

Identification of new genes involved in the spindle assembly checkpoint

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Tese de doutoramento em Ciências Biomédicas

2012

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**Identification of new genes involved in the spindle assembly
checkpoint**

Tese de Candidatura ao grau de Doutor em
Ciências Biomédicas submetida ao Instituto de
Ciências Biomédicas Abel Salazar da
Universidade do Porto

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Afiliação – Instituto de Ciências Biomédicas
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Sousa-Guimaraes, S., Sunkel, C., Malmanche, N. (2011). "*polo* is identified as a suppressor of *bubR1* nondisjunction in a deficiency screen of the third chromosome in *Drosophila melanogaster*." *G3* **1**(2): 161-169.

Reis, M., **Sousa-Guimaraes, S.**, Vieira, C., Sunkel, C. E., Vieira, J. (2011). "*Drosophila* genes that affect meiosis duration are among the meiosis related genes that are more often found duplicated." *PLoS ONE* **6**(3): e17512.

Agradecimentos / Acknowledgements

Para não me esquecer de ninguém, quero agradecer de um modo geral a todas as pessoas que me ajudaram, de alguma forma, nesta caminhada de 6 anos. Em particular, quero agradecer:

Ao meu coordenador, Professor Claudio Sunkel, pela oportunidade que me proporcionou em iniciar a minha carreira científica, pelo apoio e pelo incentivo.

Ao Nicolas Malmanche por me ter iniciado na genética de *Drosophila* e por me ter apresentado o fantástico mundo da meiose.

Ao Jorge Vieira por me ter dado a oportunidade de participar numa colaboração que me proporcionou aprender matérias e técnicas novas.

À Tália Figueiredo por me ter iniciado na Biologia Molecular.

À Tatiana Moutinho pela ajuda no laboratório, mas principalmente pela revisão da tese.

À Anabela Nunes pela ajuda na formatação e design da capa da tese.

À Paula Sampaio pelo apoio na microscopia e ao Frederico Silva pelo apoio na produção e purificação de proteína recombinante.

À Augusta Monteiro, à D. Adelaide, à Maria João Falcão e ao Sr. Carlos pelo apoio técnico.

Ao Torcato Martins pela amizade, por me ter ajudado a integrar no grupo da Genética Molecular e por me ter ajudado a desenvolver o meu poder de abstração. ☺

À Joana Marinho pela amizade, incentivo e discussões científicas.

À Sofia Pinho pela amizade, companhia e apoio técnico.

Aos passados (Inês Barbosa, Marta Neto e Telmo Henriques) e actuais (João Barbosa, Sara Silva e Eurico Sá) *Drosophilistas* pelas discussões científicas, mas principalmente pelos momentos de descontração passados na "FlyRoom".

Aos restantes membros do grupo da Genética Molecular não-*Drosophilistas* pela troca de ideias e convívio no laboratório.

Um especial agradecimento à Marta Freitas pelo ombro amigo.

À minha família, porque sem ela nada disto seria possível.

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Summary

Meiosis is a special type of cell division in which the nuclear content divides twice despite the fact that DNA is only duplicated once. Failed meiotic chromosome segregation, or nondisjunction, results in aneuploid gametes that when fertilized, lead to miscarriages and birth defects in humans. In mitosis, faithful chromosome segregation is monitored by the spindle assembly checkpoint (SAC). During the past several years, it has become clear that the SAC is also present during meiosis. This thesis focuses on BubR1, an important SAC protein. Previous work in the laboratory identified a *bubR1* allele (*bubR1*^{D1326N}), which has a point mutation in a conserved region of the kinase domain. This allele induces high frequency of meiotic nondisjunction, in a BubR1 dosage-dependent manner, indicating that the kinase activity is essential in meiosis. This nondisjunction phenotype is observed along with a premature disassemble of the synaptonemal complex, a proteinaceous structure that mediates pairing and synapsis of homologous chromosomes and promotes meiotic recombination. Taking advantage of these features, we carried out a dominant enhancer/suppressor screen in *Drosophila melanogaster* to identify genes that interact with *bubR1* meiotic activity. Using this approach, it was possible to identify two synthetic lethal *loci*, one *locus* that induces sterility and several *loci* that suppress/enhance the nondisjunction phenotype observed in *bubR1* mutants. *polo* and *Pp1-87B* were identified as two genes responsible for the suppression of nondisjunction observed in *bubR1* mutants. In the case of *polo*, the suppression of nondisjunction was observed along with the maintenance of sister chromatid cohesion and of the synaptonemal complex during prophase I progression, when *polo* is in hemizygous conditions. These results indicate that the screen allowed for the identification of relevant BubR1 meiotic interactors and further analysis will be required to identify the relevant genes that either suppress or enhance the meiotic nondisjunction phenotype in other chromosomal regions.

In the second experimental part, we analysed the rate of gene duplication of 33 genes that are involved in meiosis in 12 available *Drosophila* genome databases. Against theoretical expectations, we found that the rate of gene duplication in this group of meiotic genes is similar to the value estimated for the whole *Drosophila* genome using species of the *melanogaster* subgroup. More perplexing was to find that genes like *polo* and *mtrm* are duplicated and their duplicate appears to be functional. This is surprising given previous results indicating that Polo and Mtrm are involved in the regulation of meiotic progression in a strictly stoichiometric relationship.

Resumo

A meiose é um tipo de divisão celular especial em que o conteúdo nuclear se divide duas vezes apesar de só haver um ciclo de replicação do DNA. Deficiências na segregação dos cromossomas durante a meiose, ou não-disjunção, resultam na formação de gametas aneuploides que, quando fertilizados, levam a abortos espontâneos e malformações congénitas em humanos. Em mitose, a correcta segregação dos cromossomas é controlada pelo ponto de controlo (*checkpoint*) do fuso mitótico (SAC). Durante os últimos anos, tem-se tornado evidente que o SAC também funciona em meiose. Esta tese foca-se no estudo da proteína BubR1, um componente essencial do SAC. Em trabalhos anteriores do laboratório, foi identificado um alelo do gene *bubR1* (*bubR1^{D1326N}*), que possui uma mutação pontual numa região conservada do domínio cinase. Este alelo induz elevada frequência de não-disjunção que está associada à quantidade de BubR1 existente nas várias combinações heteroalélicas de *bubR1*, indicando que a actividade da cinase é indispensável durante a meiose. Este fenótipo de não-disjunção é observado juntamente com a remoção prematura do complexo de coesina e do complexo sinaptonémico - uma estrutura proteica que medeia o emparelhamento e a sinapse dos cromossomas homólogos, promovendo a recombinação. Aproveitando estas características, realizou-se um rastreio de *enhancers* e supressores dominantes em *Drosophila melanogaster* para identificar genes que influenciem a actividade meiótica de *bubR1*. Foi possível identificar dois *loci* letais sintéticos, um *locus* que induz esterilidade, além de vários *loci* que aumentam e suprimem a não-disjunção em mutantes de *bubR1*. *polo* e *Pp1-87B* foram identificados como dois genes supressores do fenótipo de não-disjunção observado em mutantes *bubR1*. No caso do *polo*, a supressão da não-disjunção foi observada conjuntamente com a conservação da coesão das cromátides-irmãs e do complexo sinaptonémico durante a progressão da prófase I, quando *polo* está em condições hemizigóticas. Estes resultados indicam que o rastreio realizado permitiu identificar importantes interactivos meióticos do BubR1. No entanto, são necessários estudos adicionais para identificar os genes responsáveis pelo aumento ou pela supressão do fenótipo de não-disjunção meiótica noutras regiões cromossómicas.

Na segunda parte experimental, analisámos a taxa de duplicação génica de 33 genes que estão de algum modo envolvidos na meiose, em 12 espécies de *Drosophila*, cujos genomas são conhecidos. Contrariamente ao esperado, descobrimos que a taxa de duplicação génica neste grupo de genes meióticos é semelhante à estimada para o genoma inteiro em espécies pertencentes ao

subgrupo *melanogaster* de *Drosophila*. Mais surpreendente foi descobrir que genes como *polo* e *mtrm* encontram-se duplicados e as duplicações poderão ser funcionais. Isto é espantoso tendo em conta resultados anteriores que indicam que o Polo e o Mtrm estão envolvidos na regulação da progressão meiótica numa relação estritamente estequiométrica.

Chapter 1

General Introduction

1. Cell cycle

Cell cycle is the sequence of events that leads to the reproduction of all cells. This self-reproduction is an essential feature since it is vital to the development and function of all forms of life, either for unicellular or for multicellular organisms. At the end of each cell cycle, a cell gives rise to two daughter cells and these new cells, if they undergo another round of the cell cycle, will also give rise to new daughter cells. The cycle in eukaryotic cells can be divided into two parts: interphase and mitotic (M) phase (Figure 1.1).

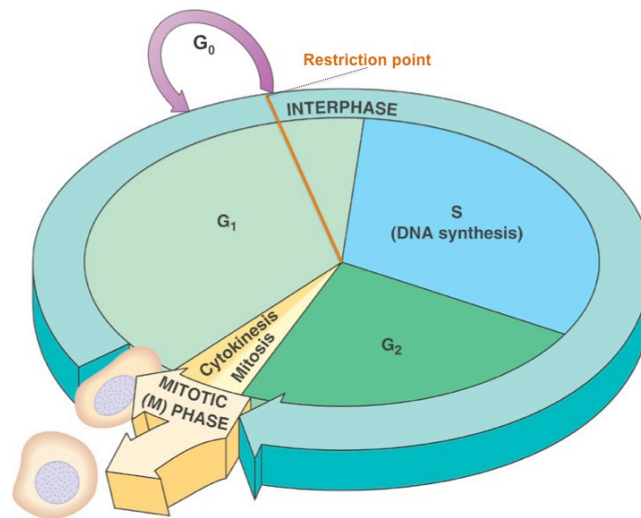


Figure 1.1. Schematic representation of the cell cycle phases. The division cycle of most eukaryotic cells is divided into two parts: Interphase (G_1 , S and G_2) and Mitotic phase (Mitosis and Cytokinesis). If growth factors are not available at the restriction point, which operates at late G_1 and controls the cell cycle progression, cells enter a quiescent stage called G_0 (Adapted from Campbell and Reece 2005).

1.1. Interphase

Interphase is the part of the cell cycle in which cells spend the most of their time and is characterized by significant metabolic activity and growth. This part of the cell cycle can be divided into three phases: G_1 (Gap 1), S (DNA synthesis) and G_2 (Gap 2) phases (Figure 1.1). G phases are intervals (gaps) between M phase and S phase, with G_1 being followed by S phase and G_2 by M phase. These intervals are important for regulatory transitions, in which the cell can control the progression to the next phase due to a variety of intracellular and extracellular signals. In Gap phases, cells are continuously growing and are metabolically active. S phase corresponds to the stage in which the DNA replication takes place.

Some types of cells are constantly proliferating, as is the case for most embryonic cells; others, such as neurons, stop dividing and others divide only occasionally, as is the case of skin fibroblasts. Many types of cells can, under certain

circumstances, exit the cell cycle during G_1 to enter a state called G_0 (the quiescent stage). In G_0 , cells are metabolic active, but growth ceases and metabolic activity is significantly reduced. Cells can re-enter the cell cycle from G_0 back into G_1 under the right environmental conditions. In late G_1 , once growth factors or other extracellular signals are present, cells can pass the so-called restriction point (or START as it is called in budding yeast) so that they become committed to entering S phase and undergo a complete round of cell cycle (reviewed by Lodish *et al.* 2000; Morgan 2007). The restriction point represents a major control point that cells use to verify whether intra and extracellular conditions are appropriate to undergo a complete round of the cell cycle.

1.2. M phase

M phase is divided into two events: mitosis, probably the most complex stage of the cell cycle and that corresponds to the nuclear division, and cytokinesis, which encompasses the division of the cytoplasm and the physical separation of the two daughter cells (Figure 1.1).

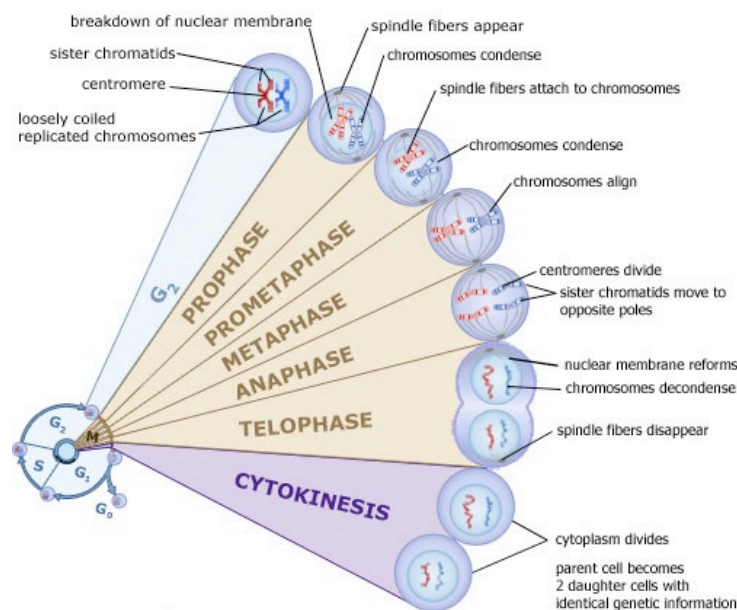


Figure 1.2. Schematic representation of the mitotic phase stages. Mitosis starts with Prophase, when chromatin condenses into chromosomes and centrosomes start to separate. At Prometaphase onset, the nuclear envelope breaks down and chromosomes start to be captured by microtubules that are growing from centrosomes at opposite sides of the cell. During Metaphase, all chromosomes align at the equator of the cell and, at Anaphase, sister chromatids move towards opposite poles. At the end of the mitotic phase chromosomes decondense, the nuclear membrane reforms around each daughter nuclei (Telophase). Subsequently, the division of the cytoplasm (Cytokinesis) generates two independent daughter cells (Adapted from www2.le.ac.uk/).

Mitosis can be divided into five stages: prophase, prometaphase, metaphase, anaphase and telophase (Figure 1.2). Preparation for mitotic chromosome segregation begins in the S phase of the cell cycle. As the DNA is duplicated cohesion is established between sister-chromatids. Cohesion is mediated throughout most of the cell cycle by the multisubunit chromosome-associated protein complex, appropriately called the Cohesin complex. This complex is composed by two proteins of the structural maintenance of chromosome (SMC) protein family, SMC1 and SMC3 that are associated with two non-SMC components, Scc1 and Scc3 (Figure 1.3). During S phase other important cytoplasmic components are also replicated, such as the centrosome. As happens with the DNA, the centrosome is only duplicated once per cell cycle so that each daughter cell receives a single centrosome. The centrosome contains two centrioles and is the main microtubule organizing center (MTOC) of the cell. Many proteins involved in the overall regulation of the cell-cycle progression are localized to the centrosomes.

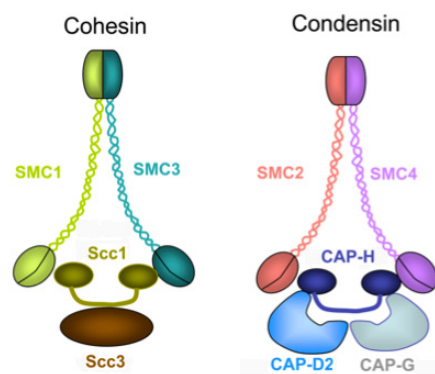


Figure 1.3. Condensin and Cohesin complexes. Both Cohesin and Condensin complexes contain two proteins of the structural maintenance of chromosome (SMC) protein family (SMC1 and SMC3 in Cohesin complex and SMC2 and SMC4 in Condensin complex) that are associated with two or three, respectively, non-SMC components (Scc1 and Scc3 in Cohesin complex and CAP-H, -D2 and -G in Condensin complex) (Adapted from Kitagawa 2009).

Prophase starts with the onset of chromosome condensation and sister-chromatid resolution. During prophase, DNA undergoes condensation, which consists in its compaction into chromosomes, each composed by two sister chromatids. Sister-chromatid resolution takes place later in prophase and is the individualization of sister chromatids and their organization around individual axes. This involves decatenation of the sister chromatid DNA by Topoisomerase II and on the removal of the Cohesin complex from the arms of the chromosomes whilst still joined at the centromeres. Centromeres are specific chromatin regions that constitute the sites for the assembly of the kinetochore - the structure that mediates spindle microtubule attachment. Chromosome condensation is mediated by the Condensin

complex, a complex composed by a heterodimer of SMC proteins, SMC2 and SMC4, and three non-SMC subunits, CAP-D2, CAP-G and CAP-H (Figure 1.3). Besides chromosome condensation and sister chromatid resolution, around the nucleus the previously duplicated centrosomes separate and move to opposite sides, where they will form the two poles of the mitotic spindle.

The beginning of prometaphase is characterised by the breakdown of the nuclear envelope. This allows microtubules that are being nucleated from both centrosomes that have migrated to opposite poles sides to search for and attach to kinetochores. Microtubules are highly dynamic polymers composed by α - and β -tubulin that continuously grow and shrink, allowing them to explore the cell during prometaphase and interact with chromosomes. Kinetochores are multiprotein complexes assembled at the centromere of each sister-chromatid and serve as the site for spindle microtubule attachment. Through the search and capture mechanism, microtubules dynamically search for and capture kinetochores. During this process chromosomes move back and forth until they attach correctly to spindle microtubules. Once the kinetochores of each sister-chromatid become stably attached to microtubules from opposite poles and become bioriented (amphitelic attachment), they can align at the center of the mitotic spindle, reaching the metaphase stage. Before chromosomes achieve proper amphitelic attachment, kinetochores can interact incorrectly with spindle microtubules, so that only one sister kinetochore is attached to a pole (monotelic attachment), with both sister kinetochores attached to the same pole (syntelic attachment) or with one sister kinetochore attached to one pole, while the other is attached to both poles (merotelic

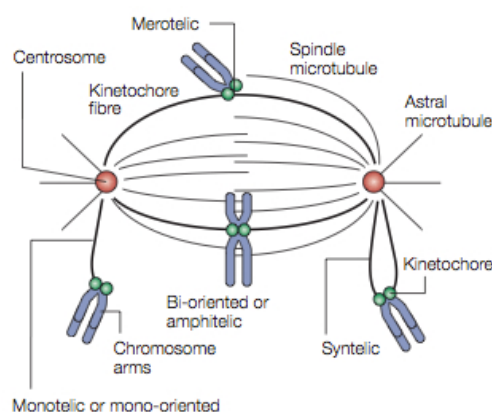


Figure 1.4. Accurate chromosome segregation requires chromosome bi-orientation. Schematic representation of a metaphase spindle with a correctly bi-oriented (amphitelic) chromosome (the sister kinetochores are attached to opposite poles) and three mal-oriented chromosomes: in monotelic (mono-oriented) attachments only one kinetochore is attached to one pole; in merotelic attachments one kinetochore is attached to both poles and in syntelic attachments both kinetochores are attached to the same spindle pole (Adapted from Keen and Taylor 2004).

attachments) (Figure 1.4). Several molecular mechanisms are involved in detecting and correcting abnormal attachments (see later section 1.4.2, on the Spindle Assembly Checkpoint). During prometaphase, forces generated at kinetochores pull chromosomes along the microtubule track toward the spindle pole, or towards the center of the cell, resulting in the generation of inter- and intra-kinetochore tension. Once all chromosomes are properly attached and positioned at the center of the cell, the cell is at metaphase.

After all chromosomes congress to the metaphase plate, anaphase starts due to the cleavage of the cohesion subunit SCC1 by the protease Separase. This destroys the cohesion between sister chromatids that had remained at the centromere and chromosomes move apart, to opposite poles of the mitotic spindle. Once sister chromatids are at opposite poles of the spindle, telophase begins with the disassembly of the spindle, the reassembly of the nuclear envelop and the decondensation of the chromosomes.

Cytokinesis is the process during which the cytoplasm and the cellular components of the parental cell, previously duplicated in interphase, are divided into the two daughter cells. In animal cells, cytokinesis is mediated by a contractile ring of actin and myosin II filaments, which forms in the cytoplasmic side of the cell membrane. The gradual contraction of the contractile ring pulls the membrane inward, forming a cleavage furrow that eventually divides the cell into two. The localization of this cleavage furrow is determined by the position of the mitotic spindle to ensure that each daughter cell receives only a single copy of the genome (reviewed by Lodish *et al.* 2000; Morgan 2007).

1.3. Regulation of the cell cycle

To coordinate the events of the cell cycle described above, eukaryotic cells have developed a highly regulated molecular machine that is driven by cycles of Cyclin-dependent Kinase (Cdk) activity working in concert with several other protein kinases, such as members of the Polo and Aurora kinases families. These molecular switches are essential mechanisms that allow the cell to maintain the proper order of events so that, for example, the cell does not proceed to G₂ phase before the DNA replication is complete.

1.3.1. Cyclin-Dependent Kinases (Cdks)

The main components of the cell cycle regulatory mechanism are the Cyclin-dependent Kinases (Cdks). Along the different phases of the cell cycle the activity of

these kinases changes (Figure 1.5). Cdks are a family of small serine/threonine protein kinases composed mainly by a catalytic core shared by all protein kinases. In *D. melanogaster* there are three Cdks that control cell cycle (Cdk1, Cdk2 and Cdk4) and humans have a fourth one, Cdk6. As their names indicate, these kinases need to bind to a cyclin (Cyc) subunit to be active. Cdk activity oscillates during the cell cycle and this oscillation is mainly due to the synthesis and eventual destruction of their regulatory subunit, the Cyclins. Depending on which Cyclin Cdks are associated with, different cell cycle events are promoted by the phosphorylation of a large number of proteins. Particular phosphorylation events are responsible for the initiation and progression of different cell cycle phases.

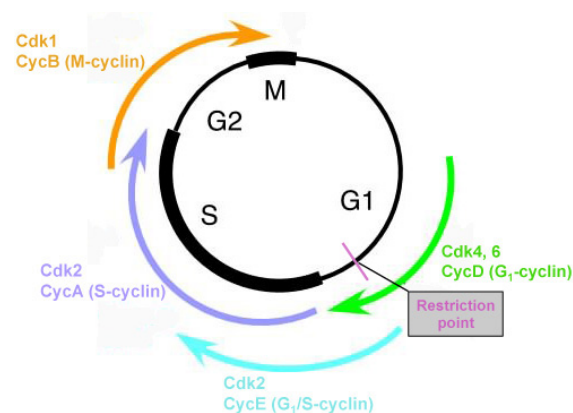


Figure 1.5. Schematic representation of the Cyclins (Cyc) and Cyclin-dependent Kinases (Cdk) complexes throughout the cell cycle. Cdk4/6-CycD complexes control progression through the G₁ restriction point. Cdk2-CycE complexes function later in G₁ and are also required for the G₁ to S transition. Cdk2-CycA complexes are then required for S phase progression and Cdk1-CycB complexes drive the G₂/M transition (Adapted from www-bcf.usc.edu).

There are four classes of Cyclins: G₁, G₁/S, S and M Cyclins. G₁ Cyclins are the only ones whose concentration depends on the rate of cell growth or on growth-promoting signals. Cdk4-CycD complexes (and also Cdk6-CycD in some cells) play a critical role in progression through the restriction point in G₁ in response to extracellular factors. Cdk inhibitor proteins (CKIs) that bind and inactivate Cdk-Cyc complexes are also important for promoting the arrest of the cell cycle in G₁ in response to unfavourable environmental conditions or intracellular signals such as DNA damage.

The G₁/S Cyclin in *D. melanogaster* and in humans corresponds to Cyclin E. Cdk2-CycE complexes are required for the G₁/S transition and initiation of DNA synthesis. The increase in CycE levels also leads to the production of CycA (a S phase Cyclin in vertebrates), resulting in the formation of the Cdk2-CycA complex

that is responsible for initiating DNA replication. CycA levels rise in S phase and stay high until nuclear envelop breakdown (NEB), in early mitosis.

CycB (the M phase Cyclin in vertebrates) levels start to rise in G₂, reach their maximum level at the beginning of mitosis and start to be degraded at metaphase-anaphase transition. Cdk1-CycB complexes are responsible for mitotic spindle assembly and contribute to the alignment of sister-chromatids in metaphase. The Cdk1-CycB complex is also known as maturation promoting factor (MPF), but this term is usually only used in meiosis.

Binding of Cyclins to Cdks only activates Cdks partially; for full activation a threonine residue near the kinase active site of the Cdks needs to be phosphorylated by another Cdk, known as Cdk7 (complexed with cyclin H) or as Cdk-activating kinase (CAK). Besides this activation by phosphorylation, Cdks can also be inhibited by phosphorylation. The inhibitory phosphorylation, in vertebrates and in *D. melanogaster*, is catalysed by two Wee1-related protein kinases, Wee1 and Myt1. When Cdks are phosphorylated by one or both of these kinases they are inactive. In order to become active again these inhibitory phosphorylation sites have to be removed by a phosphatase. In vertebrates, dephosphorylation is carried out by the Cdc25 protein family (Cdc25A, Cdc25B and Cdc25C) and in *D. melanogaster* by String. In part, the phosphorylation that activates Cdc25 and inactivates Wee1 and Myt1 (myelin transcription factor 1) is done by Cdk1 itself and also by Polo-like kinases, resulting in a positive feedback loop that activates Cdk1 rapidly during entry in mitosis (reviewed by Morgan 1997; Cooper 2000).

Once a Cdk is activated, its activity is kept at a maximum level until its role is completed. The transition from one cell cycle stage to a next one is unidirectional and irreversible. To ensure this, after a particular stage is completed, proteins that are essential for the execution of that stage are mostly degraded, so that the cell cannot go back and can only progress to the next stage. Thus, protein degradation appears to ensure unidirectionality of the process. Protein degradation is achieved through the Ubiquitin-proteasome pathway. This pathway uses the highly conserved polypeptide Ubiquitin as a tag to target proteins for degradation. Additional Ubiquitin subunits are then added to form a multiubiquitin chain. These polyubiquitinated proteins are then recognized and degraded by the 26S proteasome. Ubiquitination is a multistep process carried out by three enzymes, the Ubiquitin-activating enzyme (E1), the Ubiquitin-conjugating enzyme (E2) and the Ubiquitin ligase (E3). The E3 is the enzyme responsible for the selective recognition of appropriate substrate proteins. There are two related E3 complexes that are involved in controlling different stages of cell cycle progression: the anaphase-promoting complex or cyclosome

(APC/C) and the SCF (Skp1/cullin/F-box protein)-related complexes. APC/C with its activator Cdc20 controls metaphase/anaphase transition, since it is required for sister chromatids separation and with its other activator Cdh1, controls the levels of important mitotic regulators in G_1 . The SCF complex controls the G_1/S transition, and to some extent, the G_2/M transition (reviewed by Vodermaier 2004).

Cdk-Cyclin complexes work by phosphorylating target proteins and thereby changing their organization and function. For example, in late prophase, Cdk1-CycB complexes formed in the cytoplasm move to the nucleus, where they phosphorylate the proteins of the nuclear lamina, triggering NEB. The Cdk1-CycB complexes also promote other mitotic processes such as centrosome separation and reorganization of the Golgi apparatus. At the same time of NEB, CycA starts to be degraded (reviewed by Cooper 2000; Morgan 2007).

As the cell reaches metaphase, Cdk1-CycB activates APC/C by phosphorylating its core subunits, enhancing Cdc20 binding. This way, at metaphase/anaphase transition, Cdc20 activates the APC/C, leading to the ubiquitination and consequently proteolysis of Securin, CycB and several other proteins. Cdk1 inactivation leads to dephosphorylation of Cdk targets, spindle disassembly, exit from mitosis and cytokinesis.

As already stated, APC/C needs to be phosphorylated by Cdk1-CycB complex for Cdc20 to be able to bind it, but Cdh1 does not require phosphorylation. On the contrary, phosphorylation of Cdh1 by Cdk prevents it from binding to the APC/C from S phase to metaphase (Zachariae *et al.* 1998). So, APC/C^{Cdc20} activation results in the destruction of CycB, and thereby inactivation of Cdk1-CycB complex. This inactivation decreases APC/C phosphorylation, which leads to the release of Cdc20 from APC/C and consequently to the formation of APC/C^{Cdh1}. The binding of Cdh1 to the APC/C maintains its activity as cells enter G_1 to ensure continued destruction of CycB. As long as APC/C is active by Cdh1, cells can pause in G_1 . In *D. melanogaster* this is not the only factor that stabilizes the G_1 state. During this time, cells accumulate CycE, which is not a substrate of APC/C^{Cdh1}. Once the cell is committed to another cell cycle, by growth or extracellular factors, Cdh1 is phosphorylated by Cdk2-CycE complex. This phosphorylation leads to the inactivation of APC/C until the next mitotic exit. Besides this mechanism there is another one that maintains APC/C^{Cdh1} inactive during G_2 . This mechanism depends on the regulator of CycA-1 (Rca1) that allows the accumulation of CycA during G_2 by inhibiting APC/C^{Cdh1}. However, in vertebrates only Cdk2-CycA inhibits APC/C^{Cdh1}, but since CycA is a substrate of APC/C^{Cdc20} and APC/C^{Cdh1} it is yet unclear how CycA accumulates. Nevertheless, vertebrate cells contain an orthologue of *D.*

melanogaster Rca1, called early mitotic inhibitor-1 (Emi1) that inhibits APC/C^{Cdh1} thus allowing the accumulation of APC/C substrates at G₁/S transition. Besides APC/C^{Cdh1} inhibition, Emi1 can also inhibit APC/C^{Cdc20} in early stages of mitosis when the spindle-assembly checkpoint is not yet active. Emi1 is then targeted by the Polo-like kinase 1 (Plk1) to be degraded by SCF (reviewed by Peters 2006).

1.3.2. Polo-like kinases (Plks)

Besides Cdks, other protein kinases like Polo kinases have been found to play an essential role in the orchestration of cell division (Figure 1.6). Polo was first identified in *Drosophila melanogaster* and is highly conserved through evolution. The budding and the fission yeasts have only one Polo-like kinase (Cdc5 and Plo1, respectively), while *D. melanogaster* has two (Polo and Sak) and humans have four members (Plk1-4). Polo is closest related to yeasts Polo-like kinase and to the human Plk1. All Plks have a similar architecture, with an N-terminal kinase domain and one or two C-terminal regulatory domains, known as Polo boxes, which mediate protein interactions. Comparing the function of several Plks of different organisms lead to the conclusion that they play similar cellular roles despite of the differences in the molecular mechanisms for each pathway.

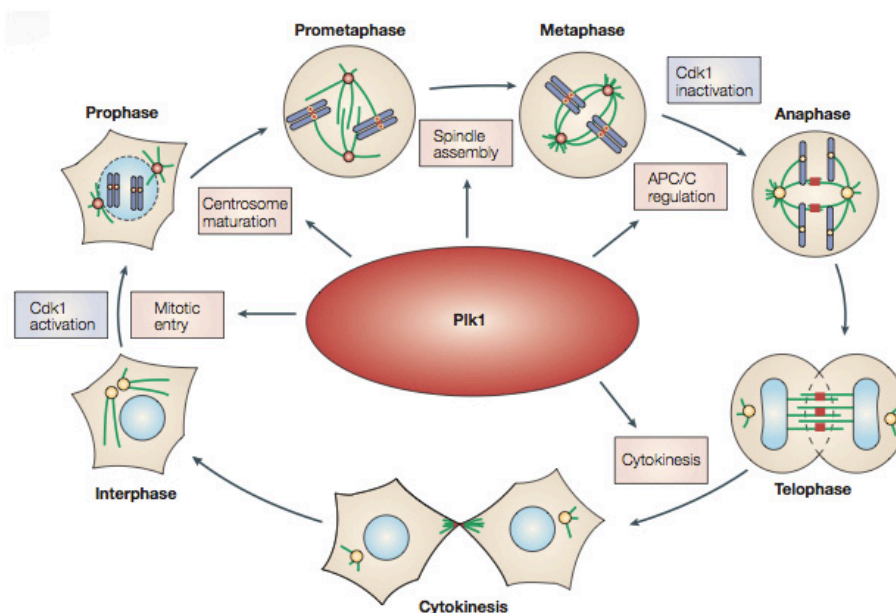


Figure 1.6. A schematic view of Plk1 functions in mammalian mitosis. The red colour is used to indicate the association of Plk1 with different structure; DNA/chromosomes are marked in blue; microtubules in green and both centrosomes and kinetochores in yellow (Adapted from Barr *et al.* 2004).

Throughout cell cycle Polo is involved in several stages of cell division, nevertheless it has only been described the roles of Polo during mitosis. At mitotic

entry, Polo activates Cdk1, directly through CycB phosphorylation at centrosomes (Toyoshima-Morimoto *et al.* 2001; Jackman *et al.* 2003) and indirectly by phosphorylating and activating Cdc25 (Roshak *et al.* 2000; Toyoshima-Morimoto *et al.* 2002) and by phosphorylating and inactivating Wee1 and Myt1 (Nakajima *et al.* 2003; Watanabe *et al.* 2004). In vertebrates, Polo kinases have been implicated in the activation of APC/C and in human cells Plk1 targets Emi1 (an APC/C inhibitor) for degradation (Hansen *et al.* 2004; Moshe *et al.* 2004; Eckerdt and Strebhardt 2006). Polo is also required to promote mitotic entry after DNA damage response (van Vugt *et al.* 2004). During prophase, Polo phosphorylates Cohesin on chromosome arms, promoting its dissociation (Sumara *et al.* 2002) and allowing for sister chromatid resolution. At spindle poles, Polo is involved in bipolar spindle formation (Sunkel and Glover 1988) as well as in centrosome maturation by recruiting the γ -tubulin ring complex (γ -TuRC) and by activating Abnormal spindle (Asp) (do Carmo Avides *et al.* 2001; Donaldson *et al.* 2001). Polo also controls the centrosomal localization of Aurora A kinase (De Luca *et al.* 2006) and inhibits an isoform of shugoshin 1 (Sgo1) that is involved in centriole cohesion and centriole separation after their replication (Wang *et al.* 2008). Additionally, *D. melanogaster* Sak (or Plk4 in humans) is essential for centriole duplication (Bettencourt-Dias *et al.* 2005; Habedanck *et al.* 2005). Polo is also involved in cytokinesis. Despite the mechanical differences of cytokinesis in yeasts and animals, Polo-like kinases are found to be indispensable for this process (Ohkura *et al.* 1995; Carmena *et al.* 1998; Petronczki *et al.* 2008).

1.3.3. Aurora kinases

Besides Plks, Aurora kinases also play an important role in the control of several mitotic events. There are three Aurora kinases, Aurora A, Aurora B and Aurora C.

Aurora A is mainly involved in centrosome maturation, being required for the correct localization and function of essential centrosomal components, which promote growth of the pericentrosomal material (PCM) and contribute for increased centrosomal microtubule nucleation activity (Terada *et al.* 2003; Mori *et al.* 2007). Aurora A is also involved in the separation of the duplicated centrosomes that will define the two poles of the mitotic spindle (Glover *et al.* 1995; Liu and Ruderman 2006). Besides these important roles of Aurora A in centrosome function, this kinase is also required for mitotic entry by promoting the G₂-activation of Plk1 (Macurek *et al.* 2008; Seki *et al.* 2008) and for Cdc25B activation at centrosomes (Cazales *et al.*

2005). Both these phosphorylation events lead to initial Cdk1-CycB activation at the centrosomes during the G₂/M transition (Lindqvist *et al.* 2005).

The Aurora family member that has a broader function in cell cycle is Aurora B. At the beginning of mitosis, this kinase is involved in the organization of chromosomes in two ways. It is essential to recruit the Condensin complex during chromosome condensation (Giet and Glover 2001) and in the process of Cohesin complex removal from chromosome arms, since Aurora B restrains the localization of Sgo1 to centromeric regions (Dai *et al.* 2006; Resnick *et al.* 2006). Aurora B is also involved in the assembly of the mitotic spindle (Sampath *et al.* 2004; Kelly *et al.* 2007). Aurora B is essential for the establishment of correct microtubule-kinetochore attachments. It has been found that this kinase promotes the destabilization of abnormal chromosome attachments (Tanaka *et al.* 2002; Lampson *et al.* 2004) and promotes kinetochore-microtubule turnover in merotelic attachments (Cimini *et al.* 2006; Knowlton *et al.* 2006). This way Aurora B plays an “indirect” role in the spindle assembly checkpoint (SAC) by creating unattached kinetochores in the presence of attachments that do not generate tension, so that they can recruit checkpoint proteins to kinetochores resulting in APC/C^{Cdc20} inhibition (Biggins and Murray 2001; Ditchfield *et al.* 2003). Additionally, Aurora B is required for efficient disassembly of the mitotic spindle during telophase (Buvelot *et al.* 2003) and to promote the correct function and localization of several proteins involved in cytokinesis (Kaitna *et al.* 2000).

Aurora C is the least known member of the Aurora protein family. There are several studies regarding Aurora C function in controlling cell cycle progression in somatic and/or tumour cells (Kimura *et al.* 1999; Chen *et al.* 2005; Yan *et al.* 2005), but until now it is only clear that Aurora-C is required for spermatogenesis and male fertility (Dieterich *et al.* 2007; Kimmins *et al.* 2007).

1.4. Cell cycle checkpoints

Checkpoints are key surveillance mechanisms that monitor cell cycle events, so that subsequent events do not initiate if problems in the completion of previous ones are encountered. During the cell cycle there are three major checkpoints (Figure 1.7).

1.4.1. G₁ Checkpoint and G₂ Checkpoint

The G₁ checkpoint and G₂ checkpoint arrest cell cycle progression in the presence of DNA damage, allowing the cell to repair the damage before DNA

replication takes place or before entry to mitosis, respectively. G_2 checkpoint also arrests cells in G_2 in the presence of unreplicated DNA and ensures that the genome is only replicated once per cell cycle.

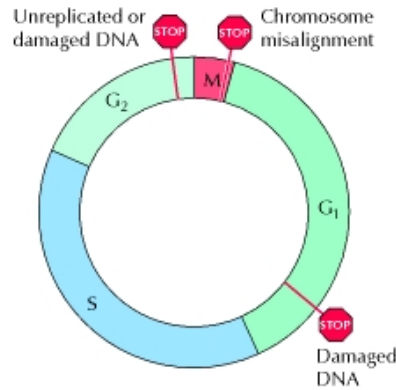


Figure 1.7. Cell cycle checkpoints. Several checkpoints monitor cell cycle progression. The presence of damaged DNA can activate G_1 and G_2 checkpoints. G_2 checkpoint can also be activated in the presence of unreplicated DNA. In mitosis, the spindle assembly checkpoint (SAC) monitor chromosome misalignment and misattached (Adapted from Cooper 2000).

Both G_1 and G_2 checkpoints can be triggered by the presence of DNA damage. Some types of DNA damage, like subtle changes in nucleotide base structure, are easily repaired due to the existence of sensor proteins that scan the genome, detect the lesion and recruit specialized enzymes to repair it. Other types of DNA damage are more extensive and more difficult to repair. In these cases, sensors activate a specialized molecular pathway called the DNA damage response. This response involves the activation of signalling pathways that diffuse the DNA damage signal to several effector proteins. Some of these proteins increase the production of DNA repair enzymes in order to repair the damages, other effector proteins inhibit cell cycle progression by triggering a system that leads to cell-cycle arrest. The DNA damage checkpoint is activated when one or the two protein kinases, ATM (Ataxia Telangiectasia Mutated) and ATR (ATM and Rad3-related) are recruited to the DNA damaged site. The activation of one or both protein kinases depends on the type of the DNA lesion as well as on the phase of the cell cycle in which the DNA damage response is triggered. The way in which these two protein kinases are recruited to the site of the DNA damage is also different for each one of these protein kinases. However, as result of their recruitment, both kinases phosphorylate regulatory proteins that ultimately activate two effector protein kinases, Chk1 (Checkpoint Kinase 1) and Chk2 (Checkpoint Kinase 2). These proteins phosphorylate Cdc25, inhibiting its interaction with Cdk2, in G_1 checkpoint, and Cdk1, in G_2 checkpoint, blocking the formation of Cdk2-CycE and Cdk2-CycA complexes in the first case and

Cdk1-CycB complex in the second. As consequence, cell cycle is arrested. However, activation of ATM and/or ATR in G₁ and G₂ checkpoints can also promote a delayed and a long-term maintenance of cell-cycle arrest, through stabilization and activation of p53 (reviewed by Cooper 2000; Morgan 2007; Brnzei and Foiani 2008).

1.4.2. Spindle Assembly Checkpoint

The Spindle Assembly Checkpoint or SAC monitors incorrect kinetochore-microtubule attachments at the metaphase-to-anaphase transition and generates a “wait-anaphase” signal capable of delaying mitotic exit in the presence of unattached or incorrectly attached kinetochores. The SAC is so sensitive that a single unattached kinetochore is enough to generate the “wait-anaphase” signal (reviewed by Musacchio and Salmon 2007). Key components of SAC were identified in two independent genetic screens in budding yeast, designed to isolate mutants for whom the mitotic arrest was overridden in the presence of microtubule depolymerising drugs. These screens allowed the identification of the Mad1, -2 and -3 (Mitotic-arrest Deficient) and Bub1 and -3 (Budding Uninhibited by Benomyl) genes (Hoyt *et al.* 1991; Li and Murray 1991). The Mad and Bub proteins are highly conserved throughout evolution (Li and Benezra 1996; Taylor and McKeon 1997; Bernard *et al.* 1998; Gorbisky *et al.* 1998; Basu *et al.* 1999) with Bub-related 1 kinase (BubR1) being the only exception. BubR1 was identified in higher eukaryotes and displays homology in its N-terminal part with the yeast Mad3 protein and in its C-terminal part with the kinase domain of Bub1 (Taylor *et al.* 1998). Additionally, the Monopolar Spindle 1 protein (Mps1) is also a component of the mitotic checkpoint (Weiss and Winey 1996).

At the beginning of mitosis, to prevent precocious activation of the APC/C, an inhibitory signal is produced that prevents the activation of APC/C until all chromosomes have become correctly attached and bi-oriented between opposite spindle poles on the metaphase plate. The complete mechanism by which SAC delays anaphase onset in the presence of unattached or incorrectly attached kinetochores is still not known. Nevertheless, it is now clear that the localization of SAC components at unattached kinetochores is essential for the production of the inhibitory signal and that the production of complexes with SAC components is important for the maintenance of the inhibition. However, it is not yet very clear if the maintenance of the inhibitory signal is due to the binding of Cdc20 to SAC components or due to the fast turnover of SAC components at unattached kinetochores (reviewed by Musacchio and Salmon 2007).

Taking into account several studies, a “two-step” model for SAC activation and its maintenance was proposed. This model suggests that in a first and kinetochore-independent step, APC/C inhibition results from the production of the mitotic checkpoint complex (MCC), a cytosolic multisubunit complex composed by several SAC proteins, including Mad2, BubR1 and Bub3, and also by the APC/C regulator Cdc20. The MCC inhibits APC/C activity at early stages of mitosis, even before the assembly of the kinetochore, since it was found to be present and active in interphase cells. So, its formation does not require unattached kinetochores. This way, MCC provides a rapid mechanism to inhibit APC/C when cells enter mitosis. The second step is kinetochore-dependent and starts as soon as NEB, with the accumulation of SAC proteins at unattached or incorrectly attached kinetochores, and with the sequestration of Cdc20 by Mad2 and BubR1 (reviewed by Chan *et al.* 2005; Musacchio and Salmon 2007; Ciliberto and Shah 2009). Once all sister kinetochores are correctly attached, aligned at the metaphase plate and under tension, the production of the inhibitory signal is stopped, which leads to APC/C activation and mitotic exit.

2. Meiosis

The cell cycle described previously corresponds to the one that occurs in embryonic and somatic cells and results in the production of two daughter cells with identical genetic composition from each other and to the parental cell. However, there is another type of cell cycle in which a parental diploid cell gives rise to four haploid cells with only one copy of the genome. This specialized cell cycle called meiosis, is restricted to germ cells, is the key to sexual reproduction and, due to recombination, will give rise to genetic diversity.

2.1. Meiotic stages

At the onset of meiosis, DNA is replicated and sister chromatid cohesion is established. But, in contrast to what happens in mitosis, two sequential rounds of cell division without intervening cytokinesis, follow the pre-meiotic S phase in order to reduce the genome copy number. Meiosis results in the production of haploid cells or gametes that prevent the doubling of the genetic material with every new generation. These sequential rounds of nuclear division are called Meiosis I and Meiosis II.

A diploid cell possesses a pair of each chromosome also called homologous. During mitosis each chromosome behaves independently of its homolog whereas, in meiosis I, the homologous chromosomes first pair with one another and normally undergo recombination, and then segregate to different daughter cells maintaining the sister chromatids together. Due to the fact that chromosome number is reduced to half, creating two haploid cells, this division is also known as reductional division. Meiosis I is followed by Meiosis II that resembles mitosis in that sister chromatids separate, segregate to opposite spindle poles and give rise to different daughter cells. This division is also known as equational division, since it originates four haploid without reduction in chromosome number (Figure 1.8). This pattern of segregation requires an extended prophase I during which chromosomes must assemble meiosis-specific axial structures, locate and align with their homologous, stabilize this alignment and undergo crossover recombination events between their DNA molecules. Prophase I is subdivided into five substages (leptotene, zygotene, pachytene, diplotene and diakinesis) based on the changes in chromosome morphology visible by light microscopy and on the progression of pairing and synapsis.

In leptotene stage (Greek "*leptos*" = thin) each homologous chromosomal DNA is found in large parallel loops or coils organized around a central axis containing meiotic cohesion, forming the chromosomal axes. Also, both sister chromatids of

each homolog are tightly bound together by Cohesin. This way, leptotene is characterized by having chromosomes that are discernibly individualized, appearing thin and thread-like. At this stage, meiotic recombination initiates with double-strand breaks (DSBs) that are formed along sister chromatids.

In zygotene stage (Greek “*zygos*” = pair) homologous chromosomes axes pair along their entire length and a protein structure, called Synaptonemal complex (SC), assembles between them. This process is called synapsis and results in the formation of a structure known as bivalents (when homologs are connected as a result of chromosome pairing and synapsis). In this stage, chromosomes appear shorter and fatter. During this stage the DSBs are repaired.

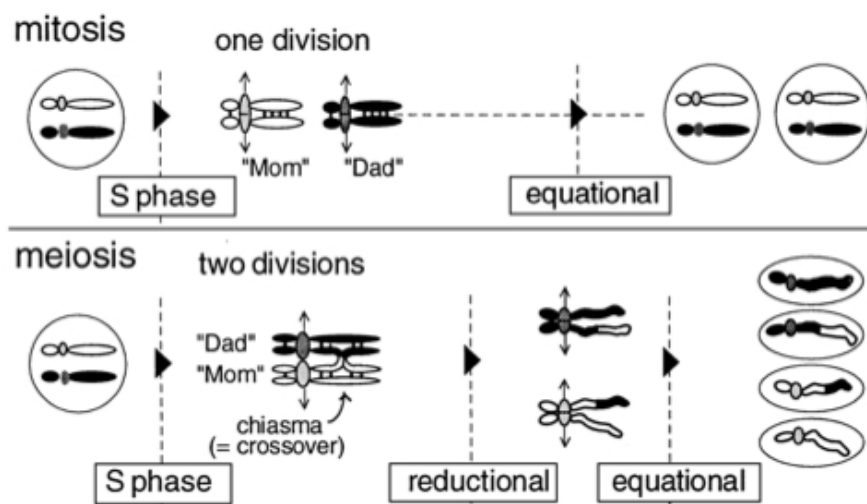


Figure 1.8. Comparison between mitotic and meiotic divisions. In mitosis, DNA replication is followed by one cell division, where chromosomes behave independently (equational segregation). In meiosis, DNA replication is followed by two sequential rounds of cell division. In the first division the homologous chromosomes, previously paired, segregate to different daughter cells (reductional segregation) and the second division is similar to mitosis, since there is the segregation of sister chromatids (equational segregation) (Adapted from Zickler and Kleckner 1998).

In pachytene (Greek “*pakhus*” = thick), SC is fully developed and meiotic recombination between homologs results in the formation of crossovers. In this stage chromosomes are shorter and thicker and tightly associated. The transition from pachytene to diplotene is often called the diffuse stage due to the dramatically decondensation of chromatin. In this “stage” the SC components are progressively disassembled resulting in the desynapsis of homologs. Despite of SC dissolution, bivalents remain connected due to the presence of cohesion between sister DNA molecules.

In the classical diplotene (Greek “*diploos*” = double) configuration, homologues are widely separated (in repulsion) but remain held together at the chiasmata (Greek

“*chiasma*”, plural “*chiasmata*” = beams arranged in a cross in the framework of a roof) and, eventually, sisters become more distinct due to chromosome condensation.

The final stage of prophase I is diakinesis (Greek “*kinesis*” = movement). During this stage, bivalents continue to shorten and compact until metaphase I, where they are nearly as short as their mitotic metaphase counterparts (reviewed in Zickler and Kleckner 1998; Petronczki *et al.* 2003; Pawlowski and Cande 2005).

2.2. Synaptonemal Complex

Page and Hawley (2004) compared the Synaptonemal complex (SC) to “railroad tracks”, where the lateral elements of SC are the two side rails connected by the transverse filaments (Figure 1.9). Electron microscopy studies (Page and Hawley 2004) revealed that at pachytene stage an electron-dense linear structure, running down the centre of the SC and known as the central element, is also visible. The SC has an important role in initiating meiotic recombination and in the maturation of recombination intermediates (single-strand invasions and double Holliday junctions) into crossover events. The SC is also required to maintain meiotic pairing and to ensure segregation in organisms that do not undergo meiotic recombination.

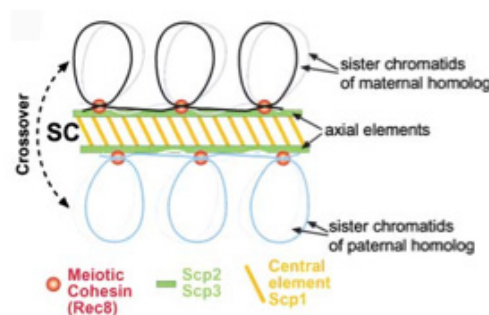


Figure 1.9. Schematic model of the synaptonemal complex structure in pachytene stage. The SC proteins include the transverse/central elements and the axial elements that will give rise to the lateral elements (Adapted from Petronczki *et al.* 2003).

The axial elements formed in leptotene are incorporated into the SC as part of the lateral elements. Besides the axial elements, several proteins were identified as being components of the lateral elements. These proteins differ between species: in mammals, are the Synaptonemal complex proteins (SCP), SCP2 and SCP3 (Offenberg *et al.* 1991); in yeasts, are Hop1 (Hollingsworth *et al.* 1990) and Red1 (Smith and Roeder 1997) and, in *Drosophila*, ORD (orientation disruptor) plays a key role in lateral elements formation and in SC maintenance (Miyazaki and Orr-Weaver 1992).

The Cohesin complex also has an important role in the formation of the lateral elements of the SC, by forming an axial chromosome core on which lateral element proteins can bind and assemble. However, some subunits of the Cohesin complex (described above) used in meiosis are replaced by meiosis-specific paralogs during pre-meiotic S phase. For example, Scc1 is replaced by a similar protein known as Rec8 (Klein *et al.* 1999; Watanabe and Nurse 1999) or C(2)M (Manheim and McKim 2003). Besides the Cohesin complex, the Condensin complex also plays a role in the assembly of the lateral elements, since it is required during meiotic prophase for axial length, compaction and chromosome individualization (longitudinal compaction). In addition to this role, which is similar to the one on mitotic chromosome condensation, the Condensin complex appears to have several meiosis-specific roles, namely in the recruitment of the SC complex and in chromosome pairing (Yu and Koshland 2003).

As it was said above, the lateral elements are connected by the transverse filaments, which are composed by proteins that have been identified in several species. Despite the fact that the amino acid sequence of these proteins is different, all of them contain in their centre an extended coiled coil-rich segment flanked at each end by large globular domains. These proteins include Zip1p in *S. cerevisiae* (Sym *et al.* 1993), SCP1 in mammals (Meuwissen *et al.* 1992) and C(3)G in *D. melanogaster* (Page and Hawley 2001).

2.3. Homologous Recombination and Crossovers Generation

In most organisms, the recombination process is initiated with the generation of DSBs (Sun *et al.* 1989). In *S. cerevisiae*, these DSBs are created by Spo11 (Keeney *et al.* 1997). *mei-W68* is the *Drosophila* SPO11 homolog (McKim and Hayashi-Hagihara 1998). *Drosophila* has another gene required for DSB formation, *mei-P22* (Liu *et al.* 2002). DSBs do not occur randomly along the chromosome but are focused at preferred sites, or recombination hotspots, which are generally found in chromatin that is relatively accessible to proteins. Spo11 is a meiosis-specific Topoisomerase-like enzyme that forms a tyrosine phosphodiester linkage with both 5' ends created by cleavage (Keeney *et al.* 1997). These 5' ends are then resected by a 5'-to-3' exonuclease creating single-stranded 3' overhangs. Afterwards, through a process called single-end invasion, the single-stranded 3' overhang invades the uncut homologous non-sister chromatid and pairs with it, creating a template for repair synthesis, displacing the other strand of the DNA duplex to form a single-stranded loop, known as a D-loop. By an unknown mechanism, recombination between sister-chromatids is suppressed. Eventually, the extension of the single

strand then leads to the expansion of the D-loop and to the pairing of the other single-stranded 3' overhang, creating as well a template for repair synthesis. The migration of the heteroduplex branch exposes the 5' ends originated from the resection, which are then ligated to the newly synthesized DNA, creating a joint molecule called a double Holliday junction (DHJ). The last step of meiotic recombination is the resolution of the DHJs into reciprocal exchanges (crossovers). Resolution involves the cleavage of a pair of strands at each end of the junction and their reciprocal ligation. The cleavages can be horizontal or vertical but, to form a crossover, one junction must be resolved horizontally and the other vertically. If both cleavages are horizontal or vertical it generates noncrossovers with patches of gene conversion that happen at the site of DSBs. Mapping analysis indirectly supports the assumption that the sites of crossovers will be the chiasmata points (reviewed in Roeder 1997; Petronczki *et al.* 2003; Morgan 2007) (Figure 1.10).

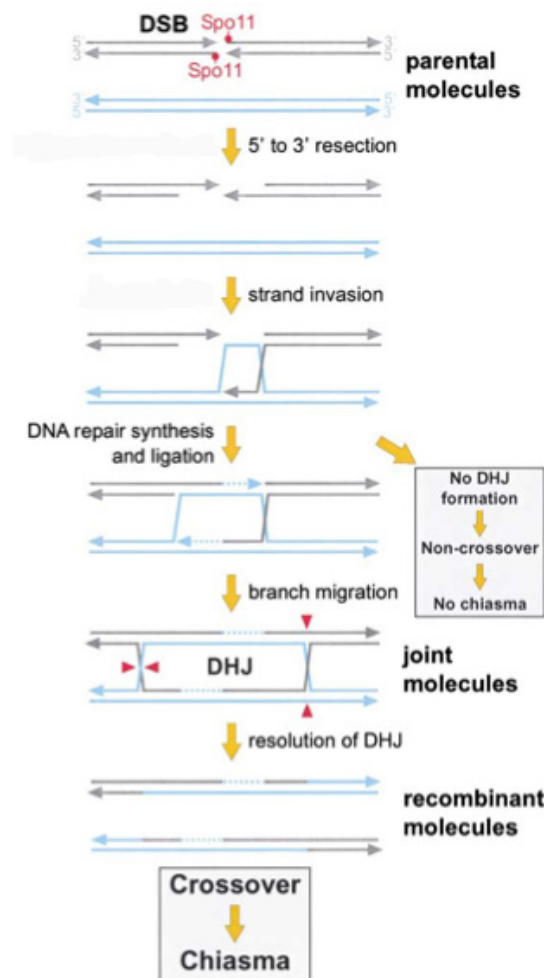


Figure 1.10. Meiotic DSB repair pathway of meiotic recombination and chiasma formation. The process is explained in the main text. “DHJ” stands for double-Holliday junction; the red arrowheads represent the way that the DHJ have to be resolved in order to produce crossovers (Adapted from Petronczki *et al.* 2003).

Genes required for the repair of all meiotic DSBs are encoded by homologs of the Rad52 epistasis group: *okra* (RAD54), *spn-B* (XRCC3); *spn-D* (RAD51C) (Ghabrial *et al.* 1998) and *spn-A* (RAD51) (Gonzalez-Reyes *et al.* 1997). Besides these DSBs repair genes, two additional classes of *Drosophila* meiotic genes are specifically required for crossing over: the precondition and the exchange classes of genes. The precondition class of genes may be involved in the process that determines the number and the distribution of crossovers. This class of genes include: *mei-218* (McKim *et al.* 1996), *mei-217* (Liu *et al.* 2000), *recombination-defective* (*rec*) (Grell and Generoso 1980) and *minichromosome maintenance 5* (*mcm5*) (Lake *et al.* 2007). The exchange class of genes may be directly involved in the part of the DSB repair pathway that generates crossovers. This class includes the endonuclease homologous to XPF/Rad1, *mei-9* (Sekelsky *et al.* 1995), *mus312* (SLX4/BTBD12) (Yildiz *et al.* 2002), *ercc1* (Radford *et al.* 2005) and *hold'em* (*hdm*) (Joyce *et al.* 2009) genes.

Not all DSBs result in DHJs because occasionally the pairing of the single-stranded 3' overhang with the uncut homologous non-sister chromatid is not stabilized. In these cases the invading strand eventually dissociates and returns to the chromatid from which came. The DSBs are then repaired creating an intact chromosome that is no longer attached to its homolog.

2.4. Prophase I checkpoints network

For prophase I to occur successfully cell cycle events that occur at the same time have to be coordinated. To achieve this, prophase I is controlled by two checkpoints: the meiotic recombination checkpoint and the pachytene checkpoint. The meiotic recombination checkpoint delays the oocyte developmental program in the presence of DSBs until damaged DNA is repaired. As in the mitotic DNA-damage response, this signal only initiates once DSBs have been formed. In *Drosophila*, oocytes with unrepaired DSBs have abnormal dorsal-ventral polarity and defects in a meiotic-specific organization of chromosomes called karyosome. These patterning defects are *mei-41*-dependent, since mutations in this gene suppress the dorsal-ventral patterning defects caused by unrepaired DSBs (Ghabrial and Schupbach 1999). Mei-41 is the *Drosophila* homolog of ATR and it is also involved in the mitotic DNA damage checkpoint (Hari *et al.* 1995).

One of the downstream targets of ATM/ATR in the mitotic DNA damage checkpoint is the *Drosophila* Chk2 homolog (DmChk2/Mnk). Mutations in *DmChk2* also suppress the phenotypes observed in mutants of DSB repair genes. So, as

happens in the mitotic DNA damage checkpoint, DmChk2 is also a target of Mei-41 in the meiotic recombination checkpoint (Abdu *et al.* 2002). However, there are several evidences suggesting that DmChk2 can also be activated by a Mei-41-independent pathway (Klovstad *et al.* 2008; Lu *et al.* 2010) (Figure 1.11).

The retarded mobility and the abnormal subcellular localization of the *Drosophila* Wee1 (dWee1) kinase, observed in DSBs repair mutant genes, is restored to wild-type in *DmChk2* mutants. So, activation of the meiotic recombination checkpoint affects dWee1 mobility and its localization through a *DmChk2*-dependent posttranslational modification. However, *dwee1* mutants are not able to suppress the dorsal-ventral patterning or the oocyte nuclear morphology defects caused by DSBs repair mutant genes. Therefore, Abdu, *et al.* (2002) suggest that two different pathways are activated by the persistence of unrepaired DSBs: one affecting dWee1 and the meiotic cell cycle progression, and a second pathway that leads to patterning defects (Abdu *et al.* 2002). Additionally, in *Drosophila*, the tumour suppressor protein p53 is expressed in the presence DSBs and this expression is also DmChk2-dependent (Lu *et al.* 2010) (Figure 1.11).

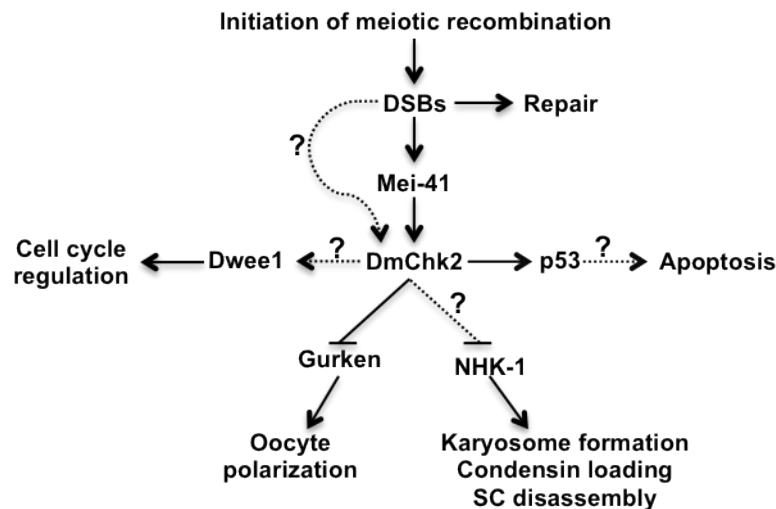


Figure 1.11. The meiotic recombination checkpoint pathway in *Drosophila* oocytes. In the presence of unrepaired DSBs formed during meiotic recombination the meiotic recombination checkpoint is activated. Mei-41 plays an important role in triggering this checkpoint. DmChk2 is activated by Mei-41, but there are evidences that it can also be activated through a Mei-41-independent pathway. Activation of DmChk2 leads to a strong reduction in *gurken* mRNA translation that results in polarity defects. Through an unknown pathway, the meiotic recombination pathway inactivates NHK-1 that is required for karyosome formation, Condensin loading and SC disassembly. DmChk2 also activates p53 that may lead to apoptosis in response to persistence DSBs. Additionally, the meiotic recombination checkpoint may affect the meiotic cell cycle progression in a Dwee1-dependent manner (Adapted from Abdu *et al.* 2002; Lancaster *et al.* 2010; Lu *et al.* 2010).

Gurken, the ligand for the Epidermal Growth Factor receptor, was shown to be a downstream effector of the meiotic recombination checkpoint required in oocyte

polarization (reviewed in Ray and Schupbach 1996). Gurken is responsible for specifying dorsal fates in *Drosophila*'s embryo and eggshell, by communicating information on oocyte polarity to adjacent follicle cells (Neuman-Silberberg and Schupbach 1993; Gonzalez-Reyes *et al.* 1997; Ghabrial *et al.* 1998). Mutations in DSB repair genes lead to improper localization and translation of *gurken* mRNA, which results in eggshells and embryos with polarity patterning defects (Ray and Schupbach 1996) (Figure 1.11).

The Nucleosomal Histone Kinase-1 (NHK-1) is a Histone 2A kinase shown to be involved in the reorganization of the oocyte nucleus, including karyosome formation, SC disassembly and Condensin loading (Ivanovska *et al.* 2005). In the presence of DSBs, the meiotic recombination checkpoint suppresses NHK-1 delaying oocyte nuclear reorganization and prophase I exit until DNA breaks are repaired (Lancaster *et al.* 2010) (Figure 1.11).

Independently of DSBs formation, defects in chromosome axis formation or abnormal SC assembly can also generate a delay in meiotic progression. This delay is triggered by the pachytene checkpoint. The pachytene checkpoint, despite of being DSB-independent, can also be activated in the presence of unrepaired DSBs. This checkpoint prevents exit from the pachytene stage of meiotic prophase I until meiotic recombination and chromosome synapsis are completed (Roeder 1997). Besides pachytene delay, pachytene checkpoint responses also include a delay in the phosphorylation of the histone variant H2Av, a chromatin response to DSBs, and a delay in choosing the oocyte between the two pro-oocytes. The checkpoint provides time for the generation of more crossovers when defects in the crossover generation pathway are present. These defects can be, for example, due to a mutation in a gene required to generate crossovers (in the exchange classes of genes) or due to heterozygosity for a chromosome rearrangement, as is the case of balancer chromosomes, which suppresses crossing over. How pachytene checkpoint facilitates more crossovers is not yet known, but might be due to an extension of the "crossover determination phase". Several studies have shown that the pachytene checkpoint is Pch2-dependent, since Pch2 is required to increase crossing over in heterozygous balancers and pachytene delays are associated with persistent Pch2 expression. In the presence of defects that cause pachytene delay, the protein Sir2 might be involved in the suppression of Pch2 degradation prior to late pachytene (Joyce and McKim 2009; Joyce and McKim 2010; Joyce and McKim 2011).

2.5. Chromosome Segregation

At meiosis I, chiasmata allow homologous chromosomes to remain connected while orienting away from each other toward opposite poles of the metaphase I spindle. In contrast to mitosis, in meiosis I the two sister kinetochores of each homolog must be attached to the same pole (syntelic attachment). How this is achieved it is not entirely known, but several studies indicate that either both kinetochores behave as a single unit with both attaching to the spindle pole or only one kinetochore is attached to the spindle whereas the other one is silenced (Goldstein 1981; Winey *et al.* 2005). In *S. cerevisiae*, sister chromatid co-orientation in meiosis I depends on the four-protein Monopolin complex (Mam1, Csm1, Lrs4 and Hrr25/casein) (Toth *et al.* 2000; Rabitsch *et al.* 2003; Petronczki *et al.* 2006). It has been proposed that this Monopolin complex crosslinks and/or co-orient sister kinetochores in meiosis I, so that they attach to microtubules extending from the same spindle pole (Monje-Casas *et al.* 2007) (Figure 1.12). Recently it was shown that the Csm1 and the Lrs4 Monopolin complex subunits form a complex and that a conserved surface path on these domains binds to two kinetochore subunits: Dsn1 (subunit of MND/Mis12 complex) and Mif2/CENP-C (Corbett *et al.* 2010). However, homologs of Monopolin have not yet been identified in other organisms.

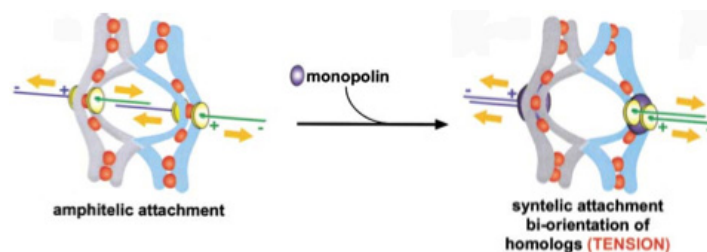


Figure 1.12. Model for microtubule-kinetochore attachments in Meiosis I. In the absence of the Monopolin complex the sister kinetochores of homologous chromosomes are amphitelic attached. The Monopolin complex promotes syntelic attachments to achieve tension (Adapted from Petronczki *et al.* 2003).

By holding maternal and paternal chromatids together, chiasmata greatly enhance the possibility to generate tension when microtubules are attached to kinetochores even in the presence of syntelic attachments. Due to this important role of chiasmata in ensuring the correct homolog segregation, crossing-over is a process tightly regulated. The frequency of crossovers is non-random and their distribution along each chromosome is controlled. Usually a single chiasma is sufficient to direct segregation but, in cases in which multiple crossovers form, they will not occur close to each other due to a phenomenon called interference that inhibits additional

crossovers in the vicinity of a previously established one. Additionally, crossovers only occur in euchromatin and normally rarely form near the centromere or the telomere. Since centromeric cohesion is maintained until anaphase II, if chiasmata were formed near the centromere they could not be resolved effectively in anaphase I (reviewed in McKim *et al.* 2002).

Nevertheless, segregation can also be achiasmate as happens for the *Drosophila* fourth chromosome or in synthetic balancer chromosomes. In these cases, segregation is ensured by the homologous achiasmate system that is based on heterochromatin homology, or by the non-homologous achiasmate system that does not require centromeric alignment and is based on parameters such as chromosome shape and chromosome size. However, achiasmate segregation can only correctly segregate one pair of large chromosomes since, equal number of chromosomes will be sent to each pole, without homology consideration, in the presence of two achiasmate autosomes (Hawley and Theurkauf 1993).

Due to the fact that during meiosis there are two consecutive rounds of chromosome segregation, sister-chromatid cohesion must be lost in a two-step process. In meiosis I cohesion is only lost on chromosome arms, while centromeric cohesion is lost in meiosis II. This system allows the separation of homologous chromosomes in meiosis I while preventing the loss of centromeric cohesion between sister chromatids, which ensures sister-chromatid congression and bipolar orientation during meiosis II. This occurs because centromeric cohesion is protected from cleavage in meiosis I by a protein called Sgo1, or Mei-S332 in *Drosophila*, which localizes at centromeres in meiosis I and associates with Rec8. This is accomplished by the Sgo1-dependent recruitment of the serine/threonine protein Phosphatase 2A (PP2A) to centromeres. It appears that PP2A counteracts Rec8 phosphorylation by Polo and/or Aurora B thus preventing Cohesin dissociation from centromeric chromatin during metaphase I. However, additional evidence, suggests that this is only part of the mechanism and that Sgo also has a role independent of PP2A (reviewed in Rivera and Losada 2006).

After spindle disassembly and cytokinesis, meiosis II initiates immediately, usually before the full chromosome decondensation and nuclear envelope reformation. As described above, DNA replication does not occur in the transition from meiosis I to meiosis II. The mechanism that prevents this is not entirely known but must be related to incomplete inactivation of Cdk1 that results in the dephosphorylation of only a subset of Cdk1 targets (Iwabuchi *et al.* 2000).

As happens in mitosis, during meiotic metaphase II kinetochores of sister-chromatids align at the metaphase plate and attach to the spindle with a bipolar

orientation. Sister-chromatid separation is achieved in anaphase II through an APC/C^{Cdc20}-dependent destruction of Securin that leads to activation of Separase and subsequently to cleavage of the Cohesin complex subunit Rec8 that is no longer protected by Sgo1.

An important difference between mitotic and most female meiotic spindles is their organization in the absence of centrosomes and centrioles in the latter. In these acentrosomal spindles, γ -tubulin ring complexes are localized at the two acentriolar poles and initiate microtubule nucleation; motor and other proteins are then responsible to organize these microtubules into a bipolar spindle. However, in *Drosophila* γ -tubulin is not present at the spindle poles. In this case it is thought that chromosomes nucleate or capture microtubules that are then shaped into a bipolar spindle, since meiotic spindle formation starts with a bulk of microtubules emanating from chromosomes (McKim and Hawley 1995; Theurkauf and Hawley 1992). The Kinesin-like protein Non-Claret Disjunctal (NCD) was the first protein identified that is involved in spindle pole formation during meiosis. In acentrosomal spindles, NCD promotes and stabilizes bipolar spindle organization by bundling spindle microtubules (Hatsumi and Endow 1992; Matthies *et al.* 1996). Subito (SUB) like NCD is a Kinesin-like motor protein that is also required to organize the poles of the acentrosomal meiotic spindle (Giunta *et al.* 2002). Additionally, No Distributive Disjunction (NOD) is another Kinesin protein involved in female meiosis. NOD is a Chromokinesin that facilitates interaction between the spindle and chromosomes and may act as a brake to prevent chromosomes from moving prematurely to the poles (Afshar *et al.* 1995; Matthies *et al.* 2001). Despite the fact that female meiotic spindle is formed in a centrosome-independent manner, two centrosomal proteins, Mini spindles (Msps) and Transforming Acidic Coiled-Coil protein (D-TACC), are found at acentrosomal poles in the female meiotic spindle of *Drosophila*. These proteins that are also associated with mitotic centrosomes, interact with each other and are both required for maintaining the bipolarity of acentrosomal spindles (Cullen and Ohkura 2001). Furthermore, Asp is also found in female meiotic spindles (Riparbelli *et al.* 2002).

2.6. Developmental Control of Oocyte Maturation and Egg Activation

In most animals, oocyte development involves meiotic arrest at two points. The first occurs at the first meiotic division in diplotene stage. During this arrest, the oocyte differentiates and undergoes growth to produce eggs capable of sustaining embryonic development upon fertilization. Human oocytes are able to remain

arrested at this stage up to 40 to 50 years. Oocyte maturation releases this arrest, allowing the oocyte to progress through meiotic divisions. In most vertebrates, the second arrest takes place at metaphase II but in insects it occurs at metaphase I whereas in marine invertebrates it is in G_1 that follows both meiotic divisions. This second arrest point coordinates the end of meiosis with fertilization (Sagata 1996). However some animals, like *Caenorhabditis elegans* only arrest meiosis once, at prophase I (Figure 1.13). In this nematode, sperm signalling releases oocytes from the meiotic arrest and promotes oocyte maturation and ovulation. Egg activation and completion of meiosis are then triggered by fertilization (Yamamoto *et al.* 2006).

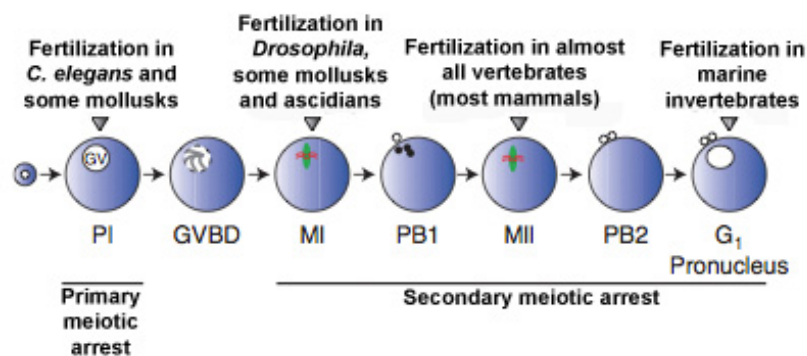


Figure 1.13. Meiotic arrests during oocyte development. The oocyte development of most animals undergoes two arrests, being the first one, for all these species, at prophase I (PI). The second meiotic arrest can be either at metaphase I (MI), at metaphase II (MII) or only at postmeiotic G_1 , depending on the species. In other species, oocyte development only arrests once at prophase I. GV, germinal vesicle; GVBD, germinal vesicle breakdown; PB1, polar body 1; PB2, polar body 2 (Adapted from Von Stetina and Orr-Weaver 2011).

2.6.1. Prophase I arrest

In most species, activation of MPF (maturation or M-phase promoting factor; Cdk1-CycB complex) is the key molecular event that promotes meiotic maturation and release from the prophase I arrest. So, to maintain the prophase I arrest MPF activity has to be kept at a low level (Sagata 1996). High levels of the cyclic adenosine 3', 5'-monophosphate (cAMP) in the oocyte are essential to keep MPF in an inactive state during this first arrest (Mehlmann 2005). High levels of cAMP promote activation of the cAMP-dependent Protein Kinase A (PKA) (Kovo *et al.* 2006) that simultaneously phosphorylates and activates Wee1 (a Cdk1 inhibitor), and inactivates Cdc25 (a Cdk1 activator). Another mechanism that maintains low MPF activity is through the presence of active APC/ C^{dh1} that restrains CycB accumulation and, therefore the MPF activation needed for the germinal vesicle breakdown (GVBD; the large nucleus arrested at prophase I is called germinal vesicle) (Reis *et al.* 2006). Cdh1 is probably activated by the phosphatase Cdc14B that counteracts

Cdk activity in mitotic cells (Schindler and Schultz 2009). APC/C^{Cdh1} activity is kept beyond GVBD in prometaphase I targeting Cdc20 for degradation, thereby preventing premature APC/C^{Cdc20} activation. So, contrarily to what happens in mitosis, during meiosis I in mouse oocytes APC/C^{Cdh1} is active prior to APC/C^{Cdc20}. APC/C^{Cdh1} activity is eventually ceased when CycB levels, and consequently MPF activity, exceed the Cdh1 threshold (Reis *et al.* 2007). Another study (Homer *et al.* 2009), identified Securin as an APC/C^{Cdh1} substrate preventing the overaccumulation of this protein and a persistent meiotic arrest. The APC/C^{Cdh1}-mediated destruction of Securin during prometaphase I was found to be BubR1-dependent, revealing an important function of BubR1 in prometaphase I. Indeed, the phenotype of oocytes BubR1-depleted is prometaphase I arrest (Homer *et al.* 2009).

Despite the fact that to date, no roles have been established for *Drosophila* cAMP in the transduction pathway during meiosis, *twine* (*twe*; the *Drosophila* germline-specific *cdc25* homolog) mutant oocytes do not progress to a normal metaphase I (Alphey *et al.* 1992; Courtot *et al.* 1992; White-Cooper *et al.* 1993), suggesting that low Cdk1 activity is also required in *Drosophila* to maintain prophase I arrest.

2.6.2. Oocyte Maturation and Metaphase (I or II) Arrest

The morphological hallmarks of meiotic maturation that encompasses the transition from the first to the second meiotic arrest are typically the GVBD, the spindle assembly and chromosome condensation and are triggered by active MPF (Jones 2004). In mammals, the onset of oocyte maturation and resumption of meiosis from prophase I arrest is mediated by Emi1, which counteracts the function of Cdc14B (Marangos *et al.* 2007), and by hormonal stimulation that leads to a drop in intra-oocyte cAMP concentration and consequent Cdc25 activation. Through these two mechanisms MPF activity rises, which leads to meiotic progression from prophase I to a second arrest at metaphase II until fertilization (Sagata 1996). This second arrest is due to an activity that has been named Cytostatic Factor (CSF). In the CSF model, the establishment of the metaphase II arrest is principally induced by the Early Mitotic Inhibitor 2 (Emi2) pathway. The Emi2 pathway, together with the c-MOS/MEK1/MAPK/p90^{Rsk} pathway, is then involved in the maintenance of the arrest by inhibiting the activity of the APC/C and keeping MPF active (reviewed in Madgwick and Jones 2007).

As it was said above, the second arrest in *Drosophila* oocytes is at metaphase I. Evidence suggests that high levels of MPF activity is also required for meiotic maturation in *Drosophila* (Xiang *et al.* 2007; Von Stetina *et al.* 2008), and CycB

degradation is essential to release from the metaphase I arrest (Swan and Schupbach 2007). In contrast to mammals, *Drosophila* MOS (DMOS) signalling is not essential for the maintenance of the metaphase I arrest, since *dmos* mutant oocytes have a normal metaphase I arrest. However MAPK phosphorylation is not completely eliminated in these mutants, suggesting that another MAPKKK may activate MAPK. Nevertheless, a role of DMOS in meiosis cannot be ruled out (Ivanovska *et al.* 2004).

Mammalian *in vitro* studies suggest that α -endosulfines have a possible role in insulin secretion (Bataille *et al.* 1999). However, in *Drosophila*, the endosulfine (Endos) phosphoprotein regulates all aspects of meiotic maturation, including the timing of GVBD (Von Stetina *et al.* 2008). Probably, Endos exerts its control on meiotic maturation by controlling MPF activity, since it controls Twine and Polo stability. Similar to the defects observed in *twe* and *cdk1* mutants, *endos* mutant oocytes do not progress from prophase I to metaphase I and display a severe delay in GVBD, in spindle formation and in chromosome congression. Additionally, in *endos* mutants there is a marked reduction in the phosphorylation of the Mitotic Protein Monoclonal 2 (MPM2) conserved epitopes (Davis *et al.* 1983). MPM2 antibody recognize conserved phosphoepitopes of mitotic proteins and in *Drosophila*, Polo kinase is required for the generation of these epitopes (Logarinho and Sunkel 1998). Using a *Drosophila in vitro* expression cloning binding screen, Von Stetina *et al.* (2008) identified a predicted E3 ubiquitin ligase, *early girl* (*elgi*) as a strong Endos-binding protein (Von Stetina *et al.* 2008). In *elgi* mutant oocytes, progression from prophase I to metaphase I and GVBD occur prematurely. This premature metaphase I in *elgi* mutants, in contrast to the failed metaphase I transition in *endos* mutant oocytes, suggests that *endos* and *elgi* play antagonistic roles in meiotic maturation. By binding and inhibiting Elgi, Endos may have a parallel role in refining the timing of meiotic maturation. Besides meiotic maturation, Endos is also required for normal oogenesis rates, stage 14 oocyte dehydration and fertility (Drummond-Barbosa and Spradling 2004).

Whereas *endos* is needed for normal levels of Polo in oocytes, Matrimony (Mtrm) prevents precocious activation of Polo (Xiang *et al.* 2007). Mtrm binds to Polo with an approximate stoichiometry of 1:1 and inhibits its activity until the complete meiotic maturation, where Polo protein exceed the amount of Mtrm protein and thus activate Twe, which in turn activates MPF that leads to meiotic maturation. Another study showed that the conserved Greatwall (Gwl) kinase, discovered in *Drosophila* (Yu *et al.* 2004), antagonizes Polo activity during meiosis (Archambault *et al.* 2007) (Figure 1.14). Additionally, it has been demonstrated that the metaphase I arrest also

results from the tension exerted on chiasmata of homologous chromosomes (McKim *et al.* 1993; Jang *et al.* 1995).

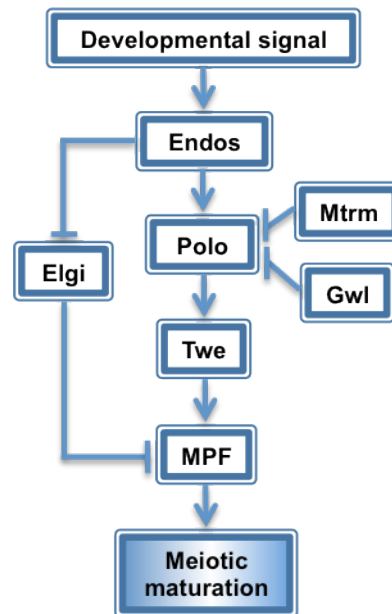


Figure 1.14. Regulatory genes that affect *Drosophila* meiotic maturation. Endos positively regulates the timing of meiotic maturation, by regulating the levels of Polo and Twe, which are required to promote MPF activation; Endos inhibits Elgi; and Polo is inhibited by Mtrm and Gwl (Adapted from Von Stetina and Orr-Weaver 2011).

2.6.3. Egg activation

The transition from mature oocyte to a developing embryo requires a series of coordinated events, collectively known as egg activation. Upon egg activation the metaphase II arrest is overcome, oocytes resume meiosis and become competent to begin the embryonic development. In mammals, egg activation is dependent on fertilization and starts with an increase of intracellular Ca^{2+} resulting in the activation of the downstream effector, Ca^{2+} /calmodulin-dependent protein kinase II (CaMKII). The direct downstream effector of CaMKII has not yet been identified but some evidence shows that CaMKII likely leads to Emi2 degradation, which leads to APC/C activation and subsequently to cycB and securin degradation (reviewed in Jones 2005). In *Drosophila*, egg activation is independent of fertilization (Doane 1960). Instead, a mechanical stimulation, via passage through the oviduct and rehydration, triggers egg activation and resumption of meiosis (Mahowald *et al.* 1983; Heifetz *et al.* 2001). In *Drosophila* egg activation no roles for CaMKII have been yet established. However, the Ca^{2+} -mediated signalling pathway has been implicated in meiosis resumption. *sarah* (*sra*) gene, which encodes the *Drosophila* Calcipressin, a member of the calcineurin regulatory family protein (RCAN), is essential for meiotic

progression after metaphase I, since *sra*-mutant oocytes arrest at anaphase I and show high levels of CycB (Horner *et al.* 2006;). In order to resume meiosis, MPF has to be inactivated through CycB degradation. Together with Fizzy (Fzy, *Drosophila* Cdc20 homolog), the *Drosophila* female meiotic-specific APC/C activator, Cortex (Cort) that is a distant member of the Cdc20 family, is also required for the completion of meiosis in the oocyte. Using double-mutant genetic analysis it was demonstrated that APC/C^{Cort} and APC/C^{Fzy} forms appear to be redundant for metaphase I-to-anaphase I transition but are essential for metaphase II-to-anaphase II transition (Pesin and Orr-Weaver 2007; Swan and Schupbach 2007).

2.7. Meiotic Spindle Assembly Checkpoint (SAC)

For many years it was thought that SAC did not exist in mammalian oogenesis. This notion came, for example, from studies done in murine oocytes, in which the delay in the anaphase onset was not detected in XO females (LeMaire-Adkins *et al.* 1997). Yet, there is now growing evidence suggesting that meiosis has a functional SAC and that many SAC components operate both in mitosis and meiosis (Cohen *et al.* 2006, Malmanche *et al.* 2006). However, it was shown that the mechanisms of the checkpoint control may be sex-specific, since oogenesis checkpoint appears to be more permissive than spermatogenesis checkpoint (Hunt and Hassold 2002). Unlike oogenesis, that becomes arrested once or twice depending on the species, spermatogenesis proceeds uninterrupted in testis of the sexually mature male.

As in mitosis, the SAC also controls APC/C^{Cdc20} activity in meiosis, preventing precocious metaphase I-to-anaphase I transition. Studies of female mouse oocytes have confirmed that SAC is indispensable for the arrest in meiosis I and prevention of precocious anaphase I onset. Mad2-depleted mouse oocytes showed precocious proteolysis of CycB and Securin resulting in shortened duration of meiosis I, while Mad2 overexpression leads to metaphase I-arrest (Wassmann *et al.* 2003; Homer *et al.* 2005) and chromosome missegregation during meiosis I (Niault *et al.* 2007). In the absence of microtubule-kinetochore interactions, Mad2 localizes to kinetochores and only disappears upon kinetochore attachment (Wassmann *et al.* 2003; Hached *et al.* 2011). Like in Mad2-depleted oocytes, the APC/C in Bub1-depleted oocytes is activated five hours earlier than control oocytes, which results in precocious Securin destruction that leads to a defective biorientation of bivalents, massive chromosome missegregation and precocious loss of cohesion between sister centromeres (McGuinness *et al.* 2009). Bub3 knockdown oocytes also present misaligned chromosomes and aneuploidy, and Bub3 overexpression inhibits metaphase-

anaphase transition in meiosis I and II. Additionally, Bub3 may be important for microtubule-kinetochore attachment (Li *et al.* 2009), as it was observed in HeLa cells (Logarinho *et al.* 2008). Besides the role of BubR1 in sustaining prometaphase I progression through a positive influence on APC/C^{Cdh1}, BubR1 is also essential within the context of the SAC, since BubR1 localizes to meiotic kinetochores after GVBD and during prometaphase I, BubR1-depleted oocytes fail to undergo the metaphase I stage arrest in the presence of low concentrations of nocodazole (Wei *et al.* 2010), microtubule-kinetochore attachments form less efficiently after BubR1 depletion (Homer *et al.* 2009; Wei *et al.* 2010) and the passage of the oocytes through meiosis I is accelerated, when a mutant form of BubR1 that cannot inhibit the APC/C *in vitro*, is overexpressed (Tsurumi *et al.* 2004). Thus, in mouse oocytes, far from being absent or ineffective, SAC appears to be important to determine the timing of APC/C activation. Also in mouse female meiosis, Hached *et al.* (2011) showed that Mps1 is required at kinetochores for proper prometaphase timing, is essential for SAC control and for chromosome alignment in meiosis I; in its absence chromosome segregation is severely impaired, leading to infertility (Hached *et al.* 2011).

Control of chromosome segregation in *Drosophila* female meiosis I is an interesting problem since the second meiotic arrest occurs, in metaphase I (and not in metaphase II, as observed for vertebrates). *mps1/ald*-mutant *Drosophila* oocytes exhibit defects in meiotic chromosome segregation, which are more severe in achiasmate homologs and result in nondisjunction (NDJ). These mutants enter anaphase I prematurely, and so there is not sufficient time for the achiasmate homologs to co-orient at metaphase I. Defects in the maintenance of the chiasmate homolog associations have also been observed in *mps1* mutants (Gilliland *et al.* 2005; Gilliland *et al.* 2007). Furthermore, NDJ is also observed in *bubR1*-mutant *Drosophila* oocytes, due to a precocious loss of Cohesin from chromosomes in prophase I (Malmanche *et al.* 2007).

2.8. Nondisjunction (NDJ)

Despite the meiotic checkpoints, errors occasionally do take place. As result, one or more pairs of homologs fail to move to opposite poles. This phenomenon is called nondisjunction (NDJ) and occurs either because the two homologs failed to pair and/or recombine, or due to a failure in chromosome segregation to opposite poles on the meiotic spindle. NDJ results in aneuploid gametes that will originate genetically abnormal embryos. Due to this fact it is not surprising that, in most organisms, meiotic NDJ is a rare event. In *Saccharomyces cerevisiae* the frequency

of NDJ is 1 in 10,000 (Sears *et al.* 1992), in *D. melanogaster* the estimated X-chromosome NDJ in the female germline ranges from 1 in 1,700 to 1 in 6,000 (Koehler *et al.* 1996) and in mouse the frequency is higher, but it does not exceed 1–2%. However, the frequency of human eggs carrying NDJ is remarkably high, about 10–30%. Most of these aneuploidy zygotes spontaneously abort during early stages of pregnancy. However some survive, such as trisomies for the sex chromosomes (XXX, XXY and XYY), trisomy 21 (Down syndrome), trisomy 18 (Edwards syndrome) and trisomy 13 (Patau syndrome). In mammals, NDJ can occur in meiosis I or in meiosis II and, although they can occur in both sexes the majority of trisomies has oocyte origin. Unlike oogenesis, spermatogenesis proceeds uninterrupted in the testis of the sexually mature male. So, the differences in meiotic progression and duration of germ cell formation may contribute to a higher susceptibility to NDJ in oogenesis compared to spermatogenesis.

Studies in human and *Drosophila* oocytes demonstrated that failed segregation of nonexchange chromosomes is the primary cause of meiosis I NDJ and that NDJ increases with age. The localisation and frequency of exchanges appears to represent an important factor in the predisposition to NDJ (reviewed in Koehler *et al.* 1996). It has been suggested that during the prolonged meiotic arrest in oocytes, Cohesin might be gradually lost and this could weaken the resistance to spindle forces, specially in chromosomes with distal chiasmata (Hodges *et al.* 2005). It has also been suggested that expression of checkpoint components is reduced in aged mammalian oocytes and this may compromise checkpoint control (Steuerwald *et al.* 2001; Baker *et al.* 2004; Hamatani *et al.* 2004). In contrast to mitotic cells and male meiosis, where SAC prevents anaphase onset until all chromosomes form stable bipolar attachments, the SAC in oocyte appears to be more permissive, since it can tolerate a small number of chromosomes that are misaligned, and thus not under tension. The reason for this to occur may be due to the fact that, in contrast to sperm, the number of oocytes is limited. So, perhaps selection has acted such that meiosis in oocytes with one or two mal-aligned chromosomes is allowed to proceed, even if there is the possibility that such univalents might still give rise to an oocyte that can produce a viable aneuploid zygote (Hawley 2011; Nagaoka *et al.* 2011).

3. *Drosophila melanogaster* as a model organism for cell cycle studies

Genetic studies using *Drosophila melanogaster* started with Thomas Morgan at Columbia University more than 100 years ago. In 1933, Morgan was awarded the Nobel Prize for his contribution to our understanding of modern genetics. Since then, *Drosophila* research has grown extensively and contributed enormously to our understanding of biology, especially in the fields of genetics and developmental biology. Some of the many advantages in using *Drosophila melanogaster* as a model organism are: it is easy and cheap to maintain in the laboratory, it has a ten-day generation time at 25°C, produces many progeny and *Drosophila* has only ~15,000 genes of which at least half have been shown to be highly conserved in *H. sapiens*. Finally, *Drosophila* has only four chromosomes making cytogenetic analysis relatively easy.

Moreover, the extensive genetic analysis already carried in *Drosophila* allows the elucidation of protein function through the generation of multiple mutant alleles that can reveal separate functions of individual proteins in multiple cellular processes. Moreover, *Drosophila* allows the study of protein function in different types of cell cycle throughout development. A surprisingly large number of developmental processes seem to be conserved between flies and vertebrates, even though they diverged ~700 million years ago. One of the most powerful aspects of *Drosophila* research has been the ability to do large-scale genetic screens. In forward genetic screens, a large number of mutant flies is generated that can be later used in screens for abnormal phenotypes in the biological process of interest. One of the most famous *Drosophila* screens was done by Nusslein-Volhard and Wieschaus (1980) and allowed the identification of 139 mutations affecting embryogenesis. For this work they were awarded the Nobel Prize in 1995.

In fact, *Drosophila* has only one main drawback, which is the fact that stocks have to be continuously maintained in the laboratory because it is not possible to freeze them (and successfully revive them afterwards).

3.1. *Drosophila melanogaster* life cycle

Drosophila egg development starts with several mitotic divisions within the central portion of the egg, the first of which occurs after fertilization and ovoposition, but before pronuclear fusion. The first 13 cycles of nuclear division are very rapid and occur synchronously without gap phases throughout in a common cytoplasm – a

syncytial egg, meaning that these nuclear divisions are not followed by cytokinesis. At the end of the eighth nuclear division, most of the nuclei migrate to the periphery of the egg where mitoses continue, although at a progressively slower rate. After the tenth division, the few nuclei that continued in the centre of the egg, the “yolk” nuclei, cease division and become polyploid. During the ninth division, about five nuclei reach the surface of the posterior pole of the embryo and become enclosed by cell membranes to generate the pole cells that will give rise to the gametes of the adult. Afterwards, at cycle 10 most of the remaining nuclei reach the periphery of the embryo to undergo four more divisions at progressively slower rates. Following cycle 13, there is a switch from maternal to zygotic control of the mitotic cycles. Additionally, the oocyte plasma membrane folds inward between the nuclei and eventually separates each somatic nucleus into a single cell. This process creates the cellular blastoderm, in which all cells are arranged in a single-layered jacket around the yolky core of the egg. After cellularization of the blastoderm, cells start to differentiate and move to form a multilayered embryo – gastrulation. At the end of embryogenesis, 21-22 hours after fertilization at 25°C, the embryo hatches as a feeding larva (Foe *et al.* 1993). The larva passes through three instars where, due to endoreplication, larval cells grow to an enormous size. The first two instars take one day each and the third instar takes two days. After this larval stage, larvae pass through a metamorphosis stage as pupa, which takes five days to give rise to an adult fly (Morgan 2007).

3.2. *Drosophila melanogaster* oogenesis

A normal *Drosophila* ovary contains 16 ovarioles, the functional units of the ovary, where oogenesis occurs (Figure 1.15). Each ovariole represents an independent string of the developing oocytes and it is divided into 14 stages. The first stage corresponds to the germarium and the other stages to the vitellarium. The germarium is in the tip of each ovariole and can be divided into four regions: region 1, 2a, 2b and 3. Region 1 contains mitotically active germline stem cells (GSCs) that are in direct contact with the somatic terminal filament (TF) cells at the apex. GSCs undergo oriented asymmetric divisions to produce a daughter stem cell, which remains associated with the TF, and a differentiated daughter, the cystoblast, which becomes displaced one cell away from the terminal filament. The cystoblast then undergoes four rounds of divisions with incomplete cytokinesis to form a 16-cell cyst interconnected by cytoplasmic bridges – the ring canals. As the cyst moves to region 2, it becomes enveloped by follicle cells (FC) to form an egg chamber. The oocyte

differentiation is a progressive process that begins in region 2a by the selection of two pro-oocytes that correspond to the first two cells of the cyst. As the cyst enters region 2b and contacts with the follicle cells, the pro-oocyte with four ring canals is selected to become the oocyte, and the other 15 cells will undergo multiple rounds of genome endoreplication, becoming polyploid nurse cells that are responsible for transcribing the RNA complement that will be used in the developing oocyte (King 1970; Spradling 1993).

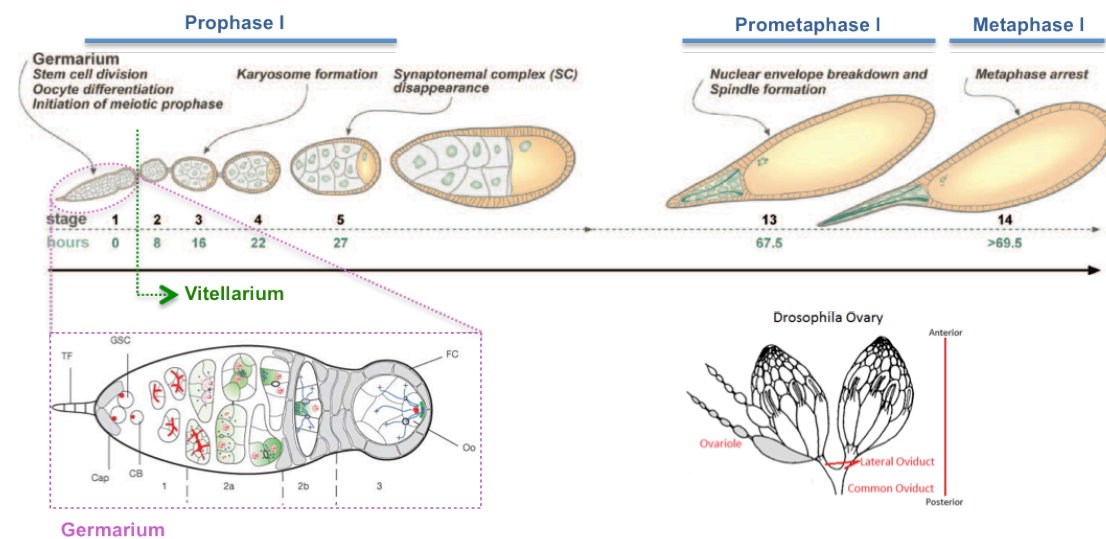


Figure 1.15. *Drosophila* ovary and oocyte development. In the right bottom corner, it is a drawing of the ovary with an ovariole in grey; in the left bottom corner is represented the first stage of the oocyte development, the germarium, which is divided into four regions along the anterior–posterior axis (1, 2a, 2b, 3); SC, red lines; Oo, oocyte. In the top is represented the oocyte development (Adapted from Xiang *et al.* 2007; Fichelson *et al.* 2009; <http://en.wikipedia.org>).

Soon after the last premeiotic division, meiotic prophase I starts, in region 2a, with homologous recombination that can be recognized through the formation of SCs and recruitment of proteins such as C(3)G. Meiosis is quickly restricted to the two pro-oocytes and then to the oocyte, as the cyst progresses into region 2b correlating this way, spatially and temporally, meiotic control and oocyte determination (Spradling 1993; Page and Hawley 2001).

When the cyst progresses from region 2b to region 3, it starts to round up and the oocyte is relocated to the posterior region of the cyst, which defines the anterior-posterior axis of the egg chamber and the future embryo. The polarized egg chamber eventually buds off the germarium to enter the vitellogenic stage 2, where joins preexisting egg chambers in a linear array to form an ovariole (King 1970; Spradling 1993).

Vitellarium is divided into 13 stages based on the morphology of the oocyte and its accompanying 15 nurse cells (de Cuevas *et al.* 1997). Around stages 5-6, the SC starts to disassemble and then oocyte development enters its first arrest until GVBD and spindle formation at stage 13 (Page and Hawley 2001). This arrest takes approximately 40 hours and allows oocyte growth at the expense of the nurse cells, which start to shrink and atrophy. In prophase I of *Drosophila* female meiosis there is no stage comparable to diplotene-diakinesis, since instead of the repulsion between homologs characteristic of these phases, during oogenesis stage 3 the chromosomal filaments begin to condense and by stage 4 they are incorporated into a dense mass known as the karyosome. The karyosome persists and becomes still more compact during stages 11 through 13. Late in stage 13, the germinal vesicle breakdown, and the karyosome is released directly into the cytoplasm to form the meiosis I spindle. At stage 14, the oocyte arrests at metaphase I until the egg passes the oviduct (King 1970; Spradling 1993).

Chapter 2

**Deficiency screen of the third chromosome to
identify BubR1 meiotic interactors**

1. Introduction

Mitosis is a process that results in the production of two identical daughter cells from a single cell. At metaphase-anaphase transition, the accuracy of chromosome segregation is ensured by the spindle assembly checkpoint (SAC) that monitors microtubule-kinetochore attachment and prevents mitotic exit until all chromosomes are attached to the bipolar spindle and under tension. The SAC proteins, Mad1, Mad2, Mad3/BubR1, Bub1 and Bub3, impose a mitotic arrest by inhibiting the activity of the anaphase-promoting complex/cyclosome (APC/C), which is essential for sister chromatid separation and mitotic exit (Li and Benezra 1996; Taylor and McKeon 1997; Bernard *et al.* 1998; Gorbsky *et al.* 1998; Basu *et al.* 1999).

In addition to its involvement in the SAC, BubR1 is also required for proper mitotic timing, capture and stabilization of kinetochore-microtubule attachment at prometaphase-metaphase transition (Basu *et al.* 1999; Ditchfield *et al.* 2003; Logarinho *et al.* 2004; Harris *et al.* 2005; Lampson and Kapoor 2005) and for mitotic arrest in the presence of DNA damage (Fang *et al.* 2006). Furthermore, BubR1 is essential to prevent early aging and infertility in mice (Baker *et al.* 2006; Hartman *et al.* 2006; Matsumoto *et al.* 2007).

In contrast to mitosis, meiosis results in the production of haploid gametes from a diploid parental cell. Meiosis involves one single round of DNA replication followed by two sequential rounds of nuclear division (meiosis I and meiosis II). During meiotic prophase I, homologous chromosomes undergo a complex series of modifications through pairing, synapsis and exchange to ensure chromosome reduction and sister chromatid separation during meiosis. Nondisjunction (NDJ), the failure to properly segregate the genome during meiosis, produces haploid cells that have unbalanced genetic composition. The vast majority of meiotic segregation errors occur during meiosis in females, and the error rate increases with advancing maternal age. The frequency of missegregation in human oocytes is remarkably high (about 10% of meiosis), and this is thought to be one reason for the high rate of miscarriages (spontaneous abortions) in early stages of pregnancy (Alberts *et al.* 2002).

In contrast to mitosis, where BubR1 has an important role in controlling the metaphase-anaphase transition, it was shown that MAD3/BubR1 has an essential and conserved function during prophase I progression in meiosis. MAD3/BubR1 is required to delay prophase I in response to non-exchange chromosomes in budding yeast (Cheslock *et al.* 2005). In *D. melanogaster*, BubR1 was shown to play an essential role during meiotic progression in both sexes and to prevent missegregation of both chiasmate and achiasmate homologous chromosomes.

During oogenesis, BubR1 is also required to maintain the synaptonemal complex (SC) (Malmanche *et al.* 2007). In mice, BubR1-depleted oocytes have reduced capacity to trigger essential meiotic arrest through destabilization of the APC/C inhibitor Cdh1 (Homer *et al.* 2009). It was also shown that overexpression of exogenous BubR1 arrests oocyte maturation during meiosis I, while dominant-negative BubR1 expression accelerates meiotic progression (Wei *et al.* 2010). These studies indicate an essential requirement for BubR1 function in timing the complex events taking place during meiotic prophase I progression.

In *D. melanogaster*, we have previously characterized a *bubR1* EMS-induced mutant allele with a point mutation in a conserved amino acid essential for its kinase activity, *bubR1*^{D1326N} (Malmanche *et al.* 2007). Using this allele, we showed that individuals with *bubR1*^{D1326N} in *trans* with two other previously characterized mutant alleles, *bubR1*¹ (Basu *et al.* 1999) and *bubR1*^{rev1} (Perez-Mongiovi *et al.* 2005), and with *Df(2R)nap9*, a deficiency uncovering *bubR1*, lack any of the described mitotic phenotypes and have a functional SAC. However, during meiosis, *bubR1*^{D1326N} *trans*-heterozygotes show meiotic NDJ for the autosome and sex chromosomes, with the frequency being BubR1 dosage-dependent. We took advantage of these sensitized genetic backgrounds (Malmanche *et al.* 2007) to perform a genetic screen to identify third chromosome deficiencies displaying suppression or enhancement of NDJ frequency for the X chromosome. For this, we used the third chromosome *D. melanogaster* deficiency kit from the Bloomington *Drosophila* Stock Center and we tested 60 % of the third chromosome euchromatin.

2. Materials and Methods

2.1. *Drosophila melanogaster* stocks

The third chromosome deficiency kit, as well as most of the mutant alleles, were obtained from the Bloomington *Drosophila* Stock Center.

2.2. Genetic screen

From the 96 deficiency stocks of the third chromosome, we were able to generate 65 deficiencies in the *bubR1*^{D1326N} genetic background. To test their effect on female X chromosome NDJ, *bubR1*^{rev1}/*bubR1*^{D1326N};Df/+ females were crossed to C(1;Y)1, v f B/O (*attached-XY*) males (Figure 2.1), and the resulting X chromosome NDJ frequency was calculated from the progeny. From these crosses, regular female progeny are *Bar* (X/C(1;Y)1, v f B) and regular male progeny are non-*Bar* (X/O), exceptional female progeny are non-*Bar* (XX/O) and exceptional male progeny are *vermilion*, *forked* and *Bar* (C(1;Y)1, v f B/O) (Figure 2.1).

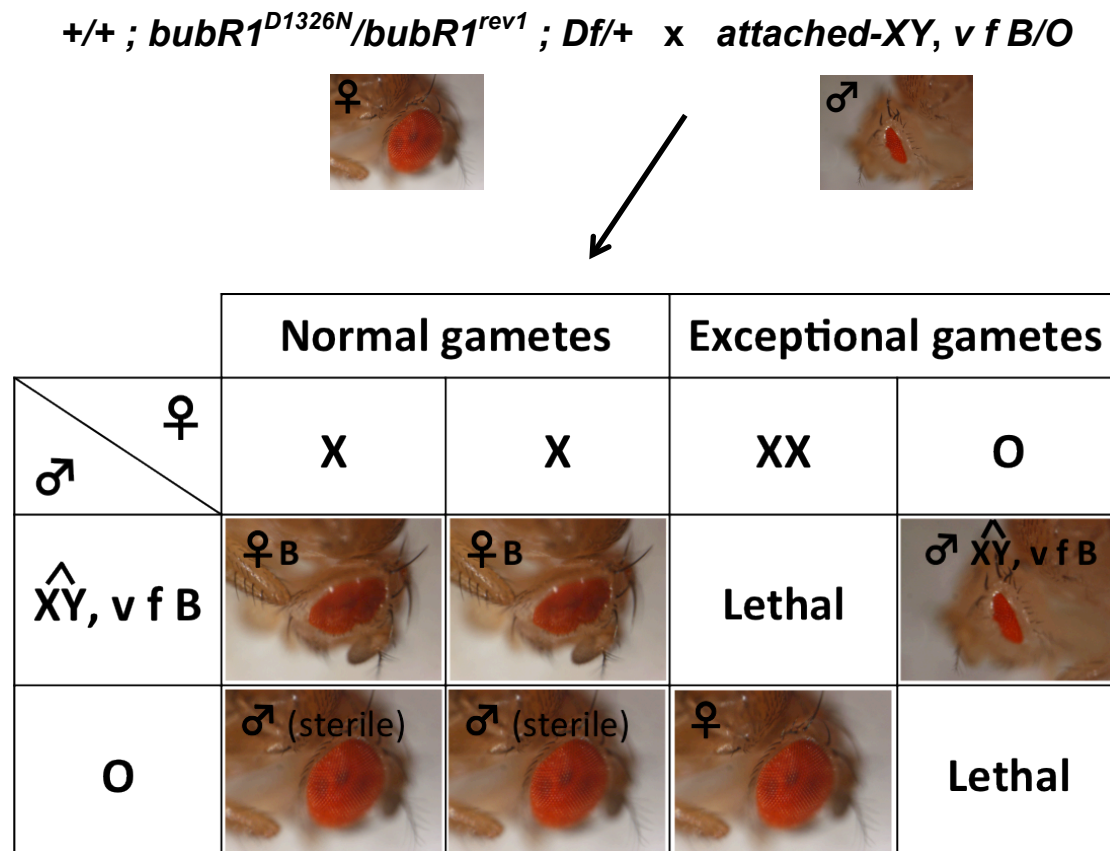


Figure 2.1. Schematic diagram of the genetic cross and the table with the different genotype outcome of the cross, done to calculate the NDJ frequency. +/+;*bubR1*^{D1326N}/*bubR1*^{rev1};Df/+ females produce normal gametes (X) and exceptional gametes (XX and O) if has occurred an NDJ event. Due to the male genotype used, it allowed for the identification of each genotype of the progeny of the cross, since it presents a distinct phenotype.

The X chromosome NDJ frequency was calculated using the following formula:

$$X_{NDJ} = \frac{2 \times \text{males and females exceptional progeny}}{\text{Adjusted total progeny}}$$

and

$$\text{Adjusted total progeny} = (2 \times \text{males and females exceptional progeny}) + \text{normal progeny}$$

The exceptional progeny was multiplied by 2 due to the lethality associated with half of the exceptional progeny (triplo-X and nullo-X).

Of the 60% of euchromatin tested, due to the presence of haplolethal and haplosterile *loci*, two deficiencies were found that were lethal with mutant *bubR1* in their background (*bubR*^{1D1326N}/*bubR1*^{rev1};Df/+), one deficiency displaying female sterility, 10 deficiencies that enhance and 19 deficiencies that suppress the NDJ frequency observed in *bubR*^{1D1326N}/*bubR1*^{rev1} females.

2.3. Immunofluorescence on ovaries

Ovaries were dissected in cold 1X PBS. Fixation was performed for 20 minutes in 2 % formaldehyde (EM grade) as described in Page and Hawley (2001), followed by 2 h permeabilization in 1X PBS - 0,5 % Triton X-100 - 10 % calf serum. Primary antibodies used were: guinea pig anti-C(3)G (Page and Hawley 2001), rat anti-SMC1 (Malmanche *et al.* 2007), rabbit anti-SMC2 (Savvidou *et al.* 2005), mouse anti-Orb (Lantz *et al.* 1994). The following secondary antibodies were used at 1:1000: anti-rabbit Alexa Fluor 488, anti-mouse Alexa Fluor 568 and anti-guinea pig Alexa Fluor 647. DNA was detected in 1X PBS containing 1 µg/ml of DAPI. Images were obtained using a Leica TCS SP2 AOBS Confocal Microscope (Leica Microsystems, Heidelberg), deconvolved using Huygens Essential (version 3.0.2pl) and processed with Adobe Photoshop 7.0.

2.4. Immunofluorescence in embryos

Wild-type and *bubR1*-mutant embryos were collected and aged at 25°C, fixed in a solution with an equal volume of heptane and 3.7 % formaldehyde in PEM buffer for 20 minutes, and then stained with 1 µg/ml of DAPI.

3. Results

3.1. Analysis of X chromosome NDJ in *bubR1*^{D1326N}/*bubR1*^{rev1} females

To determine if a specific third chromosome deficiency has an effect on *bubR1*-mutant X chromosome NDJ, first we analysed the overall variations observed in the control genotype, *bubR1*^{D1326N}/*bubR1*^{rev1}. During the screening procedure, we performed 16 rounds of X chromosome NDJ experiments, using for each group a control with the *bubR1*^{D1326N}/*bubR1*^{rev1} genotype. Within these 16 controls, the X chromosome NDJ frequency ranges from 16.60 % to 35.20 %, allowing us to calculate an average frequency of 25.82 % with a standard deviation of 4.87 % (Table 2.1). These results indicate an inherent variability in the experimental procedure that reflects uncontrollable environmental effects, as well as sampling errors. Thus, we performed a statistical analysis between the *bubR1*-mutant X chromosome NDJ frequency of a given third chromosome deficiency and the control for its specific experimental round. For that, it was used the multinomial-Poisson hierarchy model (Zeng *et al.* 2010) to take into account variations in sample size and sampling errors. Using this parameter, we identified 32 deficiencies that affect *bubR1*-mutant X chromosome NDJ with a 95% of confidence interval.

Table 2.1. X chromosome NDJ of *bubR1*^{D1326N}/*bubR1*^{rev1} females of the 16 control experiments.

Experiment	Normal progeny	Exceptional progeny		Total adjusted progeny	X chromosome NDJ
	X/XY & X/O	XX/O	O/XY		
1	1869	122	64	2241	16.60%
2	2641	280	40	3281	19.51%
3	1452	149	48	1846	21.34%
4	2885	272	153	3735	22.76%
5	1782	233	33	2314	22.99%
6	1286	111	85	1678	23.36%
7	1138	157	28	1508	24.54%
8	972	130	32	1296	25.00%
9	1545	170	107	2099	26.39%
10	737	102	34	1009	26.96%
11	1324	178	72	1824	27.41%
12	282	45	10	392	28.06%
13	631	79	49	887	28.86%
14	1116	210	44	1624	31.28%
15	775	167	22	1153	32.78%
16	681	166	19	1051	35.20%
Average					25.82% ± 4.87%

3.2. Third chromosome deficiencies modifying *bubR1*-mutant X chromosome NDJ

The third chromosome deficiency kit that we originally used included 96 deficiencies. From these we were able to generate 66 stocks in the appropriate *bubR1*^{D1326N} genetic background that covers 60% of the third chromosome euchromatin. As stated above, we performed 16 rounds of experiments to screen these 66 deficiencies (Table 2.2). Using a statistical analysis, we identified 10 deficiencies that enhance and 19 deficiencies that suppress the *bubR1*-mutant X chromosome NDJ phenotype. We also identified one deficiency (*Df(3L)ED4674*) causing sterility in *bubR1*^{D1326N}/*bubR1*^{rev1} females and two deficiencies (*Df(3L)BSC10* and *Df(3R)BSC56*) inducing zygotic developmental arrest. Following the first round of screening, we performed a second round of genetic screens using overlapping deficiencies and mutant alleles of candidate genes for three deficiencies that enhance and two that suppress *bubR1*-mutant X chromosome NDJ, for the deficiency that causes female sterility and for the two deficiencies that induce embryonic lethal phenotype. We also performed complementation tests using a number of overlapping deficiencies for the remaining enhancers and suppressors to confirm the breakpoints of the deficiencies in the stocks we established and used to test *bubR1*-mutant X chromosome NDJ.

3.3. Enhancers of *bubR1*-mutant X chromosome NDJ

In the group of deficiencies that enhance the frequency of *bubR1*-mutant X chromosome NDJ, we narrowed down to a smaller genomic region or identified candidate genes for the following deficiencies: *Df(3R)D605* (97E2;98A3-4), *Df(3L)Pc-2q* (78C5-6;78E3-79A1) and *Df(3L)BSC13* (66B12-C1;66D2-4). Since the breakpoints of some of these deficiencies had not been defined molecularly, it is likely that these regions and the genes they contain cannot be accurately defined.

Df(3R)D605: *Df(3R)D605* (97E2;98A3-4) enhances *bubR1*-mutant X chromosome NDJ by 17.19 % when compared to its own matched control. To identify a smaller genomic region, we tested four overlapping deficiencies [*Df(3R)ED6255* (97D2;97F1); *Df(3R)Exel6206* (97E1;97E5); *Df(3R)ED6237* (97E4;97E11) and *Df(3R)IR16* (97F1-2;98A)] and one deficiency covering completely *Df(3R)D605* [*Df(3R)ED6265* (97E2;98A7)]. Among these five deficiencies, only *Df(3R)ED6265* has a similar effect as *Df(3R)D605* on *bubR1*-mutant X chromosome

NDJ, indicating that the gene responsible for the observed enhancement lies within 97F1;98A4 genomic region (Table 2.3).

Table 2.2. Deficiencies that enhance/suppress X chromosome NDJ of *bubR1*^{D1326N}/*bubR1*^{rev1} females.

Deficiency	Breakpoint	Normal progeny	Exceptional progeny		Total adjusted progeny	X chromosome NDJ	Candidate gene
		X/XY & X/O	XX/O	O/XY			
Enhancers							
<i>Df(3L)Ar14-8</i>	61C5-8;62A8	993	126	28	1301	23.67%*	CG13900; Klp61F; msd1; msd5
<i>Df(3L)BSC23</i>	62E8;63B5-6	550	127	16	836	34.21%*	Mrtf; aly
<i>Df(3L)BSC33</i>	65E10-F1;65F2-6	959	196	12	1375	30.25%*	mus312; eco
<i>Df(3L)BSC13</i>	66B12-C1;66D2-4	841	281	14	1431	41.23%*	mtrm
<i>Df(3L)ri-Xt1</i>	77E2-4;78A2-4	801	109	18	1055	24.08%*	kni; Pitsire; Spc105R
<i>Df(3L)ME107</i>	77F3;78C8-9	751	149	24	1097	31.54%*	chb (Mast); pzg; Pc
<i>Df(3L)Pc-2q</i>	78C5-6;78E3-79A1	323	109	15	571	43.43%*	pzg; Pc; SAK
<i>Df(3R)ED5177</i>	83B4;83B6	462	105	16	704	34.38%*	asl
<i>Df(3R)ED5780</i>	89E11;90C1	61	22	4	113	46.02%*	cal1; ald
<i>Df(3R)D605</i>	97E2;98A3-4	274	85	15	474	42.19%*	Klp98A
Suppressors							
<i>Df(3L)pbl-X1</i>	65F6;66B7-8	780	30	19	878	11.16%*	mus312; eco; Pdp1;pbl; Arp66B
<i>Df(3L)ZP1</i>	66A17-20;66C1-5	968	81	21	1172	17.41%*	pbl; Arp66B
<i>Df(3L)66C-G28</i>	66B8-9;66C9-10	754	136	16	1058	28.73%*	mus301; RecQ4
<i>Df(3L)BSC8</i>	74D3-75A1;75B2-5	687	56	17	833	17.53%*	CycT
<i>Df(3L)ED4858</i>	76D3;77C1	566	23	13	638	11.29%*	polo
<i>Df(3L)rdgC-co2</i>	77A1;77D1	686	37	11	782	12.28%*	polo
<i>Df(3L)ED4978</i>	78D5;79A2	1712	69	27	1904	10.08%*	
<i>Df(3L)Ten-m-AL29</i>	79C1-3;79E3-8	1079	64	19	1245	13.33%*	
<i>Df(3R)Tpl10</i>	83C1-2;83D3-4 , 84A5-6;84B1-2	647	20	22	731	11.49%*	bcd
<i>Df(3R)GB104</i>	85D12;85E10	189	2	1	195	3.08%*	βTub85D; hyd; αTub85E; topi
<i>Df(3R)ED5559</i>	86E11;87B11	270	0	0	270	0.00%*	aur; ssp5; Pp1α-87B
<i>Df(3R)sbd105</i>	88F9-89A1;89B9-10	1022	106	23	1280	20.16%*	c(3)G; msps
<i>Df(3R)ED5942</i>	91F12;92B3	725	78	18	917	20.94%*	
<i>Df(3R)BSC55</i>	94D2-10;94E1-6	613	43	20	739	17.05%*	sav
<i>Df(3R)mbc-30</i>	95A5-7;95C10-11	181	8	1	199	9.05%*	Rpn9; eIF4G2; Pros26.4; CG13599; SMC1
<i>Df(3R)mbc-R1</i>	95A5-7;95D6-11	1302	32	49	1464	11.07%*	Rpn9; eIF4G2; Pros26.4; CG13599; SMC1

<i>Df(3R)Exel6202</i>	96D1;96D1	837	64	29	1023	18.18%*	
<i>Df(3R)BSC42</i>	98B1-2;98B3-5	1849	126	35	2171	14.83%*	Sce; btz
<i>Df(3R)3450</i>	98E3;99A6-8	1003	42	28	1143	12.25%*	Doa; Slu7; yemaq; dgt6; Slbp; stg

*The percentage of X chromosome NDJ is significantly different than in *bubR1^{D1326N}/bubR1^{rev1}* females (multinomial-Poisson hierarchy model, $P < 0.05$).

***Df(3L)Pc-2q*:** *Df(3L)Pc-2q* (78C5-6;78E3-79A1) enhances *bubR1*-mutant X chromosome NDJ by 18.43 % when compared to its own matched control (Table 2.2). Within the third chromosome deficiency kit, we also tested *Df(3L)ED4978* (78D5;79A2) which partially overlaps with *Df(3L)Pc-2q*. However, *Df(3L)ED4978* suppresses *bubR1*-mutant X chromosome NDJ by 6.52 % when compared to its own matched control (Table 2.2). Thus, according to the cytogenetic map coordinates for both deficiencies, we can conclude that the enhancer gene is located in the genomic region 78C5-6;78D4 while the suppressor gene is located in the genomic region 79A1;79A2.

Table 2.3. X chromosome NDJ of *bubR1^{D1326N}/bubR1^{rev1}*; *Df(3R)D605/+* and its overlapping deficiencies.

Female genotype	Breakpoint	Normal progeny	Exceptional progeny		Total adjusted progeny	X chromosome NDJ
		X/XY & X/O	XX/O	O/XY		
<i>Df(3R)D605/+</i>	97E2;98A3-4	2033	1	0	2035	0.10%
<i>bubR1^{D1326N}/bubR1^{rev1}</i>		3271	194	156	3971	17.63%
<i>bubR1^{D1326N}/bubR1^{rev1}</i> ; <i>Df(3R)D605/+</i>	97E2;98A3-4	1010	213	26	1488	32.12%*
<i>bubR1^{D1326N}/bubR1^{rev1}</i> ; <i>Df(3R)ED6255/+</i>	97D2;97F1	2720	157	61	3156	13.81%
<i>bubR1^{D1326N}/bubR1^{rev1}</i> ; <i>Df(3R)Exel6206/+</i>	97E1;97E5	1233	107	26	1499	17.75%
<i>bubR1^{D1326N}/bubR1^{rev1}</i> ; <i>Df(3R)ED6265/+</i>	97E2;98A7	990	216	118	1658	40.29%*
<i>bubR1^{D1326N}/bubR1^{rev1}</i> ; <i>Df(3R)ED6237/+</i>	97E4;97E11	2648	214	98	3272	19.07%
<i>bubR1^{D1326N}/bubR1^{rev1}</i> ; <i>Df(3R)IR16/+</i>	97F1-2;98A	1934	222	80	2538	23.80%

*The percentage of X chromosome NDJ is significantly higher than in *bubR1^{D1326N}/bubR1^{rev1}* females (multinomial-Poisson hierarchy model, $P < 0.05$).

***Df(3L)BSC13*:** *Df(3L)BSC13* (66B12-C1;66D2-4) enhances *bubR1*-mutant X chromosome NDJ by 18.24 % when compared to its own matched control (Table 2.2). Within the third chromosome deficiency kit, we also tested *Df(3L)ZP1* (66A17-20;66C1-5) which partially overlaps with *Df(3L)BSC13*. However, *Df(3L)ZP1* suppresses *bubR1*-mutant X chromosome NDJ (Table 2), suggesting that the enhancer gene should be in the region 66C1-66D4. Within this region, we identified *matrimony* (*mtrm* – 66C11) as a candidate gene. *mtrm* was previously identified in a screen of the major autosomes as being haploinsufficient for achiasmate segregation

in *Drosophila* oocytes (Harris *et al.* 2003). We tested a null allele of *mtrm*, *mtrm*¹²⁶ (Xiang *et al.* 2007) in a *bubR1*^{D1326N}/*bubR1*^{rev1} mutant female background and observed a 13.40 % increase of *bubR1*-mutant X chromosome NDJ. However, *mtrm*¹²⁶/+ females have a frequency of X chromosome NDJ of 9.45 %. Thus, it is likely that the enhancement of *bubR1*-mutant X chromosome NDJ observed in *bubR1*^{D1326N}/*bubR1*^{rev1}; *mtrm*¹²⁶/+ females is only due to an additive effect of the frequency of NDJ displayed by *mtrm* and *bubR1* mutants alone.

3.4. Suppressors of *bubR1*-mutant X chromosome NDJ

In the group of deficiencies that suppress the frequency of *bubR1*-mutant X chromosome NDJ, we narrowed down to a smaller genomic region or identified candidate genes for the following deficiencies: *Df(3R)ED5559* (86E11;87B11) and *Df(3L)rdgC-co2* (77A1;77D1).

***Df(3R)ED5559*:** *Df(3R)ED5559* (86E11;87B11) completely suppresses *bubR1*-mutant X chromosome NDJ (Table 2.2). To uncover the gene responsible for the full phenotypic rescue, we tested thirteen deficiencies that overlap with *Df(3R)ED5559* [*Df(3R)ED5516* (86D8;86E13); *Df(3R)Exel8154* (86E13;86E18); *Df(3R)Exel7310* (86E18;87A1), *Df(3R)ED5577* (86F9;87B13), *Df(3R)Exel6163* (87A1;87A4), *Df(3R)Exel8155* (87A4;87A9), *Df(3R)Exel7313* (87A9;87B5), *Df(3R)Exel7314* (87B3;87B8), *Df(3R)Exel6165* (87B5;87B10), *Df(3R)ED5591* (87B7;87C7), *Df(3R)Exel7315* (87B8;87B9), *Df(3R)BSC740* (87B9;87B10) and *Df(3R)Exel7316* (87B9;87B11)]. Our results show that *Df(3R)ED5577*, *Df(3R)Exel6165*, *Df(3R)ED5591*, *Df(3R)BSC740* and *Df(3R)Exel7316* have a similar effect, suggesting that the candidate gene is located within the genomic region 87B9;87B10 (Figure 2.2). Within this region, we identified *protein phosphatase 1 α at 87B* (*Pp1α-87B*) as a potential candidate gene. We tested *Pp1α-87B*^{87Bg-3} (or *ck19*^{e211}), which is a null allele of *Pp1α-87B*. *Pp1α-87B*^{87Bg-3} has a deletion of 165 bp upstream of the ATG initiation site that removes the TATA box and other upstream sequences (Dombradi *et al.* 1990). We were able to confirm that the *Pp1α-87B* gene is responsible for complete suppression of *bubR1*-mutant X chromosome NDJ.

As in mammals, *D. melanogaster* has two sub-types of serine/threonine Protein Phosphatase type 1 (PP1): PP1α (which is homologous to mammalian PP1α and PP1γ) and PP1β (which is homologous to mammalian PP1δ, also known as PP1β). PP1α is encoded by three genes: *Pp1α-13C*, *Pp1α-87B* and *Pp1α-96A*, whereas the PP1β sub-type is encoded by one single gene, *Pp1β-9C* (*flapwing*, *flw*). It was

described that in third instar larvae $Pp1\alpha$ -87B is the most abundant catalytic subunit of PP1 isoenzyme in *Drosophila*, contributing to approximately 80% of total PP1 activity. In $Pp1\alpha$ -87B heterozygous mutants, although total PP1 activity is reduced to 40%, viability is not affected but the $Pp1\alpha$ -87B homozygous mutant is lethal at the larval-pupal boundary (Dombradi *et al.* 1990). Neuroblasts of homozygous mutants show delayed progression through mitosis, defects in spindle organization, abnormal

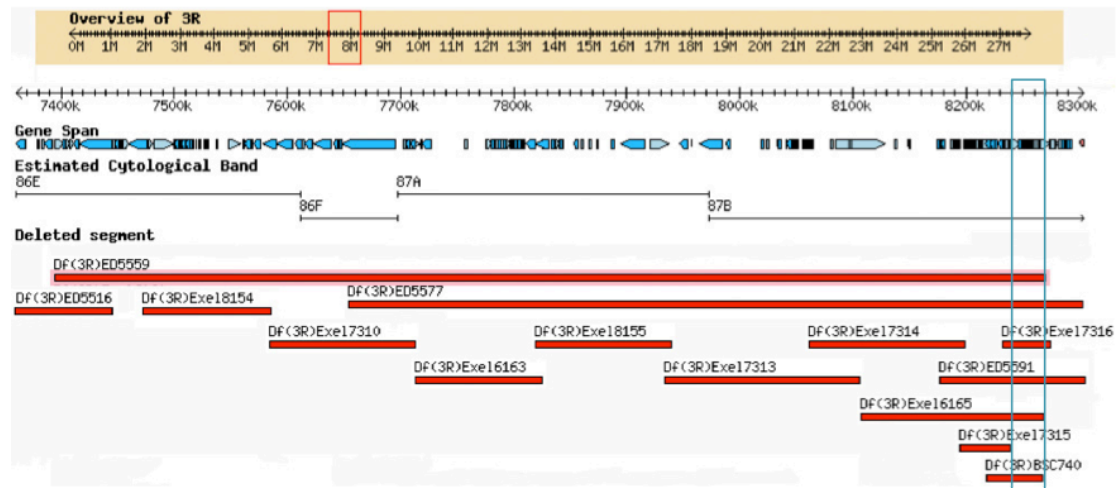


Figure 2.2. Map of the deficiencies used to refine the genomic region of *Df(3R)ED5559* that is responsible for the complete suppression of *bubR1*-mutant X chromosome NDJ. The blue rectangle represents the genomic region within which, it is the candidate gene for the phenotype observed (adapted from <http://flybase.org>).

sister chromatid segregation, hyperploidy and excessive chromosome condensation (Axton *et al.* 1990). Besides the defects observed in mutant neuroblasts, after $Pp1\alpha$ -87B knockdown S2 cells, it was observed an increase in the mitotic index, in cells with spindles of metaphase length containing uncongressed chromosomes and in cells with abnormally elongated anaphase-like spindles showing lagging chromosomes and unequal segregation. In these anaphase-like cells Mei-S332 and BubR1 remained associated with the centromere or the kinetochore moving toward the spindle poles and CycB was not degraded. Taken together, these data suggest a role for $Pp1\alpha$ -87C in regulating chromosome congression (Chen *et al.* 2007). Recently was shown that PP1 is essential to silence the spindle checkpoint by opposing to Aurora B signalling (reviewed by Lesage *et al.* 2011)

Mek1, Hop1 and Red1 are structural components of axial/lateral elements of *Saccharomyces cerevisiae* SC (Hollingsworth and Byers 1989; Smith and Roeder 1997; Blat *et al.* 2002). Mek1-dependent phosphorylation of Red1 is thought to prevent exit from pachytene, and for meiotic recombination completion Red1 has to be dephosphorylated (Bailis and Roeder 1998; de los Santos and Hollingsworth

1999; Bailis and Roeder 2000). There is evidence that Protein Phosphatase type 1, encoded by the *GLC7* gene, is required to counteract Mek1 kinase activity and to exit from the pachytene stage of meiotic prophase. Mutant cells of the *glc7-T152K* strain fail to sporulate (Tu *et al.* 1996) and, like *MEK1-C* mutants that have a constitutively active enzyme arrest in late pachytene or early diplotene (Bailis and Roeder 2000).

During mitosis, Cdk1 phosphorylates PP1 at Thr320. This phosphorylation is associated with inhibition of PP1 activity (Dohadwala *et al.* 1994). Since inhibition of PP1 by okadaic acid (OA), which inhibits both PP1 and PP2A activities but not PP2B nor PP2C activities (Ishihara *et al.* 1989), stimulates GVBD (Rime and Ozon 1990; Gavin *et al.* 1991; Schwartz and Schultz 1991; Hampl and Eppig 1995; Smith *et al.* 1998), it is possible that endogenous mechanisms of inhibiting PP1 may be linked to GVBD (Wang *et al.* 2004). Despite the fact that OA induces GVBD, it interferes negatively with the meiotic post-GVBD events: the polar body extrusion is delayed/inhibited and the increase on histone H1 kinase level is suppressed (Gavin *et al.* 1994). The strong preference of MPF kinase for histone H1 as a substrate has been used for the demonstration and semiquantitation of MPF activity in oocytes (Gautier *et al.* 1988). According to this, it was shown that PP1 might dephosphorylate Cdc25C contributing, this way, to MPF activation and progression to MII (Margolis *et al.* 2003). Cdc25C is inactive during most of the cell cycle and gets activated at the onset of the M phase, where it dephosphorylates MPF. During interphase Cdc25C has low level of phosphatase activity due to phosphorylation in some residues. The major phosphorylation site of *Xenopus* Cdc25C (XCdc25C) during interphase is Ser287 (Ser216 in human Cdc25C), which lies in a consensus 14-3-3 recognition motif that was shown to bind indeed to Cdc25C. There are several protein kinases that phosphorylate this residue, namely: Chk1, PKA, C-TAK and CaMKII. Thr138 phosphorylation, by Cdk1 in humans or by Cdk2 in *Xenopus*, seems to be important for the removal of 14-3-3 proteins and for the initial activation of Cdc25C. Around the time of entry into meiosis I (GVBD), Ser287 is dephosphorylated and it was shown *in vivo* that PP1 is likely to be the phosphatase responsible for Ser287 dephosphorylation. Afterwards, Cdc25C becomes fully activated through phosphorylation in several other residues (reviewed by Perdiguero and Nebreda 2004).

I propose the following model for the phenotype suppression of the *bubR1*^{D1326N}/*bubR1*^{rev1}-associated NDJ by the *Pp1α-87B* mutant allele. In hemizygous conditions the activity of Pp1α-87B is probably not sufficient to activate Cdc25 to normal levels, leading to a delay in prophase I progression and providing more time for *bubR1*-mutant oocytes to restore the meiotic prophase I defects

associated with the *bubR1* mutation. Alternatively, Pp1 α -87B might be involved in the dephosphorylation of BubR1-specific substrates that prevent meiotic prophase I exit.

Df(3L)rdgC-co2: *Df(3L)rdgC-co2* (77A1;77D1) suppresses *bubR1*-mutant X chromosome NDJ by 14.68 % when compared to its own matched control (Table 2.2). We identified two other overlapping deficiencies that also suppress *bubR1*-mutant X chromosome NDJ: *Df(3L)ED4858* (76D3;77C1) and *Df(3L)Exel6136* (77B2;77C6), suggesting that the responsible gene is located within the genomic region 77B2;77C1 (Table 2.4). In this genomic region, we identified *polo* (77B2-77B3) as a candidate gene and we tested three *polo* mutant alleles (*polo*¹, *polo*² and *polo*⁹). Our results show that all three alleles suppress *bubR1*-mutant X chromosome NDJ and the level of suppression increases with strength of *polo* mutations (Table 2.4). *polo*¹ corresponds to an EMS-induced point mutation in the kinase domain, is a weak hypomorph and is homozygous viable (Sunkel and Glover 1988). *polo*⁹ allele has a P-element insertion which reduces Polo level, and is homozygous third-instar larvae lethal (Donaldson *et al.* 2001). *polo*² was induced by P-M hybrid dysgenesis and is considered as the strongest *polo* allele (C.E.Sunkel, R.E. Karess and D.M. Glover, unpubl.).

Table 2.4. X chromosome NDJ of *bubR1*^{D1326N}/*bubR1*^{rev1}; *Df(3L)rdgC-co2*/+, its overlapping deficiencies, and mutant alleles.

Female genotype	Breakpoint	Normal progeny	Exceptional progeny		Total adjusted progeny	X chromosome NDJ
		X/XY & X/O	XX/O	O/XY		
<i>bubR1</i> ^{D1326N} / <i>bubR1</i> ^{rev1}		737	102	34	1009	26.96%
<i>bubR1</i> ^{D1326N} / <i>bubR1</i> ^{rev1} ; <i>Df(3L)rdgC-co2</i> /+	77A1;77D1	686	37	11	782	12.28%*
<i>bubR1</i> ^{D1326N} / <i>bubR1</i> ^{rev1} ; <i>Df(3L)ED4858</i> /+	76D3;77C1	566	23	13	638	11.29%*
<i>bubR1</i> ^{D1326N} / <i>bubR1</i> ^{rev1} ; <i>Df(3L)Exel6136</i> /+	77B2;77C6	1996	9	5	2024	1.38%*
<i>bubR1</i> ^{D1326N} / <i>bubR1</i> ^{rev1} ; <i>polo1</i> /+		438	35	17	542	19.19%*
<i>bubR1</i> ^{D1326N} / <i>bubR1</i> ^{rev1} ; <i>polo2</i> /+		1612	32	25	1726	6.60%*
<i>bubR1</i> ^{D1326N} / <i>bubR1</i> ^{rev1} ; <i>polo9</i> /+		979	36	21	1093	10.43%*

*The percentage of X chromosome NDJ is significantly lower than in *bubR1*^{D1326N}/*bubR1*^{rev1} females (multinomial-Poisson hierarchy model, P<0.05).

During meiosis *CDC5*, the yeast *polo* homolog, is required to phosphorylate and remove meiotic Cohesin from chromosome arms (Lee and Amon 2003), to form chiasmata (Clyne *et al.* 2003), to co-orient sister kinetochores and to co-segregate sister centromeres at meiosis I (Clyne *et al.* 2003; Lee and Amon 2003). In *D. melanogaster* meiosis, Polo is involved in the timing of meiotic prophase I entry, in

the restriction of meiosis to the oocyte and in the initiation/maintenance of SC (Mirouse *et al.* 2006). At later stage, Polo is required to activate Twine, the germline-specific form of Cdc25 phosphatase, which initiates the cascade of events that leads to GVBD and prometaphase I progression (Xiang *et al.* 2007). At meiosis II, Polo is essential to phosphorylate and remove MEI-S332, the Shugoshin homolog, and to allow sister chromatid segregation (Clarke *et al.* 2005).

BubR1 and Polo appear to have opposite effects during early stages of meiotic prophase I, with Polo controlling meiotic entry and SC assembly and BubR1 controlling meiotic prophase I progression and SC disassembly. To investigate at the molecular level the nature of the decrease in X chromosome NDJ phenotype by Polo kinase, we first tested if reducing the protein dosage in *bubR1*-mutant females could partially restore the meiotic prophase I phenotypes associated with the *bubR1* mutation. We analysed the nature of the SC and sister chromatid cohesion in *bubR1^{D1326N}/Df(2R)nap9;polo⁹/+* mutant combinations in region 3 of the germarium (Figure 2.3). Interestingly, while mutations in *bubR1* result in premature disassembly of the SC, in the *bubR1^{D1326N}/Df(2R)nap9;polo⁹/+* we observed the maintenance of the SC protein, C(3)G, and the sister chromatid Cohesin complex subunit, SMC1 (Figure 2.3 A and B). This suggests that decreasing Polo protein levels in *bubR1*-mutant ovarioles sufficiently delays prophase I entry and/or progression to allow for a more accurate prophase I. In addition, we investigated if a reduction in Polo levels in *bubR1*-mutant females allows higher efficiency in the specific replacement of the Cohesin complex by the Condensin complex during chiasmata formation (Yu and Koshland 2005) and SC disassembly (Ivanovska *et al.* 2005; Resnick *et al.* 2009). Thus, we analysed the localization of SMC2 Condensin complex subunit at stage 5-7, which corresponds to the pachytene-diplotene transition. In wild-type oocytes, Condensin subunit SMC2 is gradually loaded on the bivalent to fully cover the karyosome by stage 5-7 (Figure 2.3 C). However, in *bubR1*-mutant oocytes, SMC2 localizes within the nuclear space rather than being bound to the karyosome (Figure 2.3 C). In addition to decreased maintenance of the SC and of sister chromatids cohesion, mutations in *bubR1* also affect the changes in bivalent configuration at diplotene. Interestingly, decreasing Polo dosage restores Condensin loading and bivalent modifications in *bubR1*-mutant oocytes. Taken together, these results suggest that the decreased X chromosome NDJ induced by a reduction on Polo levels in *bubR1*-mutant oocytes occurs through a Polo-dependent mechanism during initial stages of meiosis.

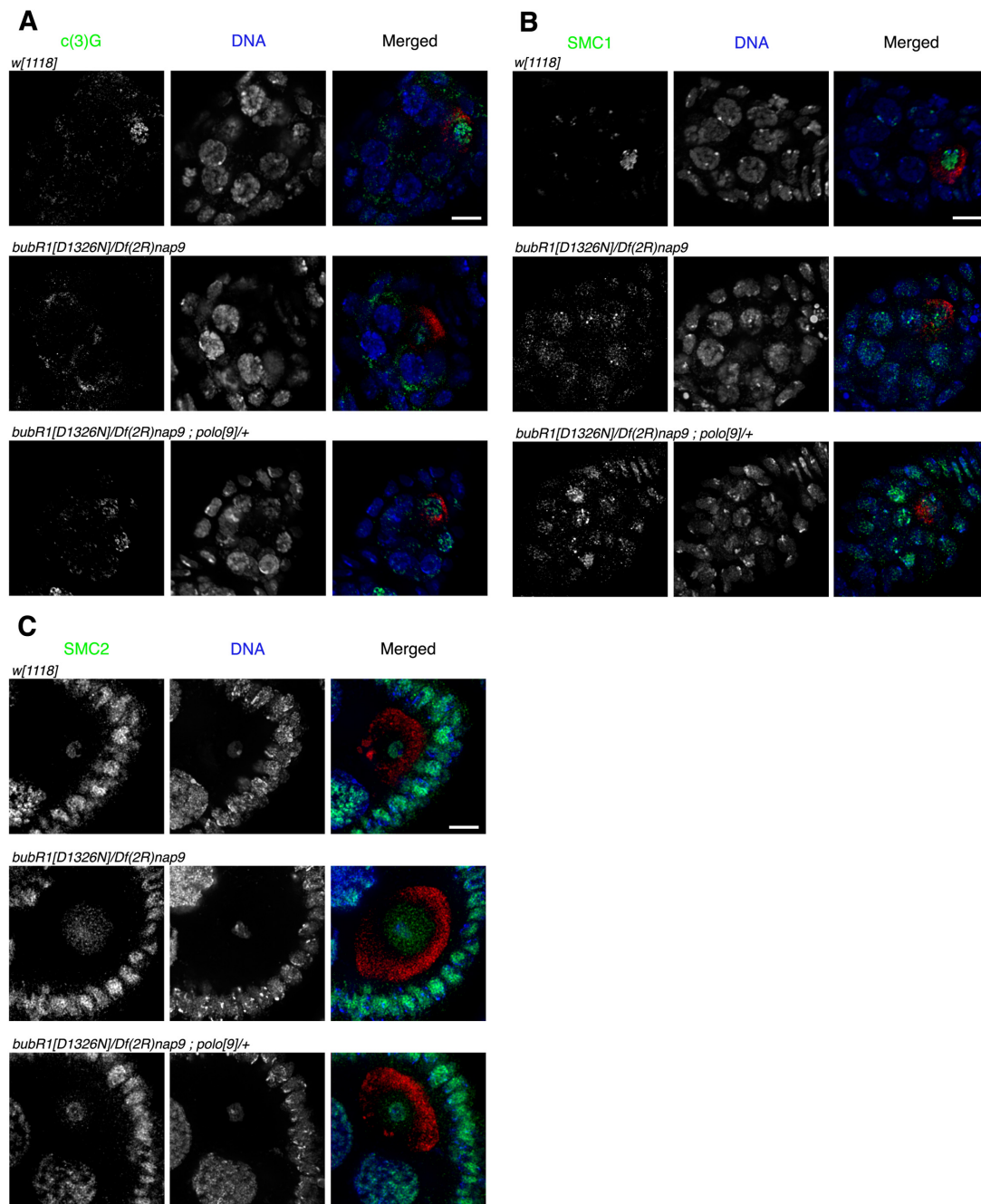


Figure 2.3. Polo is a suppressor of *bubR1*-mutant X chromosome NDJ. (A) Structure of SC in wild-type, in *bubR1^{D1326N}/Df(2R)nap9* and in *bubR1^{D1326N}/Df(2R)nap9; polo9/+* in region 3 of the germarium. C(3)G is in green, DNA is in blue, and Orb (as oocyte marker) is in red. Decreasing *polo* dosage in a *bubR1*-mutant genetic background rescues the maintenance of the SC in the oocyte nucleus comparing to *bubR1* mutants. (B) Sister chromatid cohesin subunit SMC1 in wild-type, in *bubR1^{D1326N}/Df(2R)nap9* and in *bubR1^{D1326N}/Df(2R)nap9; polo9/+* mutant in region 3 of the germarium. SMC1 is in green, DNA is in blue, and Orb is in red. Decreasing *polo* dosage in a *bubR1*-mutant genetic background rescues the maintenance of the sister chromatid cohesion in the oocyte nucleus comparing to *bubR1* mutants. (C) Condensin subunit SMC2 in wild-type, in *bubR1^{D1326N}/Df(2R)nap9* and in *bubR1^{D1326N}/Df(2R)nap9; polo9/+* mutant at the pachytene/diplotene transition (stages 5–7). SMC2 is in green, DNA is in blue, and Orb is in red. Decreasing *polo* dosage in a *bubR1*-mutant genetic background rescues the loading of condensin subunit at stages 5–7 in the oocyte nucleus during chiasmata formation comparing to *bubR1*-mutant oocyte. Scale bars = 10 μ m.

3.5. Deficiency causing sterility in *bubR1*^{D1326N}/*bubR1*^{rev1} females

Df(3L)ED4674 (73B5;73E5) causes sterility in *bubR1*^{D1326N}/*bubR1*^{rev1} mutant females suggesting a strong enhancement of the *bubR1* meiotic phenotype. To narrow down the genomic region of interest, we tested four overlapping deficiencies [*Df(3L)BSC561* (73A2;73C1); *Df(3L)Exel9004* (73D1;73D5); *Df(3L)Exel7253* (73D5;73E4) and *Df(3R)BSC414* (73E1;74C3)]. Among these four deficiencies, our results show that only *Df(3L)BSC561* causes sterility in *bubR1*^{D1326N}/*bubR1*^{rev1} mutant females, indicating the presence of a dosage-sensitive gene within the genomic region 73B5-73C1. Two potential candidate genes, *Baldspot* and *Lasp*, were found within this region, which have essential functions during spermatogenesis (Jung *et al.* 2007; Lee *et al.* 2008). Further work is required to determine which of these two genes is responsible for the sterility phenotype observed.

3.6. Identification of two deficiencies affecting somatic BubR1 kinase activity

It was previously shown that different BubR1 domains perform different functions: with the KEN box being essential for SAC activity while the kinase domain being only required for spindle formation during prometaphase (Elowe *et al.* 2010). For *Drosophila*'s BubR1 mutations in the kinase domain give rise to viable adults that lack any visible phenotype and mutant cells are able to properly segregate their genetic material during somatic cell division (Malmanche *et al.* 2007; Rahmani *et al.* 2009). Interestingly, we identified two deficiencies that induce embryonic lethality in the *bubR1* genetic background indicating that a decrease in cell cycle network components can impair cell cycle progression and zygote development in a BubR1 kinase dead context.

***Df(3L)BSC10*:** *Df(3L)BSC10* deletes the genomic region 69D4-5;69F5-7. Firstly, we characterized the stage of embryonic development arrest for *bubR1*^{D1326N}/*bubR1*^{rev1};*Df(3L)BSC10*/+. For this, we analysed embryonic collections from 0-2 hours that were aged for 3 hours before fixation. Our results showed a developmental arrest at stage 5, which corresponds to the end of the thirteenth syncytial cycle and initiation of cellularization and gastrulation (Figure 2.4). To identify candidate genes within this deficiency, we tested overlapping deficiencies covering the *Df(3L)BSC10* deleted region [*Df(3L)iro-2* (69B1-5;69D1-6); *Df(3L)Exel6117* (69D1;69E2), *Df(3L)E44* (69D2;69E3-5) and *Df(3L)ED4486* (69C4;69F6)]. However, none of the deficiencies tested induced an embryonic lethal

phenotypes, suggesting that the candidate gene is within the genomic region 69F6;69F7. Within this small region we found *RpS4* (*Ribosomal protein S4*), a gene involved in mitotic spindle elongation and organization (Goshima *et al.* 2007). Nonetheless, we cannot exclude the possibility of *Df(3L)BSC10* having another uncharacterized chromosomal aberration.

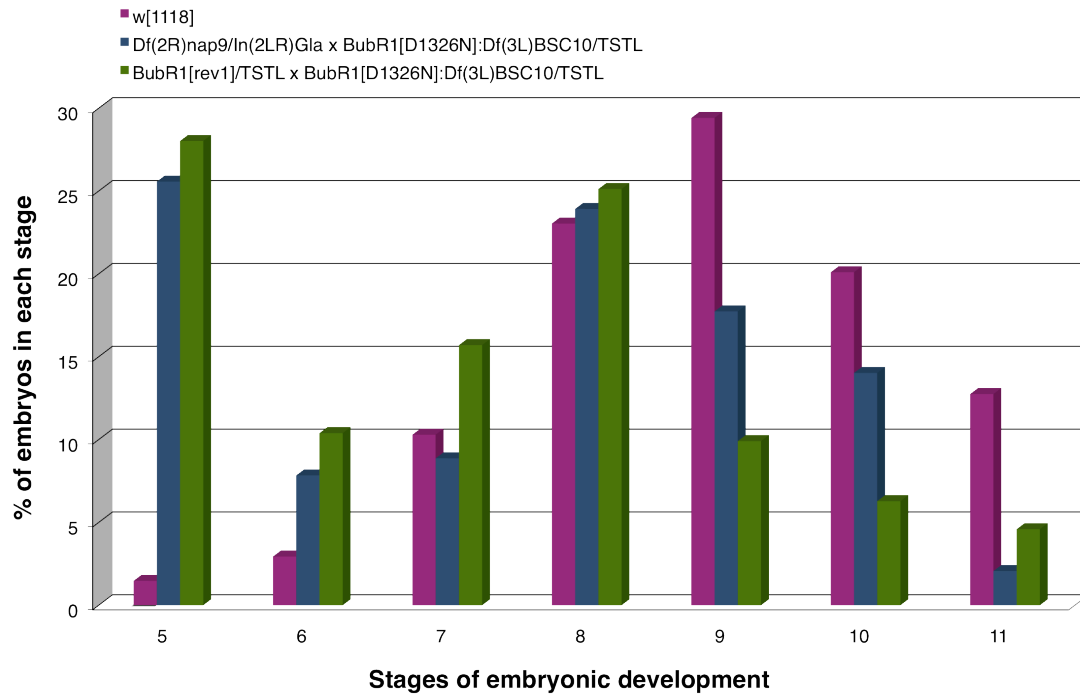


Figure 2.4. BubR1 kinase activity is essential during somatic cell cycle. The developmental arrest induced by *Df(3L)BSC10* in BubR1 kinase dead embryos takes place after the syncytial division and prevents the initialization of gastrulation.

Df(3R)BSC56: *Df(3R)BSC56* (94E1-2;94F1-2) also induces embryonic developmental arrest in *bubR1^{D1326N}/bubR1^{rev1}* mutant background. We used 5 overlapping deficiencies covering the *Df(3R)BSC56* deleted region to identify candidate genes [*Df(3R)BSC55* (94D2-10;94E1-6), *Df(3R)Exel6193* (94D3;94E4), *Df(3R)ED6103* (94D3;94E9), *Df(3R)Exel6274* (94E4;94E11) and *Df(3R)Exel6194* (94F1;95A4)]. Among these deficiencies, only *Df(3R)Exel6274* induces embryonic lethality suggesting the presence of the candidate gene within the genomic region 94E9;94E11, defined between the breakpoint of *Df(3R)ED6103* and *Df(3R)Exel6274*. Within this region, we identified *cdc16* (94E9) as a potential candidate gene. Cdc16/Apc6 is a tetratricopeptide repeat (TPR) subunit of APC/C, essential for G₂ progression. *cdc16^{RNAi}* individuals die as P5(i) early pupae and *cdc16^{RNAi}* cells show mitotic phenotypes including high mitotic index, over-condensed chromosomes in a metaphase-like arrest and failure in proper cyclin B degradation (Pal *et al.* 2007). We

therefore tested a *cdc16* allele (*cdc16*^{MB09129}) that has a transposable element inserted in an intron. However, this allele does not induce the lethality observed with *Df(3R)BSC56* suggesting that either *cdc16* is not the relevant gene or the allele we tested, which has not been extensively characterized, still produces sufficient active protein that allow embryonic development.

4. Discussion

We had previously shown that BubR1 kinase activity is essential during meiotic prophase I progression to ensure the correct timing of SC disassemble and chromosome NDJ in *D. melanogaster* female (Malmanche *et al.* 2007). Given the frequency of chromosome NDJ observed in a dose sensitive manner in *bubR1*^{D1326N} *trans*-heterozygotes, we decided to carry out a genetic screen to identify third chromosome haploinsufficient synthetic modifiers of the NDJ phenotype. We could test 66 of the 96 deficiencies that are part of the third chromosome deficiency kit generated by the Bloomington *Drosophila* Stock Centre. We could not test all of them due to the presence of haplolethal and haplosterile *loci* (Marygold *et al.* 2007) in some deficiencies. Within the 60% of the euchromatin screened, we identified enhancers and suppressors of *bubR1*-mutant X chromosome NDJ. Furthermore, we showed that Polo kinase acts as a strong suppressor of female *bubR1*-mutant X chromosome NDJ by delaying meiotic prophase I progression. Interestingly, we also found deficiencies that cause synthetic lethality or affect the fertility of mutant females. Thus, this screen proved to be highly sensitive for the identification of meiotic modifiers of BubR1 kinase activity.

Ideally, a genetic screen should be performed to simultaneously test all mutant genetic backgrounds in order to minimize variables, such as temperature, humidity and food. However, since the genotype used to test NDJ only accounts for 1/6 of the total female F₂ generation, not all the third chromosome deficiencies could be tested at the same time. Therefore, we performed 16 independent experiments. To classify the deficiencies as enhancer, suppressor or having no effect, we took into account the variation in X chromosome NDJ observed in the control genotype and performed statistical analysis using the multinomial-Poisson hierarchy model (Zeng *et al.* 2010) with a 95 % of confidence interval.

From the 66 deficiencies tested, we identified 10 deficiencies that enhance and 19 deficiencies that suppress female *bubR1*-mutant X chromosome NDJ. From these 29 hits, we were able to narrow down the genomic region to identify the genes of interest using overlapping deficiencies for 3 enhancers (*Df(3R)D605*, *Df(3L)Pc-2q* and *Df(3L)BSC13*) and for 2 suppressors (*Df(3R)ED5559* and *Df(3L)rdgC-co2*). We could identify the modifier gene for three deficiencies: *Df(3L)BSC13*, *Df(3L)rdgC-co2* and *Df(3R)ED5559*. Further studies are needed to clearly define, among the subset of candidate genes, those responsible for the modification of female X chromosome NDJ for the other deficiencies.

Despite the difficulties in identifying the gene involved in the process of X chromosome NDJ for each positive interaction, our genetic strategy allowed us to identify Polo kinase and Pp1 α -87B phosphatase as suppressors of *bubR1*-mutant X chromosome NDJ.

Our previous results (Malmanche *et al.* 2007) illustrate an essential requirement for BubR1 kinase activity in the timing of the early stages of meiotic prophase I, which suggests that BubR1 and Polo have opposite functions in early prophase I. BubR1 appears to slow down progression by maintaining the SC in place (Malmanche *et al.* 2007) while Polo accelerates the process by driving prophase I forward (Xiang *et al.* 2007). Through indirect immunofluorescence, we confirmed that Polo dosage antagonizes BubR1 function during the early stages of meiotic prophase I. We showed that Polo increases SC and sister chromatin cohesion maintenance in the *bubR1*-mutant background. In additions, we could observe the loading of the Condensin subunit SMC2 at pachytene-diplotene transition, which allows higher efficiency of bivalent reorganization during karyosome formation in this genetic setting.

Like Polo kinase, Pp1 α -87B might contribute to Cdc25 activation and this way to meiosis progression, so we can speculate that Pp1 α -87B dosage also antagonizes *bubR1*-mutant phenotype. Alternatively, Pp1 α -87B could be acting directly on a BubR1 substrate that is phosphorylated during early stages of meiosis I and normally controls meiotic progression. Further work is required to distinguish between these two possibilities.

Our genetic screen also allowed the identification of one deficiency that causes female sterility and two other deficiencies that impair BubR1 kinase activity in somatic cells and zygotic development. It has been recently shown that BubR1 kinase activity is not essential for accurate somatic chromosome segregation and animal development since BubR1 kinase dead homozygous adult flies lack any obvious phenotype and are recovered in a normal Mendelian proportion (Malmanche *et al.* 2007; Rahmani *et al.* 2009). Nonetheless, while these results strongly suggest that BubR1 kinase activity is not essential for somatic development, our findings indicate that subtle modifications in the dosage of other key components of the cell cycle machinery can trigger an essential requirement for BubR1 kinase activity.

Taken together, our results suggest that the genetic screen strategy presented here is highly efficient in recovering genes that interact with BubR1 kinase function. The screen performed allowed us to identify deficiencies showing genetic interactions, spanning from full recovery of X chromosome NDJ to sterility, the strongest effect expected for genes positively involved in BubR1 kinase activity. In

addition, the screen also allowed for the identification of deficiencies that impaired zygotic development of BubR1 kinase dead embryos suggesting a somatic phenotype. Further genetic and cytological experiments will allow the identification of genes responsible for the effects observed in the characterized deficiencies, allowing the build up of a genetic map of essential genes involved in BubR1 kinase function.

Chapter 3

Duplication of meiotic-related genes in *Drosophila*

This work was done in collaboration with the Molecular Evolution group from IBMC. My contribution to the work was to confirm the functionality of the duplicated genes.

1. Introduction

Gene duplication is one of the major sources to create genetic novelty (Ohno 1970). It can result from unequal crossing over, retrotransposition or from chromosomal or genome duplication. Unequal crossing over occurs due to significant sequence homology. The repetitive sequence region of a chromatid may not line up exactly with its corresponding region in the homologous chromosome or sister chromatid, resulting in the deletion of the sequence in one strand and its replacement with a duplication from its sister chromatid or homologous chromosome. This usually generates gene duplicates in tandem (Zhang 2003) (Figure 3.1a). Retrotransposition occurs when the messenger RNA (mRNA) is reversely transcribed into complementary DNA (cDNA) and then inserted randomly into the genome. When retrotransposition takes place the duplicated gene does not have introns nor regulatory sequences, is usually unlinked to the original gene and may contain poly A tracts and flanking short direct repeats (Long 2001) (Figure 3.1b).

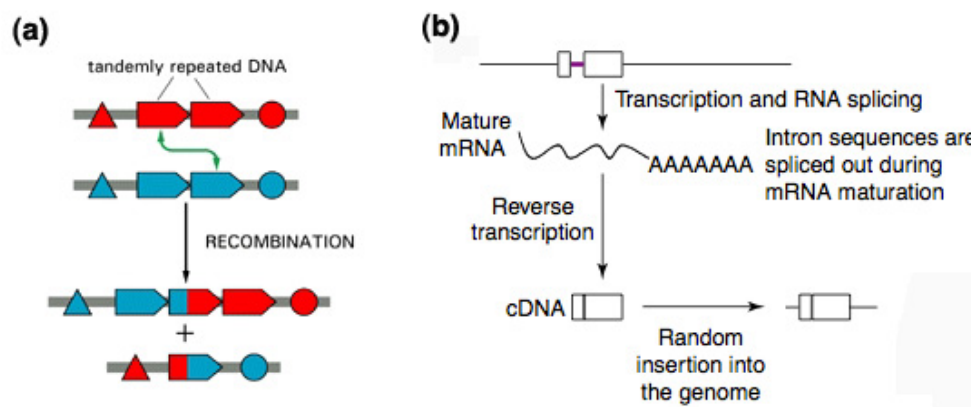


Figure 3.1. Two common modes of gene duplication. (a) Unequal crossing over that results from a recombination event in which the two recombination sites lie at non-identical locations in the two parental DNA molecules. (b) Retrotransposition, which occurs when mRNA is retrotranscribed into cDNA and is then inserted into the genome, usually in a different chromosome than the parental gene. Shapes represent exons and lines represent introns. (Adapted from Alberts *et al.* 2002 and Zhang 2003).

As happens with point mutations, gene duplication can be lost or fixed in the population. The rate at which eukaryotic genes duplicates are retained, that is, go to fixation, had been originally estimated to be 0.01 per gene per million years (Lynch

and Conery 2000). However Osada and Innan (2008), using a different method, have estimated a gene duplication rate of 0.001 per gene per million years.

Since most of the times it is not advantageous to have two identical genes, mutations that disrupt the structure and function of one of these two genes are not removed by selection. This way, the gene containing mutations progressively becomes a pseudogene, which is a gene that is neither expressed nor functional. This process is known by pseudogenization. Alternatively, the duplicated gene can acquire a new or a related function (neofunctionalization) or both duplicates can be stably maintained if their function differ in some aspects (subfunctionalization). In both cases, each daughter gene adopts part of the parental gene function. Subfunctionalization can occur by division of gene expression after duplication or at the protein function level. In the case of strongly expressed genes, extra amounts of RNA or protein products, is sometimes beneficial and so the function of the duplicated gene is conserved. In these cases divergence does not occur and the duplicated genes maintain common functions due to concerted evolution (frequent gene conversion between the duplicated genes) or purifying selection against mutations that modify gene function (reviewed in Zhang 2003).

Not all gene duplications are predicted to be equally retained. For instance, duplication of genes encoding for proteins that are part of a complex are likely deleterious (Papp *et al.* 2003; Deutschbauer *et al.* 2005; Soyer and Creevey 2010). Also, duplication of genes encoding for proteins involved in regulatory or signalling networks are rarely retained, since they likely disrupt network dynamics (Wagner 1994). Additionally, gene duplicates encoding for proteins that participate in several biochemical reactions are less likely to be retained than genes that encode proteins that participate in a single reaction (Sopko *et al.* 2006). Duplicates of genes that encode for activators are also expected to be more frequently retained than genes that encode for receptors (Soyer and Creevey 2010).

Many meiotic pathways are highly conserved across distantly related sexually reproducing eukaryotes (reviewed in Gerton and Hawley 2005) suggesting that meiotic pathways tolerate little change. This is not surprising since meiosis-related genes are known to participate in multiple pathways, to be involved in protein complexes and, when disrupted, multiple aspects of meiosis are affected. Nevertheless, the time it takes to complete meiosis is known to be very variable and environmental factors (such as temperature, nuclear DNA content and genotype) are among the most important factors in affecting meiosis duration. In the *Drosophila* genus the nuclear DNA content varies significantly (C-values between 0.12 and 0.39; <http://www.genomesize.com>), but meiosis duration has only been recorded so far in

D. melanogaster (Bennett 1977). Whether *Drosophila* meiosis-related neomorphs (meiosis-related genes with new functions) have evolved is also unknown. This is important in order to infer the degree of tolerated change of an ancient machine such as the meiotic one.

Anderson *et al.* (2009) studied 33 meiotic-related genes involved in chromosome segregation, achiasmate segregation, crossover regulation, double-strand-break formation, heterochromatin binding, recombination and/or repair, sister-chromatid cohesion, spindle assembly, and telomere maintenance. This study revealed that, in *Drosophila*, variability patterns compatible with adaptive protein divergence and polymorphism can be found in three genes somehow involved in chromosome segregation (*klp3A*, *mtrm*, and *ord*) and three genes required for telomere maintenance (*Ku80*, *mre11* and *rad50*). Nevertheless, as argued by Anderson *et al.* (2009), the observed patterns can also be explained as a consequence of fixation/persistence in *Drosophila* populations of meiotic drive elements (elements that in females influence the preferential sorting of a chromosome to the pronucleus, and consequently to the oocyte). If meiotic drive elements are common (about 18% of the meiotic genes, as surveyed by Anderson *et al.* (2009)) then such meiotic drive elements could conceivably increase the probability of fixation and, therefore, the retention of meiotic gene duplicates. It should be noted that the extent to which the amino acid variation of meiosis-related genes, within and between species, is adaptive is still unknown.

The aim of this work was to determine which of the 33 meiotic or in meiotic-related genes analysed are found duplicated. For that, it was used 12 *Drosophila* genomes available in <http://flybase.org/>: *D. melanogaster*, *D. simulans*, *D. sechellia*, *D. yakuba*, *D. erecta*, *D. ananassae*, *D. pseudoobscura*, *D. persimilis*, *D. willistoni*, *D. mojavensis*, *D. virilis* and *D. grimshawi*. The evolutionary history as well as possible functionality of each gene showing duplicates were also studied.

2. Materials and Methods

2.1. Strains

D. virilis 1051.49 (Chaco, Argentina); *D. persimilis* 14011-0111.48 (California, USA); *D. willistoni* 14030-0811.16 (Rocha, Uruguay) and *D. mojavensis* 15081-1352.00 (California, USA) were used to address expression profiles of the different genes found to be duplicated and their respective duplicates.

2.2. Genomic DNA extraction

Genomic DNA was extracted from single males using the QIAamp DNA Mini Kit from QIAGEN (Izasa Portugal, Lda.) according to the manufacturer's instructions.

2.3. RT-PCR

Total RNA was isolated from *D. virilis*, *D. willistoni*, *D. mojavensis* and *D. persimilis* whole flies (females and males) using TRIzol Reagent (Invitrogen) according to the manufacturer's instructions, and treated with *DNase I* (*RNase-Free*) (Ambion). cDNA was synthesized by reverse transcription with SuperScript III First-Strand Synthesis SuperMix for qRT-PCR (Invitrogen) and amplified by PCR using the primers shown on Table 3.1. The endogenous *ribosomal protein L32* (*RpL32*) was used as a control to check cDNA quality. No-template controls and reactions with RNA that was not reversed transcribed were performed in order to confirm the absence of genomic DNA contamination. When possible, primers were designed to encompass a region of the gene with one intron. Therefore, the cDNA amplification product is expected to have a shorter size than the amplified product from genomic DNA. The results were analysed by agarose gel electrophoresis. It should be noted that expression levels of different genes cannot be compared since, for instance, amplification product sizes are different, and primer features (such as GC content, or melting temperatures) are different. Direct sequencing was performed using as template all the PCR products obtained from cDNA amplification, to confirm the specificity of the primers designed for the duplicated genes and their respective duplicates. It should be noted that when using cDNA for a given gene and its duplicates, most PCR amplification products have different sizes.

Table 3.1. List of primers used.

Specie	Gene	Forward Primer (5'-3')	Reverse Primer (5'-3')	Product size (bp)
<i>D. mojavensis</i>	<i>RpL32</i>	AATCTCCTTGCGTTTCTT	CACTTCATCCGTCACCAG	346
<i>D. virilis</i>				
<i>D. willistoni</i>	<i>RpL32</i>	CTTCATCCGCCATCAGTC	GAACGCAGACGACCATTG	408
<i>D. persimilis</i>				
<i>D. mojavensis</i>	<i>mei-S332</i>	TTATGTAGTCACGAAAGC	AGGAAGCCAAAGTATCAC	459
	<i>mei-S332-dup</i>	TTGCTGGAGGAACGAGAA	ACGGACGAAGATGAGGTG	585
	<i>mre11</i>	GTTGGCACTGTATGGATT	AGCGGATTTTTGTCTTTG	869
	<i>mre11-dup</i>	ATCGTGGTGGTAGCAAGA	GGAGTGAAAAAGCGTGAG	729
<i>D. virilis</i>	<i>mei-S332</i>	AGGCTGAGATATTACTGA	TAGGTGACAAGGACAAAG	612
	<i>mei-S332-dup</i>	AGAAGCAACGAATAACTA	TGAAGCAGCAACTGATGT	552
	<i>cav</i>	GCCGATGAACTGGATGGT	TGCTGATGACTTTTGCTC	527
	<i>cav-dup</i>	CAAAATGAGGAGGAAGAA	GAACTGAGCCGAGCAAC	515
	<i>mtrm</i>	TTGACCGTGGACAGCATC	GTTTCTTGACAGGATTG	190
	<i>mtrm-dup</i>	TGGTCAGCATCTATTACG	GCAGTCCTCATCATTACG	511
<i>D. willistoni</i>	<i>cav</i>	AAGAACAACCAGGATTTA	CGAAGGCACAGATACATT	609
	<i>cav-dup</i>	ATGTCGCTTTTGGATTCA	GGTTTATAGGTGGATTCTC	529
	<i>mtrm</i>	CACACCAACCAAAAATGA	AGTGCCTCCAGTATGAT	510
<i>D. persimilis</i>	<i>cav</i>	AAATGGTGATGAGCAATG	GAGAACGAGGTGGTGACA	483
	<i>cav-dup</i>	GAGAAAGGCGAAAAATAC	TCCACACAGAGTTGAAG	220
	<i>polo</i>	CGGCAAAATCGATCAAA	GTCTTCAATGGTGTCTGTT	831
	<i>polo-dup1</i>	AACGGTATTGAGCAGTCG	TTCTTTTCCAGGGTCTTG	455
	<i>polo-dup2</i>	CCATTCCACCACAAAACC	CCCAGTATTCCCATCTCC	429

2.4. Datasets and sequence alignment

The *D. melanogaster* coding sequences of the 33 meiosis-related genes listed in Anderson *et al.* (2009) was retrieved from FlyBase. In order to retrieve sequences from non-melanogaster *Drosophila* species, the tblastn option with standard parameters, as implemented in FlyBase was used. The *D. melanogaster* coding sequences were used as a query. For every gene dataset, translated amino acid sequences were aligned using ClustalW (Reis *et al.* 2011).

3. Results

3.1. The vast majority (85%) of the genes involved in meiosis-related tasks are not duplicated

Of the 33 meiosis-related genes studied (those listed in Anderson *et al.* 2009), only *c(3)G* and *CG7676* (also known as *corona*) could not be found in all of the 12 publicly available *Drosophila* genomes (<http://flybase.org/>), although a non-negligible fraction is non-annotated or likely miss-annotated. For 26 out of the 33 genes analysed (*ald*, *asp*, *Axs*, *c(2)M*, *c(3)G*, *Su(var)205*, *Klp3A*, *Ku70*, *Ku80*, *mei-218*, *mei-41*, *mei-P22*, *mei-P26*, *mei-9*, *mus304*, *ncd*, *okr*, *ord*, *rad50*, *smc1*, *spn-A*, *spn-B*, *spn-D*, *subito*, *teflon* and *tefu*), there is only a single copy in the 12 *Drosophila* genomes. Therefore, we found that genus-wide up to 85% of meiosis-related genes do not have duplicates. However, nine independent gene duplications involving the genes *cav*, *mre11*, *mei-S332*, *polo* and *mtrm* were found (Reis *et al.* 2011). The 12 *Drosophila* species here analysed imply about 230 million years of independent evolution (Figure 3.2), which leads us to conclude that *Drosophila* meiosis-related genes are duplicated at a rate of 0.0012 per gene per million years. This rate is similar to that estimated for the whole *Drosophila* genome (Osada and Innan 2008).

Next, it is presented, for each gene presenting duplicates, their evolutionary history, as well as evidence that the gene duplicate(s) is functional.

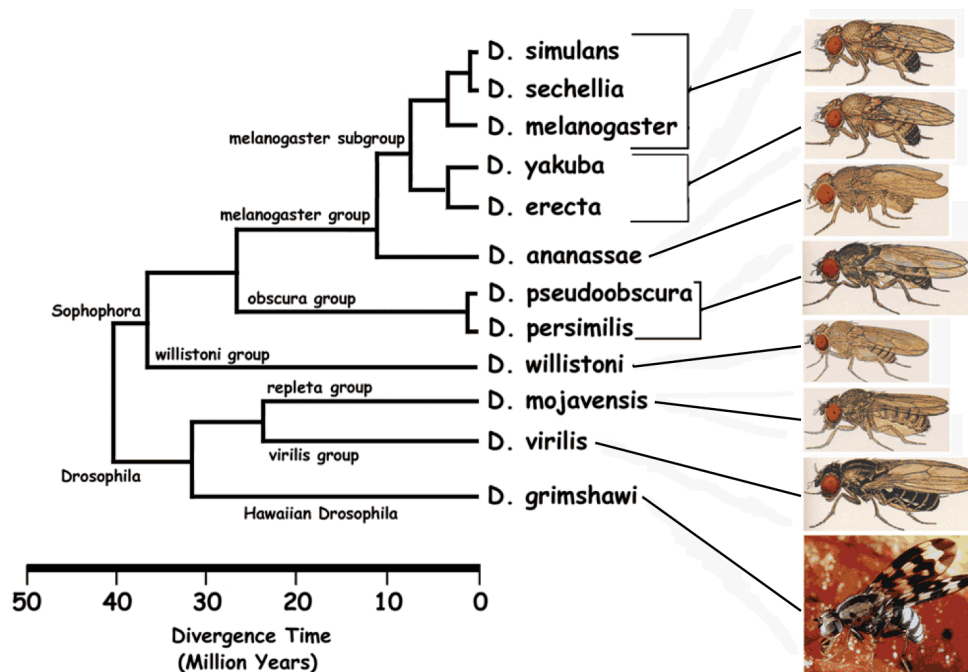


Figure 3.2. Relationship of the *Drosophila* species studied (Adapted from <http://flybase.org>).

3.2. Three independent *cav* gene duplications

Telomeres play essential roles in the maintenance of chromosome integrity by protecting linear DNA extremities from being processed as DSBs by the DNA repair machinery. In this telomere capping process, a set of proteins that associate with chromosome ends is involved in the formation of a protein complex that prevents the ligation of chromosome ends (reviewed by O'Sullivan and Karlseder 2010). Well-characterized *Drosophila* capping proteins include Heterochromatin Protein 1 (HP1, that is encoded by *Suppressor of variegation 205*, *Su(var)205*) and its partner the HP1/Origin Recognition Complex (ORC)-associated protein (or HOAP, which is encoded by *caravaggio*, *cav*) (Fanti *et al.* 1998; Shareef *et al.* 2001, Cenci *et al.* 2003). HOAP is predominantly enriched at telomeres and is required for the recruitment of other telomere proteins (Raffa *et al.* 2009; Raffa *et al.* 2010).

Phylogenetic analyses revealed the presence of three independent *cav* gene duplications: one in the *virilis* group, another in the *obscura* group (*D. persimilis* and *D. pseudoobscura*) and a third one in the *willistoni* group (Reis *et al.* 2011). Given that there is always a *cav* gene on Muller's element E (corresponding to the chromosome 3R in *D. melanogaster*), it is reasonable to assume that this would be the location of the ancestral *cav* gene. In *D. melanogaster* the *cav* gene is also localised on Muller's element E. In the four species showing two *cav* copies, the duplicated gene is on three different Muller's elements: on Muller's element A in *D. virilis*, on element B in *D. willistoni* and on element E in both *D. persimilis* and *D. pseudoobscura*. This finding is compatible with a scenario invoking three independent duplications, as suggested by the phylogenetic analyses (Reis *et al.* 2011). All *cav* gene duplicates were found to have introns, thus retrotransposition seems an unlikely explanation for the observed change in gene location.

The *D. virilis cav-dup* gene is estimated to be about 40 million years old that corresponds to the split between the *Sophophora* and *Drosophila* subgenera assuming a molecular clock for synonymous mutations. However, we were not able to find duplicates of *cav* in *D. grimshawi* or in *D. mojavensis*. *cav* and *cav-dup* are subjected to similar mutation rates but show different amino acid constraints (Reis *et al.* 2011). So, the accelerated rate of non-synonymous evolution of the *D. virilis cav-dup* gene could suggest that it is a pseudogene. Nevertheless, and according to RNA expression studies, this gene is expressed in both males and females (Figure 3.3).

The *D. willistoni cav-dup* gene is estimated to be 10 million years old, under the assumption of a molecular clock for synonymous mutations. *cav* and *cav-dup* are under similar amino acid constraint, and there is no evidence that *cav-dup* is evolving

faster than *cav* (Reis *et al.* 2011). Despite this fact, according to RNA expression studies the duplicated gene seems to be only weakly expressed in males. So, there is no evidence that it is a pseudogene (Figure 3.3).

Two *cav* genes were also found in two closely related species: *D. persimilis* and *D. pseudoobscura*. This *cav* gene duplication is estimated to be 14 million years old, under the assumption of a molecular clock for synonymous mutations. In this case, there is also no evidence that the *cav-dup* gene is evolving faster than *cav* (Reis *et al.* 2011). RNA expression studies showed that the duplicated gene is expressed in both females and males (Figure 3.3).

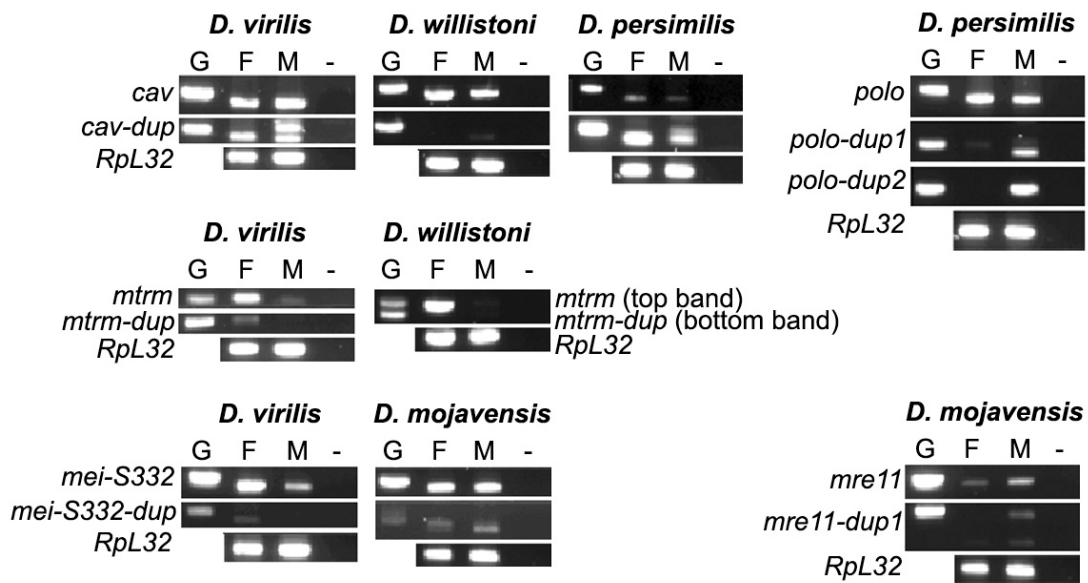


Figure 3.3. Expression patterns of genes found to be duplicated. G – genomic DNA; F – female flies; M – male flies. The cDNA of duplicated genes were sequenced in order to assure amplification specificity. In males, for genes *D. virilis cav-dup*, *D. persimilis cav-dup*, *D. mojavensis mre-11-dup* and *D. persimilis polo-dup1* a band with the size expected for an amplification from genomic DNA is observed. In order to rule out the possibility of contamination with genomic DNA, the PCR reactions were performed three times independently starting from different cDNA aliquots and identical results were obtained. The observation that when using the same aliquot, the duplicated gene shows two bands but the genes *D. virilis cav*, *D. persimilis cav*, *D. mojavensis mre-11* and *D. persimilis polo* show only one band of the expected size also shows that there is no contamination with genomic DNA.

3.3. *mre11* gene duplication in the *D. mojavensis* lineage

The *meiotic recombination 11* (*mre11*) gene encodes for the nuclease subunit of the Mre11/Rad50/Nbs (MRN) complex that is multifunctional in genome maintenance. The MRN complex is a key player in the cellular response to DSBs and is involved in almost all aspects of DNA end metabolism, including DSB detection, DSB processing and DSB-activated cell cycle checkpoint response, homologous and non-homologous recombination, meiosis and telomere maintenance. Mre11 and

Rad50 subunits are well conserved in evolution, whereas Nbs is less sequence conserved (reviewed in Assenmacher and Hopfner 2004).

In *D. mojavensis* two *mre11* copies were found, both on Muller's element B, which corresponds to the chromosome 2L in *D. melanogaster*. The *mre11* gene in *D. melanogaster* is also localised on Muller's element B. This duplication occurred in the *D. mojavensis* lineage after the separation from the sister group *D. virilis* lineage and it is estimated to be about 15 million years old, under the assumption of a molecular clock for synonymous mutations. The two *D. mojavensis mre11* genes are subjected to similar mutation rates but to different amino acid constraints (Reis *et al.* 2011). So, the accelerated rate of amino acid evolution of the *D. mojavensis mre11-dup* could suggest that it is a pseudogene. According to RNA expression studies, the *mre11-dup* is only expressed in males (Figure 3.3).

3.4. Two *polo* gene duplications in the *obscura* group

As already explained, Polo is a protein kinase that is involved in several steps of mitosis and meiosis. Polo contains two functional domains, a N-terminal kinase domain (residues 25-277 in *D. melanogaster*) with a requirement for phosphorylation of the threonine 182 for kinase activation, and a C-terminal region that contains the polo-box domain (PBD). The Polo-box domain consists of two highly conserved Polo-box motifs, PB1 and PB2, of 64 and 69 amino acids, respectively and is exclusively found in the family of Polo-like kinases that regulate mitotic entry, spindle assembly, centrosome maturation, chromosome segregation, cytokinesis and cell cycle arrest. The Polo-box domain performs dual roles, determining subcellular localization and autoinhibitory regulation of the kinase domain. In many cases, the PBD has been shown to preferentially bind phosphothreonine/serine residues with the core consensus motif Ser-pThr/pSer-Pro/X (SSP or STP), residing in the target proteins (Elia *et al.* 2003). In substrates, the central serine/threonine residue of the PBD-binding site must be phosphorylated to ensure proper binding of Polo. PBD-binding sites frequently co-occur with Polo phosphorylation motifs.

Three *polo* genes are found in the two closely related species: *D. pseudoobscura* and *D. persimilis*. The *D. persimilis* GL25129 and the *D. pseudoobscura* GA11545 genes that are on Muller's element D (corresponds to chromosome 3L in *D. melanogaster*, the same element where *polo* is localised in this species) are orthologous. The *D. persimilis* GL25881 and the *D. pseudoobscura* GA25172 genes that are on Muller's element B (corresponds to chromosome 2L in *D. melanogaster*), are also orthologous, and are here named as *polo-dup1*. The *D.*

persimilis GL19429 and the *D. pseudoobscura* GA25958 genes that are also on Muller's element B and are also orthologous, and are named here as *polo-dup2*.

There are three predicted introns in *polo-dup1*. Therefore, retrotransposition seems an unlikely explanation for the observed change in gene location (from Muller's element D to element B). Under the assumption of a molecular clock for synonymous mutations, this *polo* gene duplication is about 6.5 million years old, so it is expected to be only found in species of the *obscura* group. These two *polo* genes are subjected to similar mutation rates but have different amino acid constraints (Reis *et al.* 2011). This observation could suggest that *polo-dup1* is a pseudogene, but according to RNA expression studies it was found to be expressed in males (Figure 3.3). The protein sequence of Polo-dup1 is very similar to Polo sequence. However, it presents several fixed amino acid changes in its kinase domain and in its Polo-boxes (Appendix B).

Under the assumption of a molecular clock for synonymous mutations, *polo-dup2* is about 12 million years old, and like *polo-dup1*, it is also expected to be found only in species of the *obscura* group. There are no introns in *polo-dup2* so, in this case, retrotransposition could be an explanation for the origin of this duplication. It should be noted that such hypothesis relies on the quality of the annotation of the *D. pseudoobscura* and *D. persimilis* genomes. *polo* and *polo-dup2* are subjected to similar mutation rates but with different amino acid constraints (Reis *et al.* 2011). Nevertheless, according to RNA expression studies, like *polo-dup1*, *polo-dup2* is expressed in males (Figure 3.3) and, thus, it is unlikely to be a pseudogene. *polo-dup2* is a truncated version of *polo*, where the last third of the coding region of the gene is missing. Therefore the protein encoded by *polo-dup2* does not have any Polo-boxes. Polo-dup2 has six additional amino acids in its kinase domain and presents more fixed amino acid changes in its kinase domain than Polo-dup1, including in T182 (Appendix B).

3.5. Two independent *mtrm* gene duplications

As already mentioned, *D. melanogaster* Mtrm protein is a meiosis-specific 1:1 stoichiometric inhibitor of the Polo kinase protein (Xiang *et al.* 2007). *mtrm* is not annotated in most *Drosophila* genomes but can always be found within one intron of the *exo70* gene. In *D. willistoni* there are two *mtrm*-like genes, one on Muller's element B (that codes for a 186 amino acids long protein) and another one on Muller's element D (that codes for a 196 amino acids long protein). Since the *D. melanogaster mtrm* gene is located on Muller's element D, it seems likely that the

duplicated gene copy is the one on Muller's element B. Therefore, the *D. willistoni* duplication is here named as *mtrm-dup*. Although the two copies are on different Muller's elements, at nucleotide level both sequences are 94% identical. Since *mtrm* does not have any intron, the possible involvement of retrotransposition in the translocation of the gene could not be assessed. This is a recent gene duplication event, estimated to be 1.6 million years old, under the assumption of a molecular clock for synonymous sites. *mtrm* and *mtrm-dup* genes seem to be under similar amino acid constraint (Reis *et al.* 2011).

Recent proteomic and bioinformatic analysis of PBD-interacting proteins have revealed that PBD-binding sites frequently co-occur with Polo phosphorylation motifs (Jang *et al.* 2002). Mtrm is no exception: it contains one functional PBD-binding site, a STP with the central threonine at position 40, and a putative Polo phosphorylation consensus motif (Asp/Glu-X-Ser/Thr-Ø-X-Asp/Glu, where X is any amino acid and Ø is usually a hydrophobic amino acid) with the phosphorylatable serine at position 137. The STP motif is not only important for Polo binding, but it is also required for proper Mtrm function (Xiang *et al.* 2007; Smith *et al.* 2008). Mtrm and Mtrm-dup from *D. willistoni* show a Polo-box domain binding motif and a typical Polo-phosphorylation motif in the same protein region as in *D. melanogaster*. Moreover, most of the other phosphorylation sites reported for the *D. melanogaster* Mtrm protein are also present in the *D. willistoni* Mtrm and Mtrm-dup proteins (S48, S52, S123 and S124) (Figure 3.4). Nevertheless, according to RNA expression studies, we could not find any evidence that the *D. willistoni mtrm-dup* is expressed (Figure 3.3). Therefore, the hypothesis that this gene is a recent pseudogene that did not have yet time to degenerate cannot be ruled out.

In *D. virilis* there are also two *mtrm*-like genes, one on Muller's element A (corresponds to the X chromosome in *D. melanogaster*) and another one on Muller's element D, being the latter the orthologous of the *D. melanogaster mtrm* gene. *mtrm* and *mtrm-dup* from *D. virilis* are also intronless genes. Therefore, it is not possible to infer the role of retrotransposition in the transposition of this gene from Muller's element D to A. Bayesian phylogenetic analyses suggest that this *mtrm* gene duplication is as old as the separation of *D. grimshawi*/(*D. mojavensis*/*D. virilis*) lineages (Reis *et al.* 2011), but there is no evidence for a *mtrm* gene duplication in *D. grimshawi* nor in *D. mojavensis*. Nonetheless, *D. virilis mtrm-dup* is evolving at the same rate as the *mtrm* gene (Reis *et al.* 2011) and according to RNA expression studies, it is weakly expressed only in females (Figure 3.3).

<i>D.mel</i> Mtrm	MENSRTPTN-----KTKITLNRTPTLKER-RWNTLKVNSTSNVRCSTPIFGNFRSPNLS 52
<i>D.will</i> Mtrm	MDTIRTPKTYLQQP-NKIKLVLPRTPTKN---EWNTLQVNHINVRCSPTIFGDIRSPNLS 56
<i>D.will</i> Mtrm-dup	MDTIRTPKTYLQOPTIKIKLVLPRTPTKN---EWNTLQVNHINVRCSPTIFGDIRSANLS 57
<i>D.vir</i> Mtrm	MAEFCT-----PKDVCQLPYNRSCTD-----INTLTVDVINVRCSPTIVGKLRSNLS 47
<i>D.vir</i> Mtrm-dup	MEECRT-----PTGQHLFSYNRTPI-----LSVINKNAQNIRCSPTIFGDLRSNMS 47
	* * : * : . : : * : * : * : * : * : *
<i>D.mel</i> Mtrm	LPK-PQEEIIEPEREIKSSSPDTCSDS----NMETSLALESRRRSIKASNHSYVVNHA 159
<i>D.will</i> Mtrm	VKR-QLEEAIN-----NKSDDNNSDES----IADSSMIRESRRLSLAVSNHSYVFNHA 138
<i>D.will</i> Mtrm-dup	VKR-QLEETIN-----NKSDDNKSDES----IADSSMIRESRRGFLAVCNYSYVFNHA 139
<i>D.vir</i> Mtrm	ANP-VKKPGKEVGPLDDSNNSLNFLDSS-SASNTETSLCRESRRRSLQAENHSYVLNHA 138
<i>D.vir</i> Mtrm-dup	KPP-----GRK---LEDNRNLSLDEFMDDSGSGNSAENSLCKESRRRSLLAANHSYVINHA 130
	: . * : * : : * : * : * : * : * : *

Figure 3.4. Mtrm protein alignment of *D. melanogaster*, *D. willistoni* and *D. virilis* with their duplicated protein. In red is shown the S/T phosphorylated sites and in yellow the PBD-binding sequence, STP. “*D. mel*” stands for *D. melanogaster*; “*D. will*” stands for *D. willistoni*; “*D. vir*” stands for *D. virilis*; symbols denoting the degree of conservation observed in each column: “*” means that the residues or nucleotides in that column are identical in all sequences in the alignment; “:” means that conserved substitutions have been observed; “.” means that semi-conserved substitutions are observed.

D. virilis Mtrm and Mtrm-dup sequences show conservation of the PBD-binding site, STP with the central threonine at residue 40, and the putative Polo phosphorylation consensus motif with the phosphorylatable serine at position 137 in orthologous sites identified in *D. melanogaster* as well as the phosphorylated sites S48, S52 and S124 (Figure 3.4).

Although the *D. virilis* *mtrm-dup* gene seems to be functional, there is no data to support the assumption that this gene plays an essential role in meiosis in *D. virilis*. Indeed, it is conceivable that this gene represents a non-essential meiotic drive element that went to fixation in the common ancestor of species of the *virilis* group. Once meiotic drive element is fixed it may be difficult to lose it, since chromosomes carrying it are more represented in the next generation than chromosomes carrying alternative deleted copies of this element. Thus, such a gene could show most of the features expected for an essential gene. For *D. melanogaster*/*D. simulans*, Anderson *et al.* (2009) showed patterns of evolution at the *mtrm* gene that are compatible with both adaptive protein evolution and sequential fixation of meiotic drive elements.

3.6. Concerted evolution at the *Drosophila* subgenus *mei-S332*-like genes

Duplications in the *mei-S332* gene were also found. The *Drosophila* protein Mei-S332 is essential to maintain centromere cohesion in meiosis until the metaphase II-to-anaphase II transition. (Clarke *et al.* 2005). According to phylogenetic studies performed by Reis *et al.* (2011) this gene has been independently duplicated three times. The two copies of *mei-S332* are located on Muller’s element C (corresponds to chromosome 2R in *D. melanogaster*, the same localisation of *mei-S332* in this species), with opposite transcription orientations and at about the same distance in three species where this gene was found to be

duplicated (*D. virilis*, *D. mojavensis* and *D. grimshawi*). Thus, the finding of a similar gene arrangement in *Drosophila* subgenus suggests a unique duplication event, rather than three independent recent duplications. The little divergence observed between the two copies in each species suggests that this is a case of concerted evolution. Besides rRNA genes, concerted evolution has also been reported in other *Drosophila* genes (Wang *et al.* 1999; Somogyi *et al.* 2008; Carmon *et al.* 2010).

Dissociation of Mei-S332 from centromeres is driven by Polo kinase (Clarke *et al.* 2005). *D. melanogaster* Mei-S332 has two Polo binding sites: one SSP motif with the central serine at residue 234 and one STP motif with the central threonine at residue 331 (Clarke *et al.* 2005). The STP motif is conserved in all protein sequences, except in the duplicated copy of *D. mojavensis* (Figure 3.5), whereas the SSP motif is not conserved. In *D. melanogaster*, when the central residue of each motif is mutated to an alanine, only the T331A mutation abolishes Polo-dependent phosphorylation, in the S234A mutation it was unaffected (Clarke *et al.* 2005). This result fits our observation of a better conservation of the STP motif than the SSP motif. These findings suggest that, with the exception of the duplicated copy of *D. mojavensis*, all other duplicated genes are functional. However, according to RNA expression studies *D. mojavensis* *mei-S332-dup* gene is expressed in both sexes, whereas in *D. virilis* *mei-S332-dup* gene could only be detected in females (Figure 3.3). The expression of *D. grimshawi* *mei-S332-dup* gene was not tested, due to the fact that we could not obtain non-mutant strains of this species.

```

D.mel-Mei-S332      ---GLTPRR-----SLFNGIGKMAGSTSTPKSFLVEEMPSIRTRSRTAANKKS----- 355
D.moj_Mei-S332      AEPTLT+PRRSQ+LNFS+FN+GI+ANRPEDCSTPRQDE+TLIS+NWPLAST+KT+GK+RTRSTASGI 376
D.moj_Mei-S332-dup  -----
D.vir_Mei-S332      AGPALTPRRS+Q+LNFS+FN+GI+SNRPGDSSTPRRQE+TFTTNW+KPVSAKAKPKRTNSANS-- 395
D.vir_Mei-S332-dup  AGPALTPRRS+Q+LNFS+FN+GI+SNRPGDSSTPRRQE--TTNWIPASAKAKPKRTNSTNS-- 394
D.gri_Mei-S332      VDATLT+PRRTQ+LNFS+IFNGI+VDRPDDCSTPQRNHEMVNSERKTTMR+AKPRGKRTQSTVGC 320
D.gri_Mei-S332-dup  VDATLT+PRRTQ+LNFS+IFNGI+VDRPDDCSTPQRNHEIVNSERKTTMR+AKPRGKRTQSTVGC 320
                    *****      *  *****      . . .  ***          :          ::::      . . . .

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Figure 3.5. Mei-S332 protein alignment of *D. melanogaster*, *D. mojavensis*, *D. virilis* and *D. grimshawi* with their duplicated protein. In yellow is highlighted the conserved PBD-binding sequence, STP. “*D. mel*” stands for *D. melanogaster*; “*D. moj*” stands for *D. mojavensis*; “*D. vir*” stands for *D. virilis*; “*D. gri*” stands for *D. grimshawi*; symbols denoting the degree of conservation observed in each column: “*” means that the residues or nucleotides in that column are identical in all sequences in the alignment; “.” means that conserved substitutions have been observed; “.” means that semi-conserved substitutions are observed.

4. Discussion

Meiosis-related genes participate in multiple pathways, are involved in protein complexes and, when disrupted, affect multiple aspects of meiosis. So, it is surprising that *Drosophila* meiosis-related genes are found duplicated and retained at a rate of 0.0012 per gene per million years, similar to the value estimated for the whole *Drosophila* genome using species of the *melanogaster* subgroup (Osada and Innan 2008). In this study, nine independent gene duplications were found and these include the *cav*, *mre11*, *polo*, *mtrm* and *mei-S332* genes.

HOAP, the protein encoded by *cav*, is involved in telomere capping. *cav* was found to be duplicated three independent times: in the *virilis*, *willistoni* and in *obscura* group. These three duplications are old and the gene is expressed (*cav* duplication from *willistoni* group is only expressed weakly in males). We currently do not know the functional significance of having two *cav* gene copies in *D. virilis* with similar expression patterns. However, it has been proposed that the DNA-binding activities of HOAP and ORC function to recruit under-phosphorylated isoforms of HP1 to sites of heterochromatin nucleation (Shareef *et al.* 2003). So, taking into account this finding and the fact that the C-value of *D. virilis* is higher than the C-value of *D. melanogaster* (0.37 and 0.17, respectively; <http://www.genomesize.com>), it can be speculated that higher *cav*-related protein levels could be advantageous in species with high heterochromatin content, such as *D. virilis*. Nevertheless, this reason cannot explain the presence of a duplicate *cav* gene in *willistoni* and in *obscura* groups, since their C-values are similar to the one of *D. melanogaster*.

Mre11 is part of a complex, MRN, which is a central player in most aspects of cellular response to DNA double-strand breaks and DNA end metabolism. Against the expectations of retention of gene duplicates that codify for proteins that are part of a complex, in this study it was found two *mre11*-like genes in *D. mojavensis*. According to RNA expression studies, despite being expressed in males and in females, *mre11* expression level is higher in males than in females. This way, it was expected that the duplicated gene would balance this difference, since it is not known any reason for males to have higher levels of *mre11* than females. However, *mre11-dup* seems to be only expressed in males. Thus, this duplication instead of equalizing the differences of *mre11* expression in males vs. females, it promotes the expression imbalance. This is a puzzling result for which we do not have yet any explanation.

Polo is a protein kinase involved in several aspects of mitosis and meiosis (reviewed in Chapter 2). This way, it was expected that possible duplications would

not be retained. Nonetheless, two functional *polo* gene duplicates are observed in *D. persimilis* and in *D. pseudoobscura* and, at least in *D. persimilis*, *polo-dup1* and *polo-dup2* are both expressed in males. Since Polo plays important roles in *D. melanogaster* oogenesis, it could seem intriguing the fact that the duplicated genes are not expressed in females. According to Xiang *et al.* (2007) the precise amounts of *polo*, at least in *D. melanogaster* oogenesis, appears to be very important for the correct timing of Twine activation that leads to GVBD. Therefore having extra Polo would not be advantageous, at least in females, and this could explain why *polo* duplications are not retained in females as opposed to what we could observe in males. Indeed, according to the evolution rate tests, both *polo*-duplicated genes are subjected to similar mutation rates but to different amino acid constraints (Reis *et al.* 2011) meaning that gene duplicates are evolving faster than the original gene. It cannot be ruled out the fact that this additional amount of Polo in *D. persimilis* males may play important functions.

The *D. melanogaster* Mtrm protein is a meiosis-specific 1:1 stoichiometric inhibitor of the Polo protein kinase (Xiang *et al.* 2007). According to the theoretical predictions, possible duplications in *mtrm* would not be retained since it would disrupt stoichiometric balances. Nevertheless, two independent duplications of *mtrm* were found: one in *D. willistoni* and the other in *D. virilis*. The *D. willistoni mtrm-dup* gene seems to be a recent pseudogene and we could not find any evidence that it is expressed. However, the *D. virilis mtrm-dup* is an old gene duplication that is weakly expressed in females. It can be predicted that this extra amount of Mtrm increases the time required for the amount of Polo to exceed the available amount of inhibitory Mtrm. Thus, in *D. virilis* the duration of meiosis would be higher than in *D. melanogaster* but *D. melanogaster* is the only *Drosophila* species where meiosis duration has been recorded (about 1-2 days; Bennett 1977). Bennett (1977) has shown a linear correlation in insects between the nuclear DNA content and meiosis duration. Interestingly, among all *Drosophila* species, *D. virilis* is the species with highest nuclear DNA content.

Functional gene duplicates of *mei-S332* were also found in *D. mojavensis*, in *D. grimshawi* and in *D. virilis*. In *D. mojavensis* the level of expression of *mei-S332-dup* seems to be equal in both sexes, while in *D. virilis mei-S332-dup* seems to be only weakly expressed in females. However, the expression level of *D. virilis mei-S332* appears to be already higher in females than in males. The release of Mei-S332 from centromeres at metaphase II-to-anaphase II transition is achieved via phosphorylation by Polo kinase (Clarke *et al.* 2005). It is not known the stoichiometric balance of Polo vs. Mei-S332 required for this activity, but it was shown that in *polo*

heterozygous mutants there is a suppression of *mei-S332* mutant phenotype and *Mei-S332* fails to dissociate from centromeres at metaphase II-to-anaphase II transition, which affects chromosome segregation. Thus, it can be predicted that increasing *mei-S332* dosage without increasing *polo* dosage, *Mei-S332* dissociation from centromeres would be delayed and, as observed for *Mtrm*, this might lead to an increase in meiosis duration. Interestingly, one of the species in which *mei-S332* was found to be duplicated is in *D. virilis*, the same species that shows a functional duplication of *mtrm*. Thus, in *D. virilis* an increased duration in meiosis, triggered by extra amounts of *Mtrm* and *Mei-S332*, could be a strategy to deal with *D. virilis* large genome.

Contrarily to theoretical expectations, meiosis-related genes are found duplicated and are retained at the same rate as the average for all genes. Despite the fact that most of the genes found to be duplicated are expressed, it remains to be determined whether the duplicated genes play an essential roles in meiosis-related features in the species where they are present. It can be argued that the duplicated genes are non-essential meiotic drive gene duplicates that went to fixation. However, the possibility of subfunctionalization or neofunctionalization cannot be ruled out. Indeed, about 50% of the gene duplicates are evolving faster than the original gene, a pattern that is compatible with a short period of relaxed selection or/and acquisition of a new function. Detailed cellular and biochemical experiments must be performed in order to address this issue.

Chapter 4

General Discussion

In 1905, J.B. Farmer and J.B. Moore coined the word “meiosis”, which has Greek origin and means “reduction”. Meiosis is a common feature of all sexually reproducing organisms and, despite the fact that meiotic details are remarkably different between species (and sexes), the basic features are conserved throughout evolution. The mechanism of fertilization involves the fusion of an egg and a sperm, as Oscar Hertwig initially identified it in 1876, using sea urchin eggs. Meiosis is a specialized cell division and, due to the need of maintaining the correct ploidy of the organisms across generations, gametes have to be haploid. This was initially described in 1890 by August Weismann. Thus, during meiosis there is only a single round of DNA replication, followed by two sequential rounds of cell division. However, as Thomas Morgan observed in 1911 using *D. melanogaster*, daughter haploid cells that result from meiosis are not genetically identical to the mother cell, due to a process known as recombination. This process, which increases the genetic diversity of the offspring, also leads to the formation of chiasmata, the physical connections between homologous chromosomes during prophase I. These connections provide a physical link between homologous chromosomes that, due to the fact that kinetochores of sister chromatids bind spindle microtubules from the same spindle pole, results in the alignment of chromosomes on the meiotic spindle and in the generation of tension between homologs.

NDJ of homologs and consequent meiotic aneuploidies may take place whenever recombination fails or if the number of recombination events is severely reduced or if their localization along the chromosome arms is not optimal. These errors in meiotic chromosome segregation are thought to be the main cause of miscarriage and genetic disorders (reviewed in Hassold and Hunt 2001). Nevertheless, several lines of evidence also suggest that defects in cell cycle control, specifically in the SAC, can also increase aneuploidy levels. For example, oocytes from mice in which the levels of Mad2 are decreased present a significantly accelerated GVBD and consequently aneuploidies are observed more frequently (Niault *et al.* 2007). Also in mice, loss of Bub1 in oocytes results in chromosome NDJ at meiosis I (McGuinness *et al.* 2009). *D. melanogaster* oocytes deficient for the checkpoint protein BubR1 also have increased levels of NDJ (Malmanche *et al.* 2007).

Despite the fact that in most organisms meiotic NDJ is a rare event, it is estimated that in humans 10 to 30 % of fertilized eggs are aneuploid (trisomy or monosomy). Most aneuploidy conceptuses perish *in utero*, but some aneuploid fetuses survive to term and, as a class, aneuploidy is the most common known cause of mental retardation. A large percentage of human aneuploidies originate

from segregating errors during meiosis I, and the frequency of these errors increase with maternal age (reviewed in Hassold and Hunt 2001). Despite extensive work, the underlying causes of the age effect in aneuploidies are still unknown.

D. melanogaster is a model organism that has helped to uncover many aspects related to meiotic NDJ, including the age related effect. Since the screens for meiotic mutations in *Drosophila*, performed by Sandler *et al.* (1968) and by Baker and Carpenter (1972), that this type of approach has allowed for the identification of several genes involved in meiotic chromosomal disjunction. Indeed, the success of *D. melanogaster* as model organism is largely due to the power to perform forward genetic screens that allow for the identification of genes involved in particular biological processes. Enhancer and suppressor screens are one type of screens that can be carried out in *D. melanogaster*. These enhancer and suppressor screens rely on the fact that, for most of the genes, 50% of the wild-type protein level is usually sufficient for normal activity. However, if the organism carries a mutant allele that further reduces protein levels near its threshold, then other alterations that affect the process will result in an abnormal phenotype. Using such a sensitized genetic background it is possible to scan the genome for secondary mutations, allowing the identification of genes involved in a given pathway as dominant enhancers or suppressors.

In this work we took advantage of *D. melanogaster* genetics to carry out a dominant enhancer/suppressor screen, using the sensitized genetic background *bubR1^{D1326N}/bubR1^{rev1}*. *bubR1^{D1326N}* is a *bubR1* allele that causes meiotic NDJ in hetero-allelic combinations, while retaining SAC activity in somatic cell division. Using a third chromosome deficiency kit, we scanned most of the third chromosome and asked whether the frequency of NDJ observed in *bubR1^{D1326N}/bubR1^{rev1}* is altered. Upon the definition of genomic regions affecting the frequency of NDJ, we used smaller deficiencies and mutant alleles of candidate genes to identify the responsible *loci* for the identified dominant phenotype.

Interestingly, we were able to identify genomic regions that haploinsufficient suppressions affect sterility or viability of the sensitized genetic background. Females with *Df(3L)ED4674* in hemizygous conditions and with the sensitized genetic background are completely sterile, suggesting that *Df(3L)ED4674* contains a gene involved in oocyte development whose function requires high levels of BubR1. Additionally, we also found two genomic regions that, when combined with the sensitized genetic background in hemizygous conditions, result in synthetic lethality, so that *bubR1^{D1326N}/bubR1^{rev1};Df(3L)BSC10/+* or *bubR1^{D1326N}/bubR1^{rev1};Df(3R)BSC56/+* individuals fail to survive. Synthetic lethality is observed when two

genes that act in parallel redundant pathways or in the same essential pathway but, the combination of the effects does not ensure that the process will be executed correctly, leading to cell death, when the normal protein levels of the two genes is compromised. For *Df(3L)BSC10*, we also studied the developmental stage at which the embryos die. We observed that embryos do not proceed beyond stage 5 of the embryonic development, which corresponds to the stage where zygotic transcription begins and there is the switch from maternal to zygotic control of mitotic cycles. This reveals that BubR1 kinase activity becomes rate-limiting at this stage of development in combination with a yet unknown gene localized within *Df(3L)BSC10*.

During the course of the screen we identified 10 genomic regions that enhance and 19 genomic regions that suppress NDJ of *bubR1*-mutants. Further deficiency mapping and candidate gene approach allowed us to clearly identify two *loci* responsible for the observed effects. *polo* and *Pp1-87* were identified as suppressors of *bubR1*-mutant NDJ. Analysis of the known functions of these two genes during mitosis and meiosis suggests that the suppression is mostly likely due to their role in regulating the overall timing and progression through meiosis. In most animals, oocyte development involves meiotic arrest at two points, prophase I and metaphase I or II (depending on the species). These meiotic arrests are key to allow oocyte growth and differentiation as well as the coordination between fertilization and the completion of meiosis. Oocyte maturation releases from prophase I arrest, enabling the oocyte to progress into the meiotic divisions. Activation of MPF is the molecular key event that promotes meiotic maturation. Several evidences demonstrate that Cdc25-mediated activation of MPF is required to initiate GVBD in female meiosis (Lincoln *et al.* 2002; Xiang *et al.* 2007). Thus, Cdc25 activation appears to be one of the key steps for entry in M-phase. The activity of Cdc25 is controlled by several different phosphorylation/dephosphorylation events and by binding to regulatory proteins. During interphase Cdc25 is kept inactive, where its phosphatase activity is maintained at low levels. At the G₂/M transition, Cdc25 undergoes a major shift in its electrophoretic mobility that is correlated with an increase in its phosphatase activity (Izumi *et al.* 1992). One kinase that triggers Cdc25 activation is Polo. Studies in *Xenopus* were the first to show that Plx1 (the *Xenopus* homologue of the *Drosophila* Polo-like kinase) phosphorylate XCdc25 at multiple sites, increasing XCdc25 activity *in vitro* (Kumagai and Dunphy 1996; Qian *et al.* 2001). After this, the activation of Cdc25 by Polo-like kinases has been also reported in other organisms (Roshak *et al.* 2000), including in *Drosophila* (Xiang *et al.* 2007). However, Polo is not the only kinase involved in the process of Cdc25 activation, since inhibition of Polo only delays but does not blocks Cdc25 activation (Qian *et al.* 1998; van Vugt and

Medema 2005). In addition, Cdc25 also needs to be dephosphorylated to become fully active. Studies also in *Xenopus*, showed that PP1 is likely to be the phosphatase responsible for Cdc25 Ser287 dephosphorylation *in vivo* (Margolis *et al.* 2003). It was shown that the G₂ arrest of *Xenopus* oocytes depends on the phosphorylation of XCdc25 Ser287 by PKA during interphase (Duckworth *et al.* 2002). We identified Polo and Pp1 as dominant suppressors of the BubR1-mutant NDJ phenotype suggesting a model in which these enzymes affect the rate at which meiotic progression takes place by indirectly suppressing the NDJ phenotype. Mutations in *bubR1* that significantly reduce its activity lead to premature meiotic exit and NDJ but if concomitantly the levels of Polo and Pp1 – which are responsible for accelerating meiotic progression – are also reduced, then normal meiotic progression is restored and NDJ is suppressed. Further experiments will required to fully test this model.

In the group of deficiencies that enhance *bubR1*-mutant NDJ we found *Df(3L)BSC13*. Within this deficiency, *mtrm* was identified as the gene responsible for the dominant enhancement mutant phenotype. However, the enhancement observed appears to be only due to an additive effect of the frequency of NDJ displayed by *mtrm* and *bubR1* mutants alone. The function of the Mtrm protein is to restrain Polo function by physical interaction, with an approximate stoichiometry of 1:1 until stage 13 of *D. melanogaster* oogenesis, in order to prevent precocious activation of Cdc25 by Polo, that would lead to precocious GVBD.

Taking into account our results (Polo vs. Mtrm, Polo vs. Cdc25 and Pp187-B vs. Cdc25) it is tempting to propose that during oocyte maturation there is tight stoichiometric control of protein levels. Nevertheless, it is surprising that 15 % of the 33 genes analysed that are involved in meiosis/meiosis-related tasks are duplicated and appear to be functional in several *Drosophila* species, specially if *polo* and *mtrm* are within the group of genes that are duplicated. Thus, taking all our data into consideration, it appears that the mechanisms that control meiotic progression play a major role in regulating proper chromosome segregation.

References

- Abdu, U., M. Brodsky, et al. (2002). "Activation of a meiotic checkpoint during *Drosophila* oogenesis regulates the translation of Gurken through Chk2/Mnk." *Curr Biol* **12**(19): 1645-1651.
- Afshar, K., N. R. Barton, et al. (1995). "DNA binding and meiotic chromosomal localization of the *Drosophila* nod kinesin-like protein." *Cell* **81**(1): 129-138.
- Alberts, B., A. Johnson, et al. (2002). *Molecular Biology of the Cell*. New York, Garland.
- Alphey, L., J. Jimenez, et al. (1992). "twine, a cdc25 homolog that functions in the male and female germline of *Drosophila*." *Cell* **69**(6): 977-988.
- Anderson, J. A., W. D. Gilliland, et al. (2009). "Molecular population genetics and evolution of *Drosophila* meiosis genes." *Genetics* **181**(1): 177-185.
- Archambault, V., X. Zhao, et al. (2007). "Mutations in *Drosophila* Greatwall/Scant reveal its roles in mitosis and meiosis and interdependence with Polo kinase." *PLoS Genet* **3**(11): e200.
- Assenmacher, N. and K. P. Hopfner (2004). "MRE11/RAD50/NBS1: complex activities." *Chromosoma* **113**(4): 157-166.
- Axton, J. M., V. Dombradi, et al. (1990). "One of the protein phosphatase 1 isoenzymes in *Drosophila* is essential for mitosis." *Cell* **63**(1): 33-46.
- Bailis, J. M. and G. S. Roeder (1998). "Synaptonemal complex morphogenesis and sister-chromatid cohesion require Mek1-dependent phosphorylation of a meiotic chromosomal protein." *Genes & development* **12**(22): 3551-3563.
- Bailis, J. M. and G. S. Roeder (2000). "Pachytene exit controlled by reversal of Mek1-dependent phosphorylation." *Cell* **101**(2): 211-221.
- Baker, D. J., K. B. Jeganathan, et al. (2004). "BubR1 insufficiency causes early onset of aging-associated phenotypes and infertility in mice." *Nat Genet* **36**(7): 744-749.
- Baker, D. J., K. B. Jeganathan, et al. (2006). "Early aging-associated phenotypes in Bub3/Rae1 haploinsufficient mice." *J Cell Biol* **172**(4): 529-540.
- Barr, F. A., H. H. Sillje, et al. (2004). "Polo-like kinases and the orchestration of cell division." *Nat Rev Mol Cell Biol* **5**(6): 429-440.
- Basu, J., H. Bousbaa, et al. (1999). "Mutations in the essential spindle checkpoint gene *bub1* cause chromosome missegregation and fail to block apoptosis in *Drosophila*." *J Cell Biol* **146**(1): 13-28.
- Bataille, D., L. Heron, et al. (1999). "alpha-Endosulfine, a new entity in the control of insulin secretion." *Cellular and molecular life sciences : CMLS* **56**(1-2): 78-84.
- Bennett, M. D. (1977). "The time and duration of meiosis." *Philosophical transactions of the Royal Society of London. Series B, Biological sciences* **277**(955): 201-226.
- Bernard, P., K. Hardwick, et al. (1998). "Fission yeast *bub1* is a mitotic centromere protein essential for the spindle checkpoint and the preservation of correct ploidy through mitosis." *J Cell Biol* **143**(7): 1775-1787.
- Bettencourt-Dias, M., A. Rodrigues-Martins, et al. (2005). "SAK/PLK4 is required for centriole duplication and flagella development." *Curr Biol* **15**(24): 2199-2207.
- Biggins, S. and A. W. Murray (2001). "The budding yeast protein kinase Ipl1/Aurora allows the absence of tension to activate the spindle checkpoint." *Genes & development* **15**(23): 3118-3129.
- Blat, Y., R. U. Protacio, et al. (2002). "Physical and functional interactions among basic chromosome organizational features govern early steps of meiotic chiasma formation." *Cell* **111**(6): 791-802.
- Branzei, D. and M. Foiani (2008). "Regulation of DNA repair throughout the cell cycle." *Nature reviews. Molecular cell biology* **9**(4): 297-308.
- Buvelot, S., S. Y. Tatsutani, et al. (2003). "The budding yeast Ipl1/Aurora protein kinase regulates mitotic spindle disassembly." *The Journal of cell biology* **160**(3): 329-339.
- Campbell, N. A. and J. B. Reece (2005). *Biology, 7/E*. U.S.A., Benjamin Cummings.
- Carmena, M., M. G. Riparbelli, et al. (1998). "*Drosophila* polo kinase is required for cytokinesis." *The Journal of cell biology* **143**(3): 659-671.
- Carmon, A., M. Larson, et al. (2010). "The rate of unequal crossing over in the dumpy gene from *Drosophila melanogaster*." *Journal of molecular evolution* **70**(3): 260-265.
- Cazales, M., E. Schmitt, et al. (2005). "CDC25B phosphorylation by Aurora-A occurs at the G2/M transition and is inhibited by DNA damage." *Cell Cycle* **4**(9): 1233-1238.
- Cenci, G., G. Siriaco, et al. (2003). "The *Drosophila* HOAP protein is required for telomere capping." *Nature cell biology* **5**(1): 82-84.
- Chan, G. K., S. T. Liu, et al. (2005). "Kinetochores structure and function." *Trends in cell biology* **15**(11): 589-598.
- Chen, F., V. Archambault, et al. (2007). "Multiple protein phosphatases are required for mitosis in *Drosophila*." *Current biology : CB* **17**(4): 293-303.
- Chen, H. L., C. J. Tang, et al. (2005). "Overexpression of an Aurora-C kinase-deficient mutant disrupts the Aurora-B/INCENP complex and induces polyploidy." *Journal of biomedical science* **12**(2): 297-310.
- Cheslock, P. S., B. J. Kemp, et al. (2005). "The roles of MAD1, MAD2 and MAD3 in meiotic progression and the segregation of nonexchange chromosomes." *Nat Genet* **37**(7): 756-760.
- Ciliberto, A. and J. V. Shah (2009). "A quantitative systems view of the spindle assembly checkpoint." *The EMBO journal* **28**(15): 2162-2173.

- Cimini, D., X. Wan, et al. (2006). "Aurora kinase promotes turnover of kinetochore microtubules to reduce chromosome segregation errors." *Current biology : CB* **16**(17): 1711-1718.
- Clarke, A. S., T. T. Tang, et al. (2005). "POLO kinase regulates the Drosophila centromere cohesion protein MEI-S332." *Dev Cell* **8**(1): 53-64.
- Clyne, R. K., V. L. Katis, et al. (2003). "Polo-like kinase Cdc5 promotes chiasmata formation and cosegregation of sister centromeres at meiosis I." *Nat Cell Biol* **5**(5): 480-485.
- Cohen, P. E., S. E. Pollack, et al. (2006). "Genetic analysis of chromosome pairing, recombination, and cell cycle control during first meiotic prophase in mammals." *Endocrine reviews* **27**(4): 398-426.
- Cooper, G. M. (2000). *The Cell: A Molecular Approach*. Washington, ASM Press.
- Corbett, K. D., C. K. Yip, et al. (2010). "The monopolin complex crosslinks kinetochore components to regulate chromosome-microtubule attachments." *Cell* **142**(4): 556-567.
- Courtot, C., C. Fankhauser, et al. (1992). "The Drosophila cdc25 homolog twine is required for meiosis." *Development* **116**(2): 405-416.
- Cullen, C. F. and H. Ohkura (2001). "Mps protein is localized to acentrosomal poles to ensure bipolarity of Drosophila meiotic spindles." *Nat Cell Biol* **3**(7): 637-642.
- Dai, J., B. A. Sullivan, et al. (2006). "Regulation of mitotic chromosome cohesion by Haspin and Aurora B." *Developmental cell* **11**(5): 741-750.
- Davis, F. M., T. Y. Tsao, et al. (1983). "Monoclonal antibodies to mitotic cells." *Proceedings of the National Academy of Sciences of the United States of America* **80**(10): 2926-2930.
- de Cuevas, M., M. A. Lilly, et al. (1997). "Germline cyst formation in Drosophila." *Annual review of genetics* **31**: 405-428.
- de los Santos, T. and N. M. Hollingsworth (1999). "Red1p, a MEK1-dependent phosphoprotein that physically interacts with Hop1p during meiosis in yeast." *The Journal of biological chemistry* **274**(3): 1783-1790.
- De Luca, M., P. Lavia, et al. (2006). "A functional interplay between Aurora-A, Plk1 and TPX2 at spindle poles: Plk1 controls centrosomal localization of Aurora-A and TPX2 spindle association." *Cell Cycle* **5**(3): 296-303.
- Deutschbauer, A. M., D. F. Jaramillo, et al. (2005). "Mechanisms of haploinsufficiency revealed by genome-wide profiling in yeast." *Genetics* **169**(4): 1915-1925.
- Dieterich, K., R. Soto Rifo, et al. (2007). "Homozygous mutation of AURKC yields large-headed polyploid spermatozoa and causes male infertility." *Nature genetics* **39**(5): 661-665.
- Ditchfield, C., V. L. Johnson, et al. (2003). "Aurora B couples chromosome alignment with anaphase by targeting BubR1, Mad2, and Cenp-E to kinetochores." *J Cell Biol* **161**(2): 267-280.
- do Carmo Avides, M., A. Tavares, et al. (2001). "Polo kinase and Asp are needed to promote the mitotic organizing activity of centrosomes." *Nature cell biology* **3**(4): 421-424.
- Doane, W. W. (1960). "Completion of meiosis in unispermated eggs of Drosophila melanogaster." *Science* **132**: 677-678.
- Dohadwala, M., E. F. da Cruz e Silva, et al. (1994). "Phosphorylation and inactivation of protein phosphatase 1 by cyclin-dependent kinases." *Proceedings of the National Academy of Sciences of the United States of America* **91**(14): 6408-6412.
- Dombradi, V., J. M. Axton, et al. (1990). "Protein phosphatase 1 activity in Drosophila mutants with abnormalities in mitosis and chromosome condensation." *FEBS letters* **275**(1-2): 39-43.
- Donaldson, M. M., A. A. Tavares, et al. (2001). "Metaphase arrest with centromere separation in polo mutants of Drosophila." *J Cell Biol* **153**(4): 663-676.
- Drummond-Barbosa, D. and A. C. Spradling (2004). "Alpha-endosulfine, a potential regulator of insulin secretion, is required for adult tissue growth control in Drosophila." *Developmental biology* **266**(2): 310-321.
- Duckworth, B. C., J. S. Weaver, et al. (2002). "G2 arrest in Xenopus oocytes depends on phosphorylation of cdc25 by protein kinase A." *Proceedings of the National Academy of Sciences of the United States of America* **99**(26): 16794-16799.
- Eckerdt, F. and K. Strebhardt (2006). "Polo-like kinase 1: target and regulator of anaphase-promoting complex/cyclosome-dependent proteolysis." *Cancer research* **66**(14): 6895-6898.
- Elia, A. E., L. C. Cantley, et al. (2003). "Proteomic screen finds pSer/pThr-binding domain localizing Plk1 to mitotic substrates." *Science* **299**(5610): 1228-1231.
- Elowe, S., K. Dulla, et al. (2010). "Uncoupling of the spindle-checkpoint and chromosome-congression functions of BubR1." *J Cell Sci* **123**(Pt 1): 84-94.
- Fang, Y., T. Liu, et al. (2006). "BubR1 is involved in regulation of DNA damage responses." *Oncogene* **25**(25): 3598-3605.
- Fanti, L., G. Giovino, et al. (1998). "The heterochromatin protein 1 prevents telomere fusions in Drosophila." *Molecular cell* **2**(5): 527-538.
- Fichelson, P., C. Moch, et al. (2009). "Live-imaging of single stem cells within their niche reveals that a U3snRNP component segregates asymmetrically and is required for self-renewal in Drosophila." *Nature cell biology* **11**(6): 685-693.
- Foe, V. E., G. M. Odell, et al. (1993). *Mitosis and Morphogenesis in the Drosophila embryo: point and counterpoint*. New York, Cold Spring Harbor Laboratory Press.
- Gautier, J., C. Norbury, et al. (1988). "Purified maturation-promoting factor contains the product of a Xenopus homolog of the fission yeast cell cycle control gene cdc2+." *Cell* **54**(3): 433-439.

- Gavin, A. C., Y. Tsukitani, et al. (1991). "Induction of M-phase entry of prophase-blocked mouse oocytes through microinjection of okadaic acid, a specific phosphatase inhibitor." *Experimental cell research* **192**(1): 75-81.
- Gavin, A. C., J. C. Cavadore, et al. (1994). "Histone H1 kinase activity, germinal vesicle breakdown and M phase entry in mouse oocytes." *Journal of cell science* **107** (Pt 1): 275-283.
- Gerton, J. L. and R. S. Hawley (2005). "Homologous chromosome interactions in meiosis: diversity amidst conservation." *Nature reviews. Genetics* **6**(6): 477-487.
- Ghabrial, A., R. P. Ray, et al. (1998). "okra and spindle-B encode components of the RAD52 DNA repair pathway and affect meiosis and patterning in *Drosophila* oogenesis." *Genes Dev* **12**(17): 2711-2723.
- Ghabrial, A. and T. Schupbach (1999). "Activation of a meiotic checkpoint regulates translation of Gurken during *Drosophila* oogenesis." *Nat Cell Biol* **1**(6): 354-357.
- Giet, R. and D. M. Glover (2001). "Drosophila aurora B kinase is required for histone H3 phosphorylation and condensin recruitment during chromosome condensation and to organize the central spindle during cytokinesis." *The Journal of cell biology* **152**(4): 669-682.
- Gilliland, W. D., S. M. Wayson, et al. (2005). "The meiotic defects of mutants in the *Drosophila* mps1 gene reveal a critical role of Mps1 in the segregation of achiasmate homologs." *Current biology : CB* **15**(7): 672-677.
- Gilliland, W. D., S. E. Hughes, et al. (2007). "The multiple roles of mps1 in *Drosophila* female meiosis." *PLoS genetics* **3**(7): e113.
- Giunta, K. L., J. K. Jang, et al. (2002). "subito encodes a kinesin-like protein required for meiotic spindle pole formation in *Drosophila melanogaster*." *Genetics* **160**(4): 1489-1501.
- Glover, D. M., M. H. Leibowitz, et al. (1995). "Mutations in aurora prevent centrosome separation leading to the formation of monopolar spindles." *Cell* **81**(1): 95-105.
- Goldstein, L. S. (1981). "Kinetochore structure and its role in chromosome orientation during the first meiotic division in male *D. melanogaster*." *Cell* **25**(3): 591-602.
- Gonzalez-Reyes, A., H. Elliott, et al. (1997). "Oocyte determination and the origin of polarity in *Drosophila*: the role of the spindle genes." *Development* **124**(24): 4927-4937.
- Gorbsky, G. J., R. H. Chen, et al. (1998). "Microinjection of antibody to Mad2 protein into mammalian cells in mitosis induces premature anaphase." *J Cell Biol* **141**(5): 1193-1205.
- Goshima, G., R. Wollman, et al. (2007). "Genes required for mitotic spindle assembly in *Drosophila* S2 cells." *Science* **316**(5823): 417-421.
- Grell, R. F. and E. E. Generoso (1980). "Time of recombination in the *Drosophila melanogaster* oocyte. II. Electron microscopic and genetic studies of a temperature-sensitive recombination mutant." *Chromosoma* **81**(3): 339-348.
- Habedanck, R., Y. D. Stierhof, et al. (2005). "The Polo kinase Plk4 functions in centriole duplication." *Nat Cell Biol* **7**(11): 1140-1146.
- Hached, K., S. Z. Xie, et al. (2011). "Mps1 at kinetochores is essential for female mouse meiosis I." *Development* **138**(11): 2261-2271.
- Hamatani, T., G. Falco, et al. (2004). "Age-associated alteration of gene expression patterns in mouse oocytes." *Human molecular genetics* **13**(19): 2263-2278.
- Hampl, A. and J. J. Eppig (1995). "Translational regulation of the gradual increase in histone H1 kinase activity in maturing mouse oocytes." *Molecular reproduction and development* **40**(1): 9-15.
- Hansen, D. V., A. V. Loktev, et al. (2004). "Plk1 regulates activation of the anaphase promoting complex by phosphorylating and triggering SCFbetaTrCP-dependent destruction of the APC Inhibitor Emi1." *Mol Biol Cell* **15**(12): 5623-5634.
- Hari, K. L., A. Santerre, et al. (1995). "The mei-41 gene of *D. melanogaster* is a structural and functional homolog of the human ataxia telangiectasia gene." *Cell* **82**(5): 815-821.
- Harris, D., C. Orme, et al. (2003). "A deficiency screen of the major autosomes identifies a gene (matrimony) that is haplo-insufficient for achiasmate segregation in *Drosophila* oocytes." *Genetics* **165**(2): 637-652.
- Harris, L., J. Davenport, et al. (2005). "The mitotic checkpoint gene BubR1 has two distinct functions in mitosis." *Exp Cell Res* **308**(1): 85-100.
- Hartman, T. K., T. M. Wengenack, et al. (2006). "Mutant mice with small amounts of BubR1 display accelerated age-related gliosis." *Neurobiol Aging*.
- Hassold, T. and P. Hunt (2001). "To err (meiotically) is human: the genesis of human aneuploidy." *Nat Rev Genet* **2**(4): 280-291.
- Hatsumi, M. and S. A. Endow (1992). "Mutants of the microtubule motor protein, nonclaret disjunctional, affect spindle structure and chromosome movement in meiosis and mitosis." *Journal of cell science* **101** (Pt 3): 547-559.
- Hawley, R. S. and W. E. Theurkauf (1993). "Requiem for distributive segregation: achiasmate segregation in *Drosophila* females." *Trends in genetics : TIG* **9**(9): 310-317.
- Hawley, R. S. (2011). "Oogenesis: when most is good enough." *Current biology : CB* **21**(8): R288-290.
- Heifetz, Y., J. Yu, et al. (2001). "Ovulation triggers activation of *Drosophila* oocytes." *Developmental biology* **234**(2): 416-424.
- Hodges, C. A., E. Revenkova, et al. (2005). "SMC1beta-deficient female mice provide evidence that cohesins are a missing link in age-related nondisjunction." *Nature genetics* **37**(12): 1351-1355.

- Hollingsworth, N. M. and B. Byers (1989). "HOP1: a yeast meiotic pairing gene." *Genetics* **121**(3): 445-462.
- Hollingsworth, N. M., L. Goetsch, et al. (1990). "The HOP1 gene encodes a meiosis-specific component of yeast chromosomes." *Cell* **61**(1): 73-84.
- Homer, H., L. Gui, et al. (2009). "A spindle assembly checkpoint protein functions in prophase I arrest and prometaphase progression." *Science* **326**(5955): 991-994.
- Homer, H. A., A. McDougall, et al. (2005). "Mad2 prevents aneuploidy and premature proteolysis of cyclin B and securin during meiosis I in mouse oocytes." *Genes Dev* **19**(2): 202-207.
- Horner, V. L., A. Czank, et al. (2006). "The Drosophila calicpressin sarah is required for several aspects of egg activation." *Current biology : CB* **16**(14): 1441-1446.
- Hoyt, M. A., L. Totis, et al. (1991). "S. cerevisiae genes required for cell cycle arrest in response to loss of microtubule function." *Cell* **66**(3): 507-517.
- Hunt, P. A. and T. J. Hassold (2002). "Sex matters in meiosis." *Science* **296**(5576): 2181-2183.
- Ishihara, H., B. L. Martin, et al. (1989). "Calyculin A and okadaic acid: inhibitors of protein phosphatase activity." *Biochemical and biophysical research communications* **159**(3): 871-877.
- Ivanovska, I., E. Lee, et al. (2004). "The Drosophila MOS ortholog is not essential for meiosis." *Current biology : CB* **14**(1): 75-80.
- Ivanovska, I., T. Khandan, et al. (2005). "A histone code in meiosis: the histone kinase, NHK-1, is required for proper chromosomal architecture in Drosophila oocytes." *Genes Dev* **19**(21): 2571-2582.
- Iwabuchi, M., K. Ohsumi, et al. (2000). "Residual Cdc2 activity remaining at meiosis I exit is essential for meiotic M-M transition in Xenopus oocyte extracts." *The EMBO journal* **19**(17): 4513-4523.
- Izumi, T., D. H. Walker, et al. (1992). "Periodic changes in phosphorylation of the Xenopus cdc25 phosphatase regulate its activity." *Mol Biol Cell* **3**(8): 927-939.
- Jackman, M., C. Lindon, et al. (2003). "Active cyclin B1-Cdk1 first appears on centrosomes in prophase." *Nature cell biology* **5**(2): 143-148.
- Jang, J. K., L. Messina, et al. (1995). "Induction of metaphase arrest in Drosophila oocytes by chiasma-based kinetochore tension." *Science* **268**(5219): 1917-1919.
- Jang, Y. J., C. Y. Lin, et al. (2002). "Functional studies on the role of the C-terminal domain of mammalian polo-like kinase." *Proceedings of the National Academy of Sciences of the United States of America* **99**(4): 1984-1989.
- Jones, K. T. (2004). "Turning it on and off: M-phase promoting factor during meiotic maturation and fertilization." *Molecular human reproduction* **10**(1): 1-5.
- Jones, K. T. (2005). "Mammalian egg activation: from Ca²⁺ spiking to cell cycle progression." *Reproduction* **130**(6): 813-823.
- Joyce, E. F., S. N. Tanneti, et al. (2009). "Drosophila hold'em is required for a subset of meiotic crossovers and interacts with the dna repair endonuclease complex subunits MEI-9 and ERCC1." *Genetics* **181**(1): 335-340.
- Joyce, E. F. and K. S. McKim (2009). "Drosophila PCH2 is required for a pachytene checkpoint that monitors double-strand-break-independent events leading to meiotic crossover formation." *Genetics* **181**(1): 39-51.
- Joyce, E. F. and K. S. McKim (2010). "Chromosome axis defects induce a checkpoint-mediated delay and interchromosomal effect on crossing over during Drosophila meiosis." *PLoS genetics* **6**(8).
- Joyce, E. F. and K. S. McKim (2011). "Meiotic checkpoints and the interchromosomal effect on crossing over in Drosophila females." *Fly* **5**(2): 134-140.
- Jung, A., M. Hollmann, et al. (2007). "The fatty acid elongase NOA is necessary for viability and has a somatic role in Drosophila sperm development." *J Cell Sci* **120**(Pt 16): 2924-2934.
- Kaitna, S., M. Mendoza, et al. (2000). "Incenp and an aurora-like kinase form a complex essential for chromosome segregation and efficient completion of cytokinesis." *Current biology : CB* **10**(19): 1172-1181.
- Keen, N. and S. Taylor (2004). "Aurora-kinase inhibitors as anticancer agents." *Nature reviews. Cancer* **4**(12): 927-936.
- Keeney, S., C. N. Giroux, et al. (1997). "Meiosis-specific DNA double-strand breaks are catalyzed by Spo11, a member of a widely conserved protein family." *Cell* **88**(3): 375-384.
- Kelly, A. E., S. C. Sampath, et al. (2007). "Chromosomal enrichment and activation of the aurora B pathway are coupled to spatially regulate spindle assembly." *Developmental cell* **12**(1): 31-43.
- Kimmins, S., C. Crosio, et al. (2007). "Differential functions of the Aurora-B and Aurora-C kinases in mammalian spermatogenesis." *Molecular endocrinology* **21**(3): 726-739.
- Kimura, M., Y. Matsuda, et al. (1999). "Cell cycle-dependent expression and centrosome localization of a third human aurora/lpl1-related protein kinase, AIK3." *The Journal of biological chemistry* **274**(11): 7334-7340.
- King, R. C. (1970). *Ovarian development in Drosophila melanogaster*. New York, Academic Press.
- Kitagawa, R. (2009). "Key players in chromosome segregation in Caenorhabditis elegans." *Frontiers in bioscience : a journal and virtual library* **14**: 1529-1557.
- Klein, F., P. Mahr, et al. (1999). "A central role for cohesins in sister chromatid cohesion, formation of axial elements, and recombination during yeast meiosis." *Cell* **98**(1): 91-103.

- Klovstad, M., U. Abdu, et al. (2008). "Drosophila brca2 is required for mitotic and meiotic DNA repair and efficient activation of the meiotic recombination checkpoint." *PLoS genetics* **4**(2): e31.
- Knowlton, A. L., W. Lan, et al. (2006). "Aurora B is enriched at merotelic attachment sites, where it regulates MCAK." *Current biology : CB* **16**(17): 1705-1710.
- Koehler, K. E., R. S. Hawley, et al. (1996). "Recombination and nondisjunction in humans and flies." *Human molecular genetics* **5 Spec No**: 1495-1504.
- Kovo, M., M. Kandli-Cohen, et al. (2006). "An active protein kinase A (PKA) is involved in meiotic arrest of rat growing oocytes." *Reproduction* **132**(1): 33-43.
- Kumagai, A. and W. G. Dunphy (1996). "Purification and molecular cloning of Plx1, a Cdc25-regulatory kinase from Xenopus egg extracts." *Science* **273**(5280): 1377-1380.
- Lake, C. M., K. Teeter, et al. (2007). "A genetic analysis of the Drosophila mcm5 gene defines a domain specifically required for meiotic recombination." *Genetics* **176**(4): 2151-2163.
- Lampson, M. A., K. Renduchitala, et al. (2004). "Correcting improper chromosome-spindle attachments during cell division." *Nature cell biology* **6**(3): 232-237.
- Lampson, M. A. and T. M. Kapoor (2005). "The human mitotic checkpoint protein BubR1 regulates chromosome-spindle attachments." *Nat Cell Biol* **7**(1): 93-98.
- Lancaster, O. M., M. Breuer, et al. (2010). "The meiotic recombination checkpoint suppresses NHK-1 kinase to prevent reorganisation of the oocyte nucleus in Drosophila." *PLoS genetics* **6**(10): e1001179.
- Lantz, V., J. S. Chang, et al. (1994). "The Drosophila orb RNA-binding protein is required for the formation of the egg chamber and establishment of polarity." *Genes Dev* **8**(5): 598-613.
- Lee, B. H. and A. Amon (2003). "Role of Polo-like kinase CDC5 in programming meiosis I chromosome segregation." *Science* **300**(5618): 482-486.
- Lee, S., L. Zhou, et al. (2008). "Lasp anchors the Drosophila male stem cell niche and mediates spermatid individualization." *Mech Dev* **125**(9-10): 768-776.
- LeMaire-Adkins, R., K. Radke, et al. (1997). "Lack of checkpoint control at the metaphase/anaphase transition: a mechanism of meiotic nondisjunction in mammalian females." *J Cell Biol* **139**(7): 1611-1619.
- Lesage, B., J. Qian, et al. (2011). "Spindle Checkpoint Silencing: PP1 Tips the Balance." *Current biology : CB* **21**(21): R898-903.
- Li, M., S. Li, et al. (2009). "Bub3 is a spindle assembly checkpoint protein regulating chromosome segregation during mouse oocyte meiosis." *PLoS ONE* **4**(11): e7701.
- Li, R. and A. W. Murray (1991). "Feedback control of mitosis in budding yeast." *Cell* **66**(3): 519-531.
- Li, Y. and R. Benezra (1996). "Identification of a human mitotic checkpoint gene: hSMAD2." *Science* **274**(5285): 246-248.
- Lincoln, A. J., D. Wickramasinghe, et al. (2002). "Cdc25b phosphatase is required for resumption of meiosis during oocyte maturation." *Nature genetics* **30**(4): 446-449.
- Lindqvist, A., H. Kallstrom, et al. (2005). "Cdc25B cooperates with Cdc25A to induce mitosis but has a unique role in activating cyclin B1-Cdk1 at the centrosome." *The Journal of cell biology* **171**(1): 35-45.
- Liu, H., J. K. Jang, et al. (2000). "Two genes required for meiotic recombination in Drosophila are expressed from a dicistronic message." *Genetics* **154**(4): 1735-1746.
- Liu, H., J. K. Jang, et al. (2002). "mei-P22 encodes a chromosome-associated protein required for the initiation of meiotic recombination in Drosophila melanogaster." *Genetics* **162**(1): 245-258.
- Liu, Q. and J. V. Ruderman (2006). "Aurora A, mitotic entry, and spindle bipolarity." *Proceedings of the National Academy of Sciences of the United States of America* **103**(15): 5811-5816.
- Lodish, H., A. Berk, et al. (2000). *Molecular Cell Biology*. New York, W. H. Freeman and Company.
- Logarinho, E. and C. E. Sunkel (1998). "The Drosophila POLO kinase localises to multiple compartments of the mitotic apparatus and is required for the phosphorylation of MPM2 reactive epitopes." *Journal of cell science* **111 (Pt 19)**: 2897-2909.
- Logarinho, E., H. Bousbaa, et al. (2004). "Different spindle checkpoint proteins monitor microtubule attachment and tension at kinetochores in Drosophila cells." *J Cell Sci* **117**(Pt 9): 1757-1771.
- Logarinho, E., T. Resende, et al. (2008). "The human spindle assembly checkpoint protein Bub3 is required for the establishment of efficient kinetochore-microtubule attachments." *Mol Biol Cell* **19**(4): 1798-1813.
- Long, M. (2001). "Evolution of novel genes." *Current opinion in genetics & development* **11**(6): 673-680.
- Lu, W. J., J. Chapo, et al. (2010). "Meiotic recombination provokes functional activation of the p53 regulatory network." *Science* **328**(5983): 1278-1281.
- Lynch, M. and J. S. Conery (2000). "The evolutionary fate and consequences of duplicate genes." *Science* **290**(5494): 1151-1155.
- Macurek, L., A. Lindqvist, et al. (2008). "Polo-like kinase-1 is activated by aurora A to promote checkpoint recovery." *Nature* **455**(7209): 119-123.
- Madgwick, S. and K. T. Jones (2007). "How eggs arrest at metaphase II: MPF stabilisation plus APC/C inhibition equals Cytostatic Factor." *Cell division* **2**: 4.
- Mahowald, A. P., T. J. Goralski, et al. (1983). "In vitro activation of Drosophila eggs." *Developmental biology* **98**(2): 437-445.

- Malmanche, N., A. Maia, et al. (2006). "The spindle assembly checkpoint: preventing chromosome mis-segregation during mitosis and meiosis." *FEBS Lett* **580**(12): 2888-2895.
- Malmanche, N., S. Owen, et al. (2007). "Drosophila BubR1 Is Essential for Meiotic Sister-Chromatid Cohesion and Maintenance of Synaptonemal Complex." *Curr Biol*.
- Manheim, E. A. and K. S. McKim (2003). "The Synaptonemal complex component C(2)M regulates meiotic crossing over in Drosophila." *Curr Biol* **13**(4): 276-285.
- Marangos, P., E. W. Verschuren, et al. (2007). "Prophase I arrest and progression to metaphase I in mouse oocytes are controlled by Emi1-dependent regulation of APC(Cdh1)." *The Journal of cell biology* **176**(1): 65-75.
- Margolis, S. S., S. Walsh, et al. (2003). "PP1 control of M phase entry exerted through 14-3-3-regulated Cdc25 dephosphorylation." *The EMBO journal* **22**(21): 5734-5745.
- Marygold, S. J., J. Roote, et al. (2007). "The ribosomal protein genes and Minute loci of Drosophila melanogaster." *Genome Biol* **8**(10): R216.
- Matsumoto, T., D. J. Baker, et al. (2007). "Aging-associated vascular phenotype in mutant mice with low levels of BubR1." *Stroke* **38**(3): 1050-1056.
- Matthies, H. J., H. B. McDonald, et al. (1996). "Anastral meiotic spindle morphogenesis: role of the non-claret disjunctional kinesin-like protein." *The Journal of cell biology* **134**(2): 455-464.
- Matthies, H. J., R. J. Baskin, et al. (2001). "Orphan kinesin NOD lacks motile properties but does possess a microtubule-stimulated ATPase activity." *Mol Biol Cell* **12**(12): 4000-4012.
- McGuinness, B. E., M. Anger, et al. (2009). "Regulation of APC/C activity in oocytes by a Bub1-dependent spindle assembly checkpoint." *Curr Biol* **19**(5): 369-380.
- McKim, K. S., J. K. Jang, et al. (1993). "Mechanical basis of meiotic metaphase arrest." *Nature* **362**(6418): 364-366.
- McKim, K. S. and R. S. Hawley (1995). "Chromosomal control of meiotic cell division." *Science* **270**(5242): 1595-1601.
- McKim, K. S., J. B. Dahmus, et al. (1996). "Cloning of the Drosophila melanogaster meiotic recombination gene mei-218: a genetic and molecular analysis of interval 15E." *Genetics* **144**(1): 215-228.
- McKim, K. S. and A. Hayashi-Hagihara (1998). "mei-W68 in Drosophila melanogaster encodes a Spo11 homolog: evidence that the mechanism for initiating meiotic recombination is conserved." *Genes & development* **12**(18): 2932-2942.
- McKim, K. S., J. K. Jang, et al. (2002). "Meiotic recombination and chromosome segregation in Drosophila females." *Annu Rev Genet* **36**: 205-232.
- Mehlmann, L. M. (2005). "Stops and starts in mammalian oocytes: recent advances in understanding the regulation of meiotic arrest and oocyte maturation." *Reproduction* **130**(6): 791-799.
- Meuwissen, R. L., H. H. Offenberg, et al. (1992). "A coiled-coil related protein specific for synapsed regions of meiotic prophase chromosomes." *The EMBO journal* **11**(13): 5091-5100.
- Mirouse, V., E. Formstecher, et al. (2006). "Interaction between Polo and BicD proteins links oocyte determination and meiosis control in Drosophila." *Development* **133**(20): 4005-4013.
- Miyazaki, W. Y. and T. L. Orr-Weaver (1992). "Sister-chromatid misbehavior in Drosophila ord mutants." *Genetics* **132**(4): 1047-1061.
- Monje-Casas, F., V. R. Prabhu, et al. (2007). "Kinetochore orientation during meiosis is controlled by Aurora B and the monopolin complex." *Cell* **128**(3): 477-490.
- Morgan, D. O. (1997). "Cyclin-dependent kinases: engines, clocks, and microprocessors." *Annual review of cell and developmental biology* **13**: 261-291.
- Morgan, D. O. (2007). *The Cell Cycle: Principles of Control*. London, New Science Press Ltd.
- Mori, D., Y. Yano, et al. (2007). "NDEL1 phosphorylation by Aurora-A kinase is essential for centrosomal maturation, separation, and TACC3 recruitment." *Molecular and cellular biology* **27**(1): 352-367.
- Moshe, Y., J. Boulaire, et al. (2004). "Role of Polo-like kinase in the degradation of early mitotic inhibitor 1, a regulator of the anaphase promoting complex/cyclosome." *Proceedings of the National Academy of Sciences of the United States of America* **101**(21): 7937-7942.
- Musacchio, A. and E. D. Salmon (2007). "The spindle-assembly checkpoint in space and time." *Nat Rev Mol Cell Biol* **8**(5): 379-393.
- Nagaoka, S. I., C. A. Hodges, et al. (2011). "Oocyte-specific differences in cell-cycle control create an innate susceptibility to meiotic errors." *Current biology : CB* **21**(8): 651-657.
- Nakajima, H., F. Toyoshima-Morimoto, et al. (2003). "Identification of a consensus motif for Plk (Polo-like kinase) phosphorylation reveals Myt1 as a Plk1 substrate." *The Journal of biological chemistry* **278**(28): 25277-25280.
- Neuman-Silberberg, F. S. and T. Schupbach (1993). "The Drosophila dorsoventral patterning gene gurken produces a dorsally localized RNA and encodes a TGF alpha-like protein." *Cell* **75**(1): 165-174.
- Niault, T., K. Hached, et al. (2007). "Changing Mad2 levels affects chromosome segregation and spindle assembly checkpoint control in female mouse meiosis I." *PLoS ONE* **2**(11): e1165.
- O'Sullivan, R. J. and J. Karlseder (2010). "Telomeres: protecting chromosomes against genome instability." *Nature reviews. Molecular cell biology* **11**(3): 171-181.

- Offenberg, H. H., A. J. Dietrich, et al. (1991). "Tissue distribution of two major components of synaptonemal complexes of the rat." *Chromosoma* **101**(2): 83-91.
- Ohkura, H., I. M. Hagan, et al. (1995). "The conserved *Schizosaccharomyces pombe* kinase *plp1*, required to form a bipolar spindle, the actin ring, and septum, can drive septum formation in G1 and G2 cells." *Genes & development* **9**(9): 1059-1073.
- Ohno, S. (1970). *Evolution by Gene Duplication*. New York, Springer-Verlag.
- Osada, N. and H. Innan (2008). "Duplication and gene conversion in the *Drosophila melanogaster* genome." *PLoS genetics* **4**(12): e1000305.
- Page, S. L. and R. S. Hawley (2001). "c(3)G encodes a *Drosophila* synaptonemal complex protein." *Genes Dev* **15**(23): 3130-3143.
- Page, S. L. and R. S. Hawley (2004). "The genetics and molecular biology of the synaptonemal complex." *Annu Rev Cell Dev Biol* **20**: 525-558.
- Pal, M., O. Nagy, et al. (2007). "Structurally related TPR subunits contribute differently to the function of the anaphase-promoting complex in *Drosophila melanogaster*." *J Cell Sci* **120**(Pt 18): 3238-3248.
- Papp, B., C. Pal, et al. (2003). "Dosage sensitivity and the evolution of gene families in yeast." *Nature* **424**(6945): 194-197.
- Pawlowski, W. P. and W. Z. Cande (2005). "Coordinating the events of the meiotic prophase." *Trends Cell Biol* **15**(12): 674-681.
- Perdiguerro, E. and A. R. Nebreda (2004). "Regulation of Cdc25C activity during the meiotic G2/M transition." *Cell Cycle* **3**(6): 733-737.
- Perez-Mongiovi, D., N. Malmanche, et al. (2005). "Maternal expression of the checkpoint protein BubR1 is required for synchrony of syncytial nuclear divisions and polar body arrest in *Drosophila melanogaster*." *Development* **132**(20): 4509-4520.
- Pesin, J. A. and T. L. Orr-Weaver (2007). "Developmental role and regulation of cortex, a meiosis-specific anaphase-promoting complex/cyclosome activator." *PLoS genetics* **3**(11): e202.
- Peters, J. M. (2006). "The anaphase promoting complex/cyclosome: a machine designed to destroy." *Nat Rev Mol Cell Biol* **7**(9): 644-656.
- Petronczki, M., M. F. Siomos, et al. (2003). "Un menage a quatre: the molecular biology of chromosome segregation in meiosis." *Cell* **112**(4): 423-440.
- Petronczki, M., J. Matos, et al. (2006). "Monopolar attachment of sister kinetochores at meiosis I requires casein kinase 1." *Cell* **126**(6): 1049-1064.
- Petronczki, M., P. Lenart, et al. (2008). "Polo on the Rise-from Mitotic Entry to Cytokinesis with Plk1." *Dev Cell* **14**(5): 646-659.
- Qian, Y. W., E. Erikson, et al. (1998). "Activated polo-like kinase Plx1 is required at multiple points during mitosis in *Xenopus laevis*." *Molecular and cellular biology* **18**(7): 4262-4271.
- Qian, Y. W., E. Erikson, et al. (2001). "The polo-like kinase Plx1 is required for activation of the phosphatase Cdc25C and cyclin B-Cdc2 in *Xenopus* oocytes." *Mol Biol Cell* **12**(6): 1791-1799.
- Rabitsch, K. P., M. Petronczki, et al. (2003). "Kinetochores recruitment of two nucleolar proteins is required for homolog segregation in meiosis I." *Developmental cell* **4**(4): 535-548.
- Radford, S. J., E. Goley, et al. (2005). "*Drosophila* ERCC1 is required for a subset of MEI-9-dependent meiotic crossovers." *Genetics* **170**(4): 1737-1745.
- Raffa, G. D., G. Siriaco, et al. (2009). "The *Drosophila* *modigliani* (*moi*) gene encodes a HOAP-interacting protein required for telomere protection." *Proceedings of the National Academy of Sciences of the United States of America* **106**(7): 2271-2276.
- Raffa, G. D., D. Raimondo, et al. (2010). "Verrocchio, a *Drosophila* OB fold-containing protein, is a component of the terminin telomere-capping complex." *Genes & development* **24**(15): 1596-1601.
- Rahmani, Z., M. E. Gagou, et al. (2009). "Separating the spindle, checkpoint, and timer functions of BubR1." *J Cell Biol* **187**(5): 597-605.
- Ray, R. P. and T. Schupbach (1996). "Intercellular signaling and the polarization of body axes during *Drosophila* oogenesis." *Genes & development* **10**(14): 1711-1723.
- Reis, A., H. Y. Chang, et al. (2006). "APCcdh1 activity in mouse oocytes prevents entry into the first meiotic division." *Nat Cell Biol* **8**(5): 539-540.
- Reis, A., S. Madgwick, et al. (2007). "Prometaphase APCcdh1 activity prevents non-disjunction in mammalian oocytes." *Nat Cell Biol* **9**(10): 1192-1198.
- Reis, M., S. Sousa-Guimaraes, et al. (2011). "*Drosophila* genes that affect meiosis duration are among the meiosis related genes that are more often found duplicated." *PLoS ONE* **6**(3): e17512.
- Resnick, T. D., D. L. Satinover, et al. (2006). "INCENP and Aurora B promote meiotic sister chromatid cohesion through localization of the Shugoshin MEI-S332 in *Drosophila*." *Dev Cell* **11**(1): 57-68.
- Resnick, T. D., K. J. Dej, et al. (2009). "Mutations in the chromosomal passenger complex and the condensin complex differentially affect synaptonemal complex disassembly and metaphase I configuration in *Drosophila* female meiosis." *Genetics* **181**(3): 875-887.
- Rime, H. and R. Ozon (1990). "Protein phosphatases are involved in the in vivo activation of histone H1 kinase in mouse oocyte." *Developmental biology* **141**(1): 115-122.

- Riparbelli, M. G., G. Callaini, et al. (2002). "A requirement for the Abnormal Spindle protein to organise microtubules of the central spindle for cytokinesis in *Drosophila*." *Journal of cell science* **115**(Pt 5): 913-922.
- Rivera, T. and A. Losada (2006). "Shugoshin and PP2A, shared duties at the centromere." *BioEssays : news and reviews in molecular, cellular and developmental biology* **28**(8): 775-779.
- Roeder, G. S. (1997). "Meiotic chromosomes: it takes two to tango." *Genes & development* **11**(20): 2600-2621.
- Roshak, A. K., E. A. Capper, et al. (2000). "The human polo-like kinase, PLK, regulates cdc2/cyclin B through phosphorylation and activation of the cdc25C phosphatase." *Cellular signalling* **12**(6): 405-411.
- Sagata, N. (1996). "Meiotic metaphase arrest in animal oocytes: its mechanisms and biological significance." *Trends in cell biology* **6**(1): 22-28.
- Sampath, S. C., R. Ohi, et al. (2004). "The chromosomal passenger complex is required for chromatin-induced microtubule stabilization and spindle assembly." *Cell* **118**(2): 187-202.
- Savvidou, E., N. Cobbe, et al. (2005). "Drosophila CAP-D2 is required for condensin complex stability and resolution of sister chromatids." *J Cell Sci* **118**(Pt 11): 2529-2543.
- Schindler, K. and R. M. Schultz (2009). "CDC14B acts through FZR1 (CDH1) to prevent meiotic maturation of mouse oocytes." *Biology of reproduction* **80**(4): 795-803.
- Schwartz, D. A. and R. M. Schultz (1991). "Stimulatory effect of okadaic acid, an inhibitor of protein phosphatases, on nuclear envelope breakdown and protein phosphorylation in mouse oocytes and one-cell embryos." *Developmental biology* **145**(1): 119-127.
- Sears, D. D., J. H. Hegemann, et al. (1992). "Meiotic recombination and segregation of human-derived artificial chromosomes in *Saccharomyces cerevisiae*." *Proceedings of the National Academy of Sciences of the United States of America* **89**(12): 5296-5300.
- Sekelsky, J. J., K. S. McKim, et al. (1995). "The *Drosophila* meiotic recombination gene *mei-9* encodes a homologue of the yeast excision repair protein Rad1." *Genetics* **141**(2): 619-627.
- Seki, A., J. A. Coppinger, et al. (2008). "Bora and the kinase Aurora a cooperatively activate the kinase Plk1 and control mitotic entry." *Science* **320**(5883): 1655-1658.
- Shareef, M. M., C. King, et al. (2001). "Drosophila heterochromatin protein 1 (HP1)/origin recognition complex (ORC) protein is associated with HP1 and ORC and functions in heterochromatin-induced silencing." *Mol Biol Cell* **12**(6): 1671-1685.
- Shareef, M. M., R. Badugu, et al. (2003). "HP1/ORC complex and heterochromatin assembly." *Genetica* **117**(2-3): 127-134.
- Smith, A. V. and G. S. Roeder (1997). "The yeast Red1 protein localizes to the cores of meiotic chromosomes." *The Journal of cell biology* **136**(5): 957-967.
- Smith, G. D., A. Sadhu, et al. (1998). "Transient exposure of rhesus macaque oocytes to calyculin-A and okadaic acid stimulates germinal vesicle breakdown permitting subsequent development and fertilization." *Biology of reproduction* **58**(4): 880-886.
- Smith, S. K., S. L. Jaspersen, et al. (2008). "Matrimony ties Polo down: can this kinase get free?" *Cell Cycle* **7**(6): 698-701.
- Somogyi, K., B. Sipos, et al. (2008). "Evolution of genes and repeats in the Nimrod superfamily." *Molecular biology and evolution* **25**(11): 2337-2347.
- Sopko, R., D. Huang, et al. (2006). "Mapping pathways and phenotypes by systematic gene overexpression." *Molecular cell* **21**(3): 319-330.
- Soyer, O. S. and C. J. Creevey (2010). "Duplicate retention in signalling proteins and constraints from network dynamics." *Journal of evolutionary biology* **23**(11): 2410-2421.
- Spradling, A. C. (1993). *Developmental genetics of oogenesis. In The Development of Drosophila melanogaster*. New York, Cold Spring Harbor Laboratory Press.
- Steuerwald, N., J. Cohen, et al. (2001). "Association between spindle assembly checkpoint expression and maternal age in human oocytes." *Molecular human reproduction* **7**(1): 49-55.
- Sumara, I., E. Vorlaufer, et al. (2002). "The dissociation of cohesin from chromosomes in prophase is regulated by Polo-like kinase." *Mol Cell* **9**(3): 515-525.
- Sun, H., D. Treco, et al. (1989). "Double-strand breaks at an initiation site for meiotic gene conversion." *Nature* **338**(6210): 87-90.
- Sunkel, C. E. and D. M. Glover (1988). "polo, a mitotic mutant of *Drosophila* displaying abnormal spindle poles." *J Cell Sci* **89** (Pt 1): 25-38.
- Swan, A. and T. Schupbach (2007). "The Cdc20 (Fzy)/Cdh1-related protein, Cort, cooperates with Fzy in cyclin destruction and anaphase progression in meiosis I and II in *Drosophila*." *Development* **134**(5): 891-899.
- Sym, M., J. A. Engebrecht, et al. (1993). "ZIP1 is a synaptonemal complex protein required for meiotic chromosome synapsis." *Cell* **72**(3): 365-378.
- Tanaka, T. U., N. Rachidi, et al. (2002). "Evidence that the Ipl1-Sli15 (Aurora kinase-INCENP) complex promotes chromosome bi-orientation by altering kinetochore-spindle pole connections." *Cell* **108**(3): 317-329.
- Taylor, S. S. and F. McKeon (1997). "Kinetochore localization of murine Bub1 is required for normal mitotic timing and checkpoint response to spindle damage." *Cell* **89**(5): 727-735.

- Taylor, S. S., E. Ha, et al. (1998). "The human homologue of Bub3 is required for kinetochore localization of Bub1 and a Mad3/Bub1-related protein kinase." *J Cell Biol* **142**(1): 1-11.
- Terada, Y., Y. Uetake, et al. (2003). "Interaction of Aurora-A and centrosomin at the microtubule-nucleating site in Drosophila and mammalian cells." *The Journal of cell biology* **162**(5): 757-763.
- Theurkauf, W. E. and R. S. Hawley (1992). "Meiotic spindle assembly in Drosophila females: behavior of nonexchange chromosomes and the effects of mutations in the nod kinesin-like protein." *The Journal of cell biology* **116**(5): 1167-1180.
- Toth, A., K. P. Rabitsch, et al. (2000). "Functional genomics identifies monopolin: a kinetochore protein required for segregation of homologs during meiosis i." *Cell* **103**(7): 1155-1168.
- Toyoshima-Morimoto, F., E. Taniguchi, et al. (2001). "Polo-like kinase 1 phosphorylates cyclin B1 and targets it to the nucleus during prophase." *Nature* **410**(6825): 215-220.
- Toyoshima-Morimoto, F., E. Taniguchi, et al. (2002). "Plk1 promotes nuclear translocation of human Cdc25C during prophase." *EMBO Rep* **3**(4): 341-348.
- Tsurumi, C., S. Hoffmann, et al. (2004). "The spindle assembly checkpoint is not essential for CSF arrest of mouse oocytes." *J Cell Biol* **167**(6): 1037-1050.
- Tu, J., W. Song, et al. (1996). "Protein phosphatase type 1 interacts with proteins required for meiosis and other cellular processes in *Saccharomyces cerevisiae*." *Molecular and cellular biology* **16**(8): 4199-4206.
- van Vugt, M. A. and R. H. Medema (2005). "Getting in and out of mitosis with Polo-like kinase-1." *Oncogene* **24**(17): 2844-2859.
- Vodermaier, H. C. (2004). "APC/C and SCF: controlling each other and the cell cycle." *Current biology : CB* **14**(18): R787-796.
- Von Stetina, J. R., S. Tranguch, et al. (2008). "alpha-Endosulfine is a conserved protein required for oocyte meiotic maturation in Drosophila." *Development* **135**(22): 3697-3706.
- Von Stetina, J. R. and T. L. Orr-Weaver (2011). "Developmental control of oocyte maturation and egg activation in metazoan models." *Cold Spring Harbor perspectives in biology* **3**(10).
- Wagner, A. (1994). "Evolution of gene networks by gene duplications: a mathematical model and its implications on genome organization." *Proceedings of the National Academy of Sciences of the United States of America* **91**(10): 4387-4391.
- Wang, S., C. Magoulas, et al. (1999). "Concerted evolution within a trypsin gene cluster in Drosophila." *Molecular biology and evolution* **16**(9): 1117-1124.
- Wang, X., J. E. Swain, et al. (2004). "Endogenous regulators of protein phosphatase-1 during mouse oocyte development and meiosis." *Reproduction* **128**(5): 493-502.
- Wang, X., Y. Yang, et al. (2008). "sSgo1, a major splice variant of Sgo1, functions in centriole cohesion where it is regulated by Plk1." *Developmental cell* **14**(3): 331-341.
- Wassmann, K., T. Nialt, et al. (2003). "Metaphase I arrest upon activation of the Mad2-dependent spindle checkpoint in mouse oocytes." *Curr Biol* **13**(18): 1596-1608.
- Watanabe, N., H. Arai, et al. (2004). "M-phase kinases induce phospho-dependent ubiquitination of somatic Wee1 by SCFbeta-TrCP." *Proceedings of the National Academy of Sciences of the United States of America* **101**(13): 4419-4424.
- Watanabe, Y. and P. Nurse (1999). "Cohesin Rec8 is required for reductional chromosome segregation at meiosis." *Nature* **400**(6743): 461-464.
- Wei, L., X. W. Liang, et al. (2010). "BubR1 is a spindle assembly checkpoint protein regulating meiotic cell cycle progression of mouse oocyte." *Cell Cycle* **9**(6).
- Weiss, E. and M. Winey (1996). "The *Saccharomyces cerevisiae* spindle pole body duplication gene MPS1 is part of a mitotic checkpoint." *The Journal of cell biology* **132**(1-2): 111-123.
- White-Cooper, H., L. Alphey, et al. (1993). "The cdc25 homologue twine is required for only some aspects of the entry into meiosis in Drosophila." *Journal of cell science* **106** (Pt 4): 1035-1044.
- Winey, M., G. P. Morgan, et al. (2005). "Three-dimensional ultrastructure of *Saccharomyces cerevisiae* meiotic spindles." *Mol Biol Cell* **16**(3): 1178-1188.
- Xiang, Y., S. Takeo, et al. (2007). "The inhibition of polo kinase by matrimony maintains G2 arrest in the meiotic cell cycle." *PLoS Biol* **5**(12): e323.
- Yamamoto, I., M. E. Kosinski, et al. (2006). "Start me up: cell signaling and the journey from oocyte to embryo in *C. elegans*." *Developmental dynamics : an official publication of the American Association of Anatomists* **235**(3): 571-585.
- Yan, X., L. Cao, et al. (2005). "Aurora C is directly associated with Survivin and required for cytokinesis." *Genes to cells : devoted to molecular & cellular mechanisms* **10**(6): 617-626.
- Yildiz, O., S. Majumder, et al. (2002). "Drosophila MUS312 interacts with the nucleotide excision repair endonuclease MEI-9 to generate meiotic crossovers." *Molecular cell* **10**(6): 1503-1509.
- Yu, H. G. and D. E. Koshland (2003). "Meiotic condensin is required for proper chromosome compaction, SC assembly, and resolution of recombination-dependent chromosome linkages." *The Journal of cell biology* **163**(5): 937-947.
- Yu, H. G. and D. Koshland (2005). "Chromosome morphogenesis: condensin-dependent cohesin removal during meiosis." *Cell* **123**(3): 397-407.

- Yu, J., S. L. Fleming, et al. (2004). "Greatwall kinase: a nuclear protein required for proper chromosome condensation and mitotic progression in *Drosophila*." *The Journal of cell biology* **164**(4): 487-492.
- Zachariae, W., M. Schwab, et al. (1998). "Control of cyclin ubiquitination by CDK-regulated binding of Hct1 to the anaphase promoting complex." *Science* **282**(5394): 1721-1724.
- Zeng, Y., H. Li, et al. (2010). "Statistical analysis of nondisjunction assays in *Drosophila*." *Genetics* **186**(2): 505-513.
- Zhang, J. (2003). "Evolution by gene duplication: an update." *Trends in Ecology and Evolution* **18**(6): 292-298.
- Zickler, D. and N. Kleckner (1998). "The leptotene-zygotene transition of meiosis." *Annual review of genetics* **32**: 619-697.

Appendixes

Appendix A

D. melanogaster Spc105R antibody production

D. melanogaster Spc105-related (Spc105R) does not have significant homology with proteins of known structure and other methods of tertiary structure prediction were inconsistent and/or of low accuracy. So, using the prediction for secondary structure of the protein we chose a region of 197 amino acids (from the amino acid 997 to the 1193) on the basis of the following criteria: the beginning and the end of the region cannot have any secondary predicted structure and cannot be buried in the tertiary structure of the protein. To construct the expression plasmid the selected region was amplified by PCR using as template DNA from S2 cells with the following primers: forward, 5' CGCGGATCCTCAAATGATCAGCCAAAG 3' and reverse, 5' CCGCTCGAGTTAGCCCTTCACCGC 3'. Agarose gel electrophoresis was used to confirm the PCR product size. The product was then purified using a Gel Extraction Kit (Quiagen). After digestion with BamHI and XhoI restriction enzymes, the PCR product was inserted in the GST expression vector pGEX-4T-3 (GE Healthcare), using the T4 DNA ligase (Figure A.1). Constructs were then confirmed by sequencing. For protein expression, the plasmid was transformed in protein expression host cells, *E. coli* BL21. Small-scale protein expression was then tested in several conditions.

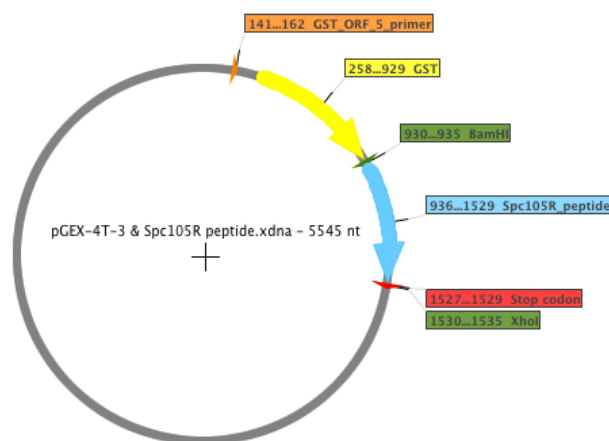


Figure A.1. Graphic map of the construct pGEX-4T-3 with the Spc105R recombinant peptide.

For large-scale protein expression, a single colony was inoculated in LB medium with 100 µg/mL of ampicillin, which was grown overnight at 37 °C. For protein production, we used 10 liters of LB medium into which the starting culture was added. The culture was grown until the optical density was 0.6 (600 nm). Protein

expression was then induced for 3 hours with 0.5 mM of Isopropyl β -D-1-thiogalactopyranoside (IPTG). After centrifugation and resuspension in the lysis buffer (1 mM of DTT, 0.2 mg/ml of lysozyme, 10 μ g/ml DNase I, 1 mM MgCl₂ and 1 mM of PMSF in 1X PBS) the samples were frozen at -80°C. The samples were then thaw quickly, sonicated and centrifuged at 35000 g for 30 minutes at 4°C. Since the recombinant protein was in the soluble fraction, the samples were immediately applied to the GSTrap HP affinity column. Using the AKTAprime plus chromatography system the protein was eluted using the following elution buffer: 10 mM reduced glutathione, 50 mM Tris-HCl pH 8.0. Since the eluted fractions did not contain exclusively the recombinant protein, different chromatography fractions were run on SDS-PAGE gel, and the band that corresponded to the recombinant protein was excised. The protein was then extracted from the gel and was used for immunization into rats. After five protein boosts, the serum was collected and antibody specificity for Spc105R was confirmed by the appearance of a band at the predicted size (223 KDa) that was not observed in the pre-immune serum or in Spc105R RNAi protein extracts. The antibody was also tested using immunofluorescence.

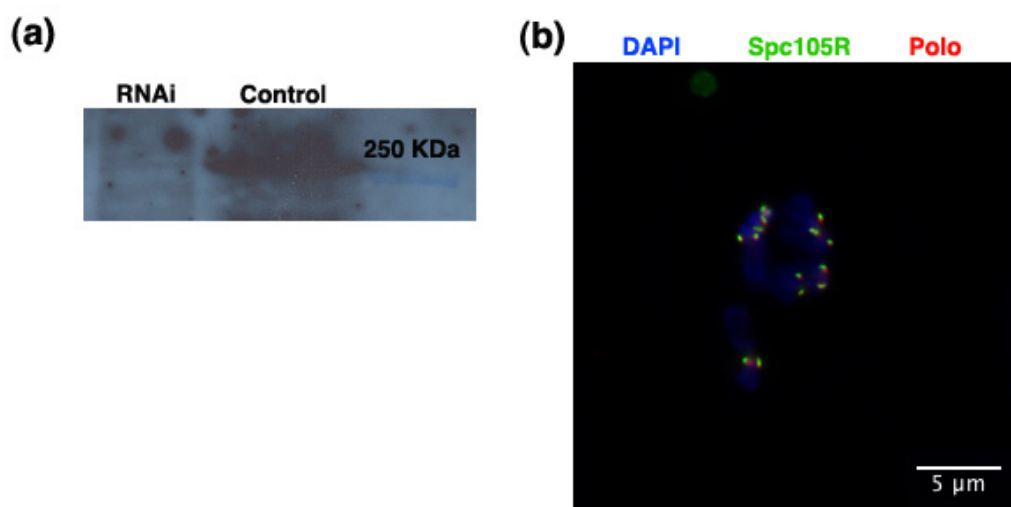


Figure A.2. Immunocytochemistry of the rat anti-Spc105R antibody. (a) For the western blot test, it was used 50 μ g of total protein extract from *Drosophila* S2 cells in control and Spc105R RNAi conditions with an antibody dilution of 1:2000. The antibody performance was proven by the appearance of a band around 250 kDa (the predicted size of Spc105R is 223 kDa) in the control extracts and its absence in the Spc105R RNAi extracts; (b) For the immunofluorescence test, it was used *Drosophila* S2 cells treated with MG132. The extraction and the fixation were performed simultaneously with 3.7% formaldehyde and the antibody dilution used was 1:2000. The Spc105R antibody co-localizes with Polo.

Appendix B

Polo alignments

Polo kinase domain (shown in yellow and T182 in green)

<i>D.mel</i> _Polo	MAAKPEDKST-DIPDRLVDINQKRTYKRMRFFGKGGFAKCYEIIDVETDDVFAGKIVSKK	59
<i>D.per</i> _Polo	MAAKPEDKST-DIPDRLFDSNQRRTYKRMRFFGKGGFAKCYEIIDVETDDVFAGKIVSKK	59
<i>D.per</i> _Polo-dup1	MAEKPEDINT-DIPYCLYDSNLRRTYKRLRFFGKGGFAKCYEIMDVETDDVFAGKIVSKK	59
<i>D.per</i> _Polo-dup2	MTAAPEDKTTTHVVPDSLFDPHRGRTYKRGRRFFGKGGFARCYEIIDVHTDDVFACKIVSKK	60
<i>D.pseu</i> _Polo	MAAKPEDKST-DIPDRLFDSNQRRTYKRMRFFGKGGFAKCYEIIDVETDDVFAGKIVSKK	59
<i>D.pseu</i> _Polo-dup1	MAEKPEDIHT-DIPYCLYDSNLRRTYKRLRFFGKGGFAKCYEIMDVETDDVFAGKIVSKK	59
<i>D.pseu</i> _Polo-dup2	MTAAPEDKTTNVVPDSLFDPHRGRTYKRGRRFFGKGGFACCYEIIDVHTDDVFACKVVSCK	60
	*: *** * : * * : : **** *****:****:*.***** *:****	
<i>D.mel</i> _Polo	LMIKHNQKEKTAQ-----EITIHRSNLNHPNIVKFHNYFEDSQNIYIVLELCKKRSMML	113
<i>D.per</i> _Polo	LMIKHNQKEKTAQ-----EITIHRSNLNHPNIVKFHNYFEDVQNIYIVLELCKKRSMML	113
<i>D.per</i> _Polo-dup1	LIIKHQKEKTAQ-----EITIHRLDHPNIVKFHNYFEDVQNIYIVLELCKKRSLMEL	113
<i>D.per</i> _Polo-dup2	LIMKPSQEEETAPEEIAVEISIHRLNHRSLVKFFSCFDDIDNTYIVLELCKKRSMSEL	120
<i>D.pseu</i> _Polo	LMIKHNQKEKTAQ-----EITIHRSNLNHPNIVKFHNYFEDVQNIYIVLELCKKRSMML	113
<i>D.pseu</i> _Polo-dup1	LIIKHQKEKTAQ-----EITIHRLDHPNIVKFHNYFEDVQNIYIVLELCKKRSLMEL	113
<i>D.pseu</i> _Polo-dup2	LIMKPSQEEETAPEEIAVEISIHRLNHRSLVKFFSCFDDIDNTYIVLELCKKRSMSEL	120
	: .*:.*:***: **:*:*.*: .*****. *: * : *****:***: **	
<i>D.mel</i> _Polo	HKRRKSITEFECRYIIYQIIQGVKYLHDNRRIHRDLKLGNLFLNDLLHVKGIDFGLATRI	173
<i>D.per</i> _Polo	HKRRKSITEFECRYIIYQIIQGVKYLHDNRRIHRDLKLGNLFLNDMLHVKGIDFGLATRI	173
<i>D.per</i> _Polo-dup1	HKRRGCITEYECRYIIYQIIQGVKYLHDKHIHRDLKLGNLFLDDMLHVKGIDFGLATLI	173
<i>D.per</i> _Polo-dup2	QRRRKIIITEFECRYIITQIIIEGVQYLHNRIHRDLKLANLFLNDMLHVKGIDFGLATRI	180
<i>D.pseu</i> _Polo	HKRRKSITEFECRYIIYQIIQGVKYLHDNRRIHRDLKLGNLFLNDMLHVKGIDFGLATRI	173
<i>D.pseu</i> _Polo-dup1	HKRRGCITEYECRYIIYQIIQGVKYLHDKHIHRDLKLGNLFLDDMLHVKGIDFGLATLI	173
<i>D.pseu</i> _Polo-dup2	QRRRKIIITEFECRYIITQIIIEGVQYLHNRIHRDLKLANLFLNDMLHVKGIDFGLATRI	180
	:** ***:***** **:***:***:*****:*****:*****:***** *	
<i>D.mel</i> _Polo	EYEGERKKTLCGTPNYIAPEILTKKGHSFEVDIWSIGCVMYTLLVGQPPFETKTLKDTYS	233
<i>D.per</i> _Polo	EYEGERKKTLCGTPNYIAPEILTKKGHSFEVDIWSIGCVMYTLLVGQPPFETKTLKDTYS	233
<i>D.per</i> _Polo-dup1	AYEGERKKTLCGTPNYIAPEILNKQHSFEVDIWSIGCVMYTLLVGQPPFETKSLRNTYS	233
<i>D.per</i> _Polo-dup2	EFEGDRRYSVCGSPNYIAPEILTKKGHSFEADIWSIGCVMYTLLAGRPPFHHKTRREGIFS	240
<i>D.pseu</i> _Polo	EYEGERKKTLCGTPNYIAPEILTKKGHSFEVDIWSIGCVMYTLLVGQPPFETKTLKDTYS	233
<i>D.pseu</i> _Polo-dup1	AYEGERKKTLCGTPNYIAPEILNKQHSFEVDIWSIGCVMYTLLVGQPPFETKSLRDTYS	233
<i>D.pseu</i> _Polo-dup2	EFEGDRRYSVCGSPNYIAPEILTKKGHSFEADIWSIGCVMYTLLAGRPPFHHKTRREGIFS	240
	:**:* :*:*****:*****:*****:*****:*****:*****:***** *	
<i>D.mel</i> _Polo	KIKKCEYRVPSYLRKPAADMVIAMLQPNPESRPAIGQLLNFEFLKGSQVPMFLPSSCLTM	293
<i>D.per</i> _Polo	KIKKCEYRVPSYLRKPAADMVISMQLQPNPESRPAIGQLLNDFLKGSKVPTFLPSSCLTM	293
<i>D.per</i> _Polo-dup1	RIKKCEYRVPSYMRKPAADMVMAMQLQPDPSCRPAIGQLLNFEFLKVSVPFTFLPISCLTM	293
<i>D.per</i> _Polo-dup2	NIKKGQYSVPSWLTISAADMVLSMLQLDPERRPAIGQLLNCSFLKAAKVPQFLPSFCLTM	300
<i>D.pseu</i> _Polo	KIKKCEYRVPSYLRKPAADMVIAMLQPNPESRPAIGQLLNDFLKGSKVPTFLPSSCLTM	293
<i>D.pseu</i> _Polo-dup1	RIKKCEYRVPSYLRKPAADMVIAMLQPDPSCRPAIGQLLNDFLKVSVPFTFLPISCLTM	293
<i>D.pseu</i> _Polo-dup2	NIKKGQYSVPSWLTISAADMVLSMLQLDPERRPAIGQLLNCNFLKAAKVPQFLPSFCLTM	300
	.*** :* ***: .*:***:***:*. ***** .*** :* ** *** *	

Polo-box1

<i>D.mel</i> _Polo	YSDKYFGYQLCDEGIGVMFNDTTKLILLPNQINVHFIDKDGKETYMTTDDYCKSLDKMKMLLS	461
<i>D.per</i> _Polo	YSDKYFGYQLCDEGIGVMFNDTTKLILLPNQINVHFIDKDGKESYMTTDDYCKTLDKMKMLLS	460
<i>D.per</i> _Polo-dup1	YSDKYFGYQLCDEGIGIMFIDTTKLILLHPNQINVHFIDKDGKESYMTTDDYCKTLEKKMKMLLS	452
<i>D.pse</i> _Polo	YSDKYFGYQLCDEGIGVMFNDTTKLILLPNQINVHFIDKDGKESYMTTDDYCKTLDKMKMLLS	460
<i>D.pse</i> _Polo-dup1	YSDKYFGYQLCDEGIGIMFNDTTKLILLHPNQINVHFIDNDGKESYMTTTEYCKTLEKKMRLLS	452
	*****:***:***** *****:****:*****:***:*.***:***	

Polo-box2

<i>D.mel</i> _Polo	FRTTCAVVMHLTNGSVQLNFSHDMKLILCPRMSAITYMDQEKNFRTYRFSTIVENGVS KDLYQKIRYAO	564
<i>D.per</i> _Polo	FRTTCAVVMHLTNGSVQLNFSHDMKLILCPRMSAITYMDHEKNFRTYRFSTIVENGVS KDLYQKIRYAO	563
<i>D.per</i> _Polo-dup1	FRTTCAVVMHLTNGTQIINFSDHMKLILCPRQSAITYIDREKNFRTYRFSTIAEKGVSKDVYPKIRYAO	555
<i>D.pse</i> _Polo	FRTTCAVVMHLTNGSVQLNFSHDMKLILCPRMSAITYMD-----	556
<i>D.pse</i> _Polo-dup1	FRTTCAVVMHLTNGTLQINFSDHMKLILCPRQSAITYIDREKNFRTYRFSTIAEKGVSKDVYPKIRYAO	555
	*****:***:***** *****:***:*****:***:*****:*	

Legend: “*D. mel*” stands for *D. melanogaster*; “*D. per*” stands for *D. persimilis*; “*D.pse*” stands for *D. pseudoobscura*; symbols denoting the degree of conservation observed in each column: “*” means that the

residues or nucleotides in that column are identical in all sequences in the alignment; “.” means that conserved substitutions have been observed; “.” means that semi-conserved substitutions are observed; “RED” highlights a substitution observed in Polo vs. Polo-dup sequences; “GREEN” highlights a different substitution in Polo vs. Polo-dup sequences; “BLUE” highlights a substitution in *D. mel* Polo sequence vs. the others sequences.

Appendix C

Abbreviations

ald: altered disjunction
APC/C: anaphase-promoting complex or cyclosome
Asp: Abnormal spindle
ATM: Ataxia Telangiectasia Mutated
ATR: ATM and Rad3-related
Bub: Budding Uninhibited by Benomyl
BubR1: Bub-related 1 kinase
c(2)M: crossover suppressor on 2 of Manheim
Ca²⁺: calcium
CAK: Cdk-activating kinase
CaMKII: Ca²⁺/calmodulin-dependent protein kinase II
cAMP: cyclic Adenosine 3', 5'-MonoPhosphate
cav: caravaggio
Cdk: Cyclin-dependent Kinase
cDNA: complementary DNA
Chk1: Checkpoint Kinase 1
Chk2: Checkpoint Kinase 2
cort: cortex
CSF: cyostatic factor
Cyc: Cyclin
D-TACC: Transforming Acidic Coiled-Coil protein
D. melanogaster: *Drosophila melanogaster*
DAPI: 4',6'-diamidino-2-phenylindole
DHJs: double Holliday junctions
DNA: DeoxyriboNucleic Acid
DSBs: double-strand breaks
E1 enzyme: ubiquitin-activating enzyme
E2 enzyme: ubiquitin-conjugating enzyme
E3 enzyme: ubiquitin-protein ligase
elgi: early girl
Emi1: Early Mitotic Inhibitor-1
EMS: ethyl methane sulfonate
endos: endosulfine
FC: follicle cells
flw: flapwing
fzy: fizzy
G₁ phase: Gap 1 phase
G₂ phase: Gap 2 phase
GSCs: germline stem cells
GVBD: germinal vesicle breakdown
Gwl: Greatwall
hdm: hold'em
HOAP: HP1/ORC-associated protein
HP1: Heterochromatin Protein 1
M-phase: mitotic phase
Mad: Mitotic-arrest Deficient
MCC: mitotic checkpoint complex
mcm5: minichromosome maintenance 5
MI: meiosis I
MII: meiosis II

MPF: maturation promoting factor
MPM2: Protein Monoclonal 2
Mps1: Monopolar Spindle 1
mre11: meiotic recombination 11
MRN complex: Mre11/Rad50/Nbs complex
mRNA: messenger RNA
msps: mini spindles
MTOC: microtubule organizing center
Mtrm: Matrimony
Myt1: myelin transcription factor 1
NCD: Non-Claret Disjunctional
NDJ: nondisjunction
NEB: nuclear envelop breakdown
NHK-1: Nucleosomal Histone Kinase-1
nod: no distributive disjunction
OA: okadaic acid
ORC: Origin Recognition Complex
ORD: orientation disruptor
PBD: polo-box domain
PCM: pericentrosomal material
PCR: polymerase chain reaction
PKA: cAMP-dependent Protein Kinase A
Plk(s): Polo-like kinase(s)
PP: protein phosphatase
qRT-PCR: quantitative real time polymerase chain reaction
Rca1: regulator of CycA-1
RCANs: regulators of calcineurin
rec: recombination-defective
RNA: Ribonucleic acid
RNAi: RNA interference
RpL32: ribosomal protein L32
RpS4: Ribosomal protein S4
rRNA: ribosomal RNA
S phase: DNA synthesis phase
SAC: spindle assembly checkpoint
SC: Synaptonemal Complex
SCF complex: Skp1/cullin/F-box protein-related complex
SCP: synaptonemal complex proteins
Sgo1: shugoshin 1
SMC: structural maintenance of chromosome
sra: sarah
Su(var)205: Suppressor of variegation 205
sub: subito
TF: terminal filament
TPR: tetratricopeptide repeat
twe: twine
γ-TuRC: γ-tubulin ring complex

Appendix D

Recipes

PEM buffer pH 6.9

0.1 M PIPES
1 mM MgCl_2
1mM EGTA

Phosphate-Buffered Saline (PBS) pH 7.4

137 mM NaCl
2.7 mM KCl
10 mM Na_2HPO_4
1.8 mM KH_2PO_4

