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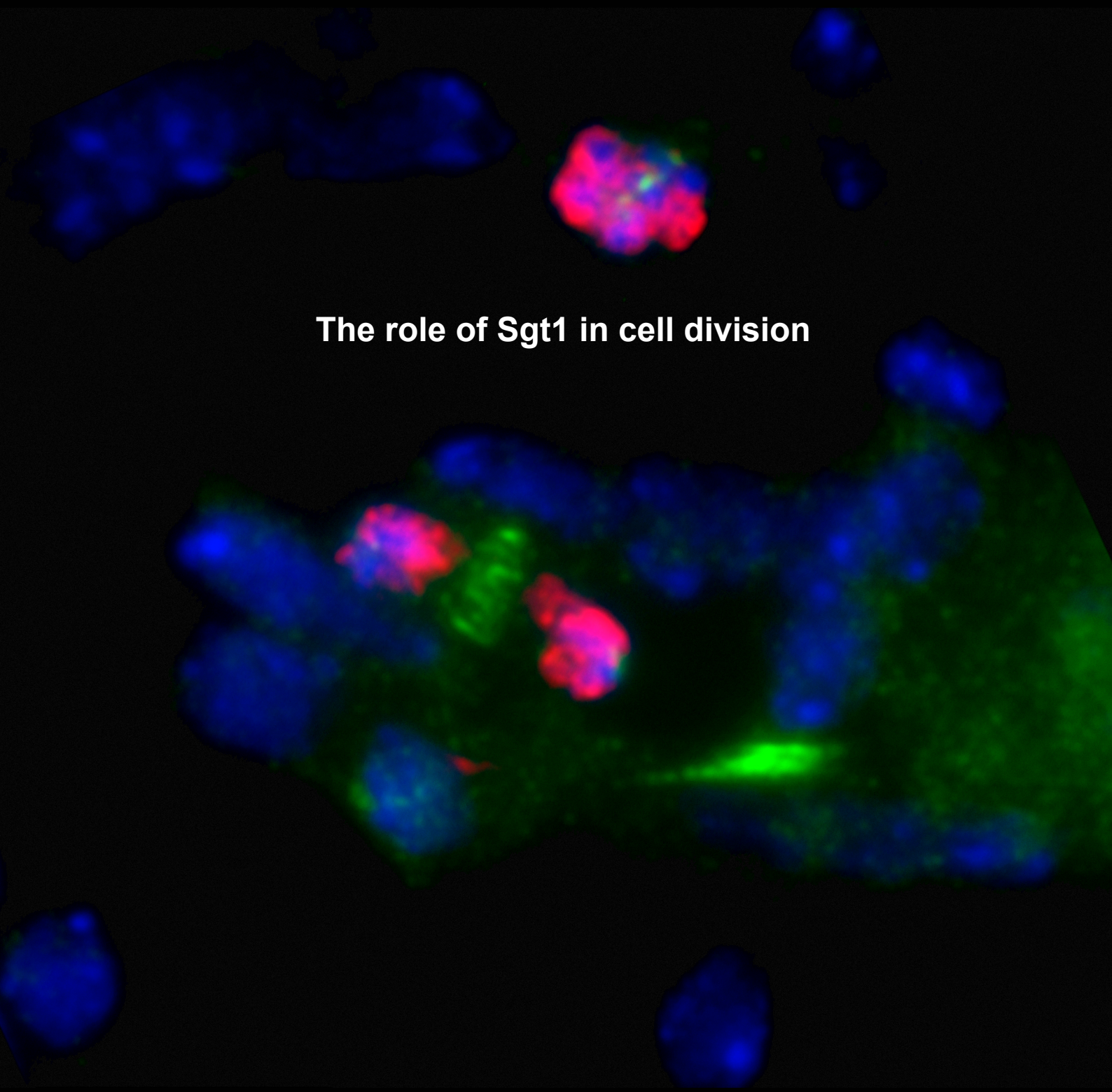

The role of Sgt1 in cell division

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Tese de doutoramento em Ciências Biomédicas

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The role of Sgt1 in cell division

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Summary

To ensure equal distribution of the genetic material, cell orchestrates several processes in order to achieve proper chromosome segregation. One of those processes is the binding of microtubules to a specific chromosome region, the kinetochore. The work reported in this thesis aims to further our understanding on the regulation of the kinetochore assembly pathway, chromosome segregation as well as to study the regulation of a major mitotic regulator Polo kinase. The first part focuses on the analysis of an identified mutant allele of *sgt1* in Drosophila and characterized its function. Previous work in yeast and human cells showed that Sgt1 interacts with Hsp90 to form a complex essential for kinetochore assembly. Analyses of the mutations in *sgt1* show that overall kinetochore assembly and spindle assembly checkpoint are not affected. However, we found that mutations in *sgt1* severely compromise the organization and function of the mitotic apparatus. In these cells, centrosomes fail to mature and pericentriolar material components do not localize normally resulting in highly abnormal spindles. Interestingly, a similar phenotype was previously described in Hsp90 mutant cells and correlated with a decrease in Polo protein levels. In *sgt1* mutant neuroblasts we also observe a decrease in overall levels of Polo. Overexpression of the kinase results in a substantial rescue of the centrosome defects, consequently most cells form normal bipolar spindles and progress through mitosis normally even in the absence of Sgt1. Taken together, these findings suggest that Sgt1 is involved in stabilization of Polo allowing normal centrosome maturation, entry and progression through mitosis. The second part of our work focused in the mechanisms that monitor chromosome segregation during anaphase and cytokinesis. The NoCut checkpoint was suggested to ensure that no DNA is present in the spindle midzone when cytokinesis takes place. However, whether this is achieved by alterations in spindle length or chromosome condensation remains an open question. To address this, we used Drosophila strains that carry re-arranged extra long chromosomes that could not be segregated by a normal mitotic spindle. These compound chromosomes are composed of two whole second chromosomes fused by Y chromosome heterochromatin and that have a single functional centromere. Using appropriate fluorescent markers for chromatin and tubulin, we studied *in vivo* and in fixed material the alterations in mitosis that must take place to allow the segregation of these chromosomes and cell viability. We found that independently of cell type, cells extend the time between nuclear envelop breakdown to anaphase. The last chapter of this thesis describes the Drosophila phenotype of a phosphomimicking mutation in the

mitotic regulator Polo kinase. Expression in eye or wing imaginal discs of Polo-T182D results in lethality and organ reduction of the adult organism. These phenotypes will serve as starting point for a future search of Polo interactors affected by the expression of the mutation Polo-T182D.

Resumo

De forma a assegurar uma distribuição igual do material genético, a célula orquestra vários processos de modo a conseguir uma segregação apropriada dos cromossomas. Um desses processos é a ligação dos microtúbulos a uma região específica dos cromossomas, o cinetocóro. O trabalho apresentado nesta tese tem como objectivo aumentar o nosso conhecimento na regulação da formação do cinetocóro, segregação cromossómica bem como estudar a regulação de um regulador mitótico, a cinase Polo. A primeira parte desta tese foca-se na análise e caracterização de um alelo mutante de *sgt1* em Drosophila. Trabalho prévio em levedura e células humanas mostrou que Sgt1 interage com Hsp90 para formar um complexo essencial para a formação do cinetocóro. As análises das mutações em *sgt1* mostram que a organização global do cinetocóro e o ponto de controlo do fuso mitótico não estão afectados nestas células. No entanto, nós descobrimos que essas mutações em *sgt1* comprometem severamente a organização e função do aparelho mitótico. Nestas células, os centrossomas falham a maturação e os componentes do material pericentriolar não localizam normalmente, resultando em fusos altamente anormais. De forma interessante, um fenótipo similar foi anteriormente descrito em células mutantes para Hsp90 tendo sido correlacionado com uma diminuição dos níveis da proteína Polo. Em neuroblastos de mutantes *sgt1* também observámos uma diminuição nos níveis totais de Polo. A sobre-expressão desta cinase resulta numa significativa recuperação dos defeitos do centrossoma, consequentemente a maioria das células forma fusos bipolares normais e progride em mitose normalmente mesmo na ausência de Sgt1. No seu conjunto, os dados aqui descritos sugerem que Sgt1 está envolvida na estabilização de Polo. Esta estabilização permite uma maturação normal dos centrosomes assim como a entrada e progressão em mitose. A segunda parte deste trabalho incide nos mecanismos que vigiam a segregação dos cromossomas durante anáfase e citocinese. Um desses mecanismos é o ponto de controlo NoCut, o qual assegura que não há ADN na zona média do fuso, local onde a citocinese tem lugar. No entanto, se isto é alcançado por alterações no comprimento do fuso ou uma condensação extra do cromossoma continua a ser uma questão por resolver. Para responder a esta questão usámos estirpes de Drosophila que contêm cromossomas extra longos re-arranjados, os quais poderão não ser segregados por um fuso normal. Estes cromossomas compostos têm apenas um centrómero funcional e são constituídos por dois segundos cromossomas unidos por heterocromatina do cromossoma Y. Para estudar *in vivo* e material fixado as alterações em mitose que

têm que ocorrer para permitir a segregação dos cromossomas compostos e consequente viabilidade celular, usámos marcadores fluorescentes apropriados para cromatina e tubulina. Observámos que independentemente do tipo celular, as células extendem o período de tempo entre a quebra do envelope nuclear até anáfase. O ultimo capitulo desta tese descreve o fenótipo em Drosophila de uma mutação que mimetiza a fosforilação do regulador mitótico, a cinase Polo. Expressão de Polo-T182D nos discos imaginais do olho ou da asa resulta em letalidade e redução do órgão no organismo adulto. Estes fenótipos servirão de base para uma pesquisa futuro para encontrar proteínas que interagem que estão afectadas pela expressão da mutação Polo-T182D.

PART I

GENERAL INTRODUCTION

1. Cell cycle

1.1. Overview

"Omnis cellula e cellula" are the Latin words that were proposed by Rudolf Virchow in 1855 to present the theory that every cell is preceded from other pre-existing cell. This theory emerges upon acceptance of the concept that the cell is the basic unit of life and that all organisms are constituted by cells presented by Schleiden(1838) and Schwann(1839) (Turner 1890). These theories served as the base of the first detailed description of cell division made by Flemming in 1879 (Flemming 1965). In his work for the first time the stainable material in the interphase nucleus is referred as "chromatin" (meaning stainable material) and he described chromatin shortening and thickening as originating threads referred as "chromosomes" (meaning stainable bodies) that align in the equatorial plane of the cell, and then split into two halves and move to opposite sides of the cell (Flemming 1965). He was the first refer nuclear division as **mitosis** (from the greek word "mitos" referring to the threads) (reviewed by Paweletz 2001).

More than 100 years have passed since first description of mitosis was made and the studies of the diverse mechanisms that underlie this process are still challenging the scientific community around the World.

1.2. The cell cycle

Cell cycle is the process that all cells must undergo in order to duplicate its basic constituents including DNA, allowing the cell to prepare for division. Through this process a single parental cell gives rise to two genetically identical daughter cells. Thus, cell proliferation is an essential process for the development of multicellular organisms by the consecutive multiplication and accurate division of its unique founder cell. It is also the mechanism that allows most adult multicellular organisms to renew old and damaged cells and to maintain tissue homeostasis. To maintain genomic stability during cell proliferation, cells go through a series of highly ordered events during the cell cycle that can be divided into two main phases: a long phase, called interphase, which includes the period between two cell division events, and a shorter phase, mitosis, where the nuclear division takes place. After nuclear division, the formation of two daughter cells is completed by division of the cytoplasm - a process

called cytokinesis. During most of the time cells are in interphase and although morphologically indistinguishable, different biochemical processes take place allowing the characterization of diverse stages in the cell cycle. The first period in the cell cycle is called G_1 (or gap 1) and results from a previous cycle of proliferation. If cells are not committed to immediate rounds of proliferation they can transiently exit the cell cycle into a phase called $G_{0,}$ characterized by low levels of metabolic activity. Resumption of the cell cycle can be achieved in the presence of factors that promote cell proliferation, as mitogens.

During G_1 cells grow and after reaching an appropriate size cells can enter S phase, the period where DNA replication takes place. During DNA replication the enzymatic machinery faithfully copies the DNA molecules and both copies of replicated DNA are held together by the incorporation of a multiprotein complex called Cohesin. After DNA replication, cells enter a second period called G_2 (or gap2 phase (Fig. 1). During this phase the cell initiates the preparation the final stage of the cell cycle, which leads to the segregation of chromosomes, during a process called mitosis (reviewed by Lodish 2003; Morgan 2007).

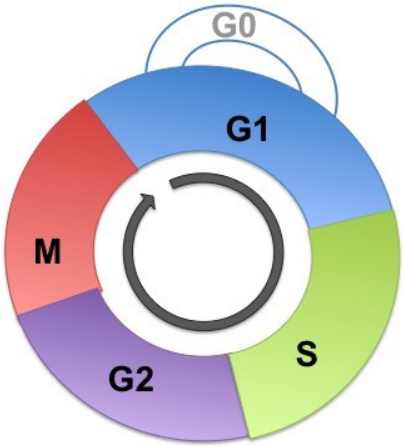

Figure 1: Schematic representation of the eukaryotic cell cycle. The main events of the cell cycle are DNA replication (S-phase), and chromosome segregation and consequent nuclear division (M-phase or mitosis). Between these main events exist the gap phases (G_1 and G_2). It is also represented here the G0 stage that is characterized by a more or less prolonged stage of nondividing cells;

1.3. Mitosis

Mitosis is a cell cycle phase characterized by some of the most dynamic and drastic alterations in cellular organization. Mitosis is usually sub-divided into 5

consecutive phases: prophase, prometaphase, metaphase, anaphase and telophase (Fig. 2). During prophase two major events can be easily visualized and include overall reorganization of the cytoskeleton and condensation of chromatin into compact mitotic chromosomes (Approx. 10000x compaction). The reorganization of cytoskeleton microtubules is conducted by separation and migration to opposite sides of the cell of the two previously duplicated MTOCs (Microtubule Organizing Centres), which in animal cells are called centrosomes. Centrosomes consist in a pair of centrioles positioned orthogonally to each other that are surrounded by the pericentriolar material (PCM) (Fig. 3). Centrosomal maturation occurs during G_2 and plays a major role in promoting the conversion of the highly stable microtubules found in interphase to highly dynamic microtubules present in mitosis.

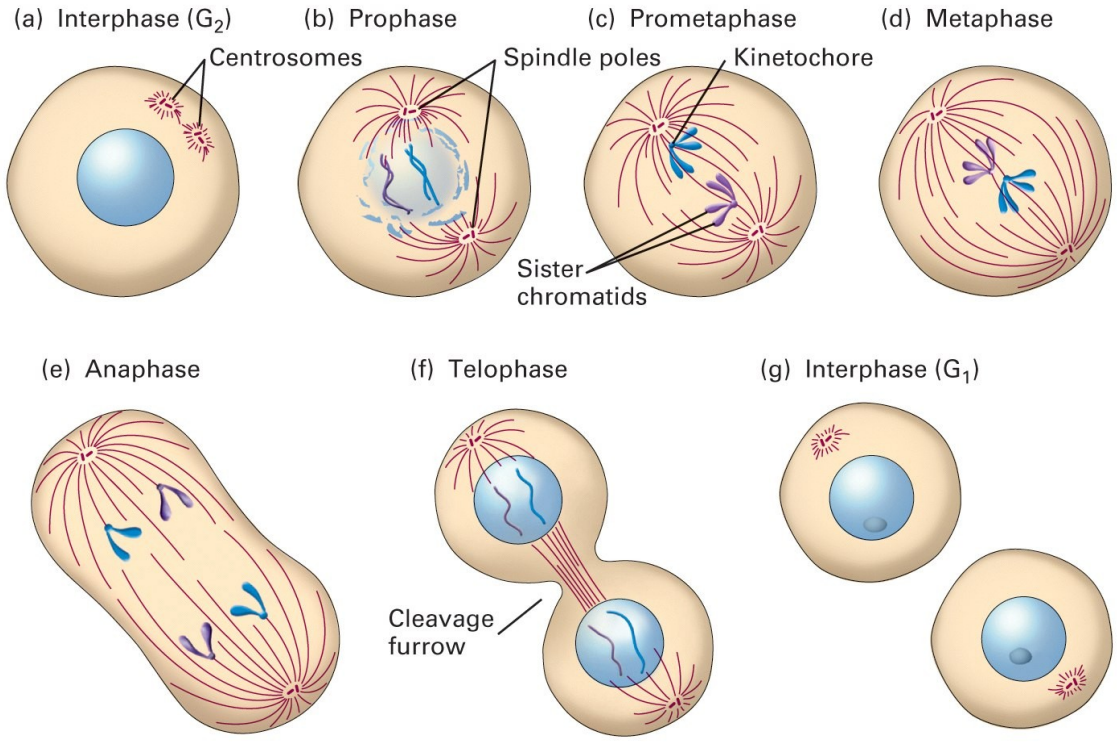

Figure 2: Mitosis in an animal cell. While in G_2 (a) chromosomes, each containing a sister chromatid, are dispersed and not visible as distinct structures. As prophase is initiated (b) centrosomes move towards opposite sides of the cell and chromosome condensation start to be visible. When nuclear envelope breaks down, prometaphase (c) starts and chromosome condensation is completed; each chromosome can be visualized as a structure composed of two sister chromatids held together at the centromeric region. During prometaphase, chromosomes are captured by microtubules growing from opposite poles, a process that contributes to chromosome congression and alignment at the metaphase plate (d). At anaphase onset (e) each chromatid pair separates and segregates to opposite poles of the cell. By the end of mitosis, in telophase (f), chromosomes decondense and the nuclear membrane re-forms

around the daughter nuclei. Cytoplasm division, or cytokinesis, occurs concomitantly with the later mitosis stages, giving rise to two daughter cells (g) (Adapted from Lodish 2003).

The next mitotic stage is called prometaphase and starts with Nuclear Envelope Breakdown (NEBD). This allows microtubules nucleated by centrosomes to invade the nuclear space and marks the initiation of the construction of the mitotic spindle. Microtubules are polymers composed of Tubulin subunits that exist as heterodimers of α-tub and β-tub. These dimers bind head-to-tail to form a linear protofilament and aggregation of 13 protofilaments forms the microtubule. Characterized as polar structures, microtubules plus ends present a GTP-binding cap that is hydrolysed as new dimers are incorporated at the tip of the microtubule. Microtubules will eventually form the mitotic spindle, a bipolar structure in which the minus or slowly growing ends are focused at centrosomes whereas the plus or fast growing ends of microtubules extend away from centrosomes. Thus, plus ends of microtubules can initiate contact with chromosomes in a process known as “Search and Capture” (Kirschner and Mitchison 1986) that will eventually lead to stable microtubule chromosome interactions. Stable interactions between microtubules and chromosomes take place at a very specific chromosomal structure called the kinetochore. The kinetochore is a multiprotein complex located at the surface of each sister chromatid at a region called the centromere that corresponds to the primary constriction of condensed chromosomes (Fig 3). As a result of the interaction with spindle microtubules the chromosomes will eventually move towards the cell centre, a process known as chromosome congression.

Metaphase occurs when all chromosomes are aligned at the equatorial plane of the mitotic spindle so that each sister chromatid of each chromosome is attached to microtubules emanating from opposite poles of the spindle. Only when metaphase is reached and all chromosomes are under tension that chromosome segregation can occur. This is initiated by the physical separation of sister chromatids as a result of proteolytic cleavage of one of the subunits of the Cohesin complex that holds sister chromatids together. At this stage sister chromatids can begin migration to opposite sides of the spindle, a process known as anaphase. Finally, during telophase, the nuclear envelope reassembles and surrounds each group of segregated chromatids, which then decondense giving rise to two daughter nuclei. At the same time, the cell cytoplasm starts division by a process known as cytokinesis. This is initiated with the ingression of the cleavage furrow normally at the centre of the cell perpendicular to the long axis of the mitotic spindle and terminates when the two daughter cells finally separate (reviewed by Lodish 2003; Morgan 2007).

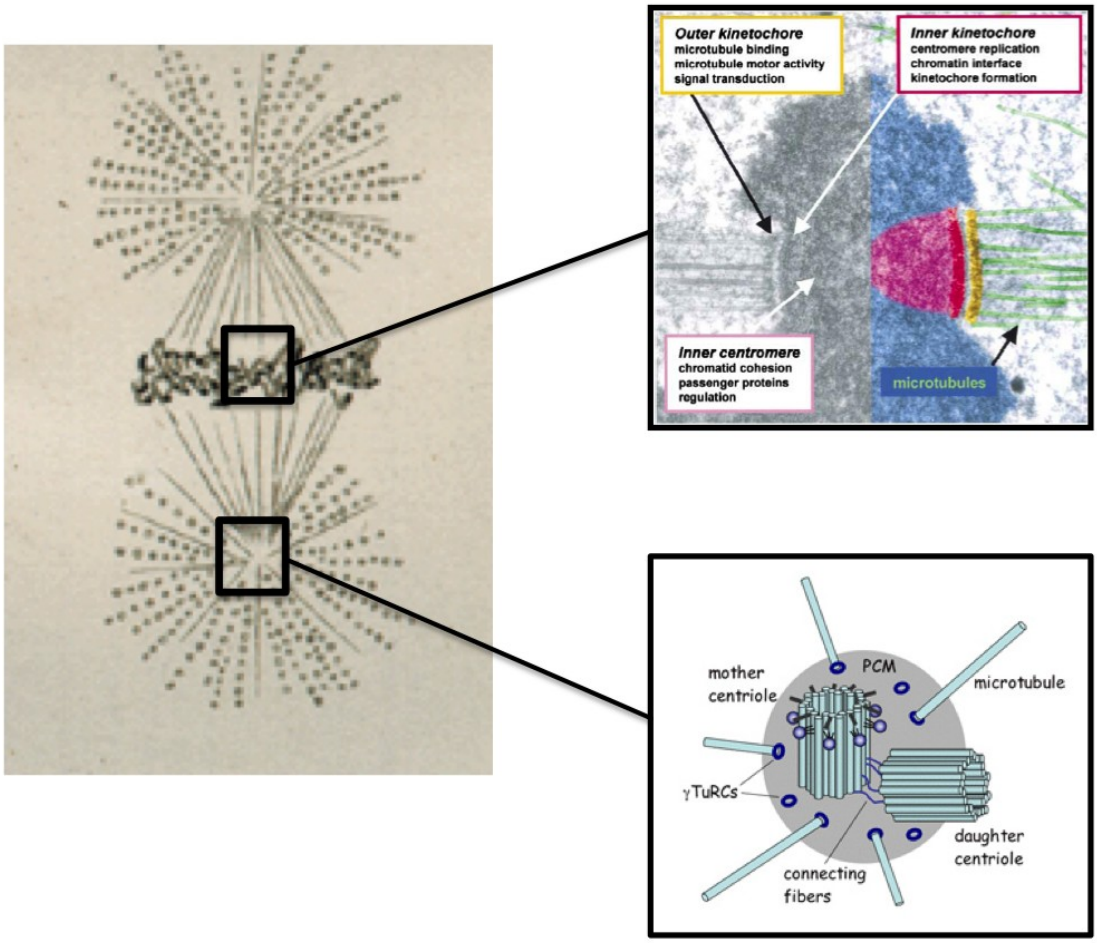

Figure 3: A drawing of the mitotic apparatus (adapted from (Paweletz 2001)); Chromosome-microtubule attachment and centrosomes are highlighted in the top and bottom insets, respectively; Top inset: electron microscopy photograph of a chromosome; chromatid arms are coloured in blue, the centromeric region of the chromosome is coloured in pink, in red the inner region of the kinetochore is coloured in red, the outer kinetochore is coloured in yellow and in green are coloured microtubules attaching the kinetochore (Adapted from Cleveland et al. 2003); On the bottom panel, we can see a scheme of a centrosome; the basic unit of the centrosomes, the pair of centrioles in the middle, and the PCM or pericentriolar material where the γTuRCs attach and start to promote the growing of nascent microtubules are represented (Adapted from www.irbbarcelona.org/files/Image/luders_fig3.jpg);

2. Cell cycle regulation

The cell cycle is orchestrated by unique enzymatic complexes that phosphorylate and degrade specific target proteins. Protein degradation is achieved by the addition of ubiquitin-polymeric chains to the target protein, signalling them to be degraded by the abundant 26S proteasome complex. Ubiquitination of a substrate requires an ubiquitin-

activating enzyme (E1), an ubiquitin-conjugating enzyme (E2) and an ubiquitin-ligase enzyme (E3). Two related E3 complexes involved in the degradation of mitotic regulators are: the SCF (Skp1/Cullin/F-box protein) and the APC/C (Anaphase-Promoting Complex/Cyclosome). APC/C activation in mitosis occurs through its interaction with Cdc20/Fizzy that triggers sister chromatid separation and the first steps of the mitotic exit. In late mitosis and during G_1 APC/C is activated by Cdh1/Hct1/Fizzy-related in order to complete mitotic exit and to maintain the cell cycle machinery in a resting state during early G_1 (reviewed by Morgan 2007). Significant advances in our knowledge of the regulatory mechanisms have been made with the identification of the main effectors of cell cycle system: cyclin-dependent kinases (Cdks) and their regulators.

2.1. Cdks (Cyclin-dependent kinases)

Cyclin-dependent kinases are protein complexes that possess a serine/threonine kinase catalytic subunit (Cdk) and a cyclin regulatory subunit. Higher eukaryotes have several Cdks but Cdk1 and Cdk2 appear to be the major catalytic subunits of the regulatory complexes during cell cycle transitions (reviewed by Morgan 1997). Cdk activity and specificity towards its substrates depends primarily on the association with different cyclin regulatory subunits. Cell cycle regulation of Cdk activity is mostly achieved by modulating cyclin levels at different stages of the cell cycle. Therefore, cyclins are regulated at the transcriptional level allowing a stage-specific accumulation of cyclins and a transient Cdk maximum activity, followed by rapid degradation of cyclin once the cell enters the next step of the cell cycle (Fig. 4). Thus, different cyclins are produced at different cell-cycle stages resulting in the formation of different cyclin-Cdk complexes that temporarily determine which target proteins will be phosphorylated by Cdks. The control over the activity of Cdk-cyclin complex ensures that cells replicate its DNA only once and that chromosomes undergo proper segregation to their daughter cells following mitosis (reviewed by Diffley 2004). Although cyclin expression/degradation works with a satisfactory efficiency, cells also regulate Cdks activity by phosphorylation of the catalytic subunit at specific cell cycle stages. In G_1 and S-phase, Cdks are also regulated by the expression or activation of CKI (Cdk inhibitor) and CAK (Cdk-activating kinase) (reviewed by Pines 2006). As a result of the combination of these regulatory processes, precise Cdk-cyclin complexes are activated at different times through the cell cycle which are then responsible for changes in the

biochemical status of cell division machinery in order to activate specific factors that carry out each cell cycle event. Hence, the orchestrated mechanisms that control Cdk activity are of major importance in several crucial steps of cell cycle.

The first regulated step is the G_1/S transition and it is considered as a decisive starting point for cells to proliferate. In a resting cell, APC/C^{Cdh1} keeps mitotic Cdks inactive. However, G_1 cyclins (cyclin D in vertebrates), in response to mitogens, induce transcription of all the machinery required for DNA synthesis, so that G_1/S cyclin (cyclin E) accumulates and activates Cdk2. In turn, the Cdk2/cyclin E complex inactivates APC/C^{Cdh1} and promotes destruction of CKIs. After this point the cell is committed to proliferate. Then G1/S-Cdk activate the accumulation of S-cyclins (cyclin A in vertebrates) allowing the formation of the S-Cdk complex that triggers DNA replication at the onset of S phase (reviewed by Diffley 2004). After DNA is completely replicated, cells enter G_2 and prepare for entry into mitosis. To do so, cells accumulate the M-cyclin (Cyclin B in vertebrates) leading to the activation of M-Cdk complexes. The M-Cdk is mainly dependent on Cdk1, whose activation depends on binding to Cyclins A/B, as well as, on the removal of two inhibitory phosphates. These inhibitory phosphorylations are performed by the kinases Wee1 and Myt1, and only at G_2/M transition are removed by the Cdc25 phosphatase (reviewed by Perry and Kornbluth 2007). Once activated, Cdk1-Cyclin B can now phosphorylate and regulate a large number of targets, promoting the changes that occur in mitosis, such as reorganization of the cytoskeleton, chromosome condensation, nuclear envelope breakdown and assembly of the mitotic spindle (reviewed by Morgan 1997).

At the metaphase to anaphase transition, the APC/C^{Cdc20} is activated inducing first the destruction of cohesion between sister chromatids. Also, at this stage S cyclins and M cyclins are prompted for degradation by the APC/C^{Cdc20} leading to the subsequent inactivation of Cdks. To complete late mitotic stages APC/C^{Cdc20} induces degradation of its own activator Cdc20. The formation of APC/C^{Cdh1} promotes completion of mitosis and cytokinesis so that cell can now reach the next G_1. Cells will remain in G_1 until new mitogenic signals are received or if prompted for differentiation (reviewed by Pines 2006).

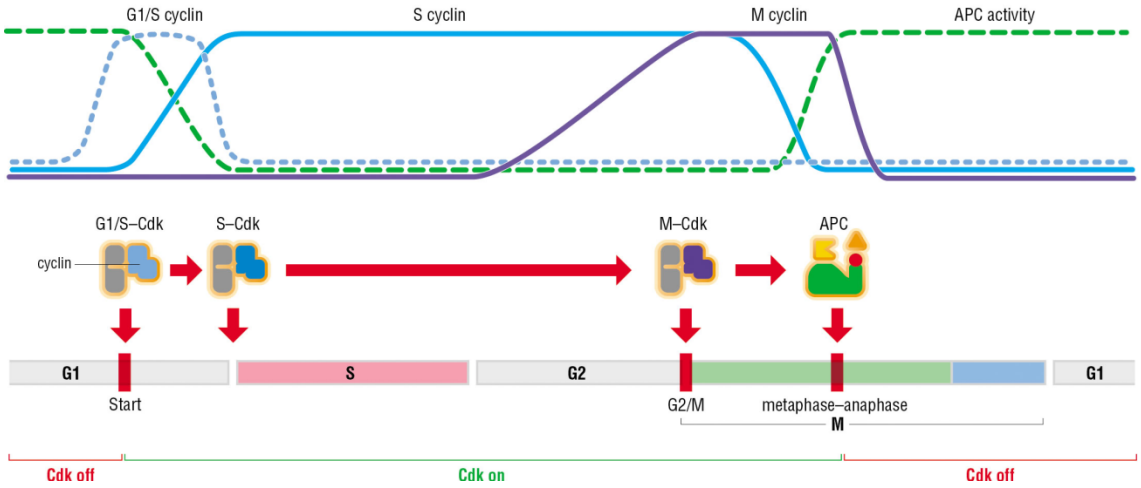

Figure 4: Regulation of Cdk activity throughout cell cycle. The dashed green line represents APC/C activity: APC/C activity drops when the cell is committed to divide (G_1/S transition) and remains very low until anaphase is triggered; activity of the G_1/S-Cdk is represented with dashed light blue line and peaks at the G_1/S transition; light blue line represents S-Cdk complex activity, required for DNA replication; dark blue line represents activity of M-Cdk mainly active in G_2/M transition until metaphase, where the levels of M-cyclin began to drop do to its degradation by the APC/C;(Adapted from Morgan 2007)

2.2. Other important mitotic kinases (Auroras and Polo)

Given that during mitosis the genome does not show any significant expression (Gottesfeld and Forbes 1997), protein degradation of the various cyclins leads to irreversible transitions. Therefore, post-translational modification of proteins involved in mitosis is one of the most important strategies used for reversible control during cell division. Together with Cdks, there are other kinases that play key regulatory roles, particularly in mitosis, namely Aurora kinase family and Polo-like kinases.

2.2.1. Aurora kinases

Auroras are part of an evolutionary conserved Threonine/Serine protein kinase family. Whereas there is only one Aurora protein in yeast (the founding member Ipl1 in S. Cerevisiae and Ark1 in S. Pombe), metazoans have at least two Aurora genes: *aurora-a* and *aurora-b*. In mammals there is another member of this family, Aurora C (Bernard et al. 1998), shown to perform Aurora B functions in the mammalian testis (Tang et al. 2006).

Aurora C kinase function seems to be restricted to meiotic tissues however both Aurora A and B have been shown to have important roles during mitosis. Aurora A localizes to centrosomes in interphase and spindle poles during mitosis. It was firstly described in Drosophila where it was shown to be required for centrosomes maturation and separation (Glover et al. 1995; Giet et al. 2002). Further studies showed that Aurora A is also involved in promoting mitotic entry (Marumoto et al. 2002; Hirota et al. 2003), spindle assembly and asymmetric cell division (Berdnik and Knoblich 2002). As part of the EXTAH complex (Eg5, XMAP215, Tpx2, Aurora A and HURP) is was also shown to be important for acentrosomal spindle assembly pathway (Sauer et al. 2005; Koffa et al. 2006). Hence, Aurora A plays a major role in mitotic spindle assembly (Barr and Gergely 2007).

On the other hand, Aurora B is very important to control sister-chromatid structure and kinetochore-microtubule attachments. Together with its CPC (Chromosome Passenger Complex) partners – the proteins INCENP, Survivin and Borealin, Aurora B has three main functions: (1) regulation of chromosome condensation and resolution (Giet and Glover 2001); (2) a key role in the correction of erroneous microtubule-kinetochore attachments (Ditchfield et al. 2003; Hauf et al. 2003); (3) coordination of chromosome segregation and cytokinesis (Norden et al. 2006; Ruchaud et al. 2007).

2.2.2. Polo kinase

For many, Polo is the major mitotic regulator since it has major functions during mitotic entry, spindle organization, mitotic progression and mitotic exit. Polo-like kinase family consist in highly conserved proteins. In yeast exists as a single gene (cdc5), whereas in metazoans there are at least two Plks genes (Drosophila has Polo and Plk4 also known as SAK) and in Humans there are four Plks genes described to date (reviewed by Archambault and Glover 2009). All these genes are transcribed into proteins that share a common domain, the PBD (Polo-box domain), required for interaction with other proteins (Lowery et al. 2005). Polo binding to its substrates often requires prior phosphorylation of target proteins by Cdks or Auroras kinases in order to direct Polo to its targets (Elia et al. 2003; Elia et al. 2003). This cooperation demonstrates how interdependent these kinases are to carry out their main functions. The activity of Polo in mitosis starts very early, at mitotic entry (Hagan 2008). At this stage Polo participates together with Cdk1 in the activation of Cdc25 (String in Drosophila), the Cdk1-activating phosphatase, and at the same time inhibits Myt1

(Inoue and Sagata 2005) and phosphorylates Wee1 promoting its degradation. Hence, Polo activity is required for full activation of Cdk-M cyclins complexes (Kumagai and Dunphy 1996; Mulvihill et al. 1999; Watanabe et al. 2004; Hagan 2008).

Since its identification, Polo and Aurora A have been shown to be responsible for the organization and maturation of centrosomes (Sunkel and Glover 1988; Glover et al. 1995). Studies done in metazoans show that Polo is required for centrosomal maturation: directly - through centrosomal recruitment of the γ-tubulin ring complex (γ-TuRC) and activation of Abnormal spindle (Asp) (do Carmo Avides et al. 2001; Donaldson et al. 2001), and indirectly – through the control of centrosomal localization of Aurora A kinase (De Luca et al. 2006). The role of the Polo family in centrosome regulation is not restricted to centrosome maturation. Recently, another member of the Polo family Plk4 in Humans and SAK in Drosophila was demonstrated to be essential for centriole duplication (Bettencourt-Dias et al. 2005; Habedanck et al. 2005). Cells from Drosophila mutants for Plk4 lose their ability to duplicate centrioles, and lose the centrioles through development. Overexpression of Plk4 leads to supernumerary centrioles (Rodrigues-Martins et al. 2007) in a similar fashion to the SkpA mutant (Murphy 2003), and in fact the control of centriole duplication has been shown to be dependent on the targeting to degradation of Sak by Slimb (as SkpA), a component of the SCF ubiquitin ligases (Cunha-Ferreira et al. 2009).

Polo is targeted for degradation by the APC/C^{Cdh1} complex, but just before its degradation Polo works to promote successful mitotic exit (Eckerdt and Strebhardt 2006). Plo1 (*S. pombe* Polo homologue) and Cdc5 (*S. cerevisiae* Polo homologue) mutants show defects in late events of mitosis, demonstrating that Polo is an upstream activator of SIN (Septation Initiation Network) and MEN (Mitotic Exit Network) which are, respectively, the mechanisms of mitotic exit (reviewed by Archambault and Glover 2009). In turn studies in higher eukaryotes suggest that Polo is responsible for the creation of a phosphodegron (degradation signal) in Emi1, a protein that inhibits APC/C (Hansen et al. 2004; Moshe et al. 2004). Then Emi1 is targeted to degradation and APC/C becomes active to target Securin to degradation, the inhibitor of Separase, releasing Separase to induce progression to anaphase (Lenart et al. 2007). Targeting of Polo to the central spindle in late mitosis depends on its interaction with PRC1 (Protein Regulator of Cytokinesis 1) (Neef et al. 2007) and MKLP2 (Mitotic Kinesin-Like Protein 2) (Neef et al. 2003). At this location in late mitotic stages, Polo recruits the RhoA GTPase exchange factor Ect2 (Burkard et al. 2007), leading to activation of the RhoA GTPase that, in turn, will trigger the assembly of cytokinetic actomyosin ring and ingression of the cytokinetic furrow (Petronczki et al. 2007).

Due to its involvement in several key stages of the cell cycle, Polo itself is regulated in several ways. Polo levels are regulated both at the level of transcription and also degradation, as levels of transcripts peak at G2/M transition (Uchiumi et al. 1997) and degradation of Polo is promoted by the APC/C^{cdh1} at mitotic exit (Lindon and Pines 2004). While Polo protein levels remain high in mitosis, different levels of Polo activity can be found. Modulation of Polo activity is achieved in two ways: (1) indirectly, as Polo substrates require priming phosphorylation by other kinases in order to interact with Polo; hence, priming phosphorylation events work as a switch in targeting Polo to specific substrates or structures: (2) directly, as different kinases can phosphorylate well conserved residues in Polo protein leading to its full activation (Jang et al. 2002). The two main Polo phosphorylation sites are the Thr210 and the Ser137 residues (Qian et al. 1999). Phosphospecific antibodies raised against these residues show that its phosphorylation is sequential in mitosis: while Thr210 is phosphorylated in early mitotic stages, Ser137 is phosphorylated at late mitotic events. In fact these phosphorylations have mitotic relevance as phosphomimicking mutations T210D and S137D causes different phenotypes. Polo-T210D causes a mitotic arrest dependent on spindle assembly checkpoint, while Polo-S137D induces premature activation of the APC/C to orchestrate the mitotic exit (van de Weerdt et al. 2005). Both mutations promote Casein's phosphorylation by Plk1 (the human homologue) and a premature mitotic entry. Phosphoblocking mutation S137A arrests cells in mitosis in a metaphase-like configuration for long periods. One can postulate that at mitotic entry Plk1 has to be phosphorylated at Thr210 in order to organize the mitotic apparatus and, after metaphase alignment, this residue should be dephosphorylated and a new residue should be phosphorylated, the Ser137, in order to activate APC/C^{cdh1} and exit mitosis properly.

3. Cell cycle checkpoints

Although the cell cycle is driven by robust mechanisms that promote unidirectional progression, these mechanisms need to be monitored throughout the different events since errors can occur due to internal fluctuations or external aggressions. These surveillance mechanisms are collectively called checkpoints and actively control cell cycle progression although they are not themselves necessarily intrinsic to the molecular processes that drive the cell cycle (Weinert and Hartwell 1989). Checkpoints guard critical cell-cycle transitions by ensuring that the previous phase is completed

and error-free before the cell is allowed to proceed to the next step. Checkpoint activation arrests the cell before initiation of the next step in the cycle providing time for repair mechanisms to correct these errors. Checkpoints appear to monitor all the major transitions during the cell cycle.

3.1. Interphase checkpoints

The main objective of the interphase checkpoints is to maintain genomic integrity. In order to maintain DNA integrity, these checkpoints hold progression through the cell cycle and give sufficient time to repair any DNA damage.

During G_1, the DNA damage checkpoint monitors DNA structure and prevents transition through the G_1/S transition. This checkpoint is essential since the presence of DNA damage in template strands would lead to fixation of mutations during DNA replication. Once the cell has passed the G_1/S transition other checkpoint components monitor the completion of DNA replication before allowing the cell to progress through the G_2/M transition into mitosis.

Both transitions involve the DNA damage checkpoint. This checkpoint detects DNA lesions such as single strand DNA or DNA double strand breaks, stops cell cycle progression and triggers DNA repair (reviewed by Elledge 1996). In the presence of DNA damage, ATM (Ataxia Telangiectasia mutated) (Pandita et al. 1995) and ATR (ATM-Rad3 related) kinases phosphorylate p53 leading to increased levels of this transcription factor. To hold the cell cycle, p53 will promote transcription of its target p21, a Cdk inhibitor (CKI) that in turn will inhibit the activity of Cdk2-cycE, preventing DNA replication. If DNA damage occurs in G2, ATM activates the kinases Chk1 and Chk2 that inhibit Cdc25 by phosphorylation, so that it is unable to activate Cdk1-CycB and consequent mitotic entry is halted. In addition, kinases that modulate Cdc25 activity such as Plks and Wee1 may also be inhibited by ATM/ATR (Niida and Nakanishi 2006; Clarke and Allan 2009; Reinhardt and Yaffe 2009; Dai and Grant 2010).

3.2. Spindle assembly checkpoint (SAC)

Once cells have entered mitosis, chromosome condensation occurs and the mitotic spindle is built. However, prometaphase chromosomes must establish stable

and correct interactions with microtubules before segregation of chromatids can occur. Interestingly, if the spindle is prevented from forming, for example, by exposure to microtubule depolymerising drugs during mitosis, cells arrest and sister chromatids do not separate. This observation suggested that cells are able to sense the absence of (correct) interactions between the spindle and chromosomes and, consequently, delay sister chromatid separation and anaphase onset. A major advance in the characterization of this checkpoint came from two parallel genetic screenings in budding yeast. These studies searched for mutants that are unable to arrest in the presence of a microtubule depolymerising drug and were named as *bub 1-3* (**b**udding **u**ninhibited by **b**enomyl) and *mad 1-3* (**m**itotic **a**rrest **d**efective) (Hoyt et al. 1991; Li and Murray 1991). These proteins were shown to be conserved throughout evolution as well as its functions in holding cells in mitosis in the absence of a proper microtubule-kinetochore attachment. The only exception was the identification in higher-eukaryotes of the Bub1-related kinase (BubR1) which shows homology in its N-terminal part to the yeast Mad3 protein and in its C-terminal part with the kinase domain of Bub1 (Taylor et al. 1998). These were the first set of molecular components of the spindle assembly checkpoint or SAC. Since its discovery, the SAC has been seen as an essential surveillance mechanism that uses the kinetochore as platform to monitor interaction between kinetochores and microtubules (attachment), as well as to ensure that tension between sister kinetochores is established (an indirect way of certify that sister chromatids are attached to opposite poles). Several independent experiments with cultured cells demonstrated that a single unattached kinetochore is able to send a “wait anaphase signal allowing enough time to correct errors in kinetochore-microtubule interaction before the beginning of chromosome segregation in anaphase (McIntosh 1991; Rieder et al. 1995). Other studies have elucidated which SAC proteins are involved in monitoring attachment or tension. The results are consistent with the idea that Mad2 is the key protein in monitoring attachment while BubR1, either directly or indirectly, monitors tension (Logarinho et al. 2004; Malmanche et al. 2006). However, current models return to the idea that only unattached kinetochores are able to maintain SAC activity. Accordingly, the mitotic delay attributed to lack of tension indeed corresponds to unattached kinetochores that recruit Mad2 and block anaphase onset (reviewed by Musacchio and Salmon 2007). Such unattached kinetochores are promptly generated by Aurora B kinase when tension is not exerted across the kinetochore pair – a process known as “correction mechanism”.

At the molecular level, APC/C inhibition is proposed to occur in two steps (Chan et al. 2005; Orr et al. 2007). It was shown that at the beginning of mitosis APC/C inhibition involves the formation of a complex including BubR1, Bub3 and Mad2 called the Mitotic

Checkpoint Complex (MCC) that sequesters the APC/C activator Cdc20 (Sudakin et al. 2001; Tang et al. 2001). Formation of the MCC complex was shown to be independent of unattached kinetochores as it occurs before NEBD, and is seen as a timer that allows Cyclin B accumulation and consequently entry into mitosis (Lopes et al. 2005). The second step of inhibition is related to monitoring microtubule-kinetochore binding. In the presence of an unattached kinetochore, checkpoint proteins Mad2 and BubR1 are targeted to this structure with a fast turnover, reinforcing the activity of the MCC and thus inhibiting the metaphase-anaphase transition (Howell et al. 2004; Shah et al. 2004). Recently, it was shown that this mechanism acts mainly through Mad2, a SAC protein that may be present in two different conformations, Mad2-open (Mad2-O) and Mad2-closed (Mad2-C). Free cellular Mad2 exists mainly in the Mad2-O conformation. However, upon binding to Mad1, a Mad1-Mad2-O complex is formed and targeted to unattached kinetochores (Luo et al. 2002; Sironi et al. 2002). At unattached kinetochores, this complex was shown to promote the conversion of Mad2-O to the closed form (Mapelli et al. 2007) that has affinity for Cdc20 binding (Yang et al. 2008). The Mad2-C-Cdc20 complex is released from the kinetochore and thought to promote cytoplasmic conversion of the free Mad2-O molecules into the closed Mad2 form. Such pathway allows the formation of new complexes with Mad2-C-Cdc20, leading to the cytosolic amplification of the inhibitory signal. In fact, it was shown that Mad2-C-Cdc20 complex can form a full MCC complex by binding the BubR1:Bub3 both in unattached kinetochore and cytoplasm (Essex et al. 2009; Kulukian et al. 2009). Although the proteins involved in the formation of the “wait” anaphase signal are well known and characterized, the dynamics and the precise mechanism that shut down SAC signalling is still under debate. Removal of checkpoint proteins from the kinetochore by the Dynein complex is the most accepted model (Howell et al. 2001; Wojcik et al. 2001).

3.3. NoCut checkpoint

DNA damage checkpoint and SAC are not the only checkpoints that monitor events during the cell cycle. One of the first attempts to identify mitotic genes was a genetic screen in *S. pombe*, which searched for temperature sensitive mutants that undergo uncoordinated mitosis. Some of the mutants identified were able to initiate cytokinesis without prior completion of nuclear division, resulting in cleavage of the nucleus by the septum; therefore, these mutants were described as *cut* (*cell untimely torn*) mutants (Hirano et al. 1986; Samejima et al. 1993). The uncoordination

phenotype between chromosome segregation and cytokinesis was accepted until recently. However, studies in *S. cerevisiae* clearly showed that abscission and completion of cytokinesis is prevented by a so-called NoCut checkpoint in the presence of either DNA within the septum (Mendoza et al. 2009) or spindle elongation impairment (Norden et al. 2006). This NoCut pathway activity was demonstrated to be associated to the activity of the annilin-related proteins Boi1 and Boi2 under the control of the mitotic kinase Ipl1 (AuroraB kinase in metazoans) (Norden et al. 2006). Thus, it was suggested that the NoCut pathway is active in every division as mutants for *boi1* and *boi2* prematurely complete cytokinesis (reviewed by Mendoza and Barral 2008). Furthermore, the NoCut checkpoint seems to be conserved as recent studies in a human cell line corroborates the previous studies done in *S.cerevisiae* (Steigemann et al. 2009). Although pioneer studies in *S.pombe* clearly show the *cut* phenotype, it does not mean that NoCut pathway is not working in this organism. Further identification and analysis of the *cut* genes showed that some of them correspond to proteins that are also involved in the formation of the NoCut response (Yanagida 1998; Mendoza et al. 2009). So, the *cut* phenotype observed *S.pombe* is due to genes that not only have a role in chromosome segregation but also in the NoCut pathway. A similar *cut* phenotype is observed in other organisms when both DNA segregation and NoCut pathway are compromised (Mendoza et al. 2009).

4. Kinetochore assembly pathway

Kinetochore is a term that came from the Greek ‘kineto-’ that means ‘move’ and ‘-chore’ meaning ‘means for distribution’, and, as the name says, kinetochores are the structures responsible for chromosome movement. Together with its function in microtubule binding and chromosome movement, kinetochores are also the structures that serve as platform for the formation of the “wait” anaphase signal, as previously described.

The kinetochore is composed by a number of multi-protein complexes that assemble in an interdependent manner at a specific chromosome locus called the centromere. The centromere was originally described as the primary constriction region of chromosomes. Nowadays it is defined as the DNA sequence where kinetochores will assemble. Since, centromeres also function in specifying the place where kinetochore will assemble and this chromosomal locus defines attachment and movement of chromosomes. Moreover, centromere ensures sister chromatid cohesion until

metaphase-anaphase transition (reviewed by Chan et al. 2005). Eukaryotic centromeres are highly variable in size and sequence and DNA sequence is not conserved between different species and even between different chromosomes, as in the case of Drosophila (Henikoff et al. 2001). Despite these divergences, all the eukaryotic kinetochores are built on a fundamental chromatin packed unit, the nucleosome, where histone H3 is replaced by a variant: CenH3 (also known as Cse4p in budding yeast, CID in Drosophila and Cenp-A in Humans) (reviewed by Chan et al. 2005). These modified nucleosomes act as the centromere identifier complex that is required for the assembly of multiple complexes in a hierarchal order to form the whole structure of the kinetochore (reviewed by Chan et al. 2005).

Several clues to unravel kinetochore assembly came from studies in budding yeast that only binds a single microtubule but it is highly complex and presents the conserved functions of the kinetochore. The centromeric DNA in the budding yeast is a 125-bp region that contains three conserved elements CDE I, CDE II and CDE III. To the CDE III element bind in a sequence specific manner a complex with a molecular mass of 240 kDa - the CBF3 complex - that is composed of proteins Ndc10p, Ctf13p, Cep3p and Skp1p. The assembly of CBF3 complex is required for the association of the other known kinetochore proteins with centromeric DNA (McAinsh et al. 2003). An initial step in the kinetochore assembly pathway in budding yeast involves the chaperone Hsp90 and its co-chaperone protein Sgt1 (Kitagawa et al. 1999).

4.1. Chaperones and co-chaperones

Chaperones are a family of proteins crucial for cell homeostasis, cell cycle control and response to environmental stresses, as DNA damage, heat shock and other physical injuries. Therefore, these proteins have three main functions: (1) to promote the correct folding of newly synthesized polypeptide; (2) for transport and activation of client proteins to certain structures or through membranes; and (3) in stress response to situations that challenge cellular homeostasis. The temporal and spatial control of chaperone functions is made by different interactions with diverse co-chaperones. One example of these interactions is the role of Sgt1 in directing Hsp90 to the kinetochore assembly pathway (Bansal et al. 2004).

4.2. Sgt1

Sgt1 was firstly described as a supressor of Skp1, a component of the SCF ubiquitin ligase complex. Several different domains are recognized in the Sgt1 protein: the tetratricopeptide repeat domain (TPR), the CHORD or p23-like central domain (CS) and the Sgt1-specific domain (SGS). Both TPR and CS domains are important for binding of Sgt1 to the chaperone Hsp90 and, in this complex plays an essential role for kinetochore assembly in yeast (Kitagawa et al. 1999), plant disease resistance pathway (reviewed by Muskett and Parker 2003) and in inflammatory responses and kinetochore assembly in higher eukaryotes (Steensgaard et al. 2004; Mayor et al. 2007).

The importance of Sgt1 in cell cycle progression was first highlighted when yeast cells carrying a Sgt1 temperature sensitive allele were shown to delay the G_1/S and G_2/M transitions. Analysis of yeast mitotic cells revealed that the G_2/M transition phenotype was caused by impaired kinetochore assembly pathway demonstrating an important role in cell division (Kitagawa et al. 1999). Currently, it is thought that dimers of Sgt1 (Bansal et al. 2009) bind to the chaperone Hsp90 and that this complex increases the ability of Sgt1 to associate and activate Skp1, directing it to Ctf13 an essential kinetochore component. Subsequently, Ctf13 binds other components of the CBF3 complex which forms the core of the yeast kinetochore and is the first step in the assembly of kinetochores in *S.cerevisiae* (Lechner and Ortiz 1996). Sgt1 has also been shown to be important for kinetochore assembly in Human cells (Steensgaard et al. 2004), as depletion of Sgt1 by RNA interference in HeLa cells results in abnormal kinetochore structure and a number of outer kinetochore proteins - such as Hec1, CENP-E, CENP-F and CENP-I - fail to accumulate. Such abnormal kinetochore structure results in abnormal microtubule-kinetochore attachment and failure in chromosome congression (Steensgaard et al. 2004). Interestingly, kinetochore accumulation of proteins involved in the spindle assembly checkpoint (SAC), such as BubR1, Mad1 and Mad2, is also impaired and, although cells are delayed in prometaphase, SAC is significantly weakened (Steensgaard et al. 2004). Similar effects upon kinetochore assembly were also demonstrated after both depletion or inhibition of Hsp90, phenotypes that are suppressed by Sgt1 overexpression (Niikura et al. 2006). Taken together these results suggest that the Sgt1-Hsp90 complex has a conserved role in the kinetochore assembly pathway in these species.

Although there is a consensus that Sgt1 and Hsp90 interact, different studies have shown that this interaction occurs through different domains in different species so that

in yeast, Sgt1 interacts with Hsp90 mostly by the TPR Domain (Bansal et al. 2004; Lingelbach and Kaplan 2004) while in metazoans it uses the CS Domain (Lee et al. 2004). Importantly, it has been shown that the stability of Polo is directly linked to Hsp90 activity (de Carcer et al. 2001). While the role of Polo at kinetochores is still under debate, it is known to be essential for centrosome maturation and spindle organization for which its interaction with Hsp90 appears to be essential (de Carcer et al. 2001). However, it is interesting that the mitotic phenotypes resulted from mutation or depletion of Sgt1 in yeast and human share common features, including delay in mitotic progression, abnormal spindle function and failure in chromosome congression, suggesting that SAC is, at least, partially active. Thus, it is essential to determine whether Sgt1 is also required for the stability and localization of Polo homologues in yeast and human cells, given that inhibition of Hsp90 decreases the activity of Plk1 in HeLa and in some tumour cells (de Carcer 2004).

4.3. Hsp90

Firstly described in yeast, Hsp90 is a protein of 90KDa found to be upregulated when cells are exposed to heat shock (Finkelstein and Strausberg 1983). Later, it was shown that it is one of the most abundant proteins also in unstressed cells and it is highly conserved throughout evolution (Csermely et al. 1998). Hsp90 is characterized as having three main distinct functional domains and one charged domain, a linker that confers mobility to the N-terminal protein domain (Fig. 5). The three main domains consist in a N-terminal ATPase domain, a middle domain that is highly charged and implicated in client protein binding, and a C-terminal dimerization domain that also binds ATP. Studies showed that through its C-terminal Hsp90 constitutively forms and works as a homodimer (reviewed by Hahn 2009). Co-chaperones containing the TPR domain were shown to interact with Hsp90 through an EEVD peptide motif present at the C-terminal end of the protein that directs the complex to a specific subset of client proteins. Yet, binding of co-chaperones is not exclusive of this motif as recent studies showed that Sgt1 and Cdc37 co-chaperones can also associate with Hsp90 through its N-terminal domain (Lee et al. 2004; Roe et al. 2004; Zhang et al. 2008).

The interaction of Hsp90 with a variety of client proteins and its involvement in cellular pathways has interested the scientific community for many years. The fact that Hsp90 levels often are 2- to 10-fold higher in several cancer types also contributes to the relevance of studies on Hsp90 function (Ferrarini et al. 1992; Gress et al. 1994;

Yano et al. 1996), and makes of Hsp90 a desirable target for anti-cancer drug development. Upregulation of Hsp90 was found to be fundamental in stabilization of many oncogenic proteins such as Her2/ErbB2, Akt, Raf-1, Hif-1alpha, hormone receptors, Survivin, Cdk4, mutant p53 and hTERT (for access to the continuous growing list, visit http://picard.ch) (Fig. 7).

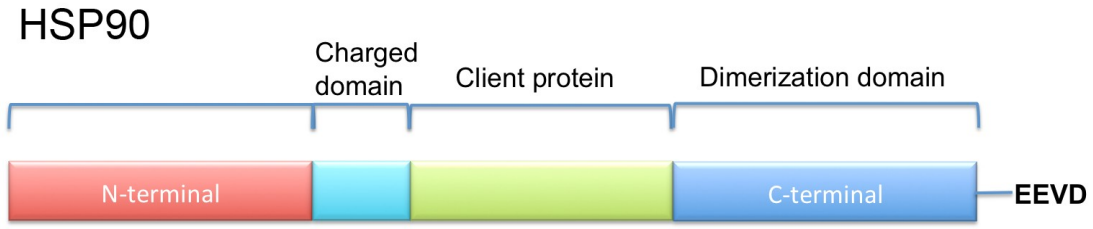

Figure 5: Schematic structure of Hsp90. Functional domains of Hsp90 are represented: N-terminal region (red), middle region (green) and C-terminal homodimerization region. The charged domain between the N-terminal and middle domains serves as a flexible linker. The middle domain is the responsible to bind the client proteins. The C-terminal domain ends in an EEVD-peptide motif which is recognized by co-chaperones carrying a TPR (tetratricopeptide repeat) domain. ATP can bind both N- and C-terminal.

Inhibition of Hsp90 leads to simultaneous degradation of these oncogenic proteins by a ubiquitin-dependent proteasome pathway (Miller et al. 1994). The first known Hsp90 inhibitor, Geldanamycin (GA), emerged in a search for benzoquinone ansamycin antibiotics from *Streptomyces hygrocopicus* in 1970 (DeBoer et al. 1970). Recent efforts were made in order to develop analogues and find new Hsp90 inhibitors because Geldanamycin has several clinical limitations. Thus, several derivatives were developed such as 17-allylamino-17-demethoxygeldanamycin (17-AAG) and 17-dimethylamino-ethylamino-17-demethoxygeldanamycin (17-DMAG). Interestingly Hsp90 present in cancer cells has 100x more affinity to 17-AAG than Hsp90 present in non-transformed cells (Kamal et al. 2003). Therefore, Hsp90 inhibition can be an attractive and effective target in transformed cells that may circumvent the current chemotherapy treatments. Given the high sensitivity of Hsp90 in cancer cells and the number of oncogenic pathways regulated by Hsp90, it has been an almost perfect target for anti-cancer treatment alone or in combination with other agents or radiations (Camphausen and Tofilon 2007). Indeed, Geldanamycin derivatives show promising results in clinical trials (Solit et al. 2007; Solit et al. 2008; Fukuyo et al. 2010) and, at low nanomolar concentrations, have potent anti-cancer activity in several human xenograft models for different cancer types (Banerji et al. 2005; Hollingshead et al. 2005).

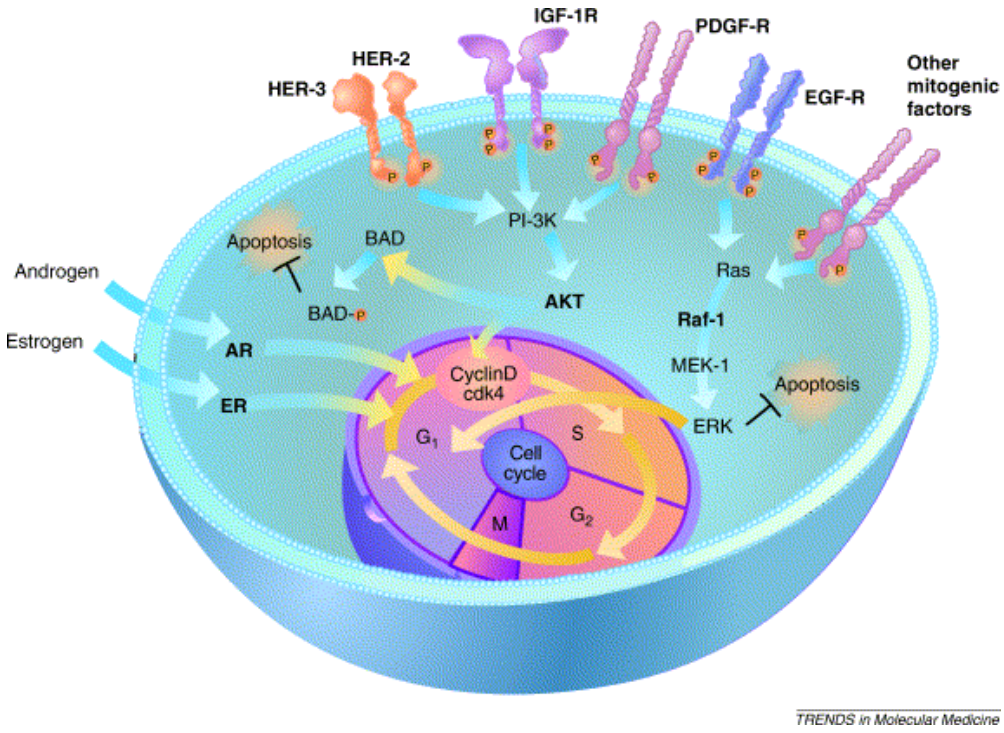

Figure 6: Hsp90 client proteins regulate multiple signal transduction pathways that are deregulated in cancers. Hsp90 client proteins (shown in bold) include key components of the mitogenic signaling that drives cell-cycle proliferation, as well as survival signal transduction pathways that inhibit apoptosis. (Adapted from Kamal et al. 2004);

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

5.1. Overview

Drosophila melanogaster has been used as a model system for genetics since it was first introduced by Morgan at Columbia University in the early 1900s. As a genetic model organism, the fruit fly has been used to unveil several cellular processes and has played a central role in developing models for the genetic basis of embryonic development. Drosophila genome contains about 14,000 genes, half of the number of genes present in *Homo sapiens*. Yet, it contains many genes that have human homologues and that are involved in a variety of human diseases. The Drosophila genome is fully sequenced and there are numerous molecular and genetic techniques that have allowed major advances in our understanding of basic biological process.

5.2. Drosophila life cycle

Early development in Drosophila is very well studied. Upon fertilization, the zygote nucleus undergoes a series of rapid and synchronous divisions called the nuclear multiplication stage. During this stage nuclei replicate their DNA and undergo mitosis without intervening gap phases or cytokinesis. These nuclear cycles take about 10 minutes and syncytial divisions are maintained until the end of division 13, when cellularization occurs. This process consists in a massive cytokinesis and leads to individualization of nuclei into a well organized layer of single cells. After cellularization, zygote gene expression becomes significant allowing the transition from maternal products previously deposited in the egg to new proteins produced by the zygote. During subsequent stages of development, cells in the embryo will continue to proliferate and give rise to all embryonic layers that will transform into a complex multicellular organism (Foe 1989).

After embryogenesis, the embryo hatches as a first instar larva that grows continuously in size, mainly by polyploidization, reaching the second and third instars. After larval development methamorphosis takes place - pupa stage (Fig. 7). At the beginning of pupariation most of the precursors of the adult structures that where specified during embryogenesis and that grew in sacs of cells - the imaginal discs – during larval development, originate the adult structures of the fly (Fig. 8).

5.3. Advantages of using *Drosophila melanogaster* to study cell cycle

The fact that Drosophila has been used as a model system for many years allows us to take advantage of a large variety of available tools. Its genome is fully sequencing (Adams et al. 2000) and several projects that aimed at establishing a collection of well-defined mutations for every gene in the fly are available (Spradling et al. 1995; Spradling et al. 1999). Most of these mutations were induced by insertions of transposable elements and are currently precisely mapped to the genome so that nature of every mutation is well defined. Thus, these mutant collections are valuable tools that allow the genetic analysis of any Drosophila gene.

In the cell cycle field, studies in the fly can provide physiological significance to the absence of a precise gene, which is a major advantage when compared to studies performed in human (transformed) cell lines.

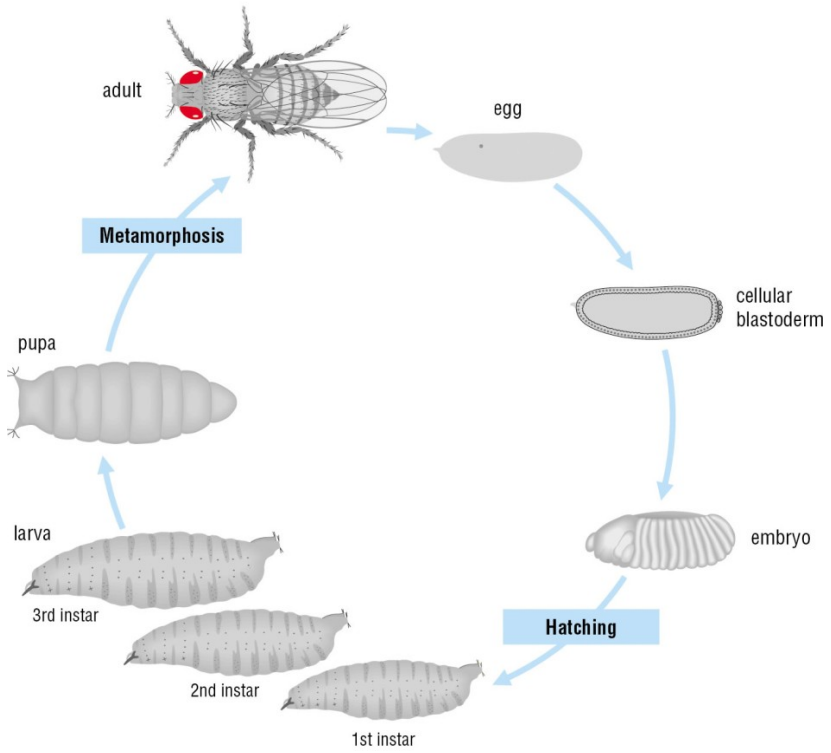

Figure 7: Life cycle of *Drosophila melanogaster*. Early syncytial divisions are followed by cellularization, resulting in the single layered cellular blastoderm, which is transformed into a segmented, multilayered embryo. Hatching of the embryo gives rise to the larva that grows through three instars before originating a pupa. Metamorphosis then leads to formation of the adult fly. (Adapted from Morgan 2007);

A very important methodology developed in Drosophila has been the possibility of controlling gene expression both in time and/or tissue using the GAL4/UAS system (Brand and Perrimon 1993). This system is based in the identification of random insertions of transposable elements that contain right next to one of the inverted repeats the coding sequence for the yeast GAL4 gene. In many of the lines carrying these insertions they have landed next to different genomic enhancers so that now the GAL4 gene is only activated in a particular tissue and at a particular developmental stage. Therefore, if one introduced a desired gene under the control of UAS sequences and combines this with a particular GAL4 Driver it is possible to express our gene of interest in virtually any Drosophila tissue at any developmental stage. In addition, it is also possible to induce somatic clones within virtually any tissue in Drosophila using the yeast- derived FLP/FRT system (Theodosiou and Xu 1998; Ryder and Russell 2003). By controlling the formation of a clone or the expression of a specific gene, it is possible to study the effect of a lethal mutation in a developing tissue within the context

of an otherwise normal organism. Finally, it is also possible to perform large scale screens to identify genetic interactions, using as a read out a specific tissue (Dietzl et al. 2007).

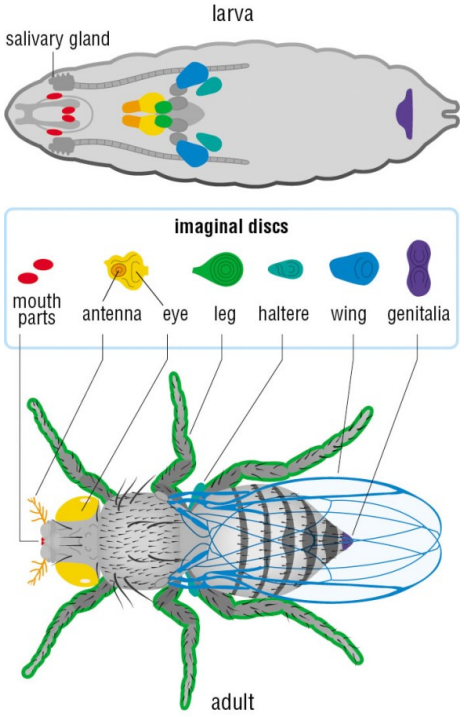

Figure 8: Imaginal discs and the consequent structure in the adult fly; (Adapted from Morgan, 2007)

5.4. Embryos and neuroblasts as mitotic model tissues

The two main Drosophila tissues used to study mechanisms that control cell cycle are developing embryos and larval neuroblasts (Fig. 9). Both systems allow good cytology and several cell cycle parameters can be studied. A combinational approach using these systems allows a full characterization of the genes involved in mitotic regulation. The selection of the tissue depends on the cell cycle function to be studied, as some aspects of the division cycles in embryos and neuroblasts are different. Early embryonic development is a suitable model to study mitosis since gap phases are not present at this stage of development. Moreover using embryos as a model allows visualizing in a single experiment several nuclei, which can be followed *in vivo* over a short period, this allows multiple measurements during successive division cycles in each experiment. One example is the pioneer studies on the dynamic behaviour of the Polo protein kinase which was done in the early embryo (Moutinho-Santos et al. 1999).

In addition, it is possible to induce different levels of inhibition of a target protein or structure through the microinjection of the syncytial embryo with a compound or antibodies that will result in a gradient effect from the site of injection spreading further away (Raff and Glover 1988; Brust-Mascher et al. 2009). The main limitations of the use of embryos are the lack of cytokinesis and the fact that these are nuclei, and not individual cells, that relay on a large pool of maternal products, which might affect the interpretation of results. The characteristic fast division can also be a disadvantage as embryonic nuclei have a weak mitotic checkpoint (Perez-Mongiovi et al. 2005).

Drosophila neuroblasts are neural stem cells that after each round of division give rise to an identical (stem) cell and to a smaller daughter cell (or ganglion mother cell-GMC) that is committed to divide twice before its progeny differentiate into a neural cell. Neuroblasts have a classical cell cycle, are very large which allows very good cytology and it is possible to follow their division *in vivo*. Neuroblasts display a robust response to microtubule damage allowing studies of SAC signalling. The main disadvantages of using neuroblasts as model are the fact that many mutations in genes required for cell division are lethal before the larvae reaches the third instar and that, at this stage, some of the phenotypes observed can be a pleyotropic effect derived from a cumulative failure in previous divisions. As stem cells, neuroblasts have to divide in a specific orientation under the control of specific determinants, therefore they are considered by many as an excellent model to study cell polarity (reviewed by Gonzalez 2007).

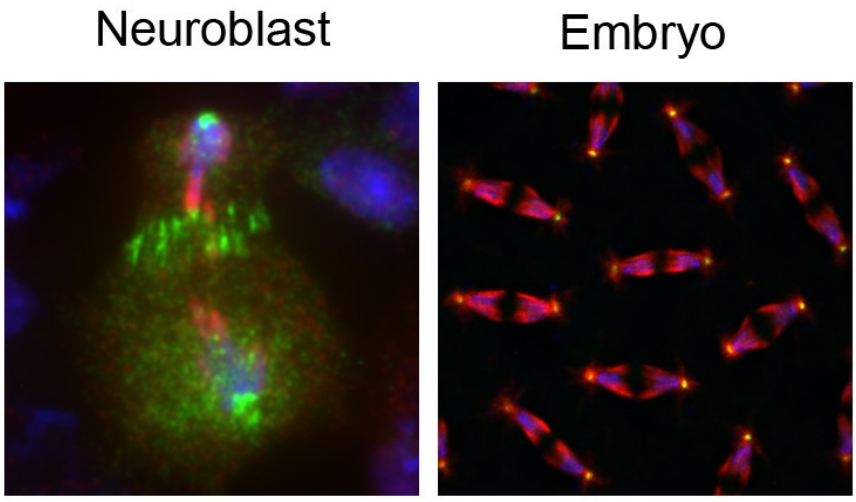

Figure 9: Anaphases in cell cycle models, neuroblasts and embryos; Neuroblast is stained for DNA (blue), for the mitotic marker PH3 (red) and for the mitotic regulator Polo (green): It is possible to observe the accumulation of Polo at the spindle central region between the stem cell (large neuroblasts) and the GMC (small cell); Embryo present the syncytial nucleus dividing synchronously, showing DNA (blue), mitotic spindle (red) and centrosomes (yellow);

In conclusion, one can say that *Drosophila melanogaster* is an excellent system to study the mechanisms that control cell cycle progression.

6. Objectives

The work presented in this thesis aims to address a number of questions. In the first chapter of the experimental work section, we present results from the study of the role of the chaperone complex Hsp90-Sgt1 in cell cycle progression, giving special emphasis to its putative role in kinetochore assembly pathway, kinetochore-dependent SAC signalling and its activity in centrosome maturation through stabilization of the Polo kinase. In the second chapter of the experimental work section, we address the relevance of chromosome size to spindle formation and mitotic timing in different type of cells and at different developmental stages. The last chapter describes the development and first stages of a genetic screen in the search for interactors of the Polo kinase.

PART II

EXPERIMENTAL WORK

Chapter 1

Sgt1, a co-chaperone of Hsp90, stabilizes Polo and is required for centrosome organization

1. Introduction

Accurate chromosome segregation requires organization and proper function of the mitotic apparatus, including correct attachment of sister chromatids to spindle microtubules. Microtubule-kinetochore interaction is highly complex and involves many factors. It is now clear that kinetochores are highly conserved and contain a large number of proteins, including internal structural components that ensure DNA binding, external components for microtubule binding and proteins involved in signalling processes, such as those required for the activation of the Spindle Assembly Checkpoint (SAC) (reviewed by Musacchio and Salmon 2007). Previously, it was suggested that in yeast and human cultured cells, assembly or at least proper localization of some kinetochore components requires the activity of the Hsp90 co-chaperone Sgt1 (Kitagawa et al. 1999; Steensgaard et al. 2004). Sgt1 was initially identified in a screen aimed to isolate suppressors of a temperature sensitive allele of *skp1* and characterized as a subunit of both the core kinetochore and SCF (Skp1-Cul1-F-Box) ubiquitin ligase complexes (Kitagawa et al. 1999). Sgt1 contains protein interaction motifs, such as the tetracopeptide repeat domain (TPR domain) and p23-like CHORD domain (also called CS domain), both found in proteins that interact with chaperones. In addition, a Sgt1-specific domain (SGS domain) required for its interaction with adenyl cyclase (Kainou et al. 2006) is also present. Human Sgt1 was shown to bind chaperone Hsp90 by its CHORD domain, very similar to the well-known Hsp90 co-chaperone p23 (Lee et al. 2004). Interaction between Sgt1 and Hsp90 was also described both in genetic and biochemical studies in yeast, and the protein complex was found to be important for assembly and turnover of the essential Centromere Binding Factor 3 (CBF3) (Bansal et al. 2004; Lingelbach and Kaplan 2004; Rodrigo-Brenni et al. 2004; Catlett and Kaplan 2006).

In human cells, severe reduction of Sgt1 levels was shown to compromise kinetochore assembly and specifically localization of SAC proteins, including Mad1, Mad2 and BubR1 (Steensgaard et al. 2004). This mislocalization results in a weak SAC response when cells were are challenged with spindle poisons that depolymerise microtubules. However, not only SAC proteins failed to accumulate, but also other constituents of the human kinetochore, including Hec1/Ndc80, CENP-E, CENP-F and CENP-I (Steensgaard et al. 2004). Interestingly, studies in human cells with an inhibitor of Hsp90 (17-AAG) suggested that this chaperone is important for kinetochore assembly, as it causes delocalization of various centromeric proteins, mitotic arrest, failure in chromosome congression and aneuploidy (Niikura et al. 2006). Moreover, it

was shown that depletion of Sgt1 causes cells to become sensitized to 17-AAG treatment, suggesting that Sgt1-Hsp90 co-chaperone is important for kinetochore assembly and function. Hsp90 has received much attention as a target for cancer therapy, given that this protein is involved in multiple pathways and is often overexpressed in a variety of tumours (Kamal et al. 2003).

In Drosophila, genetic studies showed that Hsp90 is required for the successful completion of mitosis because, in its absence, centrosomes fail to maintain their integrity (Lange et al. 2000). This centrosome phenotype was directly linked with an overall decrease in the levels of Polo, an essential mitotic kinase (de Carcer et al. 2001). Polo is involved in centrosome maturation during G_2 and is known to be involved in many other aspects of mitotic progression (Donaldson et al. 2001).

To address the function of Drosophila Sgt1, we have carried out a genetic analysis of Sgt1 in Drosophila. We identified the Sgt1 homologue and described its subcellular localization during mitosis. Analysis of mutant cells shows failure in mitotic entry. Yet, those mutant cells that do go into mitosis arrest in a prometaphase-like state with hypercondensed chromosomes. The mitotic arrest is SAC dependent and unlike previous studies in yeast and human cells in culture, we observe proper localization of SAC proteins at kinetochores. In fact, the overall kinetochore structure is not affected. Interestingly, in absence of Sgt1 function, the overall Polo protein levels are low but the kinase is still present at kinetochores. We also find that Sgt1 mutant cells fail to organize bipolar spindles and spindle poles do not have normal centrosomes even though centriole duplication is not affected. In Sgt1 mutant cells, most centrosomes fail to accumulate Polo and consequently fail to mature. Importantly, overexpression of Polo is able to significantly rescue all mitotic phenotypes resulting from the mutation in the *sgt1* gene. Our results suggest that, in Drosophila, Sgt1 is not required for normal kinetochore structure or SAC activity, but it is essential for the functional organization of centrosomes through stabilization of the Polo protein.

2. Results

2.1. Identification and subcellular localization of Sgt1 in Drosophila

The Drosophila orthologue of Sgt1 was identified through blast searches using the human Sgt1 protein sequence. A single highly conserved protein encoded by the gene CG9617 was identified with 41% amino-acid identity (Fig. 1.1). As observed for the Sgt1 family members, in the Drosophila protein the p23-like CHORD domain (also called CS domain) and the Sgt1-specific domain (SGS) are present. Although the Drosophila orthologue lacks the TPR domain present in other Sgt1 proteins, its function as a co-chaperone (Bansal et al. 2004) is unlikely to be affected, as the TPR and CS domains are both chaperone-interacting domains and may be redundant. Sgt1 has been reported in human cells to be a soluble protein without any particular localization during mitosis (Steensgaard et al. 2004).

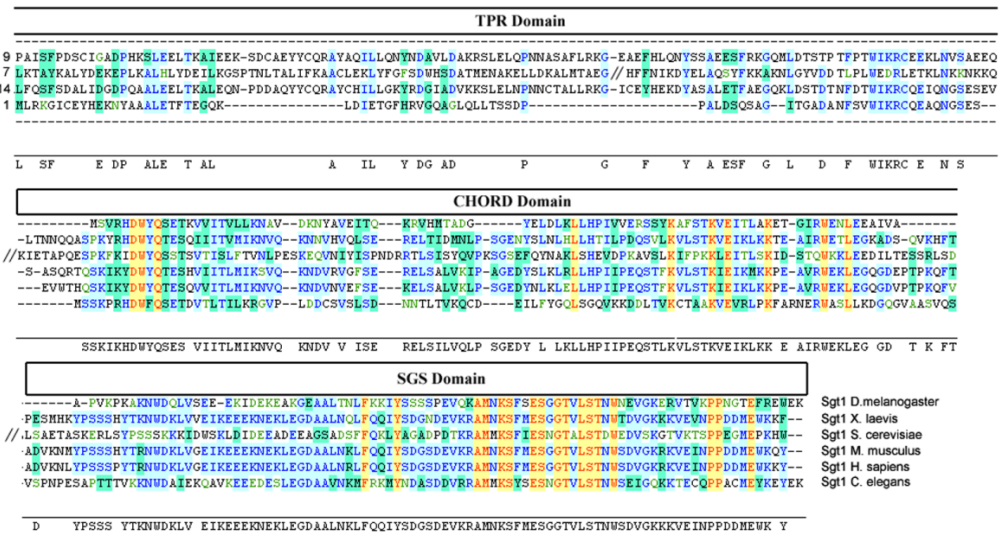

Figure 1.1: Identification of Sgt1 in *Drosophila melanogaster*. Sequence homology between Sgt1 proteins from different species is shown. Conserved domains are indicated in boxes: Tetracopeptide domain (TPR Domain), p23-like CHORD Domain and Sgt1-specific domain (SGS Domain).

To determine if Drosophila Sgt1 localizes in a similar manner, the coding sequence was tagged with EGFP, transfected into S2 Drosophila cells and expressed under the control of an inducible promoter (Fig. 1.2A–C). In asynchronous cultures, no specific accumulation of EGFP-Sgt1 is observed besides a clear localization at the mid-body during very late mitosis (Fig. 1.2A). However, if cells are arrested in mitosis with colchicine, EGFP-Sgt1 shows not only diffuse cytoplasmic staining but also strong

accumulation at centrosomes and kinetochores (Fig. 1.2B and 1.2C). Expression of EGFP-Sgt1 in interphase or of EGFP alone in either interphase or mitosis gave no specific localization (data not shown). These results indicate that, unlike yeast and human cells, Drosophila's Sgt1 shows a specific subcellular localizations at different stages of mitosis when spindle microtubules are depolymerized.

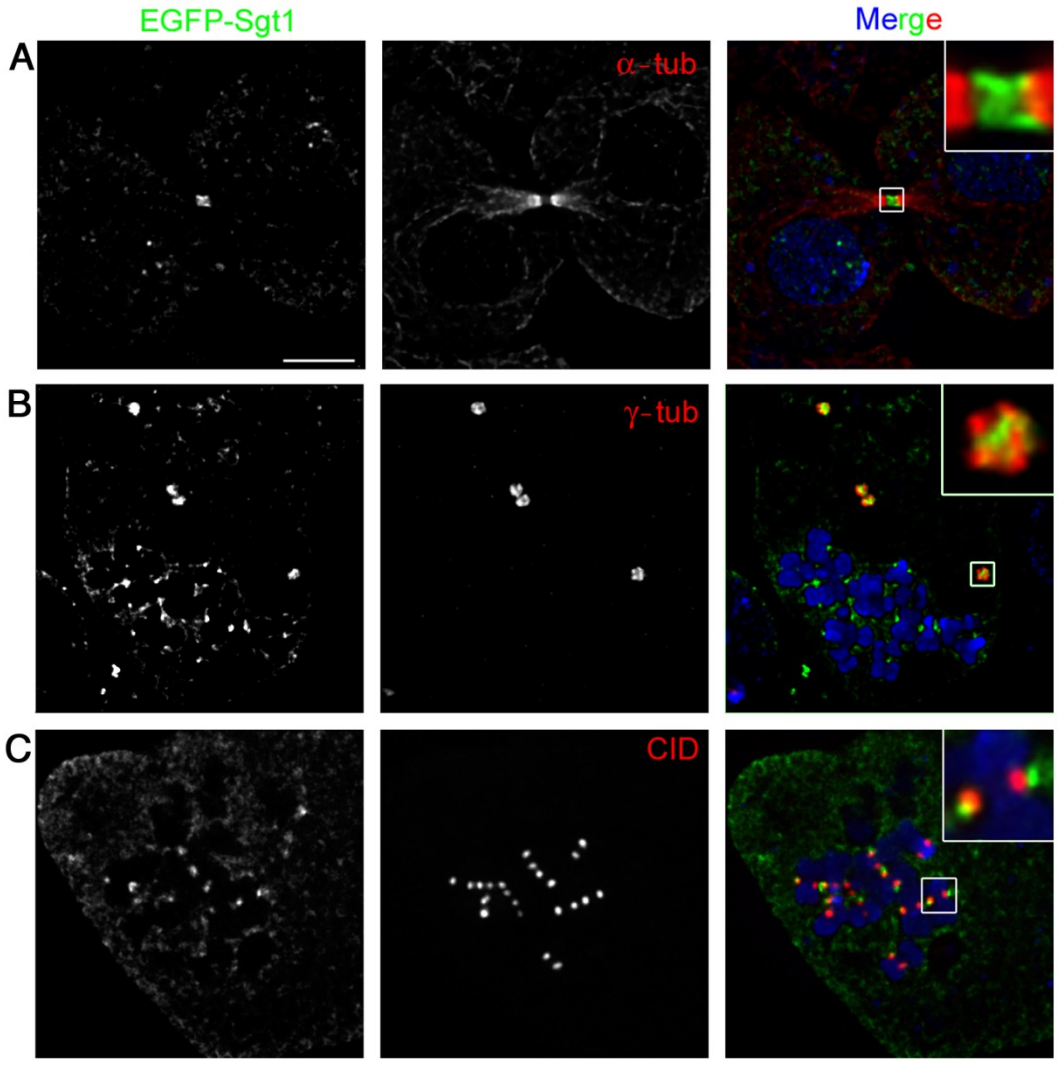

Figure 1.2: Localization of EGFP-Sgt1 in S2 Drosophila cultured cells. (A) In asynchronous cells, EGFP-Sgt1 (green) can only be detected at the cleavage furrow during late mitosis as shown by the co-staining with α-tubulin (red). When cells are treated with colchicine, we can observe **(B)** accumulation of EGFP-Sgt1 (green) at the centrosomes identified by γ-tubulin (red) and **(C)** at the outer region of the kinetochores as shown by co-staining with the centromeric marker CID (red). For all immunolocalization DNA was stained with DAPI (blue). Scale bar, 5 μm.

2.2. Identification of a *sgt1* mutant allele

To study the function of Sgt1 in Drosophila, we identified two putative mutant strains (C01268 and C01428) (Fig. 1.3A). Analysis of the nucleotide sequence indicates that the transposable-element is inserted at the same site in both strains (+55 bp). Both strains are lethal when homozygous for the insertion or as trans-heterozygotes or hemizygotes over the deficiency Df(3R)CA3, which uncovers the genomic region containing the *sgt1* gene (data not shown), suggesting that the insertions are associated with the lethality. For all other experiments reported here, we used the C01268 strain referred as $sgt1^{P1}$. To confirm that the lethality is due to the insertion, we generated precise excisions of the transposable element. In all excisions obtained, we observed a full restoration of viability.

Western blot analysis of total protein extracts from wild-type and $sgt1^{P1}$/ $sgt1^{P1}$ third instar larvae brains shows that homozygous mutant cells have highly reduced levels of Sgt1 protein, suggesting that $sgt1^{P1}$ is either a severe hypomorph or a null mutant (Fig. 1.3B).

In Drosophila cultured cells, Sgt1 localizes to specific structures during mitosis suggesting that the protein might have relevant functions during cell division. To test this hypothesis, we carried out a classical cytological analysis of third instar larval $sgt1^{P1}$ neuroblasts (Fig. 1.3C). We observed abnormal mitotic phenotypes, including prometaphases with hypercondensed chromosomes (see insets in Fig. 1.3C) and anaphases with lagging chromatids. Quantitative analysis of mitosis indicates that the mutation in *sgt1* does not have an overall effect upon mitotic index (Fig. 1.3D). Nevertheless, mitotic progression is affected: $sgt1^{P1}$mutant tissue shows a significant reduction in the frequency of prophases, a severe increase in the frequency of prometaphases and a reduction of cells exiting mitosis, when compared with controls (Fig. 1.3E). It is noteworthy that treatment of $sgt1^{P1}$neuroblasts with colchicine does not lead to accumulation of cells in mitosis, suggesting that SAC might be compromised (Fig. 1.3D).

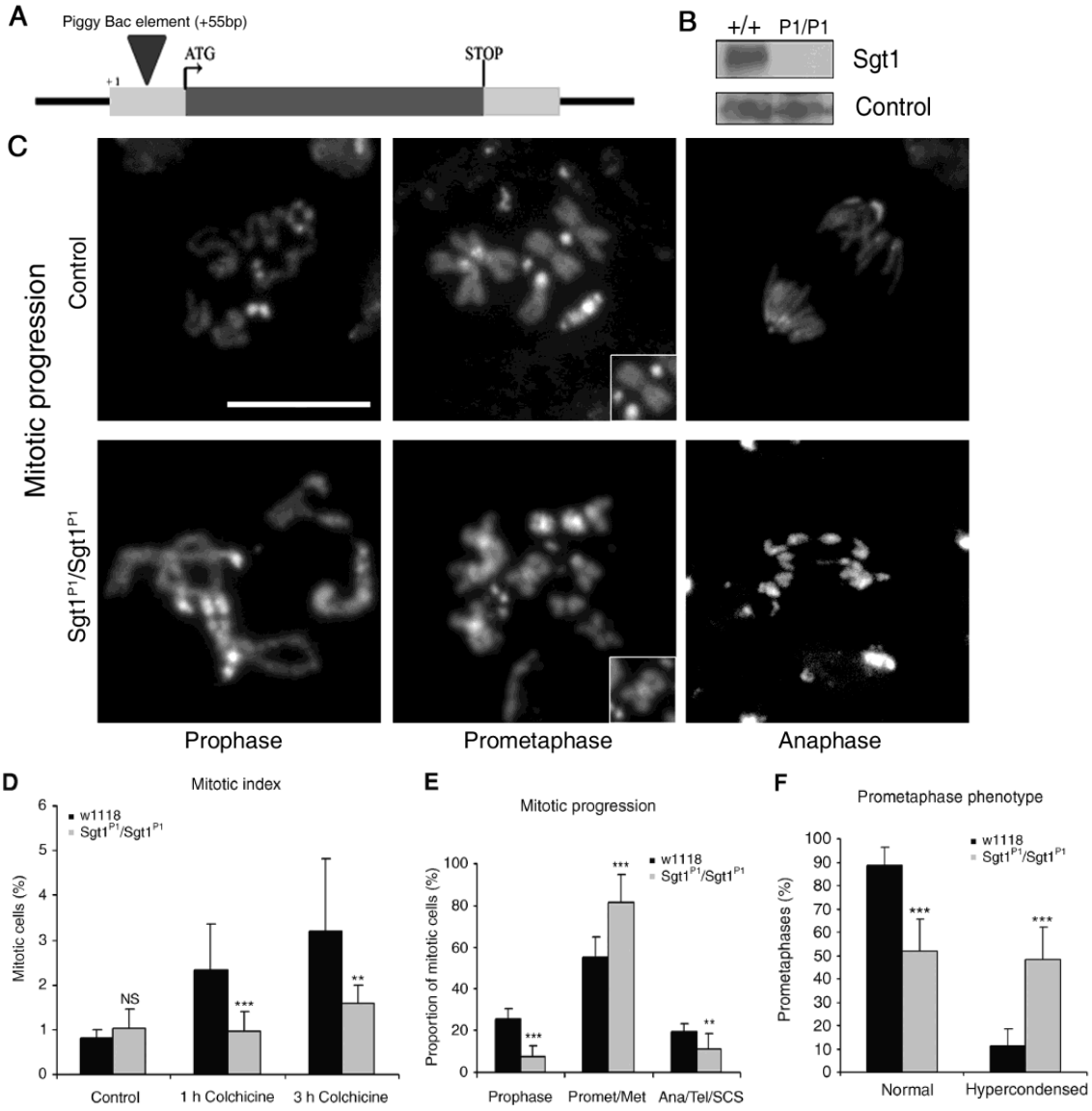

Figure 1.3: Identification and characterization of *sgt1*^{P1} alleles. **(A)** Diagram showing the transposable element insertion site in gene CG9617 of the mutant strain *sgt1*^{P1}. **(B)** Western blot of total protein extracts from control and *sgt1*^{P1} neuroblasts probed for Sgt1 and α-tubulin as a loading control. **(C)** Cytological analysis of mitotic progression of control (*w1118*) and mutant (*sgt1*^{P1}/*sgt1*^{P1}) third instar larval neuroblasts. Insets in the central panel highlight the chromosome hypercondensation phenotype; **(D)** Quantification of mitotic parameters in control and mutant neuroblasts in the absence and presence of colchicine (10µM). (n=10 brains for each condition and 2000 cells scored) **(E)** Quantification of mitotic progression in wild type and *sgt1*^{P1} neuroblasts. (n=10 brains for each condition, and 100 mitotic figures scored) **(F)** Quantification of prometaphase phenotype in neuroblasts. (n = 12 brains for each condition, and 100 prometaphases scored). Not statistically significant (ns); or significantly different: $p<0.05$ (*); $p<0.01$ (**); and $p<0.001$ (***); Bar is 5 µm.

The most striking phenotype observed by loss of Sgt1 function is a prometaphase delay/arrest with hypercondensed chromosomes, reminiscent from what is observed for control colchicine-treated control cells (Fig. 1.3F). Interestingly, when Sgt1 mutant cells are incubated in colchicine does not result in accumulation of Sgt1 mutant cells in

mitosis but sister chromatid separation was not observed, indicating that mutant cells have an active SAC. One plausible explanation for our observation is that, in the presence of microtubule poison, mutant cells are delayed before entering mitosis; eventually these cells enter mitosis and arrest in prometaphase resulting in chromosome hypercondensation. Thus, we suggest that *sgt1*^{P1} brains have a mitotic index that is not different from controls mostly because of slow mitotic progression and failure to exit mitosis.

2.3. Sgt1 and the SAC

Previous reports have shown that Sgt1 is required for overall kinetochore assembly and localization of checkpoint proteins such as BubR1, Mad1 and Mad2 (Steensgaard et al. 2004). Consequently, it was suggested that absence of Sgt1 reduces the potency of the SAC (Steensgaard et al. 2004). We analysed whether *sgt1*^{P1} mutation affects kinetochore localization of BubR1 and Bub3, two well-characterized SAC components (Logarinho et al. 2004; Lopes et al. 2005). We found that both proteins localize to kinetochores of *sgt1*^{P1} mutant cells in early prometaphase, similarly to control cells (Fig. 1.4A and 1.4B). However, we consistently observe that, in mutant cells, BubR1 and Bub3 kinetochore distribution is significantly enlarged (Fig. 1.5A and 1.5B), as described for wild type cells treated with colchicine (Fig. 1.4C). Enlargement of the kinetochore is well documented in cells treated with colchicine and might be an adaptive response in order to increase the chance of kinetochore-spindle microtubule interaction (Logarinho et al. 2004). Taken together, these results suggest that *sgt1*^{P1} mitotic cells might not be able to establish normal microtubule-kinetochore attachment.

While these observations suggest that SAC proteins can indeed localize properly in *sgt1*^{P1} cells, they do not confirm that they are functional. To address this, we constructed strains carrying the *sgt1*^{P1} mutation and *bub3*^{1}, an allele of Bub3 that renders the SAC inactive (Lopes et al. 2005). Quantitative analysis of thie double mutant (*sgt1*^{P1}; *bub3*^{1}) shows very low mitotic index (Fig. 1.5C). Furthermore, analysis of mitotic progression shows that when Bub3 expression is impaired in a *sgt1*^{P1} mutant background, cells enter mitosis more readily, do not accumulate in prometaphase and exit mitosis more frequently as shown by the increase in anaphase and telophases (Fig. 1.5D). Also, *sgt1*^{P1}; *bub3*^{1} mutant cells do not show chromosome hypercondensation, suggesting that mitotic cells do not arrest at any stage of mitosis

(Fig. 1.5E). Taken together, these observations clearly show that in *sgt1*^{P1} cells the SAC is active and is responsible for the mitotic delay observed in prometaphase.

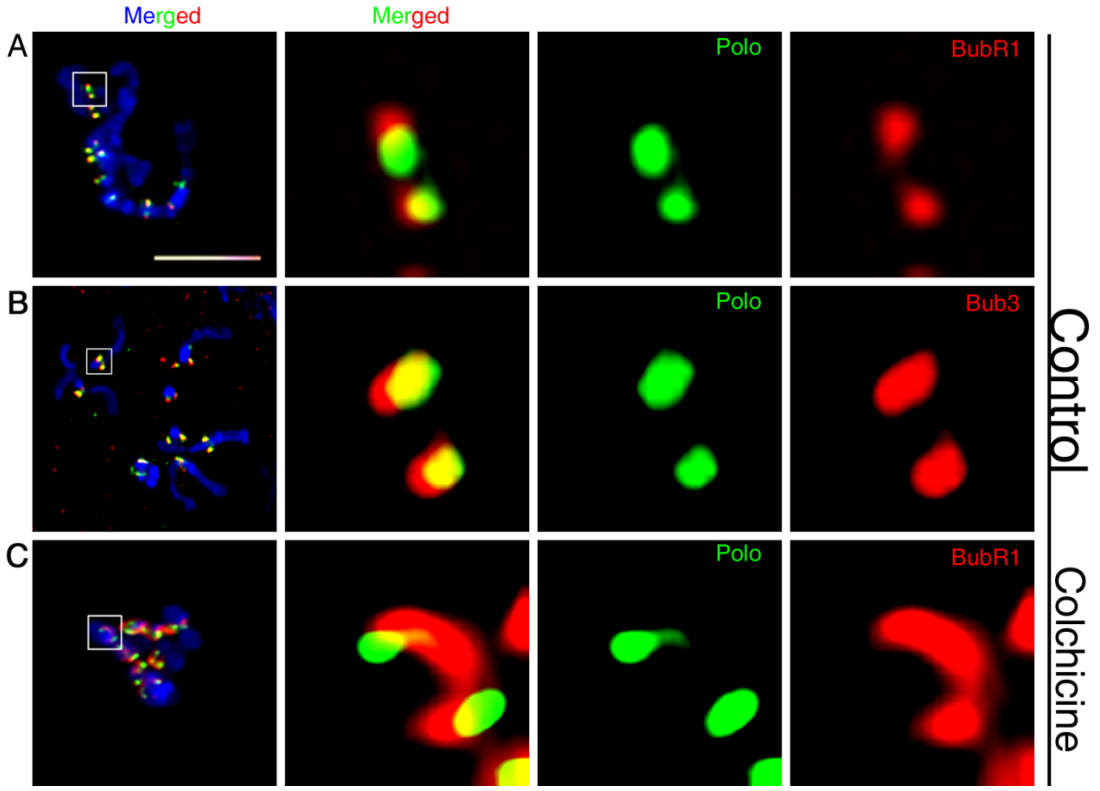

Figure 1.4: Spindle assembly checkpoint components in control neuroblasts. Immunofluorescence localization of the SAC proteins BubR1 and Bub3 in **(A-C)** control neuroblasts. In the merged image DNA (blue) is also shown and in the panels on the right the kinetochores (white box in the merged image) are shown at a higher magnification. Anti-Polo antibody was used as control but the signal had to be intensified in *sgt1*^{P1} due to the low level of this protein at the kinetochore. During prometaphase **(A,B)** control cells show clearly defined localization of both BubR1 and Bub3 **(C)** that appears expanded when incubated with colchicine to depolymerise microtubules. Scale bar, 5 µm.

2.4. *sgt1*^{P1} cells fail to progress normally through the cell cycle

Our results show that *sgt1*^{P1} cells are SAC efficient; however, they do not explain why these cells fail to accumulate in mitosis in response to spindle damage. As we do not observe neuroblasts exiting mitosis in the presence of microtubule poison, a possible explanation is that, in the absence of Sgt1, cells fail to enter mitosis and are delayed at other stages of the cell cycle. To test this hypothesis, we analysed the overall ability of third instar larval neuroblasts to accumulate Cyclin B, an essential protein required for mitotic entry (Lehner and O'Farrell 1990) (Fig. 1.6A). Quantitative

immunofluorescence analysis shows that *sgt1*^{P1} brains have significantly lower number of cyclin B-positive neuroblasts when compared with controls (Fig. 1.6B). This suggests that most mutant cells fail to reach G_2 and therefore are most likely delayed in S or G_1 phase. Accordingly, we incubated *sgt1*^{P1} neuroblasts with BrdU to ascertain whether they enter S-phase (Fig. 1.6C). Quantification of BrdU-positive cells shows that when compared with control, much fewer *sgt1*^{P1} cells incorporate BrdU (Fig. 1.6D). Both findings strongly suggest that loss of Sgt1 imposes a delay in cell cycle progression, most likely during G_1.

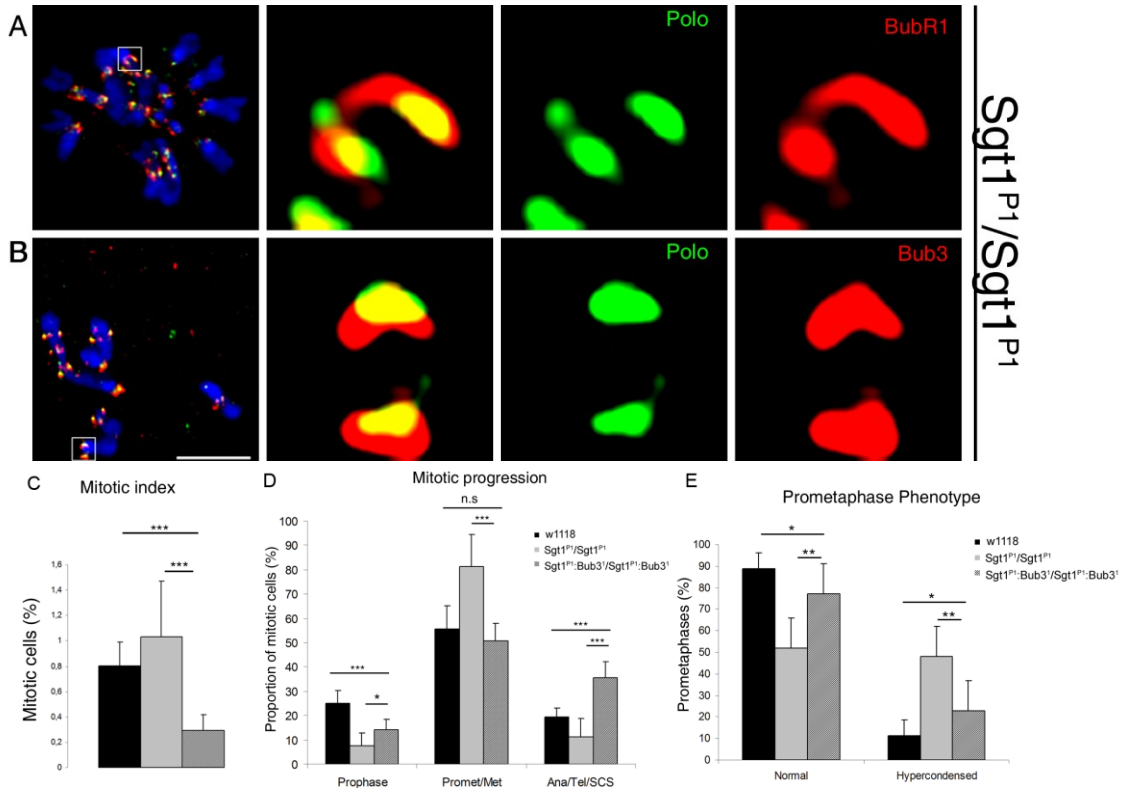

Figure 1.5: The spindle assembly checkpoint in *sgt1*^{P1} neuroblasts. Immunofluorescence localization of the SAC proteins BubR1 and Bub3 in *sgt1*^{P1} neuroblasts **(A,B)**. In the merged image DNA (blue) is also shown and in the panels on the right the kinetochores (white box in the merged image) are shown at a higher magnification. Anti-Polo antibody was used as control but the signal had to be intensified in *sgt1*^{P1} due to the low level of this protein at the kinetochore. During prometaphase **(A,B)** both BubR1 and Bub3 in *sgt1* mutant cells show a broad distribution resembling the distribution of BubR1 in control cells when incubated with colchicine to depolymerise microtubules **(Fig. 4C)**. **(C)** Quantification of the mitotic index in control, *sgt1*^{P1}*/sgt1*^{P1} and *sgt1*^{P1}*/sgt1*^{P1}*; bub3*^{1}*/bub3*^{1} mutant neuroblasts. Note that in the double mutant cells the mitotic index is significantly reduced indicative of loss of SAC activity. (n=10 brains for each condition and 2000 cells scored) **(D)** Quantification of mitotic progression in control, *sgt1*^{P1}*/sgt1*^{P1} and *sgt1*^{P1}*/sgt1*^{P1}*; bub3*^{1}*/bub3*^{1} mutant neuroblasts. (n=10 brains for each condition, and 100 mitotic figures scored) **(E)** Quantification prometaphase with hypercondensed chromosomes in control, *sgt1*^{P1}*/sgt1*^{P1} and *sgt1*^{P1}*/sgt1*^{P1}*; bub3*^{1}*/bub3*^{1} mutant neuroblasts. (n = 12 brains for each condition, where 100 prometaphases were quantified). Not statistically significant (ns) or significantly different: $p<0.05$ (*); $p<0.01$ (**); $p<0.001$ (***); Scale bar, 5 µm.

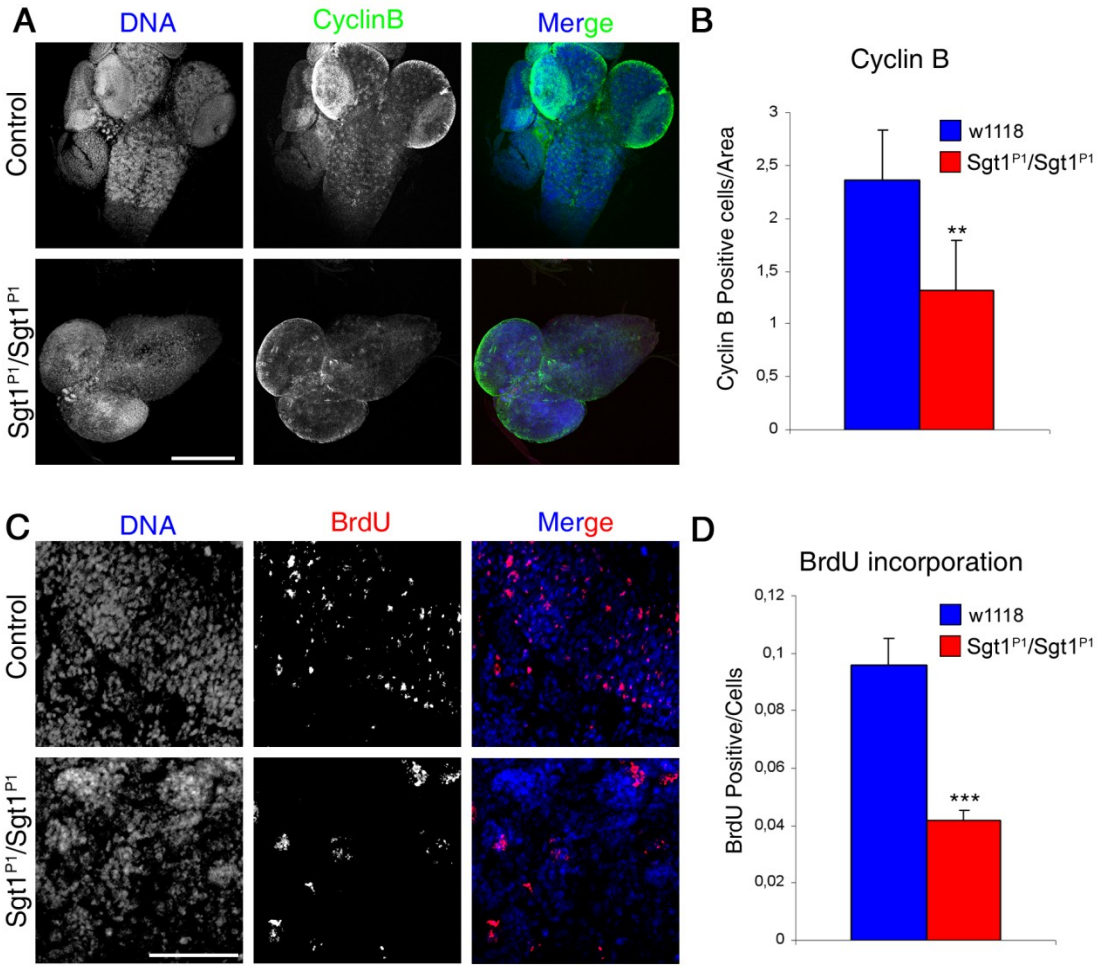

Figure 1.6: Analysis of cell cycle progression in control and *sgt1*^{P1} neuroblasts. **(A)** Brains from control *w*^{1118} or mutant *sgt1*^{P1} larvae were dissected, immunostained to reveal cyclin B (green) and counterstained for DNA (blue). **(B)** Quantification of the number of cyclin B positive cells in control and mutant brains. **(C)** Higher magnification of a random area in brains from control and mutant larvae showing BrdU (red) and DNA (blue). **(D)** Quantification of the number of BrdU positive cells in relation to the total number of cells. **(E)** Higher magnification of the boxes presented in **C** showing the threshold used to quantify the BrdU positive cells; Difference that is not statistically significant (ns); or significantly different: p<0.05 (*); p<0.01 (**); p<0.001 (***); Scale bar, 50 µm.

2.5. Kinetochore structure in *sgt1*^{P1} mutant cells

Previous work has suggested that Sgt1 is not only required for kinetochore localization of checkpoint proteins but also the overall structure of the kinetochore. Accordingly, we analysed the localization of several kinetochore proteins in *sgt1*^{P1} neuroblasts (Fig. 1.7). The kinetochore is a highly complex multi-layered structure built over CENP-A-containing centromeric heterochromatin (reviewed by Przewloka and Glover 2009). We find that CID - the Drosophila homologue of CENP-A -, the inner

kinetochore protein CENP-C, Polo and CENP-META - the Drosophila homologue of CENP-E -, all show proper kinetochore localization (Fig. 1.7). However, detailed quantitative analysis of signal intensity for the various kinetochore components shows that whereas CID, CENP-C and CENP-META remain mostly unaltered in the absence of Sgt1 (Fig. 1.7F), the level of Polo protein is significantly reduced at kinetochores when compared with wild-type control cells (Fig. 1.7F and 1.7G). This reduction in kinetochore localization can be due to either mislocalization of the protein or to a reduction in overall protein levels. To address this, we performed Western blot of total protein extracts from wild-type and *sgt1*^{P1} brains (Fig. 1.7H) and found that Polo protein levels are severely reduced (70%) in mutant cells (Fig. 1.7I). Such a decrease in Polo levels strongly suggests that Sgt1 is required for its stabilization which is in agreement with previous results showing that Hsp90, a known interactor of Sgt1 in other organisms, is required to stabilize the levels of Polo protein (de Carcer et al. 2001).

2.6. Analysis of spindle organization in *sgt1*^{P1} neuroblasts

The mitotic phenotype observed in *sgt1*^{P1} cells is similar in some aspects to the phenotype observed in Polo mutants, such as the prometaphase delay and chromosome overcondensation (Llamazares et al. 1991). Polo kinase activity is known to be responsible for centrosome maturation and spindle bipolarity (Sunkel and Glover 1988; Llamazares et al. 1991). Therefore, reduced Polo activity should lead to centrosome dysfunction and abnormal spindle formation. We isolated wild-type and *sgt1*^{P1} neuroblasts and studied their mitotic apparatus organization (Fig. 1.8). We found that whereas most control cells assemble a bipolar spindle with two well-defined centrosomes, one at each pole (Fig. 1.8A), a significant proportion (22%) of *sgt1*^{P1} neuroblasts have monopolar spindles (Fig. 1.8B and 1.8C) with either a dispersed (Fig. 1.8B) or well-focused (Fig. 1.8C) centrosome at the centre. The majority of *sgt1*^{P1} cells (78%) are able to assemble a bipolar spindle (Fig. 1.8D and 1.8E), but more than half (55%) have an abnormal number of centrosomes (30% have just one) or centrosome structure (20% with a diffuse centrosome) (Fig. 1.8F). These results indicate that loss of Sgt1 protein leads to centrosome and spindle abnormalities that are highly reminiscent of loss of Polo function.

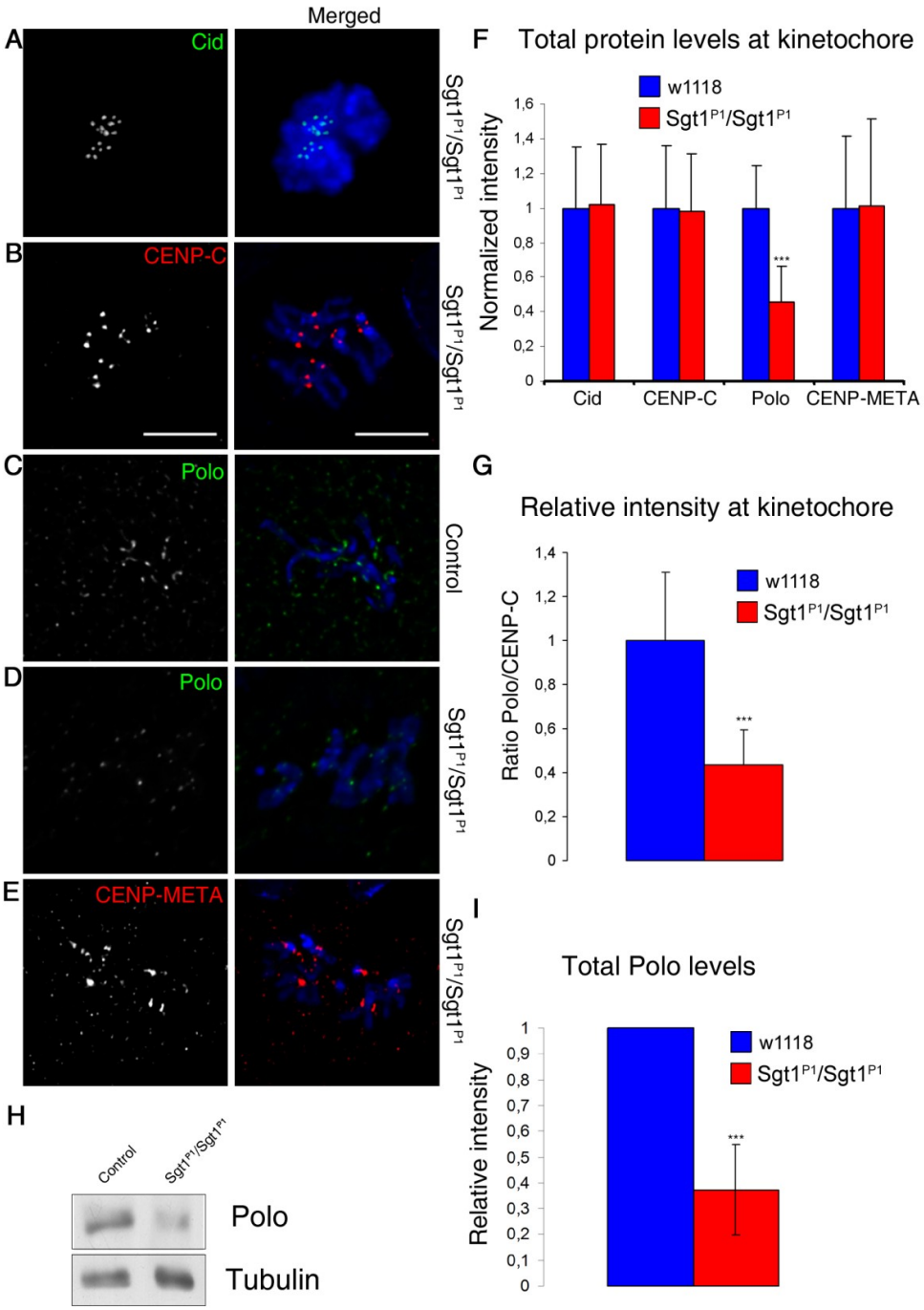

Figure 1.7: Organization of the kinetochore in absence of Sgt1. Immunolocalization of different kinetochore proteins in (**A, B, D, E**) *sgt1*^{P1} or **(C)** control neuroblasts. In the merged images CID and Polo (green), CENP-E and CENP-meta (red) and DNA (blue) are shown. Note reduced Polo signal in *sgt1*^{P1} when compared to control neuroblasts. (**F**) Quantification of the signal intensity of Cid, CENP-C, Polo and CENP-META at kinetochores in both control and *sgt1*^{P1} neuroblasts. The prometaphase cells were randomly selected and the intensities of kinetochore proteins in the sgt1 mutant cells were normalised to the average in control cells. **(G)** Quantification of the ratio between kinetochore levels of Polo and CENP-C in control and *sgt1*^{P1} neuroblasts. (**H**) Western blot of total protein extracts from control or *sgt1*^{P1} neuroblasts and (**I**) quantification of the signal showing a significant reduction in Polo protein levels in mutant brains. α-tubulin was used as a loading control. (For all the quantifications n = 200 kinetochores from 5 different preparations in each condition and a total of 20 cells) Significantly different: $p<0.001$ (***). Scale bar, 5 µm;

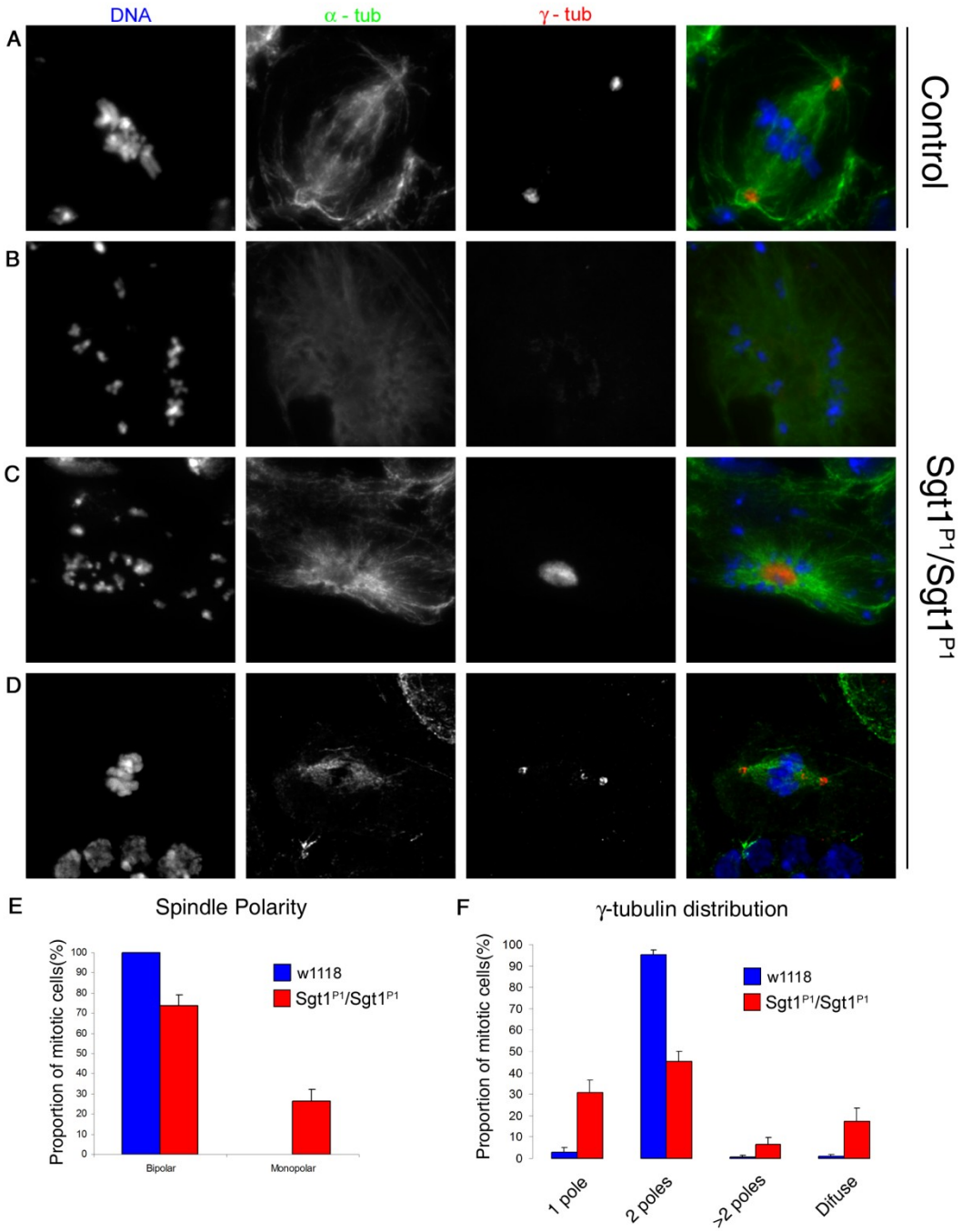

Figure 1.8: Organization of the mitotic apparatus in absence of Sgt1. (A) Control and **(B-D)** *sgt1*^{P1} neuroblasts were immunostained to reveal spindle microtubules (α-tubulin in green), centrosomes (γ-tubulin in red) and DNA (blue). **(A)** Control cells showing a normal mitotic spindle with γ-tubulin staining at both poles. Mutant cells show a variety of spindle abnormalities including **(B)** monopolar spindle with a diffuse staining of γ-tubulin, **(C)** monopolar spindle with only one centrosome, **(D)** bipolar spindle with more than two defined spots of γ-tubulin staining. **(E)** Quantification of spindle organization shows that more than 20% of mutant cells have monopolar spindles. **(F)** Quantification of centrosomes shows that most mutant cells have either less or more centrosomes as ascertained by γ-tubulin staining. (In all the conditions n=8 brains);

2.7. *In vivo* analysis of mitotic progression in *sgt1*^{P1} neuroblasts

To further analyse progression through mitosis and spindle formation in the absence of Sgt1, we generated a strain carrying the *sgt1*^{P1} mutation and fluorescently tagged centromeres (CID-mRFP) (Schuh et al. 2007) and microtubules (GFP–tubulin) (Rebollo et al. 2004). Neuroblasts were observed as primary cultures (Fig. 1.9). Wild-type neuroblasts enter mitosis with two well-defined microtubule-organizing centres (MTOCs), and the bipolar spindle forms on average 2:30 ± 0:30 minutes after nuclear envelop breakdown (NEBD). Anaphase initiates 7 ± 1:30 minutes after NEBD and the cell divides asymmetrically (Fig. 1.9A). In contrast, *sgt1*^{P1} neuroblasts show different behaviours. More than half of the cells (55%) enter mitosis with no apparent MTOC, but after NEBD are able to form a microtubule array that grows outwards, they are unable to focus at the spindle poles and mitotic progression is delayed in prometaphase for more than 1h (Fig. 1.9E). We also find some (15%) mutant cells that enter mitosis with a single MTOC, are able to form a bipolar spindle and reach metaphase with a short delay but eventually exit mitosis with a significant delay (35 min after NEBD) (Fig. 1.9C and 1.9E). We also find few cells (5%) that enter mitosis with more than two MTOCs, fail to organize a proper bipolar spindle and consequently fail to congress chromosomes and remain arrested for long periods (Fig. 1.9D and 1.9E). Finally, we observe a proportion of *sgt1*^{P1} neuroblasts (25%) that enter mitosis with two MTOCs (Fig. 1.9E). These observations indicate that, in absence of Sgt1, most cells have severe difficulties in organizing a proper bipolar spindle, do not show normal MTOCs and are mostly arrested in prometaphase.

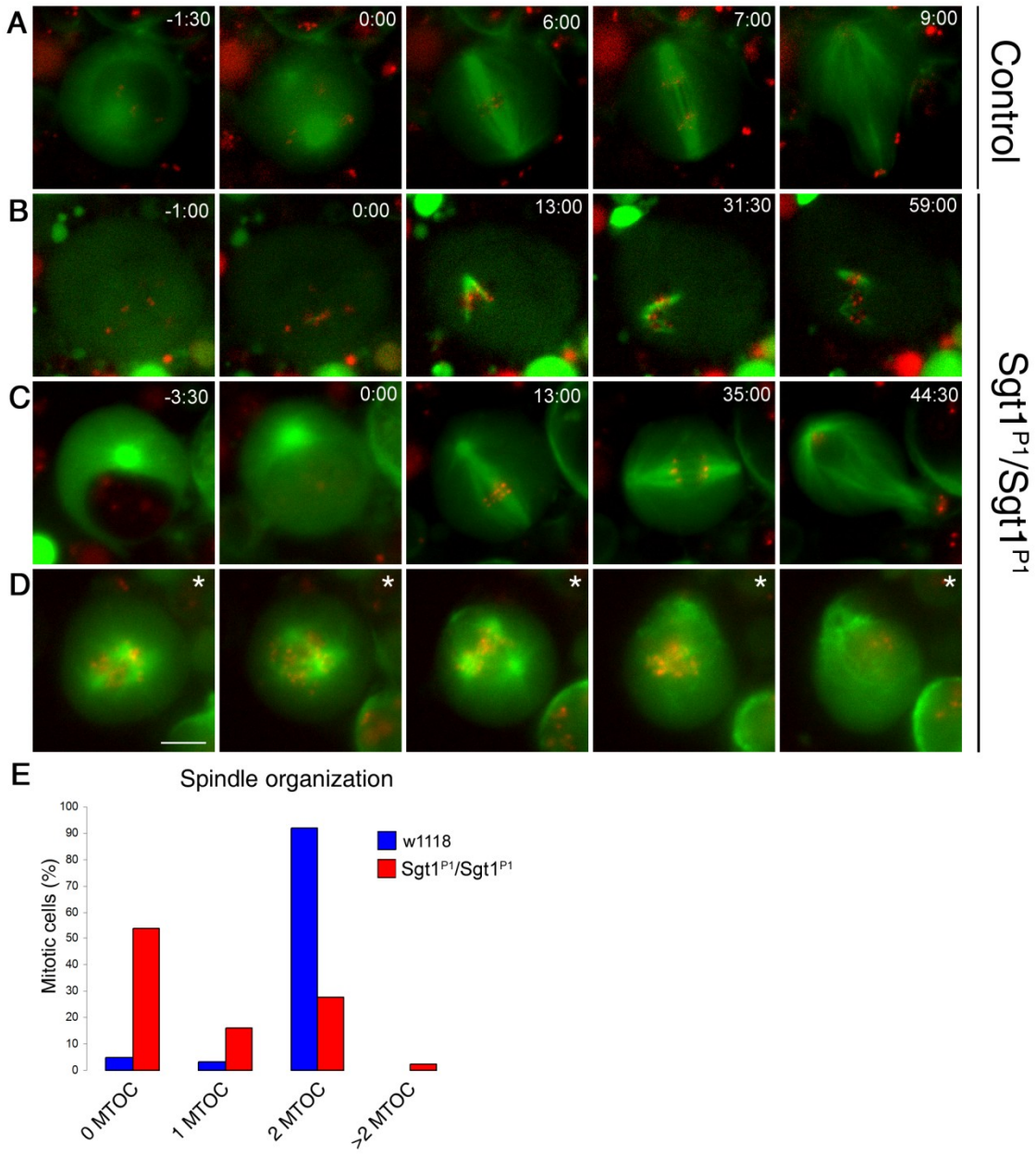

Figure 1.9: Live imaging of control and mutant neuroblasts. Primary cultures of neuroblasts were imaged by time-lapse spinning confocal microscopy to visualize spindle microtubules (GFP-α-tubulin in green) and the centromeres (Cid-mRFP in red). **(A)** Wild type cell showing two microtubule asters at opposite poles of a well-organized bipolar spindle with centromeres congressing and then separating during anaphase. **(B)** *sgt1*^{P1} cell showing a spindle without asters (MTOCs) that appears to be nucleated from the chromosomes that stays in mitosis for at least 1h after nuclear envelope breakdown. **(C)** *sgt1*^{P1} cell showing a spindle organized from a single aster that is able to enter anaphase after a delay of 35 min in metaphase. **(D)** *sgt1*^{P1} cell showing a multipolar spindle that remained in prometaphase for at least 60 min. (*) This cell was filmed in prometaphase therefore the time of NEBD could not be determined. **(E)** Distribution of the spindle organization observed by *in vivo* analysis. The number of MTOCs was determined by the presence of asters at the poles of the mitotic spindle. (Control n=65 cells; *sgt1*^{P1} n=192) Scale bar, 5 µm;

2.8. Analysis of centrosome structure in *sgt1^{P1}* cells

In order to determine whether abnormal MTOC phenotype is associated with abnormal centriole duplication, we constructed a strain carrying the *sgt1^{P1}* allele, CID-mRFP to label the centromeres and EGFP-PACT (Martinez-Campos et al. 2004) to label the centrioles, and neuroblasts were then analyzed as before (Fig. 1.10). In wild type mitotic neuroblasts most centrioles (90%) are present as pairs of two distinct dots, at each side of the metaphase plate (Fig. 1.10A). However, in *sgt1^{P1}* cells, we frequently find an irregular number of centrioles at the poles (Fig. 1.10B), or a single cluster of several EGFP-PACT labelled centrioles (Fig. 1.10C) or even cells with less than two dots of EGFP-PACT (data not shown). Quantification shows that most (90%) wild-type cells show the expected two centriole pairs and just a small proportion present less than two centriole pairs (Fig. 1.10D). However, only 40% of *sgt1^{P1}* mutant cells show a normal set of centriole pairs, approximately 40% of mutant cells has more than two centriole pairs and some cells have less than two centriole pairs (Fig. 1.10D). Moreover, when we compare the ploidy of *sgt1^{P1}* mutant cells with the number of centrioles, we observe a clear correlation between increase in centriole number and in ploidy (85% of the cells) (Fig. 1.10E). This correlation suggests that the appearance of additional centrioles is most likely to be a consequence of a failure in a previous division and a subsequent replication of the centrioles in the following cell cycle. Therefore, these observations suggest that although loss of Sgt1 affects centrosome maturation, centrioles replicate normally.

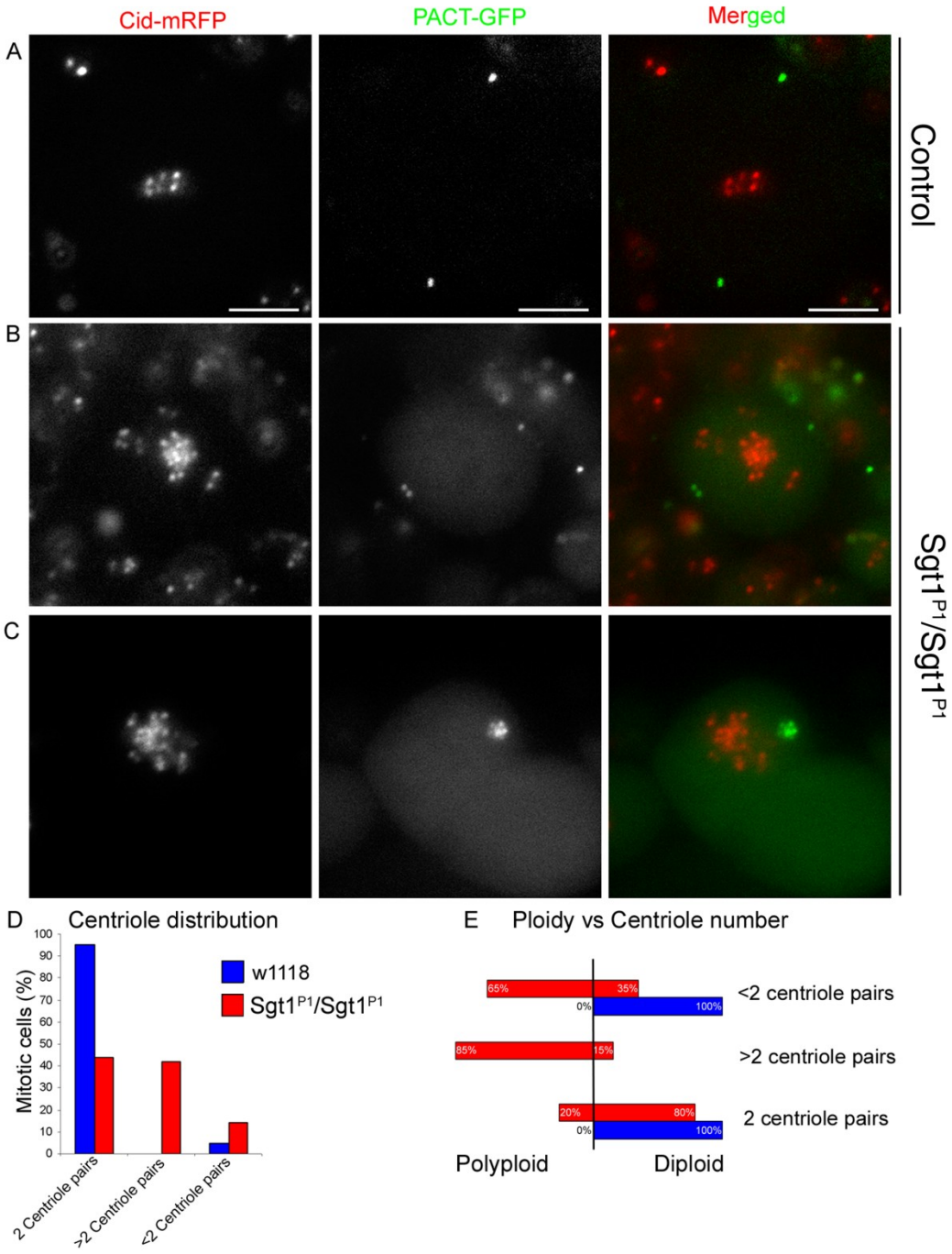

Figure 1.10: Determination of centriole numbers in *sgt*^{P1} mutant cells. *In vivo* analysis of the centrioles was carried out using PACT-GFP (green) and the centromeric marker Cid-mRFP (red). **(A)** Wild type neuroblast showing two well-defined centriolar spots at opposite sides of the metaphase plate. **(B)** *sgt*^{P1} mutant neuroblast showing irregular number of centrioles, two on one side and a single pair on the other side of the cluster of centromeres. **(C)** Polyploid cell from a *sgt*^{P1} mutant brain showing a cluster of centrioles in one side of the large centromere cluster. **(D)** Quantification of the number of centrioles in control and mutant neuroblasts. **(E)** Quantification of cellular ploidy and the number of centrioles in control and mutant neuroblasts. (control n = 40 cells; Sgt1 mutant n = 121 cells); Scale bar, 5 µm.

2.9. Overexpression of Polo rescues Sgt1 centrosome phenotype and mitotic progression

To test if Sgt1 centrosome maturation function is indeed a consequence of failure in Polo stabilization or a direct consequence of the absence of Sgt1, we overexpressed Polo protein both in *sgt1*^{P1} and in wild type flies and analysed centrosome, spindle organization and mitotic progression (Fig. 1.11). In control cells, we observed that Polo overexpression has no effect in centrosome number (Fig. 1.11B) or mitotic progression (Fig. 1.11C despite Polo protein levels are 2.5 times increased, as shown by Western blot (Fig. 1.11E). However, when Polo was overexpressed in a *sgt1*^{P1} background, we observed significant rescue of the *sgt1* mutant phenotype. Importantly, Polo levels only increase 1.25 times when Sgt1 is absent (Fig. 1.11E). The number of cells that do not show CNN staining is significantly reduced, as is the number of cells with just one centrosome, whereas cells with normal number of centrosomes and a normal bipolar spindle increase significantly when Polo is overexpressed (Fig. 1.11A and 1.11B). Moreover, overexpression of Polo in *sgt1* mutant background mostly restores normal mitotic progression, and the prometaphase arrest is recovered (Fig. 1.11C). We also analysed kinetochore organization in *sgt1*^{P1} cells after Polo overexpression. We quantified the number of cells showing at least 50% enlarged kinetochores when stained for the SAC protein BubR1 (Fig. 1.11D). We find that after Polo overexpression in the *sgt1*^{P1} mutant background, cells exhibiting BubR1 enlarged kinetochores decreases significantly (Fig. 1.11D). These observations suggest that Polo overexpression results in a significant recovery of kinetochore-microtubule attachment in accordance with the improved frequency of normal mitotic exit. Overall, these data show that the mitotic phenotypes caused by mutation in *sgt1* are recovered by Polo kinase and indicate that the observed phenotype for the *sgt1* mutant allele result from the reduction in Polo protein levels.

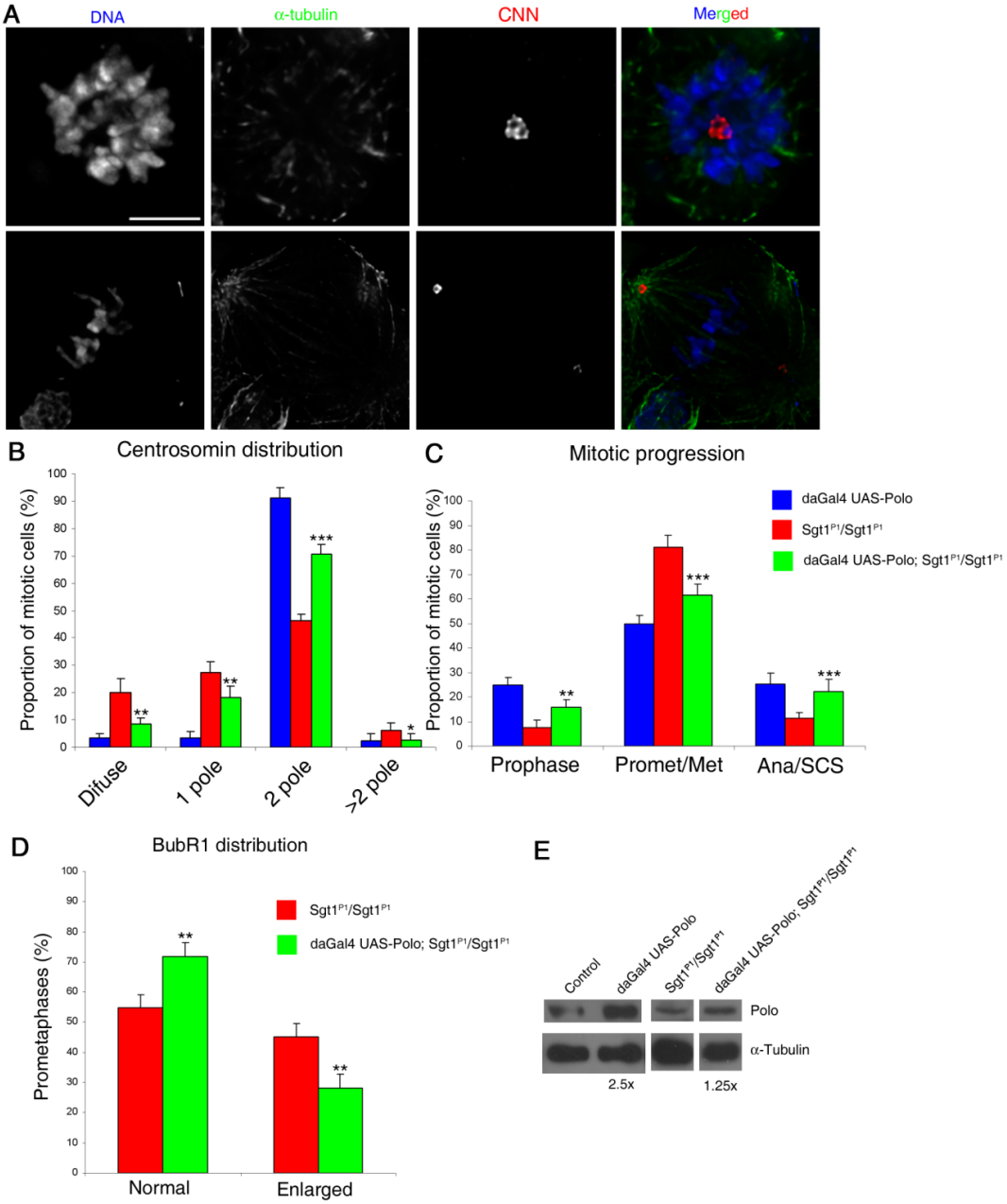

Figure 1.11: Overexpression of Polo in *sgt1*^{P1} and wild-type neuroblasts. **(A)** CNN and α-tubulin staining of neuroblasts in which Polo was overexpress using the daGAL4 driver (daGAL4:UAS-Polo; *sgt1*^{P1}/*sgt1*^{P1}). The top panel shows a cell with a monopolar spindle similar to those observed in *sgt*^{P1}/*sgt*^{P1} homozygote with one well defined pole with strong staining of CNN. In the panel below a typical Polo overexpressing cell showing now a bipolar spindle similar to controls with one defined spot of CNN at each pole. **(B)** Quantification of the CNN distribution in both control (daGAL4:UAS-Polo; +/+), Sgt1 mutant (*sgt1*^{P1}/*sgt1*^{P1}) and Sgt1 mutant overexpressing Polo. (n = 10 brains) (daGAL4:UAS-Polo; *sgt1*^{P1}/*sgt1*^{P1}). **(C)** Quantification of mitotic progression in control (daGAL4:UAS-Polo; +/+), *sgt1*^{P1}/*sgt1*^{P1} and Sgt1 mutant overexpressing Polo (daGAL4:UAS-Polo; *sgt1*^{P1}/*sgt1*^{P1}) (n=10 brains). **(D)** Quantification of the number of cells showing enlarged BubR1 kinetochore accumulation in either Sgt1 mutant (*sgt1*^{P1}/*sgt1*^{P1}) and Sgt1 mutant overexpressing Polo (daGAL4:UAS-Polo; *sgt1*^{P1}/*sgt1*^{P1}) neuroblasts. Normal staining indicated a dot-like BubR1 accumulation while enlarged indicates cells that have more than 50% of their kinetochores with BubR1 staining similar to kinetochores of wild type cells treated with colchicine. (n = 5 different brains and 200 cells scored) **(E)** Western Blot and quantification of the level of Polo in the different genotypes used. Not statistically significant (ns); or significantly different: p<0.05 (*); p<0.01 (**); p<0.001 (***); Please note that all the statistics presented correspond to the difference between Sgt1 mutant and Sgt1 mutant with overexpression of Polo; Scale bar, 5 μm.

3. Discussion

3.1. Drosophila Sgt1 protein is essential for cell proliferation

We identified the *Drosophila melanogaster* homologue of human Sgt1 protein which is encoded by the gene CG9617. This protein is partially conserved from yeast to human suggesting that it might have key functions in cellular processes. Members of the Sgt1 protein family contain three highly conserved domains in many species: TPR, p23-like CHORD and SGS domains. The TPR and p23-like CHORD domains are characteristic of proteins that interact with chaperones directing them to a specific group of proteins, named 'client proteins'. Therefore, Sgt1 was classified as a co-chaperone, as it interacts with the chaperone HSP90 through the p23-like domain. Moreover, Sgt1 is thought to form a complex that, through the TPR domain, activates Skp1, a protein involved in kinetochore assembly (Kitagawa et al. 1999; Bansal et al. 2004; Rodrigo-Brenni et al. 2004). Although, Drosophila Sgt1 lacks the TPR domain, it is unlikely that its co-chaperone function is affected, as in human cells the interaction with HSP90 is made through the p23-like CHORD domain (Lee et al. 2004), which is also conserved in the Drosophila protein. On the other hand, in Drosophila, Sgt1 protein is unlikely to interact with Skp1 and therefore might not be required for kinetochore assembly (Bansal et al. 2004; Rodrigo-Brenni et al. 2004). Our genetic analysis indicates that *sgt1* is an essential gene in Drosophila. Immunofluorescence studies show that Sgt1 localizes to the midbody in late mitosis and, in absence of microtubules, to the outer kinetochore domain and also to centrosomes. This localization pattern suggests that Sgt1 might have a function in kinetochore–microtubule interaction and in centrosome function. While our results show that Sgt1 is required for somatic cell proliferation, meiosis might also require Sgt1, as ovaries and testis are highly abundant in Sgt1 mRNA (Chintapalli et al. 2007). Cytological analysis of the mutant neuroblasts shows strong mitotic phenotypes, including disorganized metaphase plates and hypercondensed chromosomes. A low frequency of cells with lagging chromosomes during anaphase, aneuploidy and polyploidy are also observed. The strong mitotic phenotypes are consistent with the hypothesis that Sgt1 is most likely to have essential functions during cell division. Similarly, human cells depleted of Sgt1 by RNAi show similar mitotic phenotypes, including chromosome hypercondensation, failure in chromosome alignment and mitotic delay (Steensgaard et al. 2004).

3.2. Sgt1 is not required for activation or maintenance of the SAC

Studies in yeast (Bansal et al. 2004; Rodrigo-Brenni et al. 2004) and RNAi experiments in human cells (Steensgaard et al. 2004) have suggested that Sgt1 might be involved in kinetochore assembly. Furthermore, it was shown that reduction of Sgt1 levels leads to a weak SAC response and to mislocalization of checkpoint proteins. However, this weak checkpoint is able to delay mitotic cells for several hours and only when Mad2 was co-depleted, cells no longer arrest in mitosis suggesting that, even with reduced levels of kinetochore proteins, these cells have an active spindle assembly checkpoint (Steensgaard et al. 2004). These observations lead to the proposal that Sgt1 is essential for kinetochore assembly but not for the normal activation of SAC. Unlike human cells, we find that in Drosophila *sgt1* mutant neuroblasts spindle checkpoint proteins, such as BubR1 and Bub3, do accumulate normally at kinetochores. However, just like in HeLa cells (Steensgaard et al. 2004), when mutant Drosophila tissue is incubated in colchicine, the mitotic index does not increase as in controls. We propose that the mitotic index does not increase in response to microtubule depolymerisation mostly because Sgt1 mutant cells progress through the cell cycle more slowly and therefore enter mitosis less frequently than control cells. In support of this explanation, our analysis of mitotic progression revealed that mutant cells have a significant reduction in the number of prophases when compared with control cells. Moreover, *sgt1* mutant cells do not progress through the cell cycle normally, as fewer cells incorporate BrdU or accumulate cyclin B. Accordingly, genetic analysis in yeast showed that mutant cells show a delay at the G_1/S transition (Kitagawa et al. 1999). These observations strongly suggest that in the absence of Sgt1, cells are stalled early in interphase, and when they eventually enter mitosis, they become arrested in prometaphase for long periods, similarly to what was observed in human cells (Steensgaard et al. 2004). Also, we find that in Drosophila, the transient arrest caused by impaired Sgt1 function is clearly SAC dependent, as the $sgt1^{P1}$; $bub3^{1}$ double mutant does not arrest in prometaphase nor shows hypercondensed chromosomes, but does show premature sister chromatid separation and accelerated mitotic exit. Accordingly, we conclude that Sgt1 must have an essential function during interphase to promote cell cycle progression and is also required during mitosis to promote normal mitotic progression.

3.3. Sgt1 is not required for kinetochore assembly

In yeast and human cells, Sgt1 has been shown to be required for kinetochore assembly, a requirement that we do not observe in Drosophila. Interestingly, treatment of HeLa cells with 17-AAG, an HSP90 inhibitor, result in failure to localize several kinetochore proteins (Niikura et al. 2006). Nonetheless, when these cells are analysed by electron microscopy, the structure of the kinetochore is not affected and kinetochores bind microtubules normally (Niikura et al. 2006). Therefore, if Sgt1 has an important function as co-chaperone of Hsp90, this is unlikely to include important kinetochore components. Accordingly, in Drosophila *sgt1*^{P1} cells arrest in prometaphase with an active SAC response and accumulate normal levels of SAC proteins at the kinetochore. Moreover, all the structural components of the kinetochore that were analysed localized normally and at similar levels to controls, with the exception of Polo kinase (see later on this discussion). We consider three possible explanations for these divergent results between Drosophila and other species. It is possible that in Drosophila, the kinetochore assembly pathway is very different from the one described in yeast and humans, and occur through a mechanism that is Sgt1 independent. Alternatively, it is still possible that Drosophila Sgt1 may be required to stabilize only a very specific group of kinetochore proteins, different to the ones that were studied such as what was recently shown for Mis12 complex (Davies and Kaplan 2010). Finally and given that the Drosophila Sgt1 lacks the TPR domain, it is possible that other TPR-containing proteins interact with Hsp90 and are essential for kinetochore assembly. Bioinformatic analysis does indeed show that the Drosophila genome contains several proteins that contain the TPR domain however none also have the SGS domain characteristic of Sgt1-like proteins. Therefore, as in Drosophila, many proteins have been shown to diverge significantly from their yeast or human homologues or even divided the function into two separate proteins, as in the case of Separase (Jager et al. 2001), it is possible that another protein performs this kinetochore specific function.

3.4. Absence of Sgt1 results in failure of centrosome maturation

sgt1 mutant cells show severe mitotic abnormalities in the organization of the mitotic apparatus. We found significant alterations in spindle organization, namely monopolar spindles, dispersion of the PCM component, γ-tubulin and also cells that present only a single centrosome during mitosis. These observations, together with its

centrosomal localization during mitosis, suggest that Sgt1 might be required for proper organization of the centrosome. Indeed, the abnormal centrosome phenotypes are highly reminiscent to those observed after mutation or inhibition of Hsp90 function in Drosophila and human cells, suggesting that Hsp90 and Sgt1 may act in a common pathway (Lange et al. 2000). As Hsp90 has been clearly shown to be involved in the organization and function of centrosomes, and since in other organisms Sg1 is a co-chaperone of Hsp90, we speculate that the interaction between Sgt1 and Hsp90 might underline this process. Likewise, an impairment of the interaction between these two proteins would lead to abnormalities in the establishment of a stable bipolar spindle preventing proper microtubule-kinetochore interaction, which in turn would activate the SAC causing a prometaphase arrest. Moreover, this failure in centrosome organization and function is also most likely to explain the high prevalence of spindle defects observed when Sgt1 protein is absent. However, analysis of centriole components clearly shows that loss of Sgt1 function does not result in abnormal centriole duplication. We can conclude that the most significant aspect of the mutant mitotic phenotype - mitotic arrest - is most likely the result of SAC activation due to abnormal spindles that derives from abnormal maturation of centrosomes.

3.5. Sgt1 stabilizes Polo

Centrosome maturation has been shown to depend on the function of the Polo kinase (Sunkel and Glover 1988). Interestingly, we found that in the absence of Sgt1, Polo protein is destabilized and its levels reduce, both at kinetochores and in total protein extracts. As Sgt1 is a co-chaperone of Hsp90 in other organisms and depletion or inhibition of Hsp90 in Drosophila S2 cells result in low levels of Polo protein with mitotic phenotypes similar to *sgt1*^{P1} cells (de Carcer et al. 2001), we speculate that Sgt1 might be important in directing Hsp90 to Polo for its stabilization and proper physiological conformation. Indeed, when Polo is overexpressed in the absence of Sgt1, we observe that its levels do not increase as much as in controls, supporting the idea that Sgt1 is required for its stabilization. However, overexpression of Polo is sufficient for a significant recovery of the Sgt1 mitotic phenotype, suggesting that Polo is one of the proteins that cause the *sgt1* mutant phenotype. In contrast, in human cells treated with the Hsp90 inhibitor 17-AAG, Plk1 levels remain constant, suggesting that human cells might have backup mechanisms to stabilize this important kinase.

Taken together, our results suggest that in Drosophila, Sgt1 has at least two essential functions: during interphase to promote transit from G_1 to S-phase and later in mitosis for proper maintenance of Polo kinase protein levels.

Chapter 2

Monitoring chromosome segregation

1. Introduction

During the last decades there were great advances to elucidate the surveillance mechanisms that ensure genomic stability during cell division. These mechanisms are called checkpoints and ensure that all steps in a process are properly completed before the cell enters the next process. Several checkpoints, both in interphase and mitosis, have been identified. In mitosis, the most well studied checkpoint is called the spindle assembly checkpoint. This checkpoint ensures that cells only proceeds to anaphase after all chromosomes present a bipolar attachment to opposite sides of the mitotic spindle (reviewed by Malmanche et al. 2006). So far, it was thought that SAC was the only checkpoint that exists in mitosis but recently, in budding yeast, another important checkpoint called NoCut checkpoint was discovered (Norden et al. 2006). The NoCut checkpoint ensures that cytokinesis only occurs if all DNA is completely segregated to the poles of the spindle and no DNA lags behind and remains at the center of the cell during mitotic exit where the cytokinesis furrow will form. Clues to support this coordination between chromosome segregation and cytokinesis came from studies made in *S. cerevisiae* demonstrating that abscission is prevented in the presence of both unsegregated DNA (Mendoza et al. 2009) or spindle elongation impairment (Norden et al. 2006) (Fig. 2.1). The main proteins shown to be important for inhibition of cytokinesis, are the Anillin related proteins Boi1 and Boi2, that are targeted to the abscission site by the well studied mitotic kinase Ipl1 (yeast AuroraB homologue) (Norden et al. 2006). Thus, it was suggested that the NoCut pathway is active in every division as mutants for *boi1* and *boi2* prematurely complete cytokinesis (reviewed by Mendoza and Barral 2008). Furthermore, the NoCut checkpoint seems to be conserved through evolution as recent studies in a Human cell line corroborate the previous studies done in *S.cerevisiae* (Steigemann et al. 2009). Thus, in the presence of unsegregated DNA, the NoCut checkpoint monitors whether DNA is present in the spindle midzone when cytokinesis takes place. However, whether DNA is eventually removed from the spindle midzone by increasing spindle length or by inducing further chromosome condensation remains an open question.

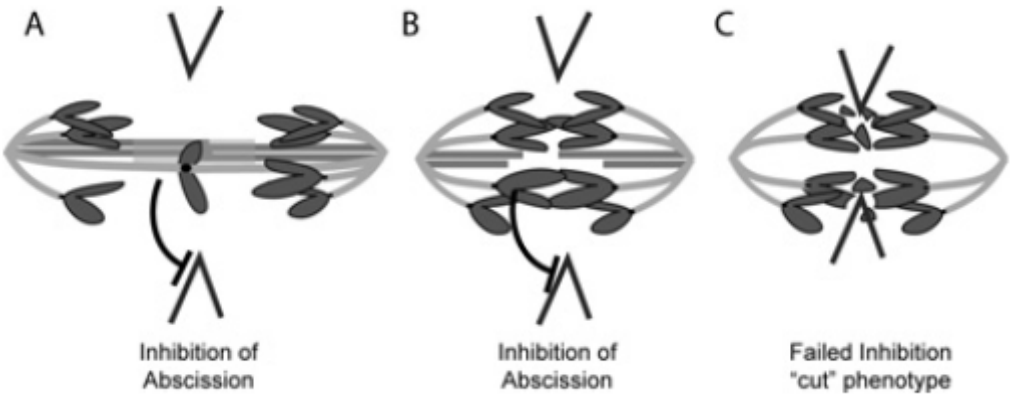

Figure 2.1: Coordination of abscission with chromosome segregation: (A) Abscission (represented by triangular blades) is inhibited by the presence of a lagging chromosome in the vicinity of the spindle midzone. Once the lagging chromosome is segregated away from the cleavage plane, abscission proceeds. **(B)** Anaphase spindle breakage, as caused by depletion of midzone components, triggers a delay in both chromosome segregation and abscission. **(C)** Inactivation of the NoCut pathway impairs the co-ordination between completion of cytokinesis and chromosome segregation. Cells with midzone defects undergo abscission with incompletely segregated chromatids. Chromosome breakage ensues (Adapted from Mendoza and Barral 2008);

Here, to pursue whether the NoCut checkpoint does exist in a complex multicellular organism and which are the mechanisms that allow the segregation of an extra long chromosome, we used a Drosophila strain that contains a chromosome constituted by two second chromosomes joined by a single centromere and so functioning as a single chromosome in which the arms have essentially double in size (Novitski et al. 1981) (Fig. 2.2). This chromosome is called a compound chromosome, and if the chromosome involved is the second chromosome is called C(2)En.

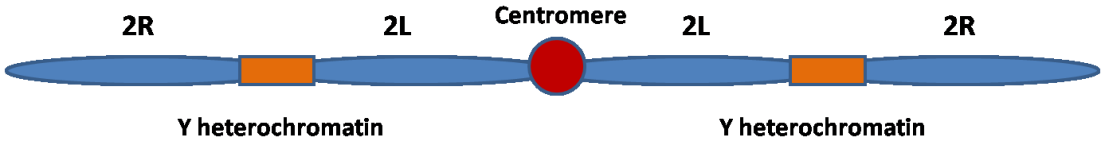

Figure 2.2: Schematic representation of the C(2)En chromosome: Chromosome arms (blue) are connected by Y heterochromatin (yellow) and shares a single centromere (red);

The main idea is that the C(2)En arms would be so long that when segregated by a normal spindle they would remain at the cell equator leading to the activation of the NoCut pathway. Then, after cytokinesis delay, the cell would have to condense more

the C(2)En or to elongate more its spindle in order to remove the DNA from the middle spindle region. Flies carrying the C(2)En are viable suggesting that to resolve the problem cells activate the NoCut checkpoint. However how do cells resolve the problem of having a chromosome that is too long and are still able to segregate the DNA properly remains an open question. To follow the segregation of the C(2)En we performed live analysis both in embryos and in stem cells of the nervous system. Here, for the first time we were able to construct a strain carrying fluorescent markers in a C(2)En background allowing us to follow both mitotic spindle and chromosomes. We found that both third instar neuroblasts and ganglion mother cells delay mitotic progression. Surprisingly, the delay in mitotic progression occurs before anaphase onset instead of the expected cytokinesis delay. Interestingly, neuroblasts carrying the C(2)En never show abnormal mitosis. However, in embryos we observed two main phenotypes that we classified as Weak or Strong mitotic failure. In early embryonic mitotic divisions, when few nuclei occupy a large cytoplasm, C(2)En nuclei divide with similar timing to wild-type. However, in cycles 12 and 13, when nuclei have to face spatial constraints, spindles have a more restricted space to expand and the spindle size became reduced. Significantly, at this stage C(2)En nuclei delay mitotic progression and spend more time during mitosis, from NEBD to anaphase onset. Apparently, in Drosophila both during the very fast embryonic nuclear divisions or in late larval somatic cells, the presence of extra long chromosomes is resolved by delaying progression through mitosis.

2. Results

2.1. C(2)En flies are viable and the spindle assembly checkpoint is efficient

In order to determine the presence of the NoCut pathway in a complex organism we hypothesized that the presence of an extra long chromosome could alter in some way mitotic progression. To further explore this hypothesis we took advantage of a pre-existing Drosophila strain that contains the two chromosomes 2 sharing a single centromere that was named C(2)En (Novitski et al. 1981). The architecture of this chromosome is 2R connected to 2L by Y heterochromatin side a common centromere (Novitski et al. 1981) (Fig. 2.2).

Previously, Sullivan and his colleagues analyzed C(2)En embryos and found that some of the rapidly dividing Drosophila nuclei sank into the embryo and were eliminated (Sullivan et al. 1993). We are interested in a different question: “How does the nuclei/cell that divides properly adapt to deal to with this long chromosome?” First we analyzed the viability of the C(2)En Drosophila stock at different developmental stages. We found that more than 95% of the embryos laid by wild-type control strain are able to hatch in 24h time. By contrast only 17,8% of the embryos deposited by C(2)En, bw^1 sp^1 stock hatch 24h after laid. This number was increased 48h after deposition to 28% however less than the 50% expected (Fig. 2.3). Hatching rate quantification show that roughly half of the C(2)En embryos fail to develop and for those that are able to hatch, they took more time to do so.

Then we analyzed a tissue enriched in mitotic cells, the third instar larvae brains. Drosophila neuroblasts are large neural stem cells because their division gives rise to a identical stem cell and to a small daughter cell committed to divide again for two times and then its progeny differentiate into a neural cell.

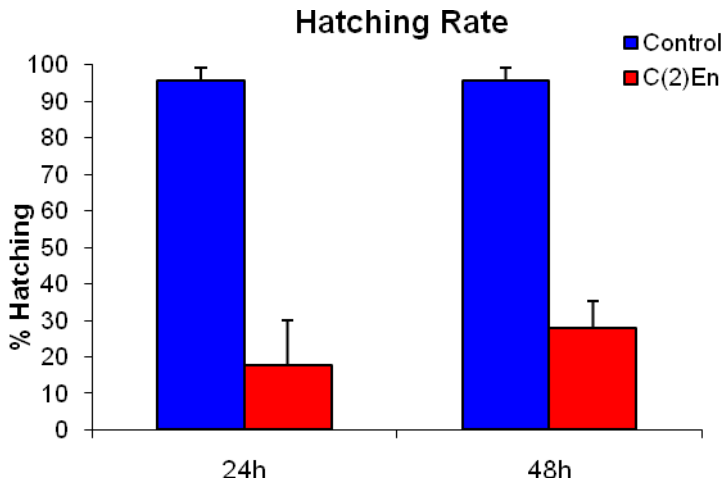

Figure 2.3: Hatching rate of Control and C(2)En embryos: Control strain is represented in blue and C(2)En strain is represented in red;

As it was described (Sullivan et al. 1993), neuroblasts do not show any obvious phenotype, chromosome segregation occurs properly and we can infer that there is not cytokinesis defect due to the absence of binucleated cells (Fig. 2.4A). To analyze if spindle assembly checkpoint (SAC) was affected in this strain we decided to challenge neuroblasts by incubating brains in the presence of the microtubule depolymerising drug - colchicine. As observed in our control strain, C(2)En containing cells accumulate in mitosis responding to the absence of kinetochore-microtubule interaction (Fig. 2.4B).

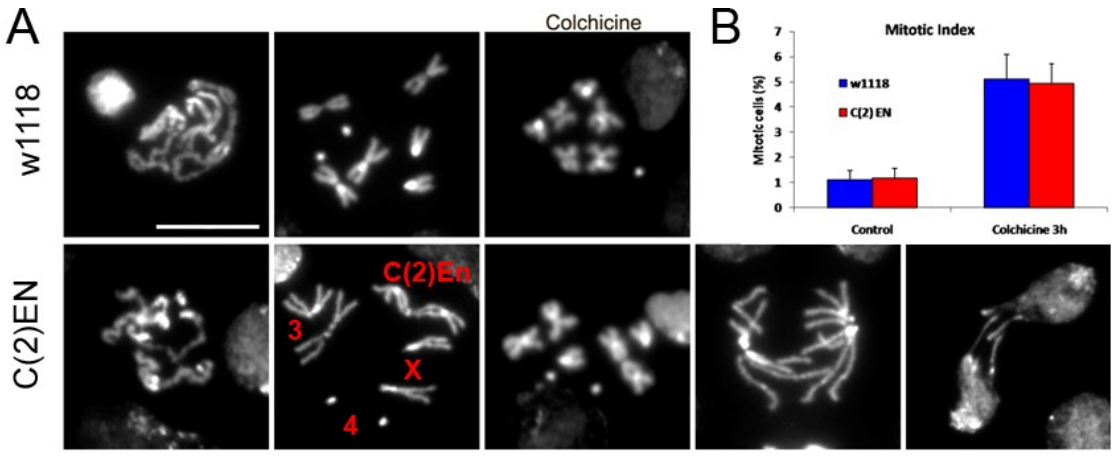

Figure 2.4: Mitotic progression of 3rd instar larvae neuroblasts: (A) Top panel – Mitotic representative figures of control w1118 neuroblasts; Bottom panel – Mitotic progression of compound chromosome containing neuroblasts (In red – reference to the two chromosomes 2 fused by a single centromere); **(B)** Mitotic index that represents the number of mitotic cells per total number of cells in the presence or absence of the microtubule depolymerising drug colchicine; Bar is 5 μm;

2.2. C(2)En cells show an increase in metaphase duration

Fixed analysis of neuroblasts showed no significant mitotic defects (Fig. 2.4). To further study the kinetics of mitotic progression and the behaviour of the mitotic structures in cells, we undertook *in vivo* analysis of these brain cells with the modified histone HisH2av labelled with RFP and the spindle-associated protein Jupiter tagged with GFP. However, given that the two copies of chromosome 2 are joined together introducing new markers into this strain is particularly difficult. When C(2)En flies produce their gametes, they contain either two chromosomes 2 or no chromosome 2 at all and when mated with other genetically normal strains the resultant zygote will have 1 or 3 chromosomes 2, which are lethal genetic combinations. The only solution to overcome this problem was to cross C(2)En females with males carrying the desired marker chromosome but where some level of chromosome nondisjunction was promoted to produce unbalanced gametes. To achieve that condition we used males with a heteroallelic combination of BubR1 (Malmanche et al. 2007) that promote chromosome nondisjunction and that contain the mitotic markers HisRFP.JupGFP in the third chromosome. Under these conditions when we crossed C(2)En bw^1 sp^1;+/+ and $BubR1^{Rev1}/BubR1^{KD}$; HisRFP.JupGFP, we were able to obtain an exceptional progeny that have the second chromosome markers bw^1 sp^1 and we search for the ones that have the fluorescence associated to the HisRFP and JupGFP. Eventually, stable C(2)En; HisRFP.JupGFP stocks were obtained (Fig. 2.5). With this technique we were able to construct strains that could allow us to follow *in vivo* the mitotic progression of C(2)En containing cells.

♀ +/+ ; C(2)EN/O ; +/+

×

♂ +/y ; $BubR1^{D1326N}/BubR1^{rev1}$; His-RFP • Jup-GFP/ His-RFP • Jup-GFP

	2nd chr Normal gametes		2nd chr Exceptional gametes	
♀ \ ♂	$BubR1^{D1326N}$	$BubR1^{rev1}$	$BubR1^{D1326N}$ $BubR1^{rev1}$	O
C(2)EN, bw^1 sp^1	Lethal	Lethal	Lethal	Viable
O	Lethal	Lethal	Lethal	Lethal

Figure 2.5: Diagram showing the genetic approach to allow *in vivo* studies in C(2)En flies; In order to obtain male gametes with a copy of the markers HisRFP.JupGFP on third chromosome, male nondisjunction was promoted by an heteroallelic combination of mutant alleles for the spindle assembly checkpoint BubR1;

First we ask whether the NoCut response is required to be activated under the presence of C(2)En. For that we followed chromosome segregation of the large neuroblasts from C(2)En and control strains carrying the fluorescent markers. We found that in the presence of the C(2)En long arms, chromatids are properly segregated in both conditions (Fig. 2.6). To measure the condensation rate of the chromatids we defined as less compaction the stack after sister chromatids separation and as the maximum compaction the stack where the nucleus reach the spindle pole. The measurements showed that the chromosomes are compacted to a similar extent despite having the C(2)En (Fig. 2.6). However, a more accurate measurement is required to determine whether C(2)En chromosome is selectively compacted when compared with other chromosomes. Putting together, these results suggest that neuroblasts have no need to activate the NoCut pathway to segregate the C(2)En. To further explore if the C(2)En have an early mitotic response we decided to follow the neuroblasts just after the NEBD until anaphase onset.

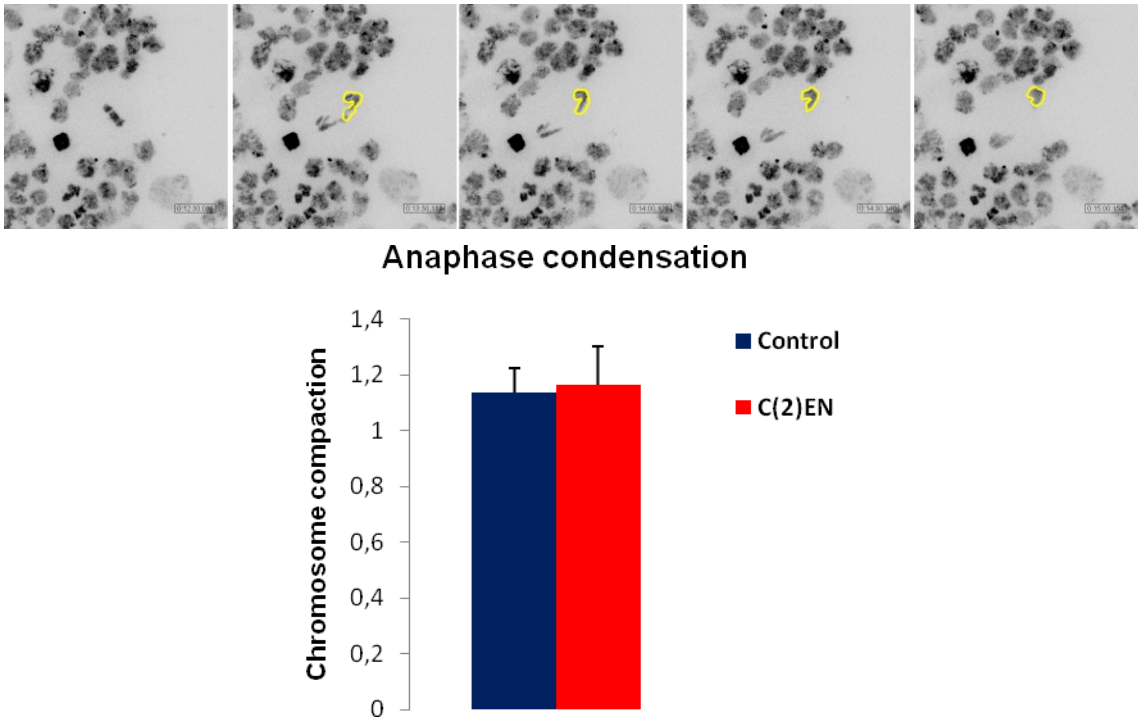

Figure 2.6: *In vivo* studies of chromosome segregation of C(2)En neuroblasts using the marker HisH2Av-RFP(DNA): Chromosome segregation of a C(2)En neuroblast showing the successful segregation of the chromatids; In yellow it is represented the area used to measure the anaphase compaction that occurs in anaphase;

For that we followed mitotic progression of the large neuroblasts from C(2)En;His-RFP:Jup-GFP and control strains carrying only the fluorescent markers. We found that similar to control cells, C(2)En cells mature both centrosomes, align their chromosomes and segregate them properly to both poles. Occasionally these cells present some delay in segregating one of the chromatids but this was always solved before cytokinesis. As the whole process occurs normally and no mitotic phenotypes were observed, we decided to study mitotic timing (Fig. 2.7A). We found that while the vast majority of control cells divide between 05:30 and 07:30 minutes, the C(2)En neuroblasts show a much broader distribution, with the majority of cells dividing within 08:30-09:30 minutes interval (Fig. 2.7B). One explanation for such difference could be that more force is required to align a chromosome with a double size. Yet, we observed that the compound-containing cells align their chromosomes with similar timing as controls: in about 3 minutes. On the other hand metaphase duration is significantly increased by about 2 minutes (from 03:30 to 05:30 on average) (Fig. 2.7C). This difference could be explained for example by a transient activation of the SAC or it could be due to an independent mechanism that detects the presence of the long chromosome and delays mitotic progression. To distinguish between these possibilities we followed the accumulation of the SAC protein BubR1 at kinetochores in control and C(2)En neuroblasts. As an internal control BubR1 levels were quantified in relation to the constitutive kinetochore protein Cenp-C (Fig. 2.8). We found no statistically significant difference between BubR1 levels in control or C(2)En cells, suggesting that this delay is not SAC dependent. However, we were not able to abrogate the SAC in C(2)En cells due to technical reasons and therefore whether the role of SAC in this processes remains to be defined.

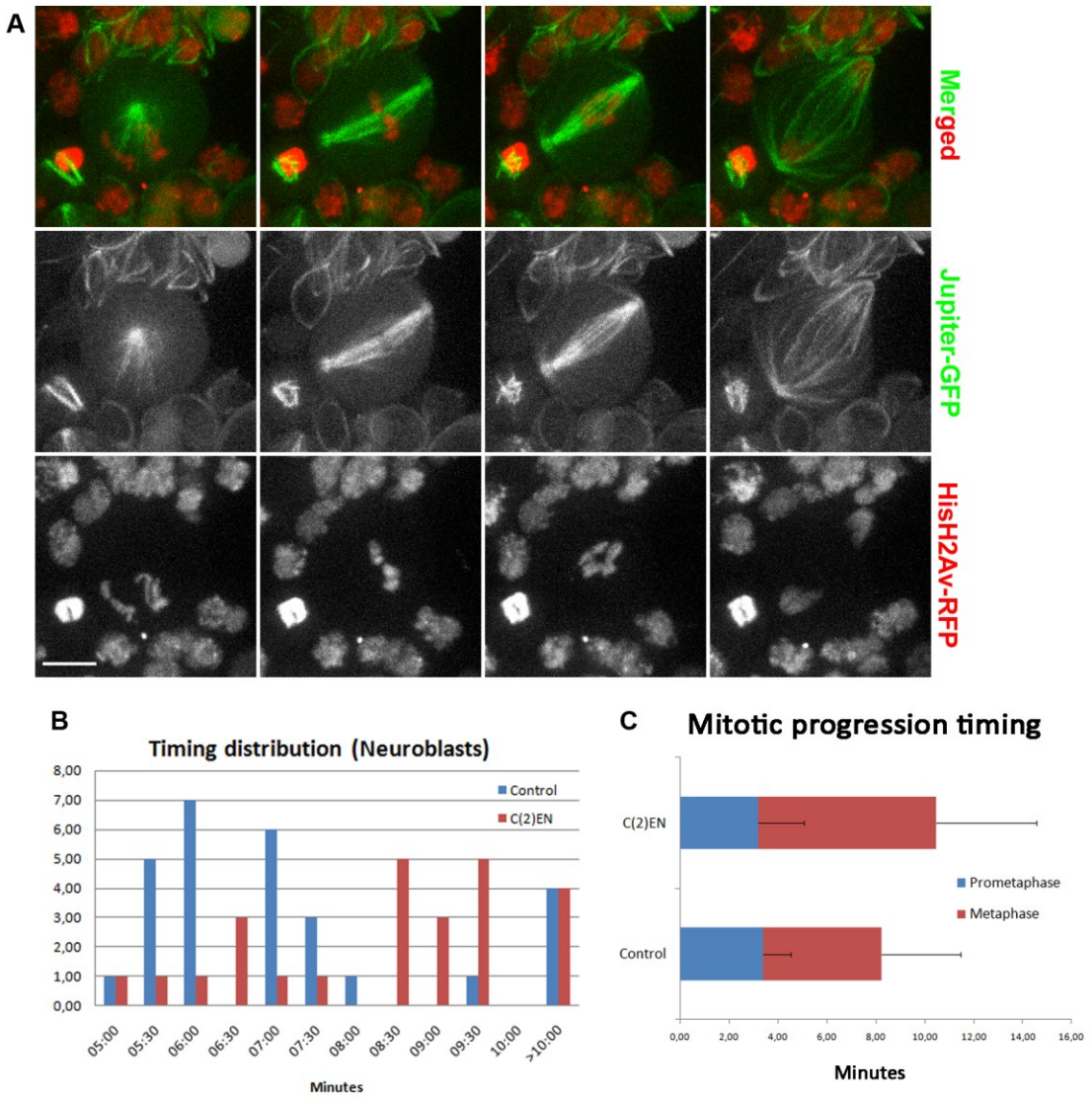

Figure 2.7: *In vivo* studies of mitotic progression of C(2)En neuroblasts using the markers Jupiter-GFP(Microtubules) and HisH2Av-RFP(DNA): (A) Mitotic progression of a C(2)EN neuroblasts showing the mitotic phases analyzed, starting at time 0:00 when nuclear envelope breakdown (first row), then metaphase alignment (second row) and anaphase progression (third and fourth row); **(B)** Distribution of NEBD-anaphase timings in both conditions; **(C)** Time spent in reaching the metaphase plate and anaphase onset; Bar is 5 μm;

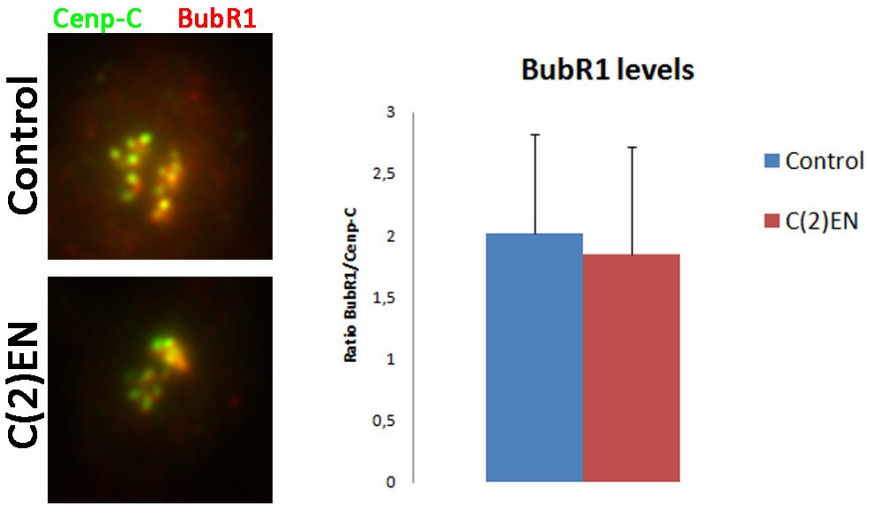

Figure 2.8: Metaphase staining of the kinetochore marker Cenp-C and spindle assembly checkpoint protein BubR1 and respective quantification of the fluorescence intensity ratios between Cenp-C and BubR1;

2.3. Mitotic progression of Drosophila C(2)En embryos

In the previous sections we showed that C(2)En neuroblasts do not show abnormal mitotic phenotypes and appear to cope with the extra long chromosome by extending metaphase timing. We then hypothesized that this timing could be important for a proper segregation, and for that we took advantage of a system where nuclei have to divide very fast: the Drosophila syncytial embryo. As described before, these embryos derived from C(2)En mothers show a strong mitotic phenotype but still at least 30% of the embryos survive. Embryos were classified into two different categories according to the severity of the mitotic defects (Fig. 2.9). Embryos of the strong phenotype are characterized by having nuclei displaying extensive motion, loss of synchrony and presence of free centrosomes as well as fusions between nuclei. The weak phenotype is characterized by the presence of some free centrosomes and a small number of nuclei that appear to sink into the embryo (Fig. 2.11). It is very likely that a proportion of the embryos of the weak phenotype are able to survive.

One possible explanation for the strong phenotype observed in the C(2)En embryos is that centrosome organization is compromised resulting in massive nuclei loss into the embryo. To further address this question we fixed these embryos and immunostained them for the centrosome constituent CP190 as well as to α-tubulin to label the mitotic spindle. We found that free centrosomes of the C(2)En embryos contain the centrosome marker CP190 similar to controls (Fig. 2.10). These

centrosomes have nucleating capacity as α-tubulin staining show an aster emanated from the centrosome (Fig. 2.10–green arrow). Hence, centrosome organization and function do not seem to be affected in compound-containing embryos.

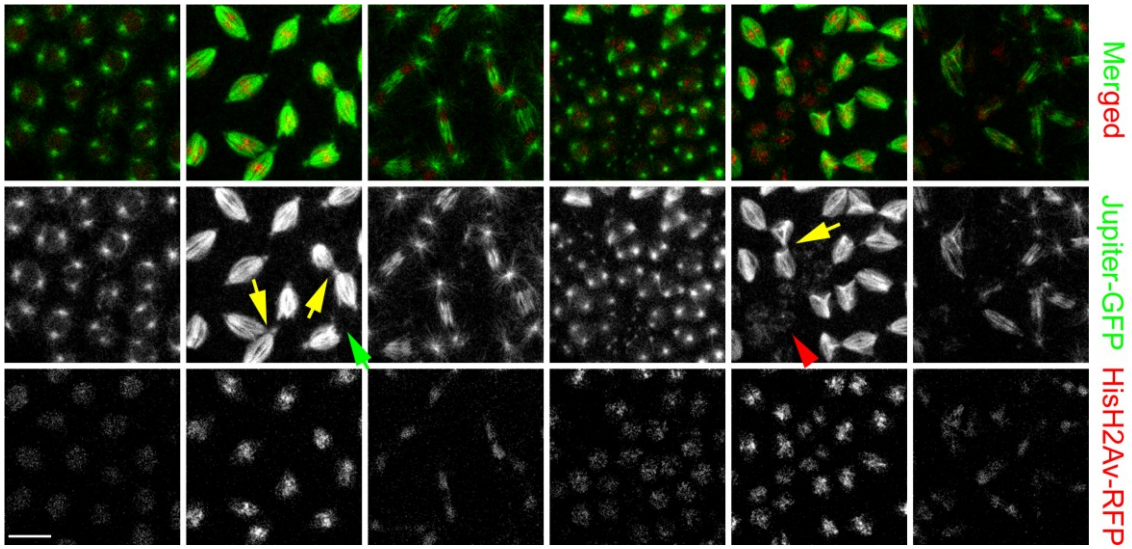

Figure 2.9: Mitotic cycles of C(2)En developing embryos that show strong phenotype: Jupiter-GFP was used to follow the mitotic spindle (green) and HisH2Av-RFP to follow the DNA (red); Green arrow point to acentrosomal side of a bipolar spindle, Yellow arrow points to the interactions between neighbour spindles and the red arrow point to the nuclei that sink into the embryo; Bar is 10μm;

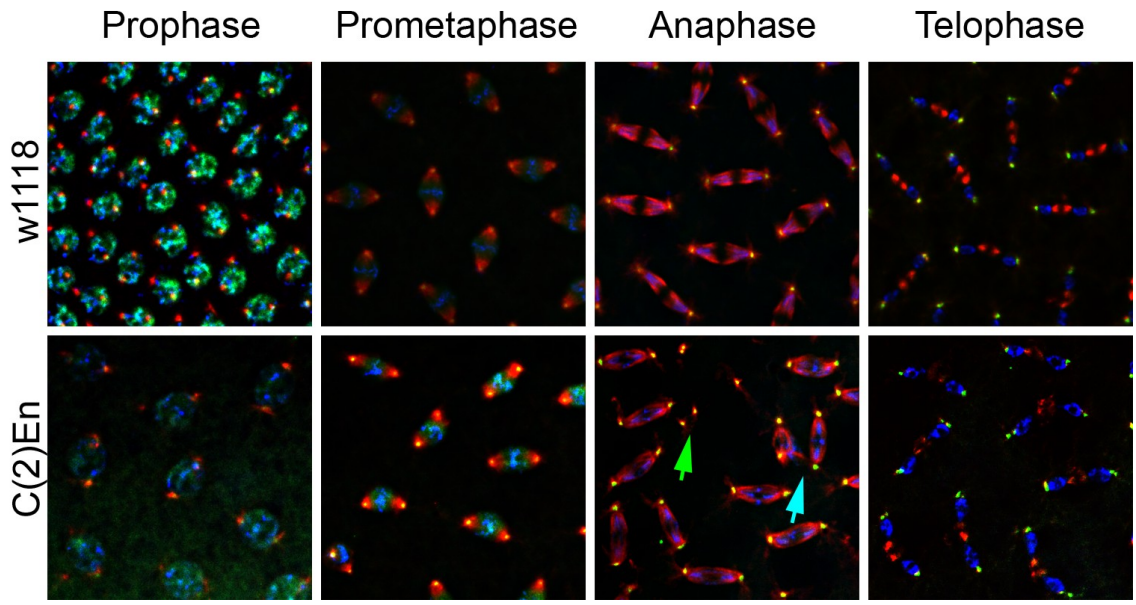

Figure 2.10: Mitotic spindle components of wild-type and C(2)En embryos: DNA (blue), the centrosome component CP190 (green) and α-tubulin as a mitotic spindle marker (red); Top panel – mitotic progression of the control (w1118) embryos; Bottom panel – mitotic progression of the C(2)En embryos; Green arrow points to free centrosomes with nucleating capacity, and the light blue arrow points to interactions between mitotic spindles;

We then focused our studies on those embryos showing the weak phenotype and asked how the nuclei adapt in this system where time and space became so limited (Fig. 2.11). We followed the nuclei between mitotic cycles 10 to 13, and found that the mitotic spindle behaves similar to control independently of the available space (Fig. 2.12-14). Both in control and C(2)En embryos, spindles increase their size and reach a plateau during metaphase and expands after anaphase onset (Fig. 2.12-14). Yet, relative to mitotic timing, though the time that C(2)En nuclei took to divide is similar to control in cycles 10 and 11 we observed a trend to increase mitotic duration when space became more limited, i. e., in cycles 12 and 13 (Fig. 2.15). Despite the observation that spindles from C(2)En embryos behaved in a similar fashion to spindles from control embryos, one can speculate that it may not be enough to segregate the C(2)En in mitotic cycles 12 and 13, therefore nuclei have to spend more time in mitosis to condense more the C(2)En and achieve a proper segregation.

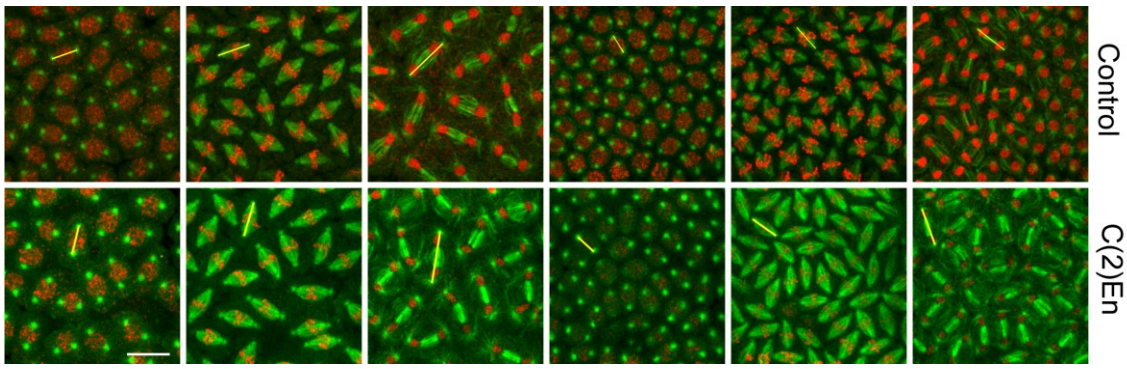

Figure 2.11: Mitotic cycles of C(2)En developing embryos that show weak phenotype: Top panel shows mitotic progression of control embryos; Bottom panel follow the mitotic division of C(2)En containing embryos; Yellow lines show how the distance between spindle poles was measured; Jupiter-GFP is shown in green and HisH2Av-RFP in red; Bar is 10μm;

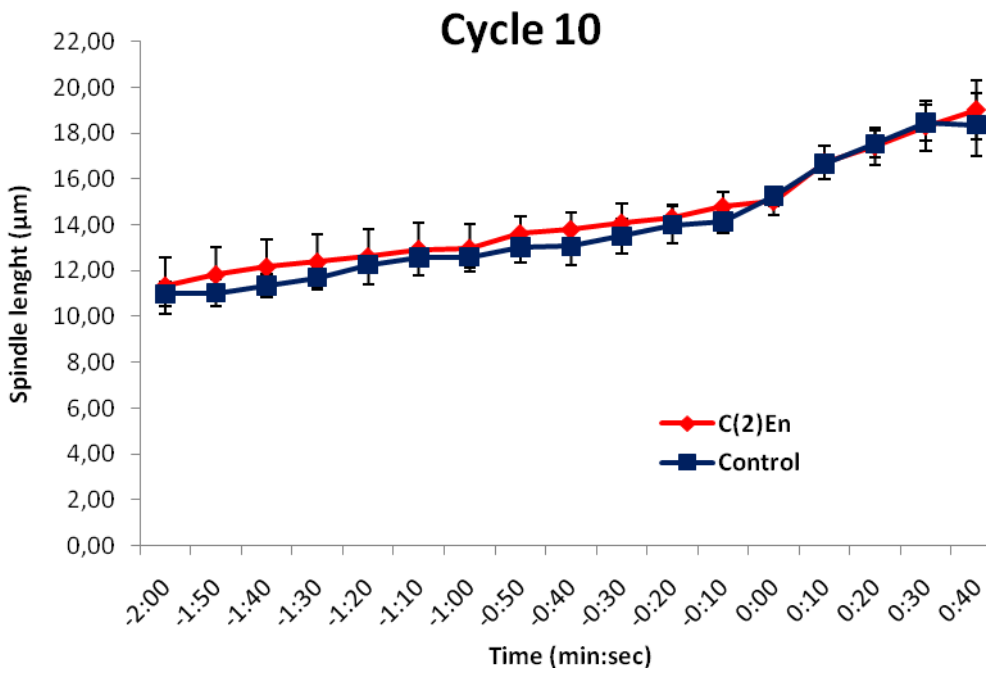

Figure 2.12: Distance between mitotic spindle poles in embryonic mitosis 10; Anaphase onset was used as reference time point (0:00); Control is represented by the blue line while the C(2)En embryo is represented by the red line;

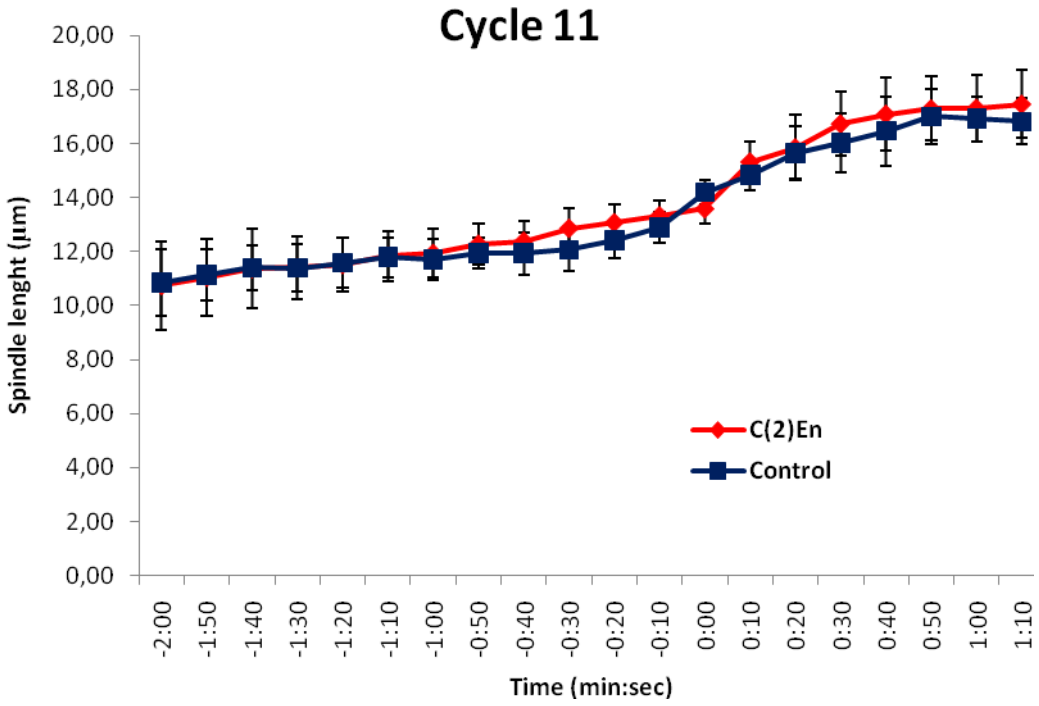

Figure 2.13: Distance between mitotic spindle poles in embryonic mitosis 11; Anaphase onset was used as reference time point (0:00); Control is represented by the blue line while the C(2)En embryo is represented by the red line;

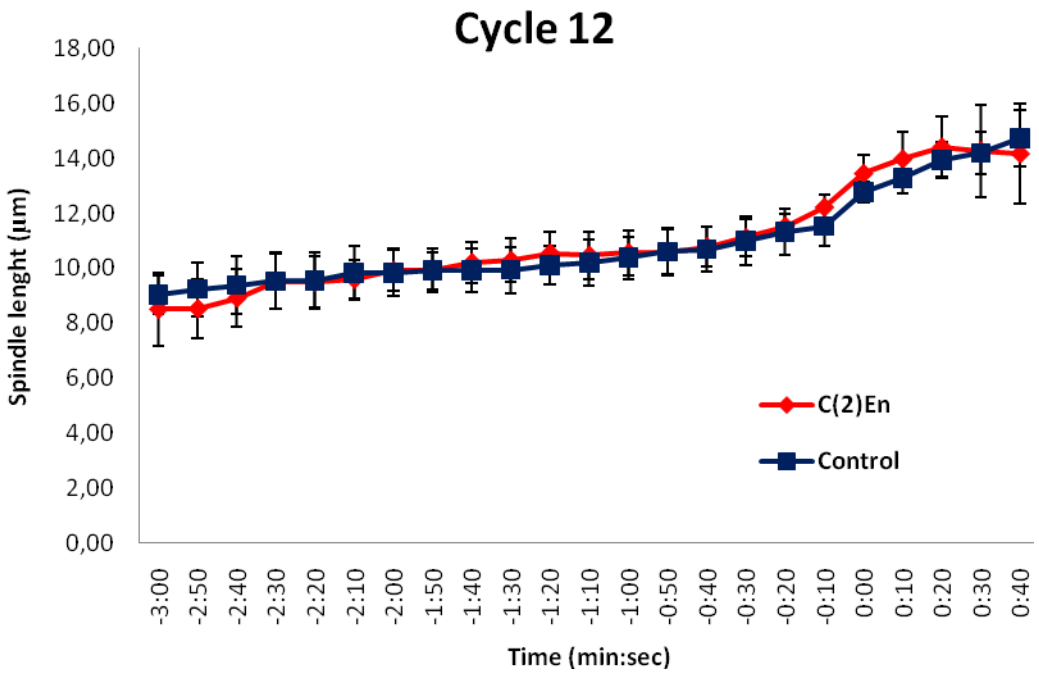

Figure 2.14: Distance between mitotic spindle poles in embryonic mitosis 12; Anaphase onset was used as reference time point (0:00); Control is represented by the blue line while the C(2)En embryo is represented by the red line;

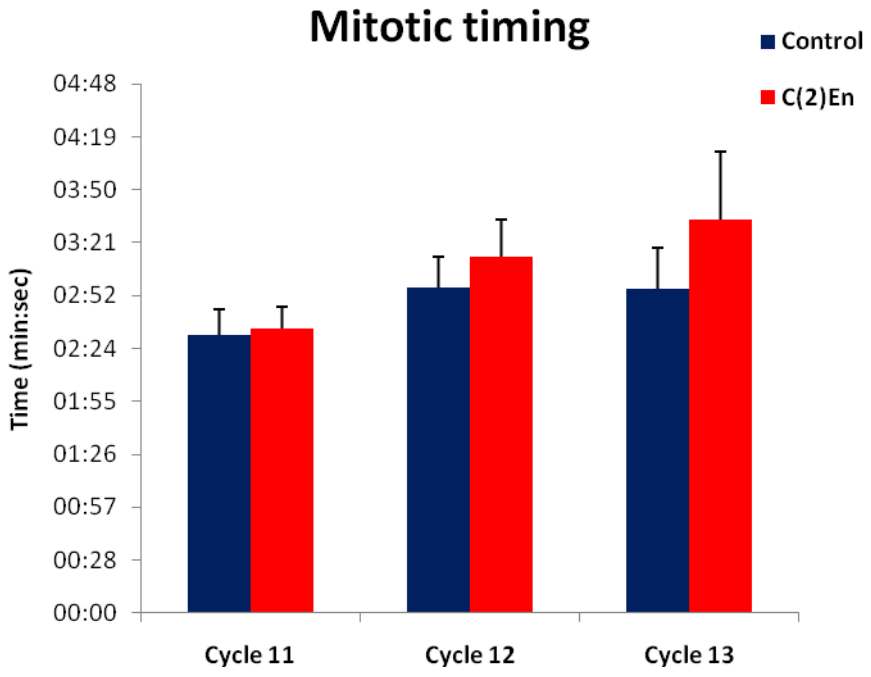

Figure 2.15: Time from NEBD to anaphase of embryonic mitosis; Control is represented by the blue line while the C(2)En embryo is represented by the red line;

Putting all the data together, we can propose that cells detect the presence of an extra long chromosome independently of cell type. Neuroblasts increase mitotic timing, specifically increasing metaphase duration. However in a system where time and space become progressively limited and all the processes have to occur very fast, nuclei containing compound chromosome do not respond to C(2)En presence until late mitotic cycles where it took more time to divide.

3. Discussion

3.1. C(2)En flies are viable and neuroblasts progress through mitosis normally

Here we wanted to study the consequences of the increase in chromosome size upon mitotic fidelity in the context of an organism. For that purpose, we used the C(2)En, a long chromosome that consists of two second chromosomes fused by a single centromere. Flies that carry the compound 2 chromosome had already been shown to be viable, however their embryos show a 10-fold increase in division errors relative to embryos with a normal karyotype; these errors do result in the elimination of the abnormal nuclei, which sink into the embryo. Yet, somatic cells such as neuroblasts do not show any phenotype and successfully segregated their chromosomes (Sullivan et al. 1993). Despite a successful segregation of the C(2)En chromosome it is possible that neuroblasts adapt in some way and allowing them to complete mitosis successfully. Interestingly, mitosis of third larval instar neuroblasts is a very efficient process since flies without the SAC essential protein Mad2 are able to survive and their neuroblasts do not show any phenotype (Buffin et al. 2007). Another possible explanation for the successful segregation of the compound 2 chromosome in this system is the fact that neuroblasts are large cells and the mitotic spindle formed is so long that can deal well with a chromosome with double size.

3.2. C(2)En Neuroblasts extend mitosis

In order to further explore mitotic progression of the neuroblasts carrying the C(2)En chromosome, we introduced into C(2)En flies chromosomes carrying fluorescent markers to study *in vivo* mitotic progression. For the first time we were able to follow the process in somatic cells of an organism and study how the cells adapt to a situation where the chromosome size was doubled. As seen in fixed preparations, we found that C(2)En neuroblasts pass through mitotic phases without showing any error. However when we looked to the kinetics of this process, we observed that C(2)En neuroblasts align their chromosomes with similar timing to wild-type but they spend more time in metaphase configuration. The extra time that these cells spent with their chromosomes aligned does not seem to be related to the spindle assembly checkpoint comparative analysis of the levels of the checkpoint protein BubR1 showed that this

protein is present at similar levels to control. This result suggests that the extra time spent in mitosis is not SAC dependent however, more work needs to be done in order to further confirm these results.

3.3. C(2)En embryos show a range of mitotic phenotypes

To extend further the studies suggesting that mitotic timing might be the major cellular response to the presence of the extra long chromosome, we analysed a stage in Drosophila development where mitosis has to occur very fast: syncytial embryo divisions. When we followed the embryonic development of C(2)En flies we found that they show two main phenotypes: a stronger phenotype, where mitotic apparatus is completely disorganized and a weak phenotype, showing some nuclei that sink into the embryo, however most of nuclei behave similarly to controls.

The stronger phenotype is characterized by randomly distributed nuclei, loss of synchrony, mitotic spindles interact with the neighbour spindles, a phenotype that is characteristic of centrosome mutants and may be the result in failure to synchronize the nuclear and the cytoplasmic cycles leaving centrosome replication uncoupled from nuclear division.. Our studies suggest that the centrosome is acting properly as it is able to localize the centrosomal marker CP190 and to nucleate microtubules. C(2)En embryonic nuclei that fail to divide duplicate its centrosomes independently of nuclear division, leading to four centrosomes surrounding a single nuclei. This situation leads to the presence of multipolar spindles and free centrosomes, similar to one that is observed after injection of the S-phase blocker aphidicolin (Raff and Glover 1988).

3.4. Late C(2)En embryos delay mitosis

Our main goal was to study how the mitotic machinery adapts and is able to cope with a long chromosome and carry out nuclear division in a very short period of time. To address that question we study in detail the timing of nuclear divisions and spindle length throughout syncytial embryonic mitotic cycles 10 to 13. Interestingly, we found that spindle size always shows the same behaviour regardless the presence or not of the compound chromosome. However, at later cycles (cycles 12 and 13) more nuclei are near the cortex and the inter-nuclei space becames more restricted. The reduction of the space causes the spindles to be smaller, and as a consequence, the C(2)En

nuclei spend more time in mitosis (nuclear envelop breakdown until anaphase onset). These data suggests that through some unknown mechanism, the Drosophila embryo can detect the presence of a long chromosome and prolong mitosis in order to segregate it, especially when space becomes limiting by the increase in number of nuclei that share a common cytoplasm.

3.5. C(2)En and NoCut

The main goal of this work was to determine whether the NoCut pathway is also present in Drosophila. Our hypothesis was that C(2)En chromosome would be too long for a normal mitosis to segregate and after anaphase onset the cell would hold cytokinesis for longer periods than wild type. Also we wanted to determined whether cellular response to the presence of the C(2)En would be to condense more the chromosome or to elongate the spindle in order to remove the DNA from the place where the cytokinesis would take place. Thus, since C(2)En flies are partly viable then different cell types of Drosophila might need to compensate in different ways to allow for the segregation of the long chromosome. Surprisingly, our studies show that the cells or embryonic nuclei adapt to the long chromosome before anaphase onset and therefore it is unlike to activate the NoCut checkpoint. One possible interpretation of these results is that cell detects the presence of a long chromosome and avoids any segregation defects by condensing their chromosomes more than control cells before anaphase. Therefore using this system we detected the presence of an early response to the presence of the C(2)En chromosome. However, some results do suggest that during cytokinesis the NoCut checkpoint does exist in Drosophila. After depletion of the condensin protein Barren a significant portion of the DNA is often left in the middle as chromatin bridges that never resolve and this causes a significant increase in the formation of binucleated cells (Oliveira et al. 2005). Barren results suggest that there is a mechanism that performs the same function of the NoCut checkpoint is similar situations.

Chapter 3

Establishment of a screening for Polo interactors

1. Introduction

Cell cycle regulation is achieved by a balance between protein synthesis and degradation as well as by post-translational modifications of the effectors that drive the process (reviewed by Pines 2006). One of the post-translational modifications most common in cell cycle regulation is the addition/removal of a phosphate group to specific residues on target proteins. Therefore studies focused on protein kinases, that catalyse the addition of the phosphate group, assume particular relevance. One of the main cell cycle kinases are the Polo kinases protein family. In mitosis Polo kinases can regulate several processes including mitotic entry, centrosome maturation and mitotic exit. Control of these processes is achieved by phosphorylation of specific target proteins (reviewed by Archambault and Glover 2009).

It has been shown that Polo itself is regulated by phosphorylation, as phosphorylation in specific residues promote its catalytic activity (Macurek et al. 2008; Seki et al. 2008). Accordingly, mutations that phosphomimick these phosphorylations result in increased Polo kinase activity (Qian et al. 1999). Phosphospecific antibodies raised against these residues in the human homologue, Plk1, show that its phosphorylation is sequential in mitosis: while Threonine 210 (T210) is phosphorylated in the early mitotic stages, Serine 137 (S137) is phosphorylated at late mitotic events. In fact, these phosphorylations have mitotic relevance as phosphomimick mutations T210D and S137D, cause mitotic arrest dependent on spindle assembly checkpoint and premature activation of the APC/C to orchestrate the mitotic exit, respectively (van de Weerdt et al. 2005). These residues are conserved among species and phosphomimicking mutations have been shown to increase the activity of Polo-kinases both in Xenopus and Human cell lines by several fold (Qian et al. 1999; Jang et al. 2002). Microinjection of mRNA encoding for S128D/T201D Plx1 into Xenopus oocytes directly induces activation of both Cdc25 and CyclinB-cdc2, supporting the idea that Plx1 is the major kinase that triggers Cdc25 activation in the G_2/M transition (Qian et al. 1999).

To study important aspects as G_2/M transition, spindle assembly and metaphase-anaphase transition regulation, we decided to take advantage of an available Drosophila strain that carries Polo-T182D under the control of an inducible promoter. Polo-T182D was mutated at the conserved residue Threonine 182 (correspondent to Threonine 210 in Plk1) to an Aspartate thus mimicking phosphorylation and leading to a constitutively active Polo kinase (Qian et al. 1999). Under these conditions we are able to determine the biological relevance of a constitutive active Polo kinase as well

as to use this dominant-negative activity to identify possible new downstream Polo interactors. To tests these effects *in vivo* we expressed Polo-T182D in the eye-antennal imaginal disc and analyzed its effects during development. Eye primordia is characterized for having a synchronous wave of cell division and differentiation, called the morphogenetic furrow, during the third instar larvae development (reviewed by Dominguez and Casares 2005). Importantly, eye defects rarely cause lethality allowing the recovery of adult flies with eye phenotypes that can be observed in the adult and easily quantified (Dietzl et al. 2007). Together with the tools that allow the study of mutations specifically in the eye disc, this model is very powerful to screen for enhancers or suppressors of specific gene mutations (Mummery-Widmer et al. 2009). Taking advantage of this system, we expressed the Polo-T182D transgene under control of imaginal disc specific drivers.

Our preliminary results showed that expression of Polo-T182D leads to lethality when expressed in the entire eye under the *eyeless*-Gal4 driver at 25ºC, and induces a strong reduction in the eye size at 18ºC. Expression of Polo-T182D in the wing imaginal disc by the *nubbin*-Gal4 driver causes severe reduction in size at both temperatures. The reduction in either eye or wing when Polo-T182D is expressed appears to occur through a mechanism that that ultimately causes apoptosis, as organ size can be restored by blocking apoptosis through the overexpression of the apoptotic inhibitor Diap1. With this approach we were able to establish the conditions to set up a screening of more than 200 putative Polo targets that can be affected by the extra activation of Polo.

2. Results

2.1. Overexpression of the mutant transgene Polo-T182D causes lethality and reduction in organ size

In order to get a deeper knowledge about the biological role of Polo in cell cycle, we used an existing Drosophila strain that carries the phosphomimicking mutant form Polo-T182D that can be expressed under the control of an inducible promoter (UAS). This inducible transgene allows overexpression in a tissue-specific manner. Therefore the first approach was to overexpress Polo-T182D in the eye-antennal imaginal disc, using the *eyeless*-Gal4 driver. When Polo-T182D is overexpressed at 25ºC, the flies follow the normal development steps, similarly to wild-type until puparium formation however they fail to eclode and die as dark pupae. Dissections of pupae showed that they had very small head or even complete absence of head development suggesting that expression of the PoloT182D at 25ºC in the whole eye imaginal disc causes generalized cell death or a failure in cell multiplication (Fig. 3.1). In order to decrease Gal4 expression in eye development flies were developed at 18ºC, so that expression of the Polo-T182D could be reduced. At this temperature we obtained viable flies, developing a normal head but showing reduced eye (Fig 3.1).

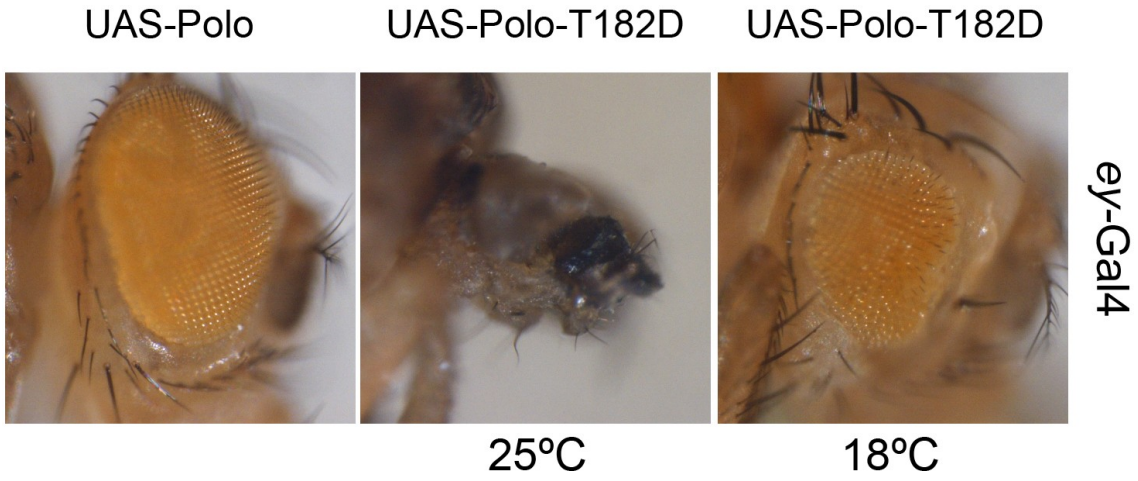

Figure 3.1: Effects on eye development caused by Polo-T182D overexpression: Polo overexpression was directed to the eye imaginal disc using the *eyeless*-Gal4 driver; UAS-Polo was used as control to the overexpression of UAS-Polo-T182D. Eye-driven expression of Polo-T182D results in lethality at 25ºC and an eye reduction at 18ºC;

To test whether Polo-T182D expression caused an eye specific phenotype or whether it has an overall effect in cellular proliferation we expressed the transgene in

others imaginal discs. Two drivers of the wing (*ms1096*-Gal4 or *nubbin*-Gal4) were used to express Polo-T182D at 18ºC and 25ºC. Unlike in the eye, expression of Polo-T182D in the wing did not result in lethality at 18ºC or at 25ºC temperature. When *nub*-Gal4 was used, wing formation was completely abolished at both temperatures and when driven by *ms1096*-Gal4, expression of Polo–T182D resulted in a very small wing at 25ºC that is slightly larger at 18ºC (Fig. 3.2).

Figure 3.2: Effects on wing development caused by Polo-T182D overexpression: UAS-Polo was used as a control and two different drivers were used to direct overexpression of mutated Polo to the wing: *ms1096*-Gal4 and *nubbin*-Gal4;

Moreover, when Polo-T182D was expressed using the *ms1096*-Gal4 driver, there was a reduction in the number of bristles in the adult scutum, a tissue also derived from the wing imaginal disc (Fig. 3.3).

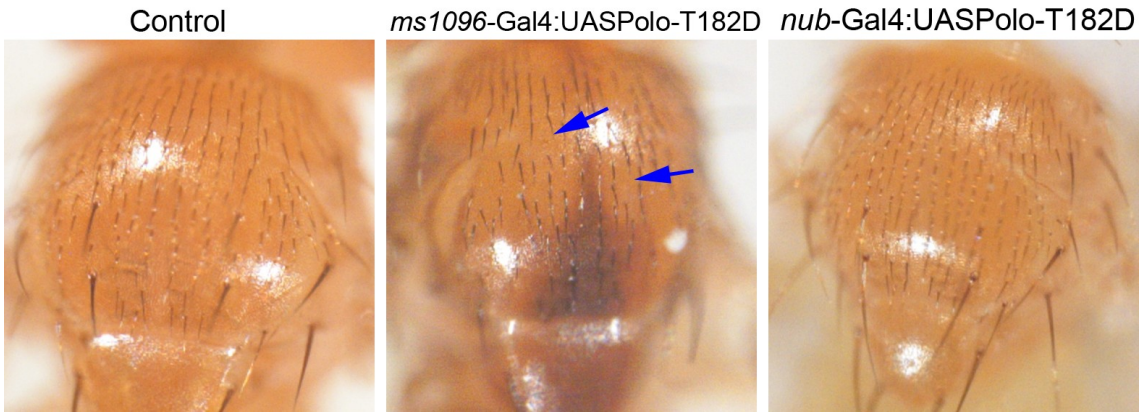

Figure 3.3: Loss of hairs in the scutum developed due to Polo-T182D expression: *ms1096*-Gal4 drives the expression of UAS-Polo-T182D to the wing pouch and also to notum which results in bristles loss;

2.2. Reduction in organ size is mainly due to apoptosis

Studies done with the equivalent mutation to T182D in Plk1 showed that this mutant form delays mitotic exit (van de Weerdt et al. 2005). To check this feature on flies we studied the development of the eye disc by immunofluorescence during third instar larval development. We found that eye primordia development is severely compromised in discs expressing the Polo-T182D transgene when compared to controls, showing a very small undifferentiated imaginal disc (Fig. 3.4).

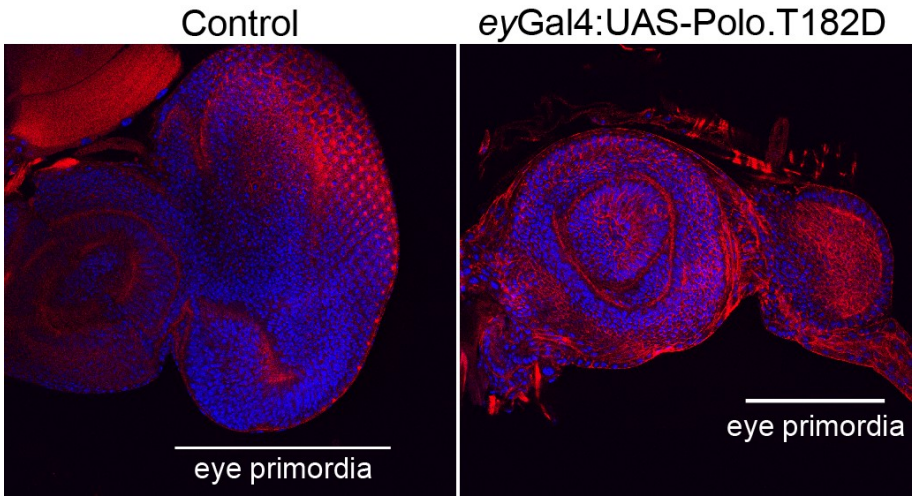

Figure 3.4: Overexpression of UAS-Polo-T182D with *ey*-Gal4 at 25ºC; Mutated Polo overexpression causes reduced third instar larvae eye imaginal discs; DNA is stained in blue and the cytoskeleton marker rhodamine phalloidin is in red;

In order to study further the cellular behaviour after expression of Polo-T182D we use the FLP/FRT-based recombination to induce clones of cells expressing Polo-T182D during early larval development. These clones also carry as a marker the GFP reporter gene. Our analysis shows that mitotic cells are not enriched in the somatic clones produced by expression of Polo-T182D suggesting that expression of the Polo-T182D protein does not specifically lead to mitotic arrest (Fig. 3.5A-D). Higher magnification images took from different clones marked with GFP showed to contain micronuclei, a feature of apoptotic cells (Fig. 3.5E and F).

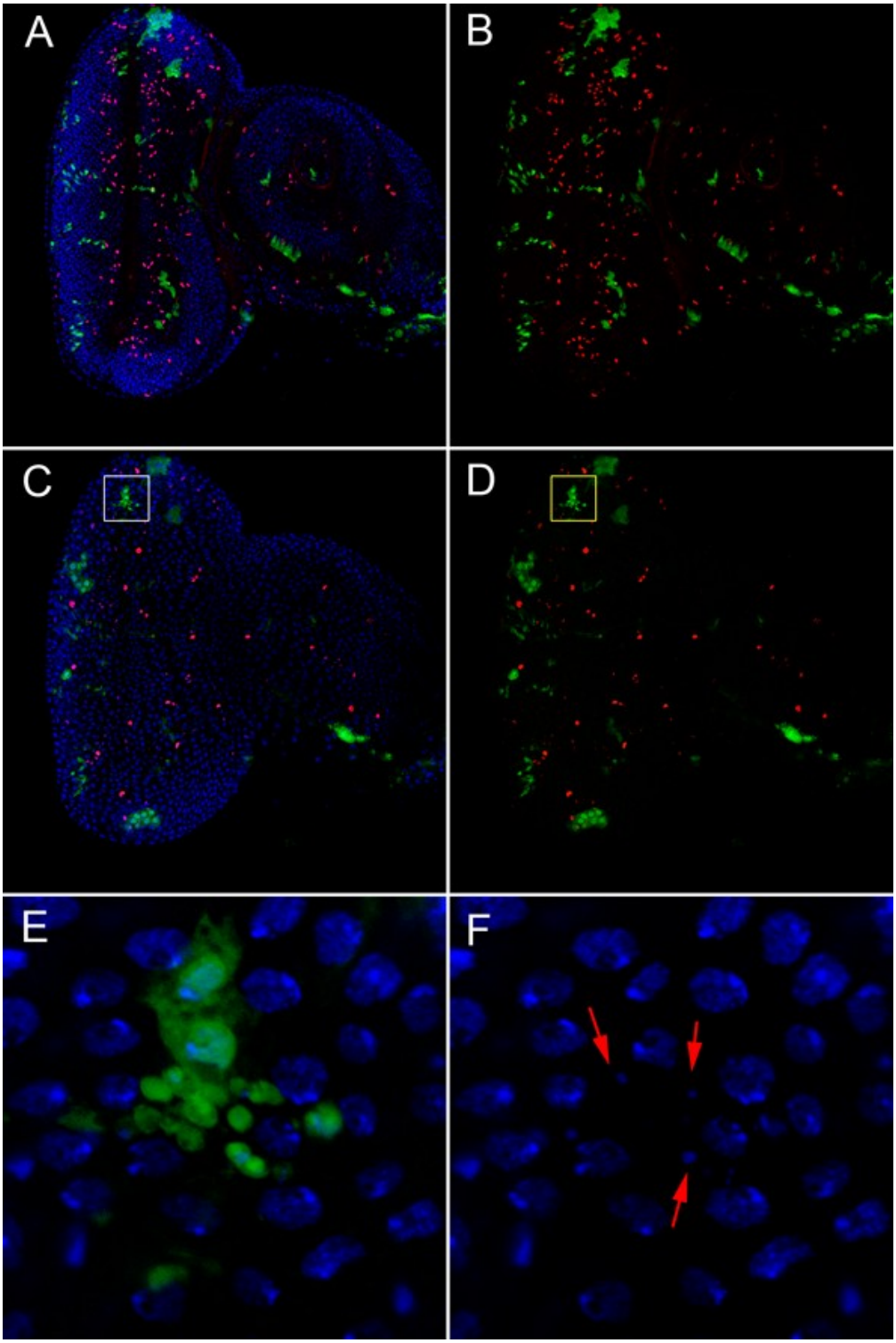

Figure 3.5: PH3 staining in third instar larvae eye imaginal discs with Polo-T182D clones induced 48h before: A and C – Merged images showing somatic clones overexpressing Polo-T182D in green, DNA is stained in blue and the mitotic marker PH3 is in red; B and D – Merged images showing clones of Polo-T182D cells in green and PH3 in red; E and F – higher magnification of the squares highlighted in figure D. Arrows point to small apoptotic bodies that often appears in Polo-T182D somatic clones;

To determine if the small micronuclei observed were result of apoptosis and consequently the cause of eye reduction, a transgene containing the apoptotic inhibitor Diap1 under the control of a UAS promoter was introduced into the strain expressing Polo-T182D. Under these conditions we were able to recover the complete size of the eye at 18ºC and rescued the lethality at 25ºC. Although the eyes observed after co-expression of the apoptosis inhibitor have the normal shape, these often show irregular shape, as the tissue differentiation does not occur properly suggesting that Polo may also have a function in tissue development and cell differentiation (Fig. 3.6).

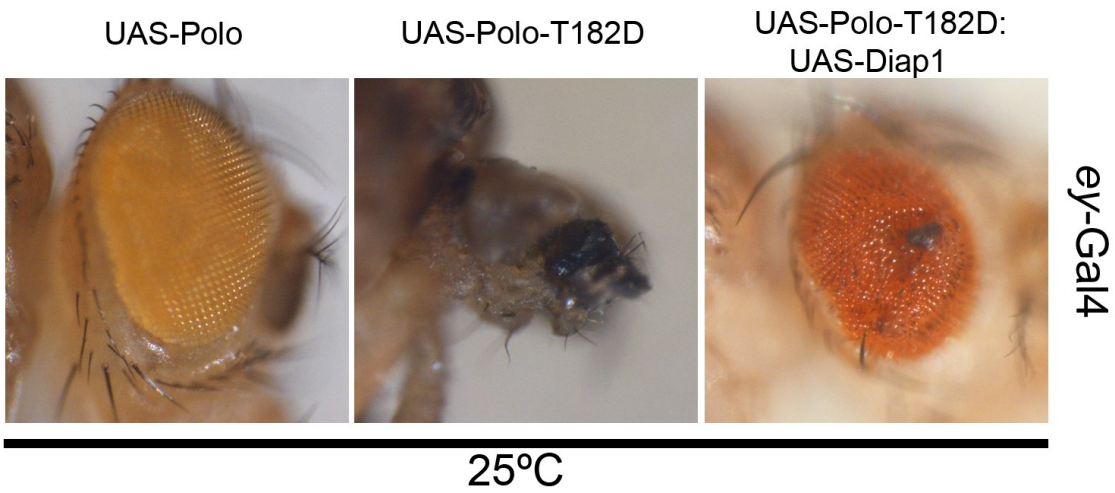

Figure 3.6: Recovery of the viability of flies that overexpress Polo-T182D during eye development: UAS-Polo was used as control, and lethality conferred by expression of UAS-Polo-T182D is recovered when apoptosis is blocked by overexpression of the apoptosis inhibitor Diap1;

All these results taken together suggest that apoptosis is the main cause of the reduction in organ size upon expression of a constitutively active form of Polo kinase. However, we do not yet know why does the hyperactivation of Polo caused by the mutation Polo-T182D results in apoptosis. Further work is required to solve this issue.

2.3. Candidate genes

While we have not yet determine why expression of Polo-T182D results in apoptosis and cell death, the various phenotypes observed either in the wing or the eye offer an excellent possibility to serve as marker in a screen designed to identify downstream effectors of Polo activity. As a first step to identify possible interactors of Polo we searched the interactions database DroId (www.droidb.org) for putative

interactors of Polo described in Drosophila or in studies/screenings done in other organisms. From our research we found a set of about 200 candidate genes, whose interactions were identified by a variety of different experimental approaches. From the interactors list, 160 are proteins with known function but 38 are proteins whose function remains to be uncovered and which represent potential targets of Polo. In order to organize the list of possible interactors a network with Polo in the centre was built and the genes were distributed according to described interactions and its biological function (Fig. 3.7). The network shows genes clustered in specific categories according to their function and each surrounded by a specific colour. The yellow group shows proteins involved in centrosome organization; the spindle assembly and spindle assembly checkpoint genes are included in the group defined by the green line; proteasome regulatory proteins by the red line; cell cycle control by the blue line; DNA binding/replication proteins by the pink line; nuclearpore/nuclear transport proteins are limited by the brown line, and an unspecific group of proteins with unknown function is shown in the bottom of the image. Therefore using this approach we can dissect which biological process may be affected by Polo hyperactivation and identify new targets of Polo.

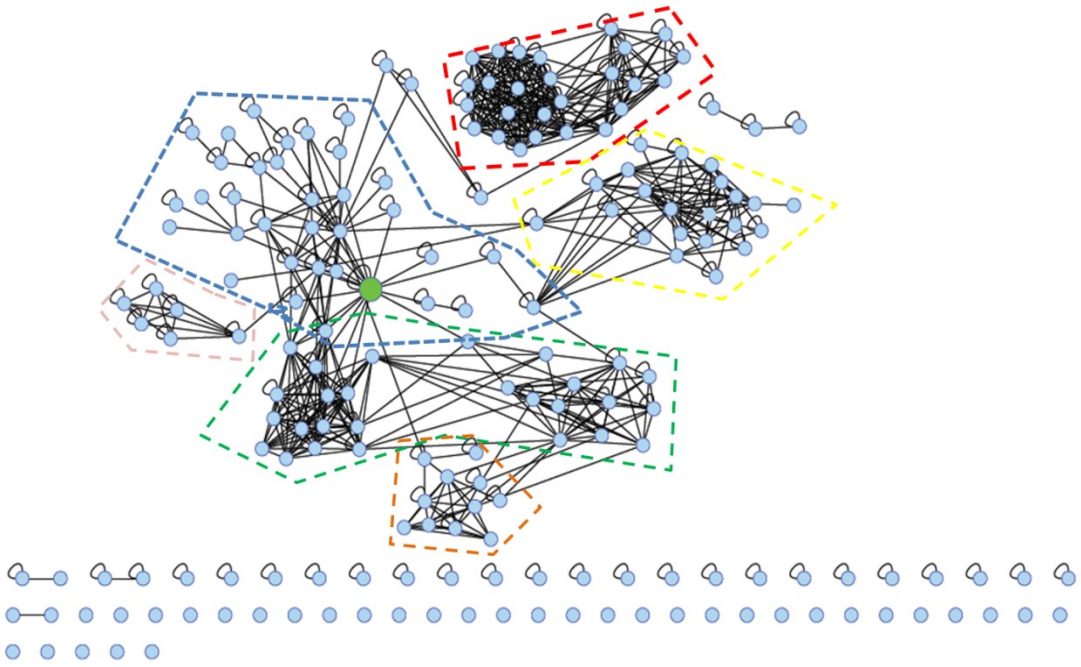

Figure 3.7: Schematic representation of the Polo interacting genes. In the yellow branch we display proteins involved in centrosome organization; the spindle assembly and spindle assembly checkpoint genes are limited by the dashed green line; proteasome regulatory proteins are put together in the red group; cell cycle control in blue; DNA binding/replication proteins in pink; nuclearpore/nuclear transport proteins are limited by the brown dashed line, and an unspecific group of proteins with unknown function is shown in the bottom of the image.

2.4. Candidate genes phenotype

As a general strategy to test the candidate genes, we decided to co-express in the same cells the Polo-T182D transgene and specific RNAis to downregulate each one of the 200 putative interactors. The majority of the RNAi constructs (198) against these proteins including Polo itself were obtained from the Vienna stock collections (http://www.vdrc.at/). We decided to add to this list a set of 21 stocks available in the lab. These stocks correspond to proteins involved in chromosome condensation, kinetochore organization and cell adhesion. These strains contain transgenes that express the appropriate RNAi under the control of an UAS sequence. First, we assess the phenotype resulting from the expression of these RNAis in the eye and in the wing alone, using for that purpose the *eyeless*-GAL4 and *nubbin*-GAL4 drivers, respectively. We then evaluated the phenotype in the adult individuals.

To analyse and classify the phenotype of each of the candidate genes we divided the observed phenotypes based on the severity of eye reduction into several categories. No eye reduction, Weak eye reduction, Medium eye reduction, Strong eye reduction and lethal (Fig. 3.8). During the course of the screening we observed that males often presented a more severe phenotype than females, under the same conditions. Therefore we decided to score males and females separately.

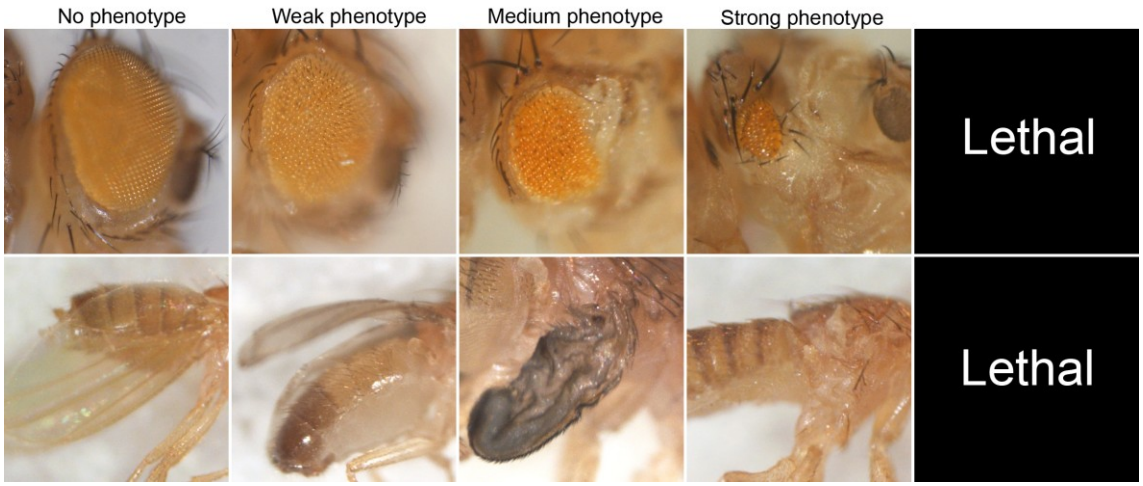

Figure 3.8: Classification of the phenotypes observed during the RNAi screening: In order to simplify the outcome of the RNAi screen we classify the phenotypes as: No phenotype, the strains that present eye and wing similar to wild type, weak phenotype the ones that present a little reduction or disorganization in organ size, medium phenotype the ones that have about half of the size of the eye/wing, strong phenotype represent the stocks that do not develop eye or wing and lethal are the ones where the flies fail to born from pupa stage;

We determined the male eye phenotype of 215 RNAi stocks. Of these 131 do not show any phenotype when the RNAi are expressed on their own, 13 have a Weak reduction in the eye size, 12 have a Medium eye reduction, 19 present a Strong eye phenotype or almost absence and 39 are lethal when expression is driven by *ey*-Gal4 at 25ºC. In the female eye phenotype we are able to score 219 RNAi phenotypes, being 32 lethal, 14 with a Strong phenotype, 20 have a Medium eye phenotype, 16 present a Weak phenotype and 136 do not show any phenotype when induced by *ey*-Gal4. It is also important to note that in one of the stocks we found one overgrowth phenotype (Fig. 3.9). This stock targets the *gig* gene (or TSC2) a tumour supressor gene involved in the insulin pathway (Gao and Pan 2001) that controls cell growth and cell proliferation (Tapon et al. 2001).

To diminish the expression of Gal4 the stocks expressing the RNAis were grown at 18ºC and we observe that the phenotypes are less severe as males of 134 RNAis strains do not show any phenotype, 8 stocks have a Weak eye phenotype, 11 with Medium eye phenotype, 16 show Strong eye phenotype and 37 are lethal. A similar analysis in females showed that 139 RNAi stocks do not show any phenotype, 14 have a Weak phenotype, 12 do have a Medium phenotype, 10 have a Strong eye phenotype and 35 are lethal (Fig. 3.10). Comparing males and females results we can observe that males often present a phenotype that is to some degree stronger than females, for example a gene that is lethal for males often present viable females but with a Strong phenotype.

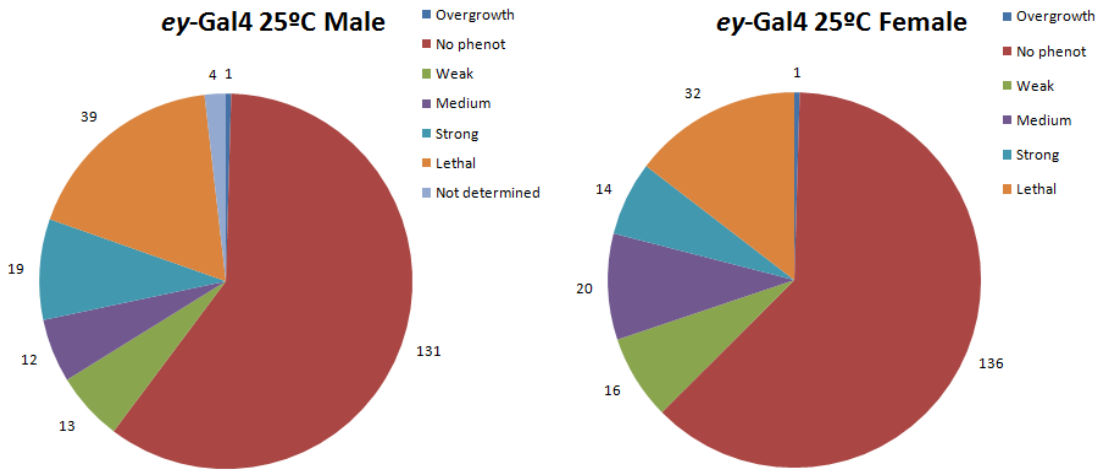

Figure 3.9: Distribution of the eye phenotypes based on the eye size at life cycle temperature 25ºC: Round graphics that shows the classification of the RNAi strains according to the severity of the phenotypes presented in figure 8; *ey*-Gal4 phenotype of the RNAi stocks is stronger in the males than in females; Not determined class that appears in the male distribution

is due to RNAi stocks that have the RNAi construct inserted in the X chromosome, therefore in our first generation screening only the females receive the RNAi construct;

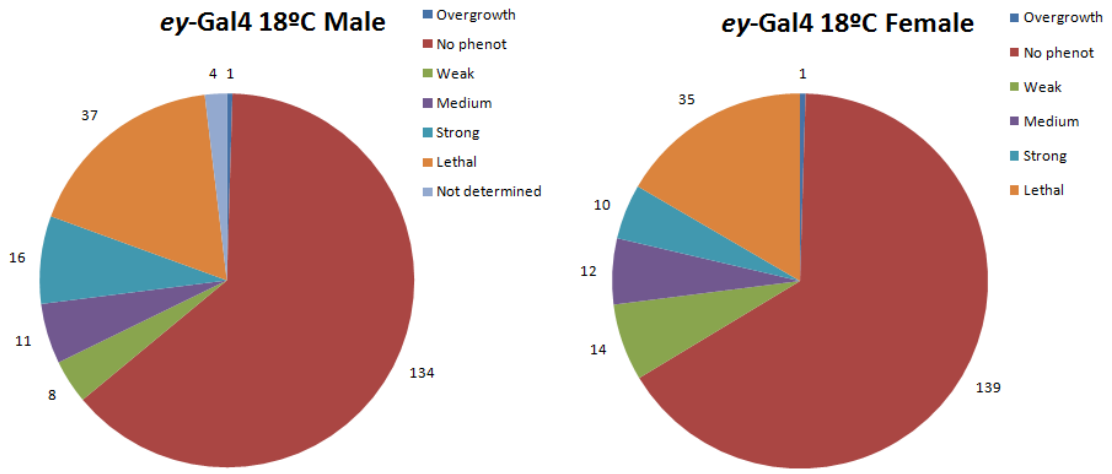

Figure 3.10: Distribution of the eye phenotypes based on the eye size at life cycle temperature 18ºC: Round graphics that shows the classification of the RNAi strains according to the severity of the phenotypes presented in figure 8; *ey*-Gal4 phenotype of the RNAi stocks is stronger in the males than in females; Not determined class that appears in the male distribution is due to RNAi stocks that have the RNAi construct inserted in the X chromosome, therefore in our first generation screening only the females receive the RNAi construct;

In order to test whether the effects we observed with the different RNAis are eye imaginal disc specific or whether they affect cell cycle in general we used the *nubbin*-Gal4 driver to express all RNAis in the wing. Similarly to what was observed in the eye, expression of the RNAis results in very different phenotypes in the wing which could be classified in terms of reduction in organ size. The wing phenotypes were divided into 5 different categories: No phenotype, Weak phenotype, Medium phenotype, Strong phenotype and lethal. Unlike when expressed in the eye-antennal disc, RNAi stocks driven by the wing promoter does not present any difference between males and females, so all the results were grouped. When flies are grown at 25ºC, we found that 93 stocks show no phenotype, 13 have a Weak phenotype, 51 present a Medium phenotype, 29 have a Strong phenotype and 30 are lethal. When grown at 18ºC, 85 stocks do not show any wing phenotype, 40 present Weak phenotype, 17 have a Medium phenotype, and the number of RNAi stocks with Strong phenotype decreased to 21 as well as the number of lethal stocks that decreased to 23 (Fig. 3.11).

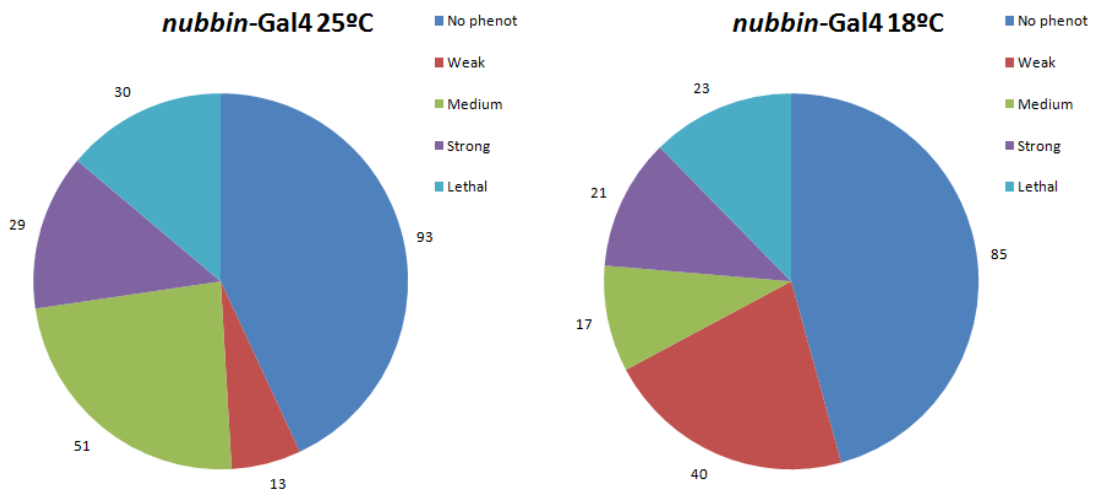

Figure 3.11: Distribution of the wing phenotypes based on the size of the wing: Round graphics that shows the classification of the RNAi strains according to the severity of the phenotypes presented in figure 8; *nubbin*-Gal4 expression is stronger at 25ºC than 18ºC, so phenotype of the RNAi stocks is stronger at 25ºC;

Together the results suggest that the use of the *nubbin*-Gal4 driver to screen for wing defects is more sensitive than using *eyeless*-Gal4, as the number of RNAi stocks that do not present phenotype at 25ºC decrease from 131/136 to 93. However due to the variety of phenotypes it is more difficult to have a proper classification of the phenotypes.

Table I: Classification of the phenotypes at 25ºC according to the eye reduction severity of the diverse RNAi groups represented in figure 7;

Categories	Proteasome	DNA binding	Cell cycle control	Nuclear transport	Centrosome organization	Spindle assembly and SAC
No phenotype	7	2	18	5	14	19
Weak phenotype	1	0	2	1	4	0
Medium phenotype	1	0	2	2	1	2
Strong phenotype	0	2	4	1	0	5
Overgrowth	0	0	1	0	0	0
Lethal	16	1	2	2	3	0

Analysing the results obtained according to the biological significance group category originally identified in the network profiles (Fig. 3.7), one can observe that the proteasome genes present the higher number of lethal genes that any other class. These genes are essential for fly development as their downregulation by RNAi even in specific imaginal discs like the eye or the wing result in lethality (Table I and II). The other categories do not present more than 3 genes that are essential for fly development, and the majority of the genes do not present any phenotype when its RNAi was driven to either tissue (Table I and II).

Table II: Classification of the phenotypes at 25ºC according to the wing reduction severity of the diverse RNAi groups represented in figure 7

Categories	Proteasome	DNA binding	Cell cycle control	Nuclear transport	Centrosome organization	Spindle assembly and SAC
No phenotype	4	1	14	4	12	13
Weak phenotype	0	0	4	0	2	3
Medium phenotype	4	3	4	2	4	4
Strong phenotype	1	0	4	3	2	7
Lethal	14	1	2	1	2	1

As a general control the RNAi against Polo was also expressed in both imaginal discs and at different temperatures. The results show that when RNAi against Polo is expressed in the wing, it presents a similar strong phenotype as observed for the expression of the mutated form. However this result does not imply that Polo mutated form is working as dominant negative since when Polo RNAi is driven to the eye, the flies die in the pupa stage at both temperatures, a very different from the outcome of expressing Polo-T182D. Together these results suggest that expression of Polo constitutively active form is different from the situation where there is no Polo.

3. Discussion

Like all other protein kinases, Polo is regulated by phosphorylation (Eckerdt and Strebhardt 2006). To analyse the effect of a specific phosphomimicking mutation in a conserved residue that was shown to have biological relevance, we express Polo-T182D in specific tissues during fly development. Although Polo is one of the mitotic regulators most studied and well understood, it is clear that there are still many unanswered questions regarding its function (reviewed by Archambault and Glover 2009). Importantly most of the studies have been done in cultured cells or cell extracts, which does not necessarily represent the in organism relevance. The activity of the human homologue Plk1 is known to be regulated by upstream kinases at a well conserved aminoacids: Threonine residue at position 210 in human, Threonine 201 in Xenopus and T182 in Drosophila. In Human cell lines T210 was shown to be phosphorylated by the Aurora A in a Bora-dependent manner (Macurek et al. 2008; Seki et al. 2008). The amino acid substitution of T210 by Aspartate results in a premature mitotic entry and a delay in mitotic exit (Jang et al. 2002; van de Weerdt et al. 2005). However, our results show that the eye reduction phenotype is not caused by a failure in mitotic exit but rather to activation of apoptosis. If apoptosis occurs after a long mitosis or as a result of an aberrant mitotic exit is an issue that need to be studied further. However, our goal in this project was to use the phenotypes caused by expression of the Polo-T182D as a read-out to search for Polo downstream interactors by co-expressing a RNAi library with all the genes that were shown to probably interact with Polo. We identified about 200 genes for which RNAi stocks were available in the VDRC collection (Dietzl et al. 2007). With this candidate gene list we then created a network to organize them into specific classes according to their function and to the functions that have been described for Polo (reviewed by Archambault and Glover 2009). The resulting network provided a clear picture of putative interactors in the proteasome, the centrosome, spindle assembly and spindle assembly checkpoint, cell cycle control, DNA binding/replication proteins, nuclearpore/nuclear transport proteins and a group of proteins with unknown function that could represent new Polo possible targets not yet described in Drosophila.

Before carrying out the screen we had to first analyze the effect upon eye and wing development of expressing RNAis for each gene individually before co-expression with Polo-T182D could be done. The results indicate that in all gene classes there are RNAis that result in no phenotype, Weak, Medium, Strong phenotypes or lethality. Interestingly, the results also show that the fly is particularly sensitive to expression of

RNAis for genes involved in proteasome function since most results in fly lethality. The reason for this is unknown but future studies will be carried to address this issue. In the meantime, the results of the preliminary screen allowed us to determine with great accuracy the individual RNAis phenotypes, an essential condition to look at co-expression with the Polo-T182D transgene.

PART III

GENERAL DISCUSSION

Cell proliferation is an essential process that ensures the maintenance of biological organization of all living organisms. It allows the maintenance of the species of single cell organisms and the formation of an entire multicellular organism from a single cell, as well as maintaining the homeostasis of their tissues and organs after its formation. In the context of this process, cell division and particularly mitosis plays a central role. Mitosis is a particularly appealing stage of cell division since there are major intracellular changes that are highly dynamic and can be easily followed by microscopy. At this stage it is possible to observe dramatic changes in the whole cell cytoskeleton as well as the equal distribution of the previously replicated genome into two daughter cells.

In order to achieve bona-fide division, mitosis has very specialized structures that ensure a very high efficiency in the main processes. One of these specialized structures is the kinetochore. It consists in a group of multi-protein complexes that assembles in a particular region of the chromosome, the centromere. Kinetochore is present in every mitotic chromosome of eukaryotic cells and many of the basic components are functionally highly conserved even though structurally might show significant divergence.

The first experimental work chapter part of the work presented in this thesis aims to understand the involvement of the Drosophila Sgt1 orthologue in the kinetochore assembly pathway and special emphasis was given to the role of the kinetochore as a platform for Spindle assembly checkpoint signalling. Studies in budding yeast showed that the complex Hsp90-Sgt1 is essential for the initial steps of kinetochore assembly (Kitagawa et al. 1999). Interestingly, this function seems to be conserved as both RNAi depletion of Sgt1 (Steensgaard et al. 2004) or treatment of human cells with an Hsp90 inhibitor resulted in a failure to localize several kinetochore proteins (Niikura et al. 2006). However, when these cells are analyzed by electron microscopy, the whole kinetochore structure does not seem affected and kinetochores bind microtubules normally (Niikura et al. 2006), suggesting that basic structure and functions of the kinetochore can be assembled without Hsp90-Sgt1. More recently it was shown that the Hsp90-Sgt1 complex interacts directly with the Mis12 complex and that this interaction is required for the stabilization of the Mis12 complex at kinetochores (Davies and Kaplan 2010). Thus, these results show that Hsp90-Sgt1 complex is essential to promote kinetochore assembly in budding yeast however the function of this complex in human cells seems to be more specific for some kinetochore proteins.

As referred above in yeast and human cells, Sgt1 has been shown to be required for kinetochore assembly. To further address Sgt1 function in Drosophila, we characterize a mutation of Sgt1 and showed that overall the structure and function of

the kinetochore in somatic cells is not affected. However, other aspects of mitosis are severely affected resulting in lethality of Sgt1 mutants. Nevertheless, detailed analysis shows that in the absence of Sgt1 all the structural components of the kinetochore analyzed localize normally and at similar levels to controls. In our work we also show that *sgt1*^{P1}cells arrest in prometaphase with high levels of SAC proteins at the kinetochore. In the absence of Sgt1, mitotic chromosomes are able to assembly the kinetochore and the kinetochore function as a platform for SAC signalling is not affected. We consider three possible explanations for these divergent results between Drosophila and other species. It is possible that in Drosophila, the kinetochore assembly pathway is very different from the one described in yeast and humans, and occur through a mechanism that is Sgt1 independent. Alternatively, it is still possible that Drosophila Sgt1 may be required to stabilize only a very specific group of kinetochore proteins, different to the ones that were studied such as what was recently shown for Mis12 complex (Davies and Kaplan 2010). Finally and given that the Drosophila Sgt1 lacks the TPR domain, it is possible that other TPR-containing proteins interact with Hsp90 and are essential for kinetochore assembly. Bioinformatic analysis does indeed show that the Drosophila genome contains several proteins that contain the TPR domain however none also have the SGS domain characteristic of Sgt1-like proteins. Therefore, as in Drosophila, many proteins have been shown to diverge significantly from their yeast or human homologues or even divided the function into two separate proteins, as in the case of separase (Jager et al. 2001), it is possible that another protein performs this kinetochore specific function.

Nevertheless, *sgt1*^{P1} cells show severe mitotic abnormalities including a highly abnormal organization of the mitotic apparatus. We find significant alterations in spindle organization and of the centrosomes. The alterations include dispersion of the PCM components and also cells containing only a single centrosome during mitosis. Putting together these observations with its centrosomal localization during mitosis raise the possibility that Sgt1 might be required for proper organization of the centrosome. Indeed, the centrosome abnormalities are highly reminiscent to those observed after mutation or inhibition of Hsp90 function in Drosophila and human cells, suggesting that Hsp90 and Sgt1 might participate in a common pathway (Lange et al. 2000). Centrosome phenotypes of Hsp90 mutants were shown to be linked to a destabilization of Polo kinase (de Carcer et al. 2001), a key protein on the process of centrosome maturation (Sunkel and Glover 1988). Therefore we analyzed the presence of Polo in various mitotic structures during cell division of Sgt1 mutant cells. We find that in these cells, Polo is destabilized and its levels are reduced, both at kinetochores and in total protein extracts. As Sgt1 is a co-chaperone of Hsp90 in other species, we speculate

that Sgt1 might act as a co-chaperone and directs Hsp90 to Polo for its stabilization and proper physiological conformation. In fact when Polo was overexpressed in the absence of Sgt1, we observe that its levels do not increase as in controls, supporting the idea that Sgt1 is required for its stabilization. Despite the low increase in Polo levels, overexpression of Polo is sufficient for a significant recovery of the Sgt1 mitotic phenotype, suggesting that Polo low levels are one of the causes for the centrosome phenotype observed. By contrast, in human cells treated with the Hsp90 inhibitor 17-AAG, Plk1 levels remain constant, suggesting that human cells might have backup mechanisms to stabilize this important kinase (Niikura et al. 2006).

Moreover, Sgt1 functions do not appear to be restricted to mitosis, as analysis of the cell cycle profile done with S-phase, and G_2/M markers show that Sgt1 mutant cells are not progressing through the cell cycle as much as the wild-type controls. Taken together, our results lead to a model where *Dm*Sgt1 has at least two essential functions, during interphase to promote transit from G_1 to S-phase and later in mitosis to promote the stability of Polo kinase.

Together with the regulation of Polo levels by Sgt1 there are other mechanisms that ensure the regulation of this important kinase. One of these mechanisms is the phosphorylation of Polo in a well-conserved residue that leads to an increase in Polo kinase activity (Qian et al. 1999; Jang et al. 2002). On the last experimental chapter of the thesis we described preliminary results in the study of the T182D mutation of Polo.

To study the role of Polo regulation at T182 residue, we have expressed a transgene that contains a phosphomimicking mutation and expressed in different cells types during development of the fly. We find that expression of Polo-T182D either in eye or wing imaginal cells results in lethality or reduction in organ size. The reduction is dependent on the levels of Polo-T182D expression since growth at lower temperatures that decrease expression of the transgene activator Gal4 results in less severe phenotypes. Reduction in organ size after expression of the Polo-T182D mutant transgene is due to apoptosis since inhibition by co-expressing the apoptotic blocker Diap1 results in significant recovery of organ size. Yet, we have not yet determined whether apoptosis occurs after a long mitotic delay or as a result of an aberrant mitotic exit. Nevertheless, our objective in this project was to use the phenotypes caused by expression of the Polo-T182D as a read-out to carry out a genetic screen. The aim of this screen is to search Polo downstream interactors by co-expressing a RNAi library with all the genes that by different approaches have been shown to be putative Polo interactors. At this stage the screen is under way.

In the second chapter we aimed to study the presence of the NoCut checkpoint (Norden et al. 2006) in Drosophila. For that propose we used a chromosome that would

be so long that a normal spindle should be unable to segregate it properly, consequently leading to the activation of this checkpoint. The chromosome that we used consists in two second chromosomes joined by a single centromere (C(2)En), resulting in a chromosome with double its length (Novitski et al. 1981). Despite of having a low hatching rate, flies carrying this chromosome are viable and do not display any phenotype. Thus, we wanted to determine how do cells of this strain cope with the extra long chromosome and which are the cellular mechanisms that allow its successfully segregation. For this we carried out our study by following mitosis of different cell types and different stages of development of flies carrying the C(2N)En *in vivo* using fluorescent markers for the DNA and mitotic spindle. Using chromosome non-disjunction we described a new method to construct strains carrying both fluorescent markers and the C(2N)En. The results show that instead of the expected cytokinesis delay that is observed in yeast (Y. Barral, personal communication), Drosophila neuroblasts carrying the C(2)En prolong mitosis, specifically at metaphase. This response appears to be spindle assembly checkpoint independent since analysis of levels of the checkpoint protein BubR1 at kinetochore do not show any alteration. One possible explanation is that the extra time provided during metaphase allows extra condensation of the chromosomes similarly to what has been observed in yeast (Y. Barral, personal communication). An alternative explanation for the successful segregation of the C(2)En in this system could rely on the fact that neuroblasts are large cells and the mitotic spindle formed is so long that can deal well with a chromosome with double size. Moreover in a stage where the time to complete mitosis is very limited, the early Drosophila embryo during syncytial division, we observe that nuclei up to cycle 11 completed mitosis in a similar time to control embryos. However when the space between nuclei became reduced at cycles 12 and 13, nuclei also took more time during mitosis. These results suggest that both cells and the late embryo syncytium respond to the presence of the extra long chromosome.

In conclusion, our studies have provided new insight into the mechanism of Polo stabilization, as well as the biological significance of mutations in the Polo-T182 residue. We also gave new insights in how cells adapt to an increase in chromosome size. Furthermore, as Polo is emerging as a new potential anti-cancer target, the data obtained here might help in the development of new anti-cancer therapies and to understand the effects of Polo inhibition.

PART IV

MATERIALS AND METHODS

Transfection of S2 cells

Drosophila S2 cells were grown to $3x10^6$ cells in 3 mL of Schneider medium (Sigma) with 10% FBS (Fetal Bovine Serum) at 25ºC and then transfected with pMT-EGFP-Sgt1 using calcium phosphate. Expression of the EGFP-Sgt1 construct was induced with $CuSO_4$ (1mM) and cells were collected after 16 hours for immunofluorescence analysis. Where indicated cells were treated with 10 µM of colchicine for the desired period. The pMT-EGFP-Sgt1 vector was generated by cloning Sgt1 full length cDNA into the HincII/SacI sites of the MCS of pMT-EGFP-C1 plasmid.

Immunofluorescence in Drosophila S2 cells

Cells were centrifuged onto slides, fixed in 3.7% methanol free formaldehyde, 0.5% Triton X-100 in 1 x PBS for 10 min followed by three washes in PBS-T (1x PBS, 0.05% Tween 20) for 5 min. For visualization of α-tubulin, cells were firstly fixed in 4 % formaldehyde in 1 x PHEM (see appendix 2 for recipe) and subsequently extracted with 0.5% Triton X-100 in 1 x PBS for 10 min Blocking was performed in PBS-TF (PBS-T, 10% FBS) for 30 min at room temperature. Primary antibody incubations were performed in PBS-TF for 1 h at room temperature followed by PBS-T wash (three times for 5 min). Incubation with fluorescent labeled secondary antibodies was according to manufacturer's instructions (Molecular Probes, The Netherlands). Slides were washed again three times with PBS-T for 5 min and mounted in Vectashield with 1 µg/ml of 4',6'-diamidino-2-phenylindole (DAPI) (Vector, United Kingdom).
Images were collected either in the Zeiss AxioImager Z1 microscope (Carl Zeiss, Germany) using an Axiocam MR ver.3.0 (Carl Zeiss, Germany) or the Leica Confocal SP2 (Leica Microsystems, Germany). Data stacks were deconvolved, using the Huygens Essential version 3.0.2p1 (Scientific Volume Imaging B.V., The Netherlands).

Drosophila stocks

Experimental work – Chapter 1:
W1118 was used as control strain (Bloomington stock center, Indiana, USA). Two mutant alleles C01268 and C01428 were obtained from Exelixis Corporation (California, USA). These lines carry a Piggy Bac transposable element insertion at nucleotide +55bp upstream of the initiator ATG of *sgt1* gene. The mutant allele *bub3*^{1}

(Lopes et al. 2005) was recombined into a chromosome carrying the C01268 mutant allele of Sgt1 to test SAC activity. For in vivo studies, strains were constructed to carry the following transgenes: Cid-mRFP (Schuh et al. 2007), GFP-Tubulin (Rebollo et al. 2004) and PACT-GFP (Martinez-Campos et al. 2004). To obtain revertants of C01268, this strain was crossed with w^{1118}; CyO, P{Tub-PBac\T}2/wg^{Sp-1} (Bloomington Stock center) and all white-eyed progeny selected. To carry out overexpression of Polo, a UAS-Polo transgene was used (Mirouse et al. 2006) and the expression induced with the ubiquitous driver *daughterless (da)* GAL4 (Wodarz et al. 1995).

Experimental work – Chapter 2:

W1118 and C(2) En, bw^1sp^1 were obtained from the Bloomington stock center (Indiana, USA). For in vivo studies, either control or compound strains were constructed to carry transgenes for expression of HisH2Av-mRFP1 (Schuh et al. 2007), Jupiter-GFP (Gift from Alain Debec) (Karpova et al. 2006). Nondisjunction phenotype was promoted by an heteroallelic combination of $BubR1^{rev1}$ (Perez-Mongiovi et al. 2005) and $BubR1^{D1326N}$ (Malmanche et al. 2007).

Experimental work – Chapter 3:

Mutated version of Polo, Polo.T182D and the drivers used for overexpression experiments during eye and wing development analysis, *eyeless*-GAL4 (Hazelett et al. 1998), *nubbin*-Gal4 (Kambadur et al. 1998) and *ms1096*-Gal4 (Capdevila and Guerrero 1994) stocks were obtained from Bloomington Stock Center (IN). To study the role of apoptosis in the Polo phenotype we used UAS-Diap1 also obtained from Bloomington Stock Center.
Identification of the Polo candidate genes was done using The Drosophila Interactions Database (DroID) that can be found at (http://www.droidb.org/) (Yu et al. 2008), and of these candidate genes the corresponding RNAi lines used were from the P-element RNAi library from the Vienna Drosophila RNAi Center (VDRC) (Dietzl et al. 2007) and their details are described in the website (http://stockcenter.vdrc.at/control/main) (See appendix 4 for complete list).

Cytological analysis

Third instar larvae brains were dissected in 0.7% NaCl solution and fixed in 45% acetic acid followed by 60% acetic acid. DNA was counterstained with DAPI in Vectashield

(Vector, UK). When required, brains were incubated with 10μM of the microtubule depolymerising drug colchicine (Sigma) prior to fixation. Mitotic index was determined as the percentage of cells in mitosis over the total cell population. At least 2000 cells were scored from different preparations. All quantifications were performed on a Zeiss Axioskop microscope (Carl Zeiss, Germany) using 1000x magnification.

Immunostaining of Drosophila neuroblasts

For immunostaining, third instar larvae brains were dissected in NaCl 0.7%, fixed in 2% formaldehyde together with 0,1% Triton X-100 in PBS and then transferred to a solution of 45% acetic acid containing 2% formaldehyde. Brains were squashed and immersed in liquid nitrogen followed by permeabilization in PBS with 0.05% Tween 20. Blocking and incubation of primary and secondary antibodies was done in PBS-BT (1% BSA and 0.1% Triton X-100). For immunofluorescence analysis of specific spindle components, brains were dissected, as previously described, and fixed in a solution of 3.7% formaldehyde in PBS then transferred to 45% acetic acid and after to 60% acetic acid. After squashing and immersion in liquid nitrogen slides were transferred to absolute ethanol at -20ºC. Permeabilization was done in PBS with 0.1% Triton. Blocking and incubation of primary and secondary antibodies was done in PBS with 1% BSA. Images were collected in a Zeiss AxioImager Z1 microscope (Carl Zeiss, Germany) using an Axiocam MR ver.3.0 (Carl Zeiss, Germany). Data stacks were deconvolved using the Huygens Essential version 3.0.2p1 (Scientific Volume Imaging B.V., The Netherlands). All the images were projected with ImageJ v.1.42 (http://rsb.info.nih.gov) and processed with Photoshop CS (Adobe Microsystems, CA).

Cytological analysis of early embryos

For cytological analysis of syncytial embryos, a 0.5-2 hours collection was obtained. Embryos were fixed in 1:1methanol:n-heptane for 5 minutes followed by three methanol washes. Embryos were then washed with PBST (0.1 % triton X-100 in PBS) and incubated with 5μg/ml of Hoechst in PBS for 5 minutes, for DNA counterstaining. After 3 washes with PBST and a final wash with PBS embryos were mounted in Vectashield (Vector, UK).

Quantitative analysis of spindle length

For quantitative analysis of spindle size in the presence of the C(2)En chromosome, Jupiter-GFP and HisH2Av-mRFP1 co-expressing embryos were collected (0.5-1.5 hours) and processed as previously described (Oliveira et al. 2007). Single stack confocal images were acquired every 10s using a Zeiss LSM510 confocal system (Carl Zeiss, Germany), equipped with a 63x/1.40 oil immersion objective, a 488 nm Ar laser and a 543 nm He/Ne laser for the excitation of GFP and mRFP1 respectively. Syncytial embryos undergoing mitosis 10-12 were used for spindle size measurements and different movies were aligned by the anaphase onset time (the first frame after sister chromatids separation was set as t=0). Quantitative analysis was performed using ImageJ v.1.42 software (http://rsb.info.nih.gov/ij/).

Antibodies

The primary antibodies used were anti-α-tubulin mouse B512 (Sigma) used at 1:1000; anti-γ-tubulin mouse GTU88 (Sigma) used at 1:250; anti-Polo mouse monoclonal MA294 (Llamazares et al. 1991) used at 1:10; anti-BubR1 Rb666 rabbit polyclonal (Logarinho et al. 2004) used at 1:2000; anti-CID rat polyclonal (Sunkel, unpublished results) used at 1:1500; anti-CENP-meta rabbit used 1:500; anti-Bub3 (Logarinho et al. 2004) used at 1:500; anti-CENP-C (Heeger et al. 2005) used at 1:4000; anti-CNN (Heuer et al. 1995) used at 1:750, CP190 (Whitfield et al. 1988) used at 1:500 and anti-phospho-histone H3 rabbit polyclonal (Upstate Biotechnology) used at 1:750. Antibodies used for immunofluorescence in whole brains were: anti-BrdU (Sigma, clone BU-33) used at 1:80, anti-cyclinB Clone F2F4 (Lehner and O'Farrell 1990) used at 1:15. Antibodies used for Western blot were: anti-Polo mouse monoclonal MA294 (Llamazares et al. 1991) used at 1:100, anti-Sgt1 used at 1:1000 and anti-tubulin DM1A (Sigma) used at 1:5000. The secondary antibodies were from Molecular Probes® and Jackson Immunoresearch and used accordingly to manufacturer instructions.

DmSgt1 antibody production

For generation of a Drosophila Sgt1 recombinant protein, the corresponding cDNA was amplified from the RH27607 clone (20-178 amino acids) (*Drosophila* Genomics Resource Centre) and the digested PCR product was subcloned into the expression vector pET-23a (Qiagen). Constructs were transformed into expression host cells, *E. coli* BL21, and several colonies were tested for protein expression. Single colonies were inoculated in LB medium with 200 µg/mL ampicilin and grown overnight at 37ºC. For a large-scale expression, 5 mL of the overnight culture were transferred into 250 mL of LB with appropriate antibiotics and cultures were grown until OD600nm = 0.6-0.7. Protein expression was induced for 6 hours after addition of 0.5 mM IPTG. The recombinant protein was mainly found in inclusion bodies and to isolate those, cells were collected by centrifuging for 30 min at 5000 rpm at 4 ºC and were resuspended in 10 ml of ice-cold sonication buffer (see appendix 2). Lysis was achieved by 15 min incubation at 37 ºC followed by sonication. Inclusion bodies were collected by centrifuging at 4500 rpm for 15 min and dissolved in 4 mL Purification Buffer (see appendix 2). Purified recombinant protein was run on SDS-PAGE, using standard procedures, and a grinded gel slice was used for rabbit immunization. Two rabbits were immunized and the respective anti-sera we designated as #1 to #2. The anti-serum #2 was affinity purified by addition of Chloroform (1:1), mixed and centrifuged 30 min at 1000 rpm twice. Antibody specificity for Sgt1 was confirmed by the appearance of the band at the predicted size (23 KDa) that was not observed in the pre-immune serum.

Protein electrophoresis and Western Blot analysis

For western blot analysis, brains were dissected from third instar larvae and collected in Sample Buffer (Appendix 2). Protein extracts were run on a polyacrilamide gel until the running front has reached the end of the gel. Proteins were transferred to a nitrocellulose membrane (Schleicher & Shuel) using a semi dry system at a 20-25V for 1:30h. The membrane was blocked overnight at 4ºC with 0.5% gelatin from cold water fish skin (FSG) (Sigma), 1% BSA and 8% low-fat milk in TBS 0.05% Tween. All primary and secondary antibodies were diluted in TBS 0,05% Tween containing 1% BSA, 0,5% FSG and membrane was incubated for 1-2h with primary antibody solution. Secondary antibodies conjugated to HRP (Amersham) were used according to the manufacturer's instructions. Blots were developed by Enhanced Chemiluminescent (ECL) method (see

appendix 2 for recipes). The membrane was then used to impress an X-ray film (Fuji Medical X-Ray Film) and the results were obtained by manual or automatic development of the film.

Brain whole mount preparations

To detect DNA replication, third instar larvae neuroblasts were dissected in PBS, incubated in 10 μM BrdU (Roche) diluted in PBS, fixed in 5% formaldehyde then washed in PBST and hydrolysed in 2.2N HCl. The tissue was then washed with 100mM Sodium Tetraborate and PBST. Preparations were blocked in PBST containing 10% FBS and then incubated with a monoclonal BrdU antibody. Anti-mouse Alexafluor 568 used at was used as secondary antibody. To detect cyclin B third instar larvae neuroblasts were dissected in 0.7% NaCl, fixed in 3.7% formaldehyde, washed and blocked in 0.7% NaCl with 10% FBS, 0.3% Triton and RNAse (10μg/ml). Preparations were incubated with primary antibody and secondary antibodies in the blocking solution and then incubated with topro3 (Molecular Probes) to label the DNA. Imaging was done using a Laser Scanning Confocal Microscope Leica SP2 AOBS SE (Leica Microsystems, Germany). For BrdU quantifications, we acquired images of the whole brains, and then used ImageJ to define a threshold for positive cells. This quantification was done in every stack acquired and then we calculated the ratio between BrdU positive cells and total number of cells.

***In vivo* studies**

Third instar larvae neuroblasts were dissected in PBS and then transferred to a drop of Schneider medium (Invitrogen) for appropriate incubation. Cells were imaged using a Spinning Disk Confocal System Andor Revolution XD (ANDOR Technology, UK). Frames were collected every 30 seconds. Time-lapse images were then treated with the microscope software IQ 1.7 (ANDOR Technology, UK).

Eye-antennal imaginal disc fixation

Larvae were dissected in cold Phosphate Buffer Saline (PBS) and then fixed in 3,7% Formaldehyde (in PBS) during 20 minutes at room temperature. After that, the imaginal discs were washed in PBT (PBS with 0,1% Triton X-100) during 30 minutes (3x10') and immunostained during 2 hours and half with the primary antibody in PBT at room temperature. Subsequently the imaginal discs were washed in PBT during 30 minutes (3x10') and stained with the secondary antibodies in PBT during one and half hours. After incubation, the discs were washed in PBT at room temperature during 15 minutes and stored in 50% Glycerol/PBS at 4ºC. Imaginal discs were further dissected in 50% Glycerol/PBS. Primary antibody used was rabbit anti-Phospho-Histone 3 1:1000 (Sigma). Alexa-coupled secondary antibodies were from Molecular Probes.
For DNA counterstaining the imaginal discs were incubated with 5µg/ml of Hoechst in PBS for 5 minutes. Cell cytoskeleton was stained with Rhodamine phalloidin (1:1000) that recognizes F-actin. Images were obtained with Laser Scanning Confocal Microscope Leica SP2 AOBS and processed with Adobe Photoshop.

Analyses of Polo interactors

The list of the described Polo interactors was obtained in the interactions database DroId (www.droidb.org) and analyses of the interaction between the putative Polo interactors were done using the Cytoscape v.2.7.0.

Statistical analyses

All the statistical analyses were performed using the SPSS for windows version 14.0 (SPSS Inc, Chicago, IL, USA). The significance levels of $p<0.05$ (*), $p<0.01$ (**) and $p<0.001$ (***) were used. Independent samples *t*-test (2-tailed) or Mann-Whitney test were used to compare the means.

PART V

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PART VI

APPENDIXES

Appendix 1

Abbreviations

17-AAG : 17-allylamino-17-demethoxygeldanamycin

17-DMAG: 17-dimethylamino-ethylamino-17-demethoxygeldanamycin

a.a.: aminoacid

Ab: Antibody

APC/C: Anaphase-Promoting Complex/Cyclosome

ATM: ataxia telanctasia

ATP: Adenosine-5'-triphosphate

ATR: ATM related

BDGP: Berkeley Drosophila Genome Project

bp: base pairs

BrdU: Bromodeoxyuridine

BSA: Bovine Serum Albumin

Bub: budding uninhibited by benzimidazole

C(2)En: Drosophila compound entire second chromosome

***C. elegans*:** *Caenorhabditis elegans*

CAKs: Cyclin-dependent kinase activating kinases

CBF3: Centromere Binding Factor 3

CDE I,II and III: Centromere DNA elements

cdc: cell division cycle

Cdks: Cyclin-dependent kinases

cDNA: complementary DNA

CENP: Centromere-associated Protein

CID: Centromere identifier

CIN: Chromosomal Instability

CKIs: Cyclin-dependent kinase inhibitor proteins

CPC: Chromosomal Passenger Complex

CS: CHORD or p23-like central domain

CUT: cell untimely torn

CyO: *Curly of Oster*

DAPI: 4',6'-diamino-2-phenylindole

***Df*:** deficiency

DNA: deoxyribonucleic acid

DSB: Double Strand Breaks

dsRNA: double stranded RNA

DTT: dithiothreitol

E. coli : *Escherichia coli*

***e.g.*:** *exempli gratia*

ECL: Enhanced ChemiLuminescence

EDTA: Ethylenediaminetetracetic acid

EEVD: Motif Glutamate-Glutamate-Valine-Aspartate

EGFP: Enhanced Green Fluorescent Protein

EM: Electron Microscopy

Exp: Experiment(s)

eyGal4: *eyeless-GAL4*

FBS: Fetal Bovine Serum

Fig.: Figure

FLP: Flippase recombination enzyme

FRT: Flippase recognition target

FSG: fish skin gelatine

g: gram

G_0: Gap phase 0

G_1: Gap phase 1

G_2: Gap phase 2

GA: Geldanamycin

Gal4: yeast transcription activator protein

GFP: Green Fluorescent Protein

GTP: Guanosine-5'-triphosphate

GMC: Ganglion mother cell

h: hour

HeLa: Human immortal cell line

HisH2Av: histone H2A variant

HRP: Horse redish peroxidase

Hsp: Heat-shock protein

IB: Immunoblotting

IF: Immunofluorescence

IPTG: isopropyl-β-D-thiogalactoside

K-fiber: kinetochore fiber

kDa: kiloDalton(s)

KT: Kinetochore

L: Liter

LB: Luria-Bertani culture medium

M phase: Mitosis

M: Molar

mAb: monoclonal antibody

Mad: Mitotic-arrest deficient

MAPs: Microtubule-Associated Proteins

MCC: Mitotic Checkpoint Complex

MCS: Multiple Cloning Site

MEN: Mitotic exit network

min: minutes

ml: milliliter

mM: miliMolar

mm: millimeter

mm:ss: minutes:seconds

mRFP1: Monomeric Red Fluorescent Protein

mRNA: messenger RNA

MT(s): Microtubule(s)

MTOC: Microtubule Organizing Center

n: number of samples in the study

***n.d.*:** not determined

NEBD: Nuclear Envelope Breakdown

nm: nanometer

nM: nanoMolar

***nub*-Gal4 :** *nubbin*-Gal4

OD: Optical density

ORF: Open Reading Frame

PBD: Polo box domain

PBS: Phosphate-Buffered Saline

PCR: Polymerase Chain Reaction

PCM: Pericentriolar Material

PH3: phosphorylated histone H3

Plks: Polo like kinases

PSCS: Premature Sister Chromatid Separation

RFP: Red fluorescent protein

RNA: ribonucleic acid

RNAi: RNA interference

RT: Room Temperature

RZZ: Rod-ZW10-Zwilch

S phase: DNA synthesis phase

***S. cerevisiae*:** *Saccharomyces cerevisiae*

S. pombe: *Schizosaccharomyces pombe*

s.d.: standard deviation

S128D: Phosphomimicking mutation in residue 128 of Plx1

Ser137D or S137D: Phosphomimicking mutation in residue 137 of Plk1

S2: Drosophila Schneider 2 cell line

SAC: Spindle Assembly Checkpoint

SCF: Skp1/Cullin/F-box ubiquitin-protein ligase

SD: Standard Deviation

SDS-PAGE: Sodium Dodecyl Sulfate-Polyacrilamide Gel Electrophoresis

SDS: Sodium Dodecyl Sulphate

sec: seconds

SGS: Sgt1-specific Domain

SIN: Septation Initiation Network

T201D: Phosphomimicking mutation in residue 201 of Plx1

T210D: Phosphomimicking mutation in residue 210 of Plk1

T182D: Phosphomimicking mutation in residue 182 of Polo

t-test: Student's *t* test

TPR: Tetracopeptide domain

Tris: Tris(hidroximethyl)aminomethane

UAS: Upstream activation sequence

UTR: Untranslated Region

VDRC: Vienna Drosophila RNAi Center

***w*:** mini-white gene

w.t.: wild-type

***X. laevis*:** *Xenopus laevis*

µg: microgram

µl: microliter

µm: micrometer

µM: microMolar

γTuRC : γ-tubulin ring complex

Appendix 2

Recipes

Protein Electrophoresis:

Stacking gel: 4% acrilamide; 125 mM Tris-HCl, pH 6.8; 0.1% SDS;

Separating gel: 12% acrylamide; 375 mM Tris-HCl, pH 8.8; 0.1% SDS;

Running buffer: 25 mM Tris, pH 8.3; 250 mM Glycine; 0.1% SDS

Transfer Buffer:

40mM glycine

50mM Tris

0.04%SDS

20%methanol

Phosphate-Buffered Saline (PBS):

137 mM NaCl

2.7 mM KCl

10 mM KH2PO4

1.8 mM Na2HPO4

Enhanced Chemiluminescent (ECL):

Solution A - 10ml Tris 100mM pH 8.5, 44 µl coumaric acid (Sigma) 90mM and 100 µl luminol (FLUKA) 250mM;

Solution B: 10ml Tris 100mM pH 8.5 and 6 µl H_2O_2 30% (Merck)

Solution A and B are mixed and incubated with the membrane at the time of ECL detection.

2x SDS-PAGE sample buffer:

100 mM Tris-HCl pH 6.8

4% (w/v) SDS

0.2% (w/v) Bromophenol blue

20% (v/v) Glycerol

200 mM DTT (dithiothreitol)

Ponceau S:

0.1% Ponceau
5% acetic acid

TBST:
50 mM Tris-HCl, pH7.5
150 mM NaCl
0.05% Tween 20

PHEM
60 mM Pipes
25 mM Hepes pH7.0
10 mM EGTA
4 mM MgSO4

LB Medium
1% tryptone
0.5% yeast extract
1% NaCl

Sonication Buffer:
20 mM Tris buffer, pH 7.5;
2 mM EDTA;
100 µg/mL lysozyme;
0,1% TritonX100

Purification Buffer:
20 mM Tris buffer, pH 7.5;
2 mM EDTA;
1 protease inhibitor cocktail per 50 mL;

Schneider's Insect Medium:
Schneider's Insect Medium, with L-glutamine and sodium bicarbonate, (Invitrogen) was supplemented with 10% (v/v) FBS (Invitrogen)

Fly Media (3L):
Mixture 1

ddH2O: 2000 ml

Molasses: 103 ml

Agar: 24 g

Mixture 2

ddH2O: 1000 ml

Cornmeal: 240 g

Yeast extract: 54 g

Flour of soy: 30 g

Malte: 60 g

After boiling mixture 1, add mixture 2 and let it boil for 35 minutes. When it cools down to 60 º C, add 17.4 ml of a solution composed by 500 ml of propionic acid and 32 ml of phosphoric acid 85%.

Appendix 3

Movie legends

Movie 1.1: Animates series of time-lapse images taken with a spinning disc confocal. Control third instar larval neuroblast from a strain expressing α-tubulin-GFP and CID-mRFP. Time 00:00 corresponds to NEBD. The cell shows two well-organized asters at opposite sides of the spindle just before NEBD that give rise to bipolar spindle at prometaphase. The centromeres congress and segregate as the cell divides asymmetrically.

Movie 1.2: Animates series of time-lapse images taken with a spinning disc confocal of a sgt^{P1}/sgt^{P1} mutant third instar larval neuroblast from a strain expressing α-tubulin-GFP and CID-mRFP. The cell enters mitosis without asters and then microtubules appear to grow from the centromeres, however a well-organized spindle is never achieved. The cell remained in this stage from more than 80 minutes and did not exit mitosis.

Movie 1.3: Animates series of time-lapse images taken with a spinning disc confocal of a sgt^{P1}/sgt^{P1} mutant third instar larval neuroblast from a strain expressing α-tubulin-GFP and CID-mRFP. The cell shows a single aster and after NEBD, the cell is able to organize a bipolar spindle, centromeres congress, and after a long delay, segregate and exit mitosis.

Movie 1.4: Animates series of time-lapse images taken with a spinning disc confocal of a sgt^{P1}/sgt^{P1} mutant third instar larval neuroblast from a strain expressing α-tubulin-GFP and CID-mRFP. When images started to be obtained, the cell was already in prometaphase and remained in this stage for more that 1 hour and did not exit mitosis nor organized a bipolar spindle. It shows a large number of centromeres indicating that cell is polyploidy and contains multiple asters that are never able to organize a proper bipolar spindle.

Movie 2.1: Animates series of time-lapse images taken with a spinning disc confocal. C(2)En third instar larval neuroblast from a strain expressing HisH2Av-RFP. Time 00:00 corresponds to the first stack of the experiment. The cell shows the segregation of the C(2)En chromatids similar to the other chromosomes.

Movie 2.2: Animates series of time-lapse images taken with a spinning disc confocal of a control mutant third instar larval neuroblast from a strain expressing Jupiter-GFP and HisH2Av-RFP. The cell enters mitosis with 2 well defined asters and forms a bipolar spindle.

Movie 2.3: Animates series of time-lapse images taken with a spinning disc confocal of a C(2)En third instar larval neuroblast from a strain expressing Jupiter-GFP and HisH2Av-RFP. The cell enters mitosis with 2 well defined asters and forms a bipolar spindle.

Movie 2.4: Animates series of time-lapse images taken with a confocal of a control embryo from a strain expressing Jupiter-GFP and HisH2Av-RFP.

Movie 2.5: Animates series of time-lapse images taken with a confocal of a C(2)En embryo from a strain expressing Jupiter-GFP and HisH2Av-RFP. This movie show what we classified as the strong phenotype, with the irregular positioning of the nuclei and the mitotic spindle phenotype.

Movie 2.6: Animates series of time-lapse images taken with a confocal of a control embryo from a strain expressing Jupiter-GFP and HisH2Av-RFP. This movie show what we classified as the weak phenotype, with a similar distribution both of the nuclei and behaviour of the mitotic spindle.

Appendix 4

Supplementary material of the experimental work chapter 3.

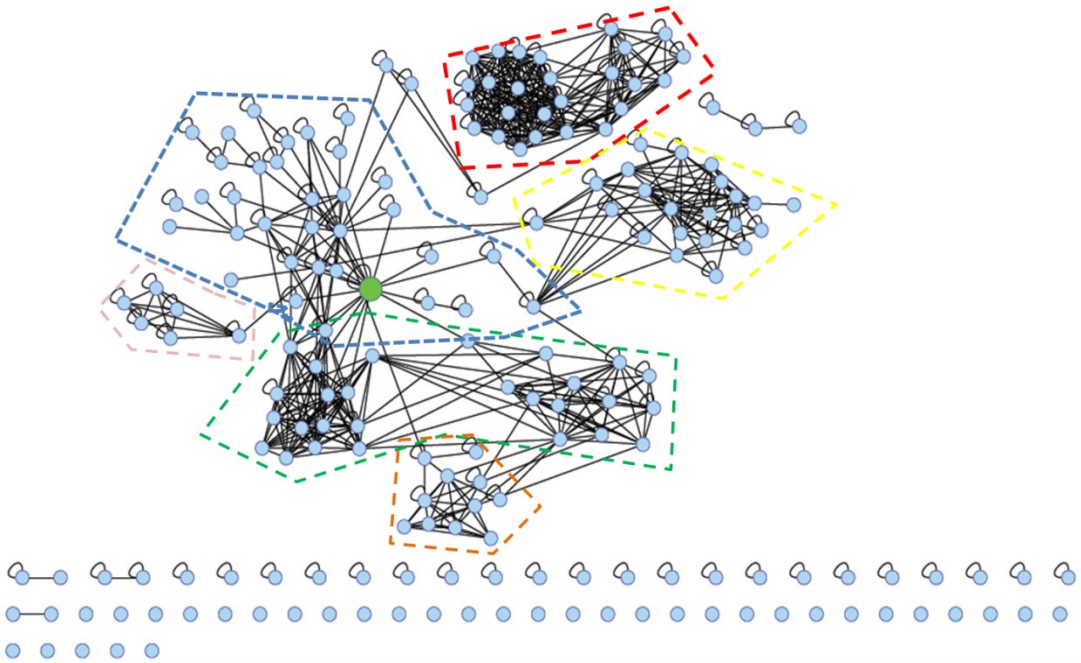

Figure A4.1: Schematic representation of the Polo interacting genes. In the yellow branch we display proteins involved in centrosome organization; the spindle assembly and spindle assembly checkpoint genes are limited by the dashed green line; proteasome regulatory proteins are put together in the red group; cell cycle control in blue; DNA binding/replication proteins in pink; nuclearpore/nuclear transport proteins are limited by the brown dashed line, and an unspecific group of proteins with unknown function is shown in the bottom of the image.

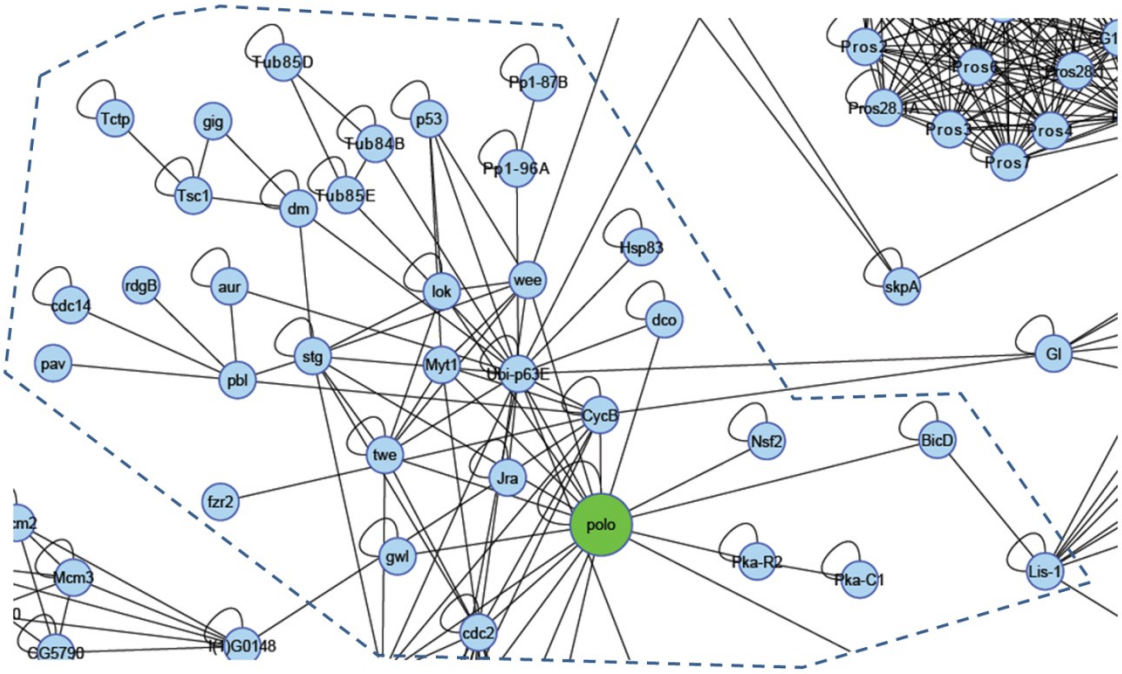

Figure A4.2: Magnification of the schematic representation of the Polo interacting genes defined in the central blue group. This group is composed by genes that are involved in the cell cycle regulation;

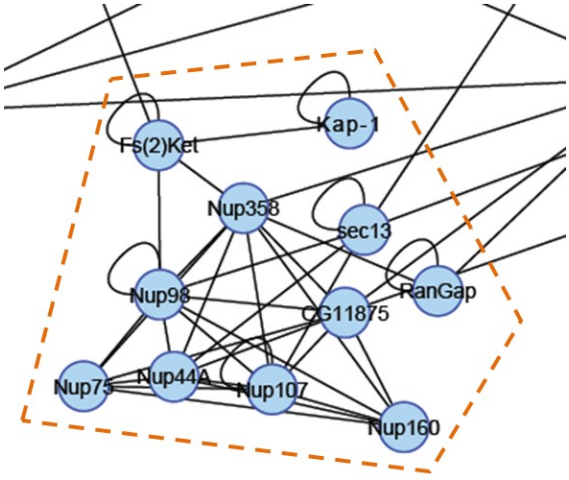

Figure A4.3: Magnification of the schematic representation of the Polo interacting genes defined in the brown group. This group is composed of proteins involved in the nuclear pore organization as well as nuclear transport proteins genes;

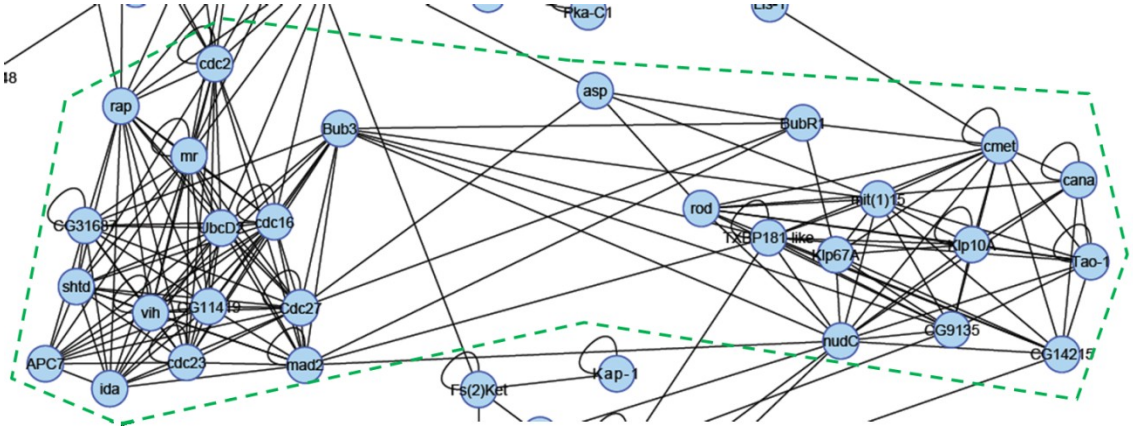

Figure A4.4: Magnification of the schematic representation of the Polo interacting genes defined by the green group. Spindle assembly and spindle assembly checkpoint genes are the majority of the genes of this group;

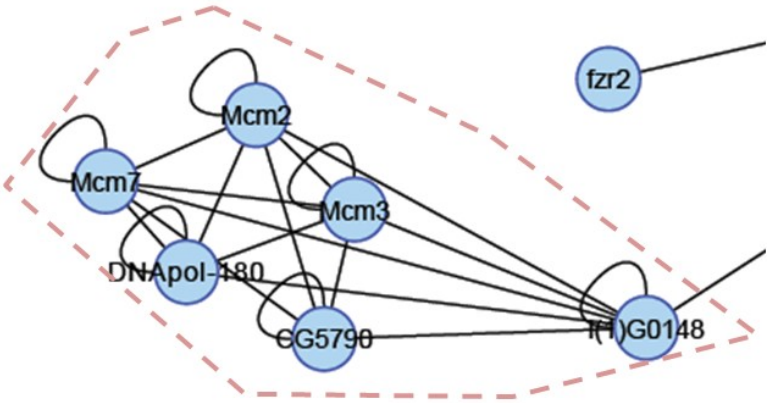

Figure A4.5: Magnification of the schematic representation of the Polo interacting genes defined in the pink branch; DNA binding and the DNA replication involved genes;

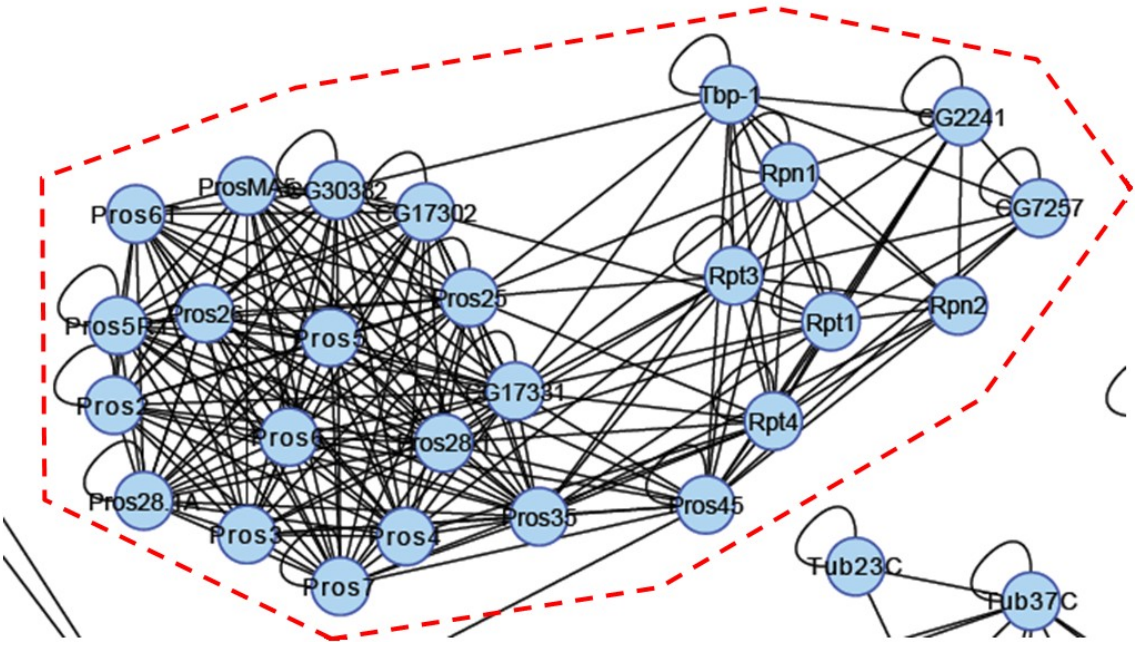

Figure A4.6: Magnification of the schematic representation of the Polo interacting genes defined in the red group. This group is composed by genes involved in the proteasome regulation;

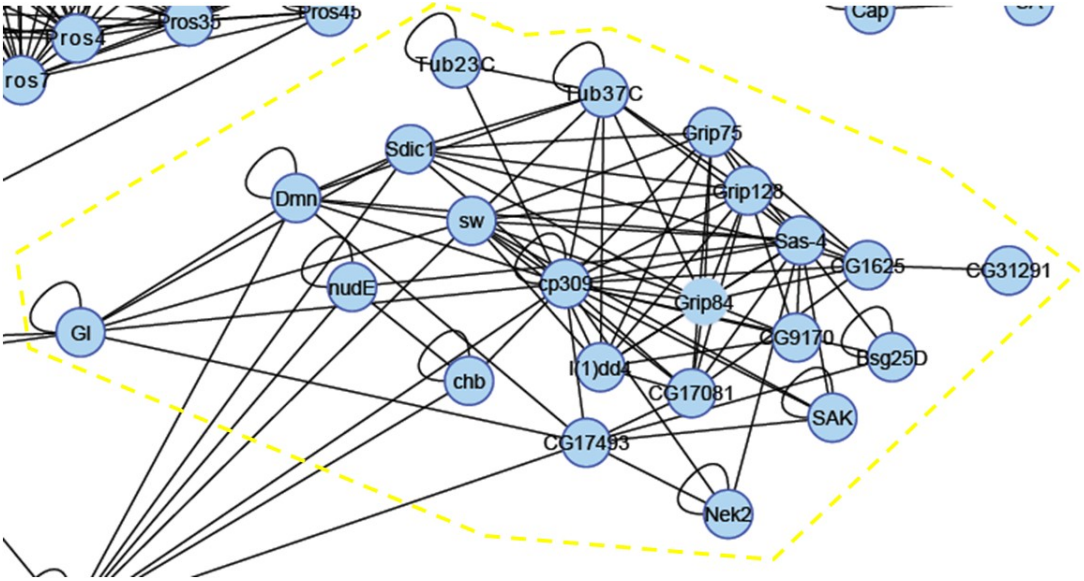

Figure A4.7: Magnification of the schematic representation of the Polo interacting genes defined in the yellow group. The yellow branch is composed by proteins involved in the centriole duplication and centrosome maturation;

Table A4.I: RNAi phenotypes with *ey*Gal4 driver

Gene	Symbol	*ey*Gal4			
		25ºC		18ºC	
		Male	Female	Male	Female
CG9170		N	N	N	N
CG11397	glu				
CG4879	RecQ5	N	N	N	N
CG6498		N	N	N	N
CG7808	RpS8	L	L	L	L
CG31196	14-3-3epsilon	N	N	N	N
CG1341	Rpt1	L	L	L	L
CG1558	Kmn1	N	N	N	N
CG32742	l(1)G0148	N	N	N	N
CG10198	Nup98	M	M	M	M
CG17566	gammaTub37C	N	N	N	N
CG11981	Prosbeta3	L	L	L	L
CG9999	RanGap	W	W	M	W
CG3510	CycB	N	N	N	N
CG1966	Acf1	N	N	N	N
CG12000	Prosbeta7	L	L	L	L
CG7186	SAK	N	N	N	N
CG8114	pbl	W	W	N	N
CG11624	Ubi-p63E	N	N	N	N
CG3917	Grip84	W	W	M	M
CG17498	mad2	N	N	N	N
CG17268	Pros28.1A	N	N	N	N
CG31361	dpr17	N	N	N	N
CG12287	pdm2	N	N	N	N
CG10637	Nak	N	N	N	N
CG32823	Sdic3	L	L	L	L
CG7538	Mcm2	S	S	S	M
CG6349	DNApol-alpha180	L	L	L	L
CG3422	Pros28.1	N	N	N	N
CG11111	rdgB	N	N	N	N
CG17256	Nek2	N	N	N	N
CG17493		N	N	N	N
CG12323	Prosbeta5	L	L	L	L
CG3342		n.d.	N	n.d.	N
CG9156	Pp1-13C	N	N	N	N
CG1560	mys	N	N	N	N
CG5790		N	N	N	N
CG1519	Prosalpha7	L	L	L	L

CG2275	Jra	N	N	N	N
CG9327	Pros29	M	M	W	W
CG33336	p53	N	N	N	N
CG7769	pic	L	L	L	L
CG1911	CAP-D2	N	N	N	N
CG4206	Mcm3	S	S	W	W
CG16983	skpA	S	S	M	M
CG2189	Dfd	N	N	N	N
CG4097	Pros26	L	L	L	L
CG5266	Pros25	L	L	L	L
CG11888	Rpn2	L	L	L	L
CG14025	Bsg25D	N	N	N	N
CG12265	Det	M	M	S	S
CG9710	nudC	S	S	L	S
CG9727		N	N	N	N
CG4379	Pka-C1	N	N	N	N
CG18000	sw	L	L	L	L
CG8440	Lis-1	M	W	W	N
CG5335		N	N	N	N
CG7425	eff	W	W	W	W
CG6975	gig	Overgrowth	Overgrowth	Overgrowth	Overgrowth
CG9476	alphaTub85E	L	M	L	L
CG10370	Tbp-1	L	L	L	L
CG10061	Sas-4	N	N	N	N
CG10250	nau	N	N		
CG1548	cathD	N	N		
CG32417	Myt1	N	N		
CG32577	disco-r	N	N		
CG6176	Grip75	N	N		
CG10938	Prosalpha5	W	W	L	L
CG7762	Rpn1	L	L	L	L
CG8548	Kap-alpha1	N	N	N	N
CG30382		N	N	N	N
CG33957	cp309	N	N	N	N
CG13623		N	N	N	N
CG6720	UbcD2	N	N	N	N
CG32371		N	N	N	N
CG10423	RpS27	L	L	L	L
CG32438	Smc5	N	N	N	N
CG32438	Smc5	N	N	N	N
CG11419		N	N	N	N
CG13380		N	N	N	N
CG9802	Cap	S	M	M	W
CG16783	fzr2	n.d.	N	n.d.	N
CG9853		N	N	N	N

CG14164		N	N	N	N
CG4262	elav	N	N	N	N
CG33484	zormin	N	N	N	N
CG33277		N	N	N	N
CG7659	tap	N	N	N	N
CG3151	Rbp9	N	N	N	N
CG4396	fne	M	M	N	N
CG7719	Gwl	N	N	N	N
CG3026	mus81	N	N	N	N
CG4800	Tctp	W	W	N	N
CG3423	SA	S	M	M	W
CG1523		N	N	N	N
CG32435	Chb	M	W	W	W
CG12306	Pólo	L	L	L	L
CG6605	BicD	N	N	N	N
CG9198	Shtd	M	M	L	M
CG3068	Aur	N	N	N	N
CG6057	SMC1	M	W	N	N
CG4494	smt3	L	L	L	L
CG10212	SMC2	S	M	L	L
CG17032		N	N	N	N
CG7911		N	N	N	N
CG1558	Kmn1	L	L	S	S
CG2508	cdc23	N	N	N	N
CG1625		N	N	N	N
CG31291		N	N	N	N
CG4904	Pros35	L	L	L	L
CG8610	Cdc27	N	N	N	N
CG4488	Wee	N	N	N	N
CG8722	Nup44A	N	N	N	N
CG10726	Barr	S	S	M	M
CG6875	Asp	N	N	N	N
CG9802	Cap	S	M	M	M
CG10988	l(1)dd4	W	N	W	W
CG15735		N	N	N	N
CG6743	Nup107	M	M	M	W
CG2331	TER94	L	L	L	L
CG1558	Kmn1	L	M	L	L
CG4738	Nup160	L	W	S	S
CG18156	Mis12	W	N	L	L
CG11494	BtbVII	N	N	N	N
CG3157	gammaTub23C	W	W	W	N
CG3000	rap	N	N	N	N
CG8269	Dmn	L	L	L	L
CG5733	Nup75	n.d.	S	n.d.	W

CG4893		N	N	N	N
CG11348	nAcRbeta-64B	N	N	N	N
CG5004		N	N	N	N
CG13330		N	N	N	N
CG3455	Rpt4	L	L	L	L
CG1489	Pros45	L	S	L	L
CG9227	tectonic	N	N	N	N
CG1258	pav	M	M	S	M
CG10895	lok	N	N	N	N
CG14427		N	N	N	N
CG1453	Klp10A	N	N	N	N
CG10212	SMC2	n.d.	L	n.d.	N
CG6147	Tsc1	N	N	N	N
CG3348		N	N	N	N
CG10104		N	N	N	N
CG9359	betaTub85D	N	N	N	N
CG11305	Sirt7	N	N	N	N
CG10850	ida	S	S	S	M
CG7917	Nlp	N	N	N	N
CG33694	cana	N	N	N	N
CG10212	SMC2	L	L	L	L
CG5133	Doc1	N	N	N	N
CG17081	Cep135	N	N	N	N
CG4965	twe	N	N	N	N
CG31989	Cap-D3	L	S	S	W
CG9802	Cap	S	M	S	M
CG7838	BubR1	N	N	N	N
CG9580	Sdic1	L	L	L	L
CG14442		N	N	N	N
CG6057	SMC1	N	N	N	N
CG2241	Rpt6R	N	N	N	N
CG31687		N	N	N	N
CG6303	Bruce	N	N	N	N
CG18497	spen	W	W	N	N
CG9623	if	N	N	N	N
CG9206	Gl	W	W	N	N
CG3412	slmb	N	N		
CG14685	Cap-H2	N	N	N	N
CG17331		L	L	L	L
CG1569	Rod	M	M	W	W
CG14215		N	N	N	N
CG10923	Klp67A	N	N	N	N
CG2048	Dco	N	N	N	N
CG9868	Prosbeta5R	N	N	N	N
CG6759	cdc16	S	M	M	M

CG7134	cdc14	N	N	N	N
CG2072	TXBP181-like	N	N	N	N
CG9201	Grip128	N	N	N	N
CG16916	Rpt3	L	L	S	M
CG9900	mit(1)15	N	N	N	N
CG3329	Prosbeta2	L	L	L	L
CG5648	Prosalpha6T	N	N	N	N
CG1877	lin19	L	S		
CG1242	Hsp83	S	W	S	S
CG17051	Dod	N	N	N	N
CG3578	Bi	N	N	N	N
CG33101	Nsf2	N	N	N	N
CG5363	cdc2	S	S	S	S
CG6773	sec13	N	N	N	N
CG11596		N	N	N	N
CG10682	vih	N	N	N	N
CG8443		N	N	N	N
CG6593	Pp1alpha-96A	L	L	N	N
CG10726	barr	S	W	S	W
CG3051	SNF1A	N	N	N	N
CG17603	Taf1	W	W	N	N
CG10573	ko	N	N	N	N
CG17291	Pp2A-29B	L	L	L	L
CG3060	mr	S	S	S	S
CG9135		N	N	N	N
CG11411	fs(1)N	N	N	N	N
CG11856	Nup358	N	N	N	N
CG2637	Fs(2)Ket	L	L	L	L
CG18543	mtrm	N	N	N	N
CG7257	Rpt4R	N	N	N	N
CG10393	amos	N	N	N	N
CG1911	CAP-D2	L	M	L	L
CG18156	Mis12	S	M	S	S
CG10798	dm	S	S	S	S
CG17134		N	N	N	N
CG8374	dmt	L	S	M	W
CG6897	bora	N	N	N	N
CG5581	Ote	N	N	N	N
CG18350	Sxl	N	N	N	N
CG14217	Tao-1	N	N	N	N
CG11875		N	N	N	N
CG8104	nudE	N	N	N	N
CG18156	Mis12	S	M	S	S
CG14444	APC7	N	N	N	N
CG1395	String	N	N	N	N

CG6392	Cenp-meta	W	N	N	N
CG7581	Bub3	M	M		

N = no phenotype; W = weak phenotype; M = medium phenotype; S = strong phenotype; L = lethal; n.d. = not determined;

Table A4.II: RNAi phenotypes with *nubbin*Gal4 driver

Gene	Symbol	*nubbin*Gal4	
		25ºC	18ºC
CG9170		N	N
CG11397	glu	S	S
CG4879	RecQ5	N	N
CG6498		M	W
CG7808	RpS8	L	L
CG31196	14-3-3epsilon	M	W
CG1341	Rpt1	L	S
CG1558	Kmn1	N	N
CG32742	l(1)G0148	M	W
CG10198	Nup98		
CG17566	gammaTub37C	N	N
CG11981	Prosbeta3	L	L
CG9999	RanGap	S	W
CG3510	CycB	N	N
CG1966	Acf1	N	
CG12000	Prosbeta7	L	L
CG7186	SAK	N	N
CG8114	pbl	S	M
CG11624	Ubi-p63E	N	N
CG3917	Grip84	N	W
CG17498	mad2	N	
CG17268	Pros28.1A	M	W
CG31361	dpr17	M	W
CG12287	pdm2	N	N
CG10637	Nak	M	W
CG32823	Sdic3	L	L
CG7538	Mcm2	M	W
CG6349	DNApol-alpha180	L	L
CG3422	Pros28.1	M	N
CG11111	rdgB	N	N
CG17256	Nek2	M	W
CG17493		N	N
CG12323	Prosbeta5	L	L

CG3342		N	N
CG9156	Pp1-13C	N	N
CG1560	mys	L	S
CG5790		N	N
CG1519	Prosalpha7	L	L
CG2275	Jra	M	M
CG9327	Pros29	L	L
CG33336	p53	N	N
CG7769	pic	L	S
CG1911	CAP-D2	N	N
CG4206	Mcm3	M	W
CG16983	skpA	L	L
CG2189	Dfd	N	N
CG4097	Pros26	L	L
CG5266	Pros25	L	L
CG11888	Rpn2	L	L
CG14025	Bsg25D	M	W
CG12265	Det	M	W
CG9710	nudC	S	M
CG9727		N	N
CG4379	Pka-C1	W	W
CG18000	sw	L	L
CG8440	Lis-1	S	S
CG5335		N	N
CG7425	eff	M	W
CG6975	gig	M	
CG9476	alphaTub85E	S	S
CG10370	Tbp-1		
CG10061	Sas-4	N	N
CG10250	nau	N	N
CG1548	cathD	M	W
CG32417	Myt1	N	N
CG32577	disco-r	M	W
CG6176	Grip75	N	N
CG10938	Prosalpha5	M	W
CG7762	Rpn1	L	L
CG8548	Kap-alpha1	N	N
CG30382		N	N
CG33957	cp309	N	N
CG13623		W	W
CG6720	UbcD2	N	
CG32371		N	N
CG10423	RpS27		
CG32438	Smc5	N	N
CG32438	Smc5	N	N

CG11419		W	W
CG13380		N	N
CG9802	Cap	M	M
CG16783	fzr2	N	N
CG9853		L	M
CG14164		N	N
CG4262	elav	N	N
CG33484	zormin	M	W
CG33277		N	N
CG7659	tap	N	N
CG3151	Rbp9	N	N
CG4396	fne	M	
CG7719	gwl	N	N
CG3026	mus81	N	N
CG4800	Tctp	W	W
CG3423	SA	M	M
CG1523		W	N
CG32435	chb	M	M
CG12306	polo	S	S
CG6605	BicD	M	
CG9198	shtd	S	
CG3068	aur	N	
CG6057	SMC1	M	
CG4494	smt3	S	
CG10212	SMC2	S	
CG17032		N	
CG7911		N	
CG1558	Kmn1	M	
CG2508	cdc23	W	W
CG1625		N	N
CG31291		N	N
CG4904	Pros35	L	L
CG8610	Cdc27	S	S
CG4488	wee	N	N
CG8722	Nup44A	M	W
CG10726	barr	S	M
CG6875	asp	M	M
CG9802	Cap	M	M
CG10988	l(1)dd4	W	W
CG15735		N	N
CG6743	Nup107	S	M
CG2331	TER94	L	L
CG1558	Kmn1	M	N
CG4738	Nup160	S	L
CG18156	Mis12	M	W

CG11494	BtbVII	M	N
CG3157	gammaTub23C	M	W
CG3000	rap	N	N
CG8269	Dmn	L	S
CG5733	Nup75	M	M
CG4893		N	N
CG11348	nAcRbeta-64B	N	N
CG5004		N	N
CG13330		N	N
CG3455	Rpt4	L	L
CG1489	Pros45	S	
CG9227	tectonic	N	N
CG1258	pav	S	S
CG10895	lok	N	N
CG14427		M	
CG1453	Klp10A	N	N
CG10212	SMC2	S	M
CG6147	Tsc1	N	N
CG3348		N	N
CG10104		N	N
CG9359	betaTub85D	N	N
CG11305	Sirt7	N	
CG10850	ida	S	
CG7917	Nlp	N	
CG33694	cana	N	N
CG10212	SMC2	S	S
CG5133	Doc1	N	N
CG17081	Cep135	N	N
CG4965	twe	N	N
CG31989	Cap-D3	L	S
CG9802	Cap	W	W
CG7838	BubR1	N	
CG9580	Sdic1	S	S
CG14442		N	N
CG6057	SMC1	M	W
CG2241	Rpt6R	N	N
CG31687		N	N
CG6303	Bruce	N	N
CG18497	spen	M	W
CG9623	if	M	W
CG9206	Gl	S	S
CG3412	slmb	M	
CG14685	Cap-H2	N	N
CG17331			
CG1569	rod	W	N

CG14215		M	W
CG10923	Klp67A	M	W
CG2048	dco	N	N
CG9868	Prosbeta5R	M	W
CG6759	cdc16	S	S
CG7134	cdc14	M	W
CG2072	TXBP181-like	N	N
CG9201	Grip128	N	N
CG16916	Rpt3	L	
CG9900	mit(1)15	N	
CG3329	Prosbeta2	L	L
CG5648	Prosalpha6T	N	N
CG1877	lin19	L	L
CG1242	Hsp83	L	L
CG17051	dod	N	N
CG3578	bi	M	M
CG33101	Nsf2	N	N
CG5363	cdc2	L	L
CG6773	sec13	N	
CG11596		M	W
CG10682	vih	N	N
CG8443		M	W
CG6593	Pp1alpha-96A	L	S
CG10726	barr	S	W
CG3051	SNF1A	M	
CG17603	Taf1	S	S
CG10573	ko	N	
CG17291	Pp2A-29B	S	
CG3060	mr	S	S
CG9135		S	S
CG11411	fs(1)N	N	
CG11856	Nup358	N	N
CG2637	Fs(2)Ket	L	L
CG18543	mtrm	N	
CG7257	Rpt4R	N	N
CG10393	amos	N	N
CG1911	CAP-D2	S	S
CG18156	Mis12	M	M
CG10798	dm	W	W
CG17134		N	N
CG8374	dmt	S	S
CG6897	bora	W	N
CG5581	Ote	N	N
CG18350	Sxl	M	M
CG14217	Tao-1	M	W

CG11875		N	N
CG8104	nudE	W	N
CG18156	Mis12	M	M
CG14444	APC7	N	N
CG1395	String	W	N
CG6392	Cenp-meta	N	N
CG7581	Bub3	N	

N = no phenotype; W = weak phenotype; M = medium phenotype; S = strong phenotype; L = lethal; n.d. = not determined;