observed in both the Cap-D3 and Cap-H2 mutants in relation to their controls (from an average of 10 per testis in controls to 5 in Cap-D3 mutant males (p-value:0.0373*) and from an average of 16 per testis in controls to 9 in Cap-H2 mutant males (p-value:0.0209*, Fig.3.9 – B). This decrease was particularly striking in *ogre*-silenced males, as three of the five assessed testes had no observable ICs whatsoever. Based on these observations we conclude that Condensin II, and particularly its regulated gene *ogre*, play an important role in the assembly of ICs and in the subsequent formation of fully mature sperm cells.

Analyzing the assembly of Individualization Complexes

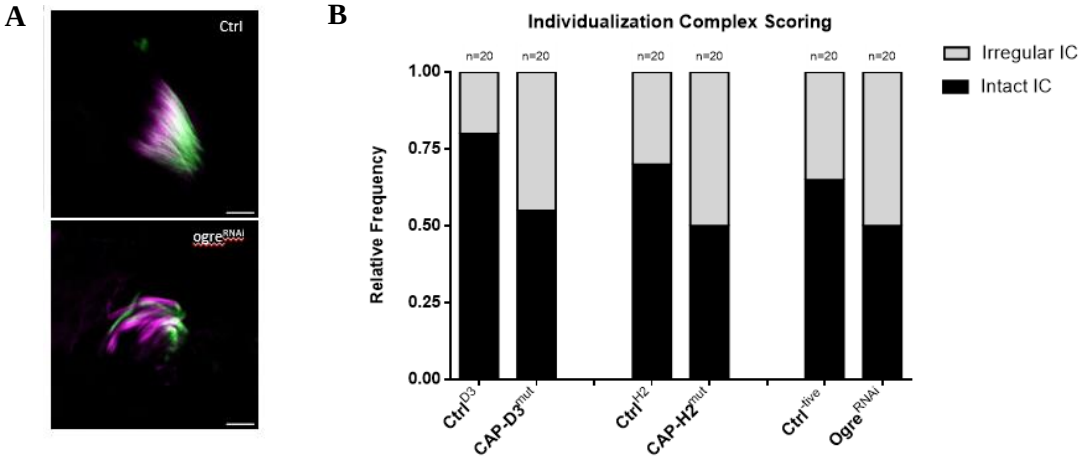

Figure 3.10 – Condensin II and its affected gene Ogre aren't needed for individualization complex maintenance. *After assessing the state of the few assembled complexes in the mutant and ogre-silenced testis we saw that they aren't particularly defective even in comparison with the respective control lines.* ***(A)*** *Illustrative images of an intact IC (top image) and what we considered an irregular (or defective) IC (bottom image). The scale bars in the images correspond to 5μM.* ***(B)*** *Quantification of the 20 individualization complexes analyzed.*

After analyzing the regulation of sperm individualization from a quantitative perspective, we next assessed this process from a qualitative point of view. Twenty ICs from each of the different tested lines were classified according to their gross morphology. Two categories were defined: intact ICs (with a regular appearance; Fig.3.10-A top image), and irregular ICs (deviations to the latter, mainly containing ICs with scattered/misaligned actin cones; Fig.3.10-A bottom image). Interestingly, we found that despite the severely reduced number of ICs in the testes of Condesin II mutants and of *ogre*-silenced males, the few remaining ones had, in about half of the cases, a normal morphology. This observation points out that the requirements of Condesin II and ogre for sperm individualization are particularly focused on the assembly of ICs, and not so much on their maintenance/function once assembled. Furthermore, the persistence of intact ICs in these lines can explain why we were still able to observe mature sperm cells inside some of the seminal vesicles. Controls such as the ones for the Cap-H2 mutants and for the *ogre* RNAi also had a sizeable number of defective ICs (6 and 7 in the 20 scored, respectively) but since the number of ICs in these testes is also significantly higher, sperm production is safeguarded. Collectively, these data indicate that the main rate-limiting step of post-meiotic development in Condensin II mutants is an impaired capacity to assemble intact ICs that ultimately leads to a block in sperm production.

Possible mechanisms through which Condensin II can regulate gene expression.

Based on our data, it is clear that Condensin II is required for proper gene expression during spermatogenesis. However, it is still unclear how this complex can influence transcription in developing male germ cells. We propose two possible mechanisms, one indirect, and the other direct. The indirect hypothesis is based on the role of Condensin II in the assembly of the meiotic chromatin organization (formation of the CTs). It is possible that in the absence of Condensin II, meiotic chromatin remains distended and dispersed inside the nucleus, resulting in an incapability of ensuring a finer control of the transcriptional dynamics of several genes. From this, genes that are necessary for male fertility, such as *ogre*, are misexpressed, ultimately contributing to the sterility observed in the Condensin II mutants. The direct hypothesis is that Condensin II plays a more active role in transcription by also binding to the promotor/enhancer regions and thus influencing gene expression. Previous studies have shown that Condensin II, or even one of its subunits (Cap-D3), can directly bind to the promotor regions of certain genes and promote or inhibit their expression (Longworth *et al.*, 2012; Kranz *et al.*, 2013). In this

scenario, Condensin II would bind to the *ogre* promotor/enhancer and directly contribute to its expression, ultimately ensuring successful spermatogenesis.

Condensin II and Ogre – a matter of individualization

One of the most interesting aspects of this work was the discovery that Condensin II mutants have their sperm production affected by the incapability of assembling ICs, a result that can be recapitulated by independently by silencing a gene (*ogre*) that is downregulated in the Condensin II mutant background. From the fertility tests to the cytology assays, *ogre*-silenced males did not only recapitulate the mutant phenotype but also its isolated absence was sufficient to explain the sterility of Condensin II mutants. The defective assembly of ICs in the *ogre* RNAi is even more severe than what is recorded in the Condensin II mutants, a possible consequence of a more pronounced downregulation of this gene in the RNAi than in the Condensin II mutants. But how can a gene responsible for gap junctions have such a relevant role in the individualization step of spermatogenesis?

It is well known that gap junctions are essential to soma-germ line communication and that this communication assures germ-line maintenance (Kidder and Cyr, 2016). Very recently, it also became clear that gap junctions mediate key events in the post-meiotic regulation of sperm differentiation (Pesch *et al.*, 2022). More specifically, the research team focused on innexin4 (also known as Zero population growth, Zpg) a well-known gap junction protein in *Drosophila* that is present in the plasma membrane of germ cells and is essential for fertility (Tazuke *et al.*, 2002). This protein interacts with innexin2 and establishes channels that allow communication between germ cells and the soma (Smendziuk *et al.*, 2015). In the paper, specific point mutations that would only affect the conformation of the channel were introduced in innexin4, thus preventing the passage of different molecules between the two compartments. It was observed that different stages of spermatogenesis were affected depending on the type of molecules that were prevented from passing, with one of those stages being sperm individualization. Indeed, some of the innexin4 mutants were not capable of correctly assembling the ICs, indicating that the assembly of these complexes requires germ cells to receive precise signals via their gap junctions. Hence, these junctions have a fundamental role in allowing the passage of signaling molecules that will communicate to the cell the need to enter the last stage of spermatogenesis.

Based on this observation, we believe that the absence / low expression of the gap junction protein ogre can also affect this communication, ultimately leading to the defects in ICs assembly recorded both in the Condensin II mutants and in the *ogre* RNAi. Therefore, our hypothesis to explain the role of ogre in spermatogenesis is that it serves an essential function in maintaining cellular communication during the final stages of spermatogenesis, thus promoting the conditions for elongated spermatids to assemble their ICs.

4 - Future Perspectives

Some of the ideas and hypotheses mentioned in the discussion would need additional experiments. With this in mind, I would like to leave a few future perspectives of work that not only would confirm our results but also take them a step further. Firstly, it would be important to validate the *ogre* RNAi via a reverse transcription quantitative real-time polymerase chain reaction (commonly referred to as RT-qPCR) test. Furthermore, it would be equally important to confirm the results seen in the *ogre*-silenced males with an alternative reagent. To do so, we could select another RNAi line for this gene or even choose a mutant line to repeat all the analyses done to validate the previously obtained results.

After these validations and in order to assess if *ogre* is indeed the gene causing the decrease in sperm production, a rescue experiment would be a suitable approach. For this, we would overexpress *ogre* in

the Condensin II mutant background and observe if this overexpression would be enough to partially rescue fertility in these flies. This experiment would help us to tie the knot on our cascade of events, while also proving the role of ogre in spermatogenesis.

To explore one of our hypotheses related to how Condensin II affects' gene expression, it would be interesting to immunoprecipitate Condensin II and sequence the DNA bound to it (Chromatin immunoprecipitation assay). This experiment would provide us with information about condensin-chromatin interactions that could ultimately support or reject a direct role of Condesin II on transcription.

5 - Conclusion

Condensins play a major role in chromatin organization and segregation during cell division. Despite this well-established role, it is important to look outside the box as these complexes appear to influence other cellular processes. In this work, we explored the non-canonical role of Condensin II in *Drosophila* spermatogenesis, confirming our hypothesis that this complex plays a role in male germ cell gene expression.

By using non-SMC mutant lines, we were able to establish a sort of trail of effects triggered by the absence of a functional Condensin II complex in spermatogenesis. In the absence of Condensin II, it is possible to observe defects in chromatin organization during meiosis, as spermatocytes fail to assemble the 3 chromosome territories (Hart *et al.*, 2008). This change occurs in a stage where the cell is producing most of the transcripts it will need for post-meiotic development (Cenci *et al.*, 1994). We could observe that 536 genes were differentially expressed in the Cap-D3 mutants, and out of these, 324 were downregulated.

From this pool of genes, we observed that *ogre*, a gene involved in the formation of gap junctions, had a major impact on male fertility. By looking at sperm production, we noted that *ogre*-silenced males recapitulated perfectly the Cap-D3 mutant phenotype of decreased spermatogenic output. We further observed that this decrease was due to defects in assembling sperm individualization complexes.

When we look at these findings as a whole, we see that the absence of Condensin II impacts both chromatin organization and gene expression, which leads to decreased capacity of assembling individualization complexes, culminating in decreased sperm production. Ogre appears to have an important role in this process since gap junctions are essential to ensure proper cellular communication in spermatogenesis.

We propose that the decreased sperm production (arising from gene expression defects) together with the previously documented aneuploidy (arising from chromosome segregation defects) actively contribute to the male sterility of Condensin II mutants. In the future, we hope to further explore these observations and put new hypotheses to the test.

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Supplementary Material

Table SM1 - List of each RNAi line used in this thesis and their corresponding gene.

Genes	Fly Lines Tested
Cap-H2	#38196/v100047/v24905
Cap-D3	#61979/#36615/v29657/v9402
SMC2	#32369
SMC4	#36069/v10937
CG3526	#62188
CG12498	v17793
Muc4B	#67940/#76072
CG14369	v102747/v8285
Ugt37A1	#58196/#62257
Cpr66Cb	v28740/v102551
CG45072	v35366
CG34436	v32771
CG15597	v13139
CG8157	v18585
CG2772	#67018
CG30288	v100420/v33700

CG7694	v25520
Ir85a	#57772
CG11893	v16356
CG10175	v101643/v1140
CG2157	v51069
dysf	#35010
CG10508	v102744/v48115
CG11905	v15401
beat-VII	#60056
CG6055	#35778
CG13643	#56017
en	v105678
CG14219	v106424
tun	v105713/v35411
ogre	#44048
CG2233	#65928
ana	#27515
GILT3	v102104

CG13946	#62336
dysc	v109928/v110019
CG31690	#44525
CG43902	#58246/#57239
Ste12DOR	v109155
mlt	v105480

Table SM2 - Drosophila melanogaster Vienna's food recipe.

Ingredients	**100mL**	**250mL**	**500mL**	**1000mL**
Molasses	4.5g	11.25g	22.5g	45g
Beet Syrup	3.0g	7.5g	15g	30g
Corn flour	5.5g	13.75g	27.5g	55g
Soy flour	2.0g	5.0g	10g	20g
Vegetable Oil (sunflower or soy)	0.2g	0.5g	1.0g	2.0g
Glucose	7.5g	18.75g	37.5g	75g
Agar-Agar	1.0g	2.5g	5.0g	10g
dH_2O	100mL	250mL	500mL	1000mL
Bavistin				

Table SM3 - Testis buffer (TB) 100 mL.

Reagents	Quantities (g)
KCl	**1,36**
NaCl	**0,27**
Tris-HCl	**0,12**
EDTA	**0,037**
PMSF	**0,017**

Table SM4 - Pre-fix Solution (4%PFA; 1 mL).

Reagents	Quantities (µL)
H_2O dd	650
PBS 10x	100
PFA (from 16% ampoules)	250

Table SM5 - PBS-T (50 mL)

Reagents	Quantities
H_2O dd	45 mL
PBS 10x	5 mL
Triton X (from 20% dilution)	250 µL

Table SM6 - Fix Solution (1 mL)

Reagents	Quantities (µL)
H_2O dd	600
PBS 10x	100
NP-40 (1% final, from 20% dilution)	50
PFA (from 16% ampoules)	250

Table SM7 - PBS 1x (50 mL)

Reagents	Quantities (mL)
H_2O dd	45
PBS 10x	5

Table SM8 - B+P Solution (10 mL)

Reagents	Quantities
H_2O dd	7.8 mL
PBS 10x	1 mL
BSA (from 10% aliquots)	1 mL
Donkey serum	100 µL
Triton X (0.3% final, from 20% dilution)	150 µL

Table SM9 - BBT Solution (10 mL)

Reagents	Quantities
PBS-T	8,9 mL
BSA (from 10% aliquots)	1 mL
Donkey serum	100 µL

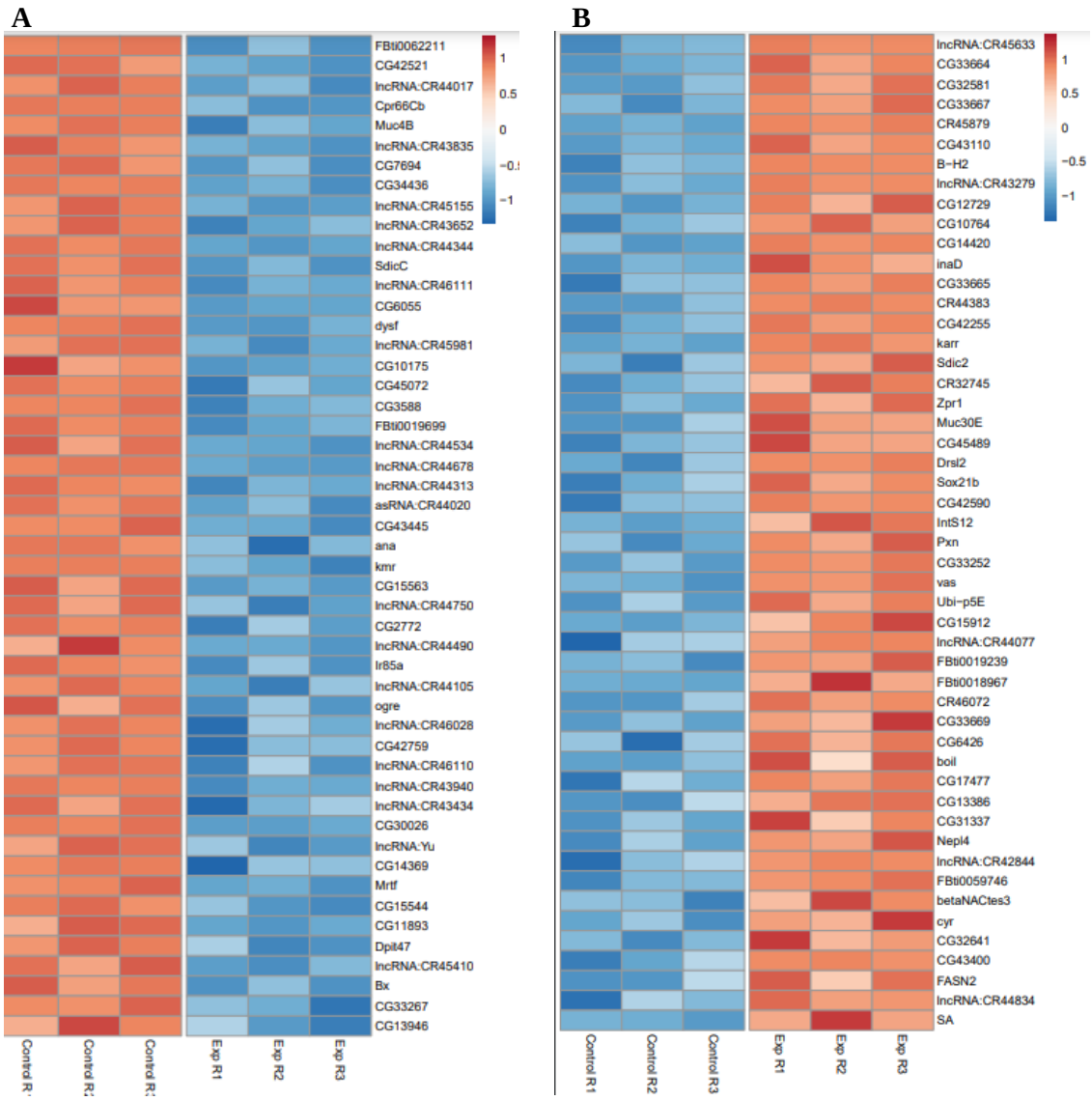

Figure SM1 – List of the 50 genes more down- and up-regulated in Cap-D3 mutants. (A) *The list of genes that were more significantly downregulated and their expression in each of the extractions.* ***(B)*** *The list of genes that were more significantly upregulated and their expression in each of the extractions.*