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Cohesion decay: quantitative analysis of partial sister chromatid cohesion

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ABSTRACT

Cell division is a highly dynamic process where sister chromatids remain associated with each other from the moment of DNA replication until the later stages of mitosis, giving rise to two daughter cells with equal genomes. The “molecular glue” that links sister DNA molecules is called cohesin, a tripartite ring-like protein complex composed of two Structural Maintenance of Chromosome proteins (Smc1 and Smc3) bridged by a kleisin subunit Rad21/Scc1, that together prevent precocious sister chromatid separation.

Accumulating evidence has suggested that cohesion decay may be the cause of segregation errors that underlie certain human pathologies. However it remains to be determined how much cohesin loss abolishes functional sister chromatid cohesion. To answer these questions, we have developed different experimental conditions aiming to titrate the levels of cohesin on mitotic chromosomes in a precise manner. Using these tools, we will determine the minimal amount of cohesin needed to confer functional cohesion.

The approaches described here take advantage of a system in *Drosophila melanogaster* where the *Tobacco Etch Virus* (TEV) protease can cleave the Rad21 subunit of cohesin leading to precocious sister chromatid separation. Firstly, we tried to express different levels of TEV protease to obtain partial loss of cohesion. However, this approach has failed to produce systematic different levels of sister chromatid separation. Most of the work was therefore focused on a second strategy, for which we established strains with different levels of cohesin sensitive/cohesin resistant to TEV protease. Strains containing different amounts of functional cohesin (TEV resistant) were tested by *in vitro* cleavage and by *in vivo* injections in embryos for their ability to promote sister chromatid cohesion. Our results reveal that removal of half of the cohesin complexes does not impair chromosome segregation, implying that chromosome cohesion is less sensitive to cohesin amounts than previously anticipated.

Keywords: cohesion, cohesin complex, cohesin fatigue/decay, chromosome dynamics, spindle assembly checkpoint, TEV system

RESUMO

Durante a divisão celular, cromatídeos irmãos permanecem associados desde a replicação do DNA até fases tardias, dando origem a duas células-filhas com material genético semelhante. A "cola molecular" que liga as moléculas de DNA irmãs denomina-se coesina, complexo proteico tripartido em forma anelar composto por duas proteínas da família *Structural maintenance of chromosomes* (SMC1 e SMC3) ligados por uma subunidade *kleisin* Rad21/Scc1 que juntos evitam a precoce separação dos cromatídeos irmãos. No entanto, perda de coesão já foi observada em células humanas paradas em mitose por longos períodos ou em oócitos envelhecidos, levando a consequências desastrosas para a célula.

Evidências sugerem que perda de coesão pode ser a causa subjacente de erros de segregação que ocorram durante a mitose ou meiose, embora continue por ser determinado quanta perda de coesina suprime a coesão entre cromatídeos irmãos e se os *checkpoints* são eficazes na deteção de baixos níveis de separação. Diferentes condições experimentais serão aplicadas para determinar os níveis mínimos de coesina necessários para conferir coesão funcional.

As abordagens descritas aqui tiraram proveito de um sistema em *Drosophila melanogaster* onde a protease *Tobacco Etch Virus* (TEV) cliva a subunidade Rad21 da coesina levando à separação precoce dos cromatídeos irmãos. Primeiramente, tentamos expressar diferentes níveis desta protease de forma a obter perda parcial de coesão. Contudo esta abordagem falhou na obtenção de diferentes níveis de separações dos cromossomas. A maioria do trabalho foi, portanto, centrada numa segunda estratégia, onde se estabeleceram linhagens com diferentes níveis de coesina sensível/coesina resistente à protease TEV. Linhagens contendo diferentes quantidades de coesina funcional (TEV resistente) foram testadas por clivagem *in vitro* e *in vivo* por injeções em embriões. Os nossos resultados indicam que remoção de cinquenta por cento de coesina não interfere com a coesão dos cromatídeos irmãos, confirmando que apenas grandes decréscimos afetam a segregação.

Palvras-chave: coesão, complexo coesina, decréscimo/fatiga de coesina, dinânmica de cromossomas, checkpoint de formação do fuso, sistema TEV

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ABBREVIATIONS

ºC – Celsius

APC/C - Anaphase-promoting complex /cyclosome

APC^{Cdc20} - Anaphase-promoting complex connected with Cdc20

ATPase – adenylpyrophosphatase

BSA - Bovine serum albumin

Cdc20 - Cell-division cycle protein 20

Cdk - Cyclin-depend kinase

CdLS - Cornelia de Lange syndrome

Cdk1 - Cyclin-dependent kinase 1

DAPI - 4',6-diamidino-2-phenylindole

DNA - Deoxyribonucleic acid

DSB - Double-strand break

G0 – Gap phase 0

G1 – Gap phase 1

G2 – Gap phase 2

GFP - Green fluorescent protein

h - Hour

HS – Heat shock

HRP - Horseradish peroxidase

M-Cdk - Mitotic cyclin-dependent kinase

Mad2 - Mitotic arrest deficient 2

Min – Minutes

MT - Microtubules

NaCl – Sodium chloride

NEBD - Nuclear envelope breakdown

Nm - Nanometre

PBS - Phosphate-buffered saline

PBS-T - Phosphate-buffered saline Tween

PSCS - Premature sister chromatid separation

PVDF - Polyvinylidene fluoride

Rad21^{TEV} – Rad21 sensitive to TEV cleavage

Rad21^{wt} – wild type Rad21 resistant to TEV cleavage

Rad21$^{wt\text{-}EGFP}$ – wild type EGFP-tag Rad21 resistant to TEV cleavage

Rad21$^{UASwt\text{-}myc}$ - wild type with a myc-tag Rad21 resistant to TEV cleavage, induced by UAS/Gal4 system

RBS - Roberts' Syndrome

Rpm – Rotation per minute

RNA - Ribonucleic acid

RT - Room temperature

RIPA - Radioimmunoprecipitation assay buffer

SAC - Spindle-assembly checkpoint

SC – Sister chromatids

SCC - Sister chromatids cohesion

SDS - Sodium dodecyl sulfate

sec - Seconds

Smc – Structural maintenance of chromosomes

TEV – *Tobacco Etch Virus*

UAS - Upstream Activation Sequence

V – Volts

wt – Wild type

WABS - Warsaw Breakage syndrome

1 INTRODUCTION

The cell cycle is the life story of a cell, a series of events that take place in a cell leading to its duplication and division, producing two daughter cells. After fertilization, successive cell divisions give rise to the development of an organism and its maintenance (growth and proliferation of cells), and without proper segregation of the genomic material the cycle would fail its goal of equal chromosome separation.

1.1 Cell cycle

The cell cycle history started more than 100 years ago with Schneider and Flemming providing pioneer descriptions about how a cell divided (Flemming, 1882; Schneider, 1873). The processes that were characterized are cell growth, deoxyribonucleic acid (DNA) replication, distribution of that material into two identical daughter cells and then its division (Cooper and Hausman, 2007). Two major parts characterize the cell cycle: M phase and interphase (figure 1.1). Most of the cell cycle's time is spent in the interphase, when uncondensed chromatin appears uniformly distributed within the nucleus.

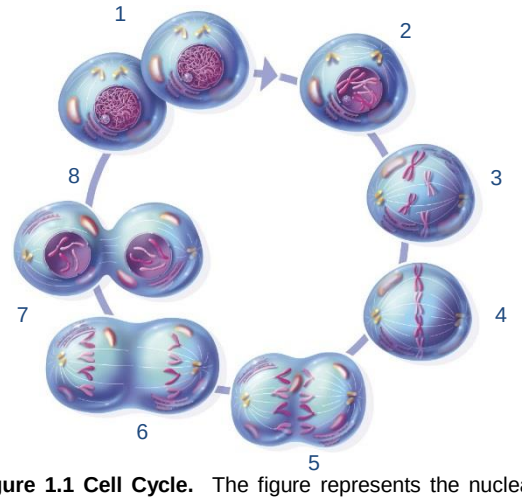


Figure 1.1 Cell Cycle. The figure represents the nuclear division (2-prophase, 3-pro-metaphase, 4-metaphase, 5-anaphase, 6-anaphase , 7-telophase) with subsequent cytokinesis (8). A interphase is represented in 1. Adapted from (Jones, 2012)

The interphase is divided into several stages: G_1 phase (gap 1) corresponding to the interval between the end of M phase and the beginning of DNA replication, when a checkpoint or regulatory transition cell commits to cell-cycle entry or not. An alternative phase (G_0) exists when a cell exits G_1 and enters a quiescent stage: remains metabolically active but does not divide anymore unless stimulated. The next stage is the S phase (DNA synthesis) when replication of DNA takes place and cohesion is established between single sisters keeping them together. In G_2 phase (gap 2) the growth continues and the cell prepares itself to enter mitosis by synthetizing proteins required during this process. Additionally, specific checkpoints or regulatory pathways exists at the G1/S and G2/M transitions to determine whether cells are ready to proceed into the next stage (Alberts et al., 2008; Cooper and Hausman, 2007).

M phase comprises two major processes; the segregation of the genetic information, and the cytoplasmic division or cytokinesis. Initial steps (prophase) include the disentangling and condensation of DNA molecules into rod-shaped structures called sister-chromatids (SC) which are attached to each other at the centromere, and the centrosome's movement to opposite sides of the nucleus, to form the mitotic spindle. Subsequently, nuclear envelope breakdown (NEBD) and chromosome's attachment to the microtubules (MTs) at their kinetochores occur (prometaphase), followed by chromosome alignment

at the equator of the spindle (metaphase) and the pulling of SCs to opposite poles (anaphase), when chromosomes start to decondense. At this point, a new nuclear envelope reassembles around each set of chromosomes (telophase) and a contractile ring divides the cytoplasmic content (cytokinesis), completing the formation of the daughter cells with an equal amount of DNA (Alberts et al., 2008; Cooper and Hausman, 2007).

Cell cycle can also be characterized from the regulatory point of view, defined by the activity of the cyclin-depend kinases (Cdks) and a cyclin subunits (Morgan, 2007). This regulation is divided into two parts: an initial increase in the activity of mitotic cyclin-dependent kinases (M-Cdk) such as the cyclin-dependent kinase 1 (Cdk1)), in the G2/M transition (Alberts et al., 2008). In onset of S phase, cyclin A is expressed being the levels maintained until early mitosis, being destroyed at prometaphase (den Elzen and Pines, 2001; Furuno et al., 1999; Gong et al., 2007).

The other part starts at the metaphase to anaphase transition, when the E3 ubiquitin-protein ligase called anaphase-promoting complex/cyclosome (APC/C) binds with the activating subunit cell-division cycle protein 20 (Cdc20) and initiates anaphase (APC^{Cdc20}). Before this transition occurs, the APC is initially inhibited by components of the spindle-assembly checkpoint (SAC), the mitotic checkpoint that prevents chromosome segregation errors. SAC entraps Cdc20 and blocks its ability to recruit substrates to the APC, through its binding to the Mitotic arrest deficient 2 (Mad2) protein. After the last pair of SC is attached to the spindle and correctly bi-oriented with proper tension, the SAC is inactivated and the APC^{Cdc20} becomes active triggering the loss of centromeric cohesion between SC and mitotic exit (Alberts et al., 2008; Sullivan and Morgan, 2007).

1.2 Cohesin Complex

DNA replication results in the duplication of each of the chromosomes producing identical sister chromatids. A key event during this process is to guarantee the "memory" of which two DNA molecules are genetically identical, until anaphase onset. This is mediated by the cohesin complex, a "molecular glue" that keeps SC together (Guacci et al., 1997; Mehta et al., 2013; Michaelis et al., 1997). This cohesive function of cohesin contributes to the correct direction of sister kinetochores and avoids the premature loss of sister chromatid due to the pulling forces of the spindle during metaphase (Losada, 2014).

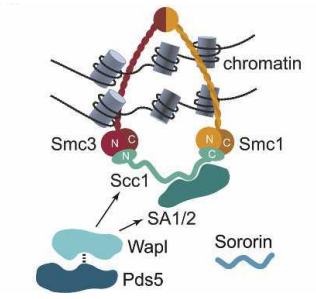


Figure 1.2 The cohesin complex. A struture like ring containing the subunits Smc1, Smc3 and Scc1/Rad21 entraps SC. Others cohesin-associated proteins are showed. From (Peters et al., 2008)

Cohesin complex is a ring-shaped complex first identified in genetics screens that aimed to identify proteins required for holding SC together (Guacci et al., 1997; Michaelis et al., 1997). It is composed by structural maintenance of chromosomes protein 1 (Smc1) and structural maintenance of chromosomes protein 3 (Smc3) which belong to the protein's family of structural maintenance of chromosomes (Smc)(figure 1.2). Members of this family are large adenylpyrophosphatases (ATPases) (Michaelis et al., 1997; Strunnikov et al., 1993); the polypeptide chains fold back on themselves around a central "hinge" domain, followed by an anti-parallel coiled-coil structure and at the other side an adenosine triphosphate (ATP) binding cassete (ABC)-like ATPase "head" domain formed by N- and C-terminal sequences.

Smc1 and Smc3 hinge domains bind to each other, whereas the ATPase heads from both SMC subunits are physically connected by a third element Scc1/Rad21 that belongs to the α-kleisins family (Haering et al., 2002; Hirano and Hirano, 2002; Melby et al., 1998). Rad21 N terminus binds to the ATPase domain of Smc3 and the C terminus binds to Smc1, and like this a ring-like structure is formed with an outer diameter of ≈50 nanometre (nm) (Haering et al., 2002). A fourth subunit associates itself with Rad21 called cohesin subunit Scc3/ stromalin antigens (Scc3/SA), being called stromalin antigens 1 and 2 (SA1 and SA2) in metazoan (Losada et al., 2000; Sumara et al., 2000).

In addition to these proteins, three more are associated with cohesin. Amongst them is Pds5 (Denison et al., 1993; van Heemst et al., 1999) characterized by numerous HEAT repeats (Panizza et al., 2000) One of the Pds5 binding partners is Wapl, which regulates cohesin interaction with chromatin. Wapl is a conserved protein from yeast to humans, being required for promoting dissociation from chromosomes. It forms a stable sub complex with Pds5, through which it binds to cohesin (Gandhi et al., 2006; Kueng et al., 2006; Rowland et al., 2009).

The third protein called sororin/Dalmatian is a small protein firstly discover as subtract of APC (Rankin et al., 2005). It is dispensable for the association of cohesin with chromatin but it is critical for stable binding of cohesin complexes to DNA during G2 phase (Schmitz et al., 2007).

The ring-like organization of cohesin has been well supported by electron microscopic data of purified vertebrate cohesin complex (Anderson et al., 2002) and by crystal structures of subcomplexes of cohesin or related Smc complexes (Haering et al., 2002; Haering et al., 2004).

More details about nomenclature check Appendixes 8.5.

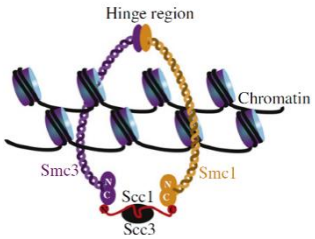


Figure 1.3 One ring/embrace model. One ring embrace the two SC. Adpated from (Mehta et al., 2012).

The discovery of the cohesin complex as a ring like-shape that could hold SC by trapping them both inside the ring (Haering et al., 2002) brought some controversy of how this could be achieved. The strongest version of the model defends that a single monomeric ring traps the SC (the ring model, figure 1.3), but others have proposed that two rings are needed for trapping individually each sister (the handcuff model).

Both models hold that cohesin rings grasps SC using a topological principle rather than physically binding to them or to nucleosomes; furthermore both predict that breaking the ring at any point should trigger dissociation from chromatin and loss of SC cohesion (SCC). This prediction has been tested through the use of TEV protease: cleavage of Rad21 at *Tobacco Etch Virus* (TEV) sites either at a mutated separase site (Uhlmann et al., 2000) or elsewhere within its central domain (Gruber et al., 2006; Oliveira et al., 2010; Pauli et al., 2008) does show that effect. These findings imply that it is not the generation of novel N or C termini that compromises cohesin's ability to hold sisters together but rather the disconnection of N- and C-terminal domains of its kleisin subunit (Gruber et al., 2006). Smc3 severing was also achieved by insertion of TEV cleavage sites within regions of low coiled-coil probability on both strands. Due to the coiled-coil nature of these region, cleavage of one strand is not enough to release the sisters but that can be achieved if two sites are inserted in parallel positions (Gruber et al., 2003; Ivanov and Nasmyth, 2005). The models also predicted correctly that cleavage of cohesin ring by TEV protease or by linearizing circular minichromosomes *in vitro* leads

to the abolishment of cohesin from coprecipitations techniques (Ivanov and Nasmyth, 2005).

The strong ring model (figure 1.3) states that this protein complex would entrap 10nm chromatin fibers topologically. The support came by different researches as where the ring and DNA after covalently closed migrates as dimers in a gel electrophoresis using protein denaturant sodium dodecyl sulfate (SDS)(figure 1.4) (Haering et al., 2008). Nevertheless, some criticism have been reported, for example the observation that SCC of circular silent-mating type (HMR) loci loops out from yeast chromosomes by site-specific recombination is lost when silencing factors are inactivated, however cohesin persists on the chromatin circles (Chang et al., 2005). Furthermore, it has been observed that cohesin can be present on chromosomes but it is not in cohesive state, as example in the N-acetyltransferase ECO1 (need it for cohesin loading) mutant, cohesin can still loads onto chromosomes but SCC couldn't be establish during DNA replication (Skibbens et al., 1999; Toth et al., 1999). Another major criticism is based on the internal diameter of the ring (≈40nm, which imposes constrains to accommodate two SC and creates a static configuration, not flexible enough for cohesin to performs its dynamics functions in for example, DNA replication or repair (Mehta et al., 2012).

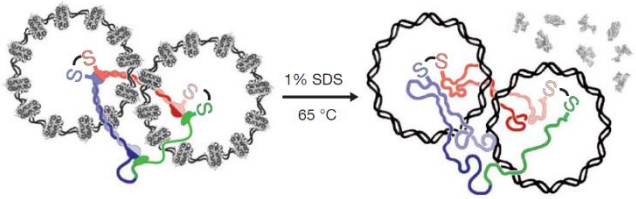


Figure 1.4 A convalently closed cohesin ring. General scheme of how after denaturation this closed cohesin continues with the DNA. Adpated from (Haering et al., 2008).

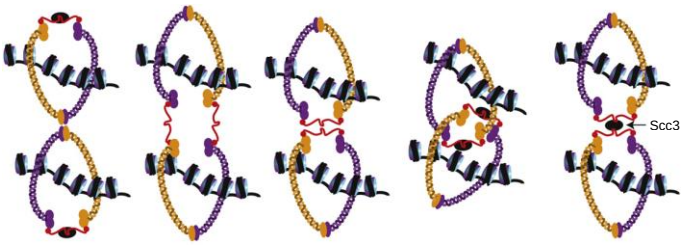


Figure 1.5 The two ring model. The handcuff is the last one on the right being the only one with some supporting information. Adapted from (Mehta et al., 2012).

The alternative models ("snap" or "handcuff" model) are based on cohesion by interactions between different cohesin rings in each SC or by topological entrapment as mention before (figure 1.5) but using two cohesin rings. However, with the exception of the handcuff model, that still relies on a topological entrapment, all the other models do not present any solid evidences. Other models have also been mention in the literature as the bracelet and the rod model (showed on figure 1.6).

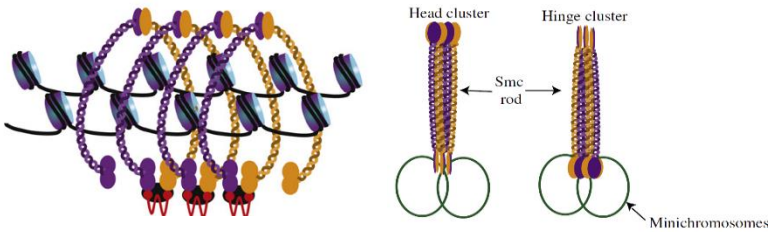


Figure 1.6 The Bracelet and the Rod model. In the left (bracelet), cohesin rings oligomerized together forming a bracelet like-shape. In the right (rod), cohesins associate with one another to form a struture like rod. Adpated from (Mehta et al., 2012).

Until de moment, the major evidences point out the ring model as the most plausible one and throughout this thesis cohesion establishment and decay will be discussed in light of this model.

1.2.1 Cohesin Cycle

- **Cohesin Loading**

To perform its functions cohesin must be bind to chromosomes before the onset of DNA replication to create a functional linkage between the nascent SC (Uhlmann and Nasmyth, 1998). No DNA binding motif was found in the cohesin's components, however genome wide mapping revealed that cohesin binds to every 10-15kb region on the chromosome arm, known as cohesin associated region (CAR) (Blat and Kleckner, 1999; Glynn et al., 2004; Laloraya et al., 2000; Lengronne et al., 2004; Megee et al., 1999; Tanaka et al., 1999). In *S. cerevisiae* the cohesin loaders have been discovered as a conserved Sister chromatid cohesion protein 2 and 4 (Scc2/Scc4) protein complex (Ciosk et al., 2000) and binding sites of this complex have been located as distinct sites than CARs, favouring the idea that cohesin first binds to Scc2/4 and then relocates to CARs. However it has been argued by experimental evidence that Scc2/4 binding sites can lie even within CARs suggesting a direct deposition (Kogut et al., 2009). Orthologs of Scc2 and Scc4 have also been found in many other organisms like fission yeast, *Drosophila*, human, *Xenopus* and *C. elegance* (Ocampo-Hafalla and Uhlmann, 2011).

Chromatin immunoprecipitation (CHIP) analysis in Drosophila showed that the Nipped B (Scc2 ortholog) is presented at the transcribed regions and its distribution overlaps with RNA polymerase II (Misulovin et al., 2008), where the distribution pattern of cohesin was found to coincide with the pattern of its loader. One explanation for these is that cohesin strongly connects with Nipped B, and the same does not happen between Scc2/4 in budding yeast (Mehta et al., 2012). Furthermore, in murine embryonic stem cells, the cohesin loader Nipped-B-like protein (Nipbl) colocalizes with the transcriptional mediator complex at the promoter regions of some expressed genes (Kagey et al., 2010). However a major fraction of mouse and human cohesin colocalizes with the CCCTC binding factor (CTCF) insulator (required for transcriptional repression) with a preference for regions in the vicinity of transcribe genes (Wendt et al., 2008). It still not clear whether and how human cohesin translocates from its loading sites to CTCF-binding sites.

An alternative model is that cohesin may dissociate from the initial loading site where Scc2/4 bind and then reloaded further downstream. This assumption is supported by Nasmyth and co-workers when reported that ATP hydrolysis (known for cohesin's association with chromatin) (Arumugam et al., 2003; Weitzer et al., 2003) is also required for the translocation of cohesin on the chromosomes (Hu et al., 2011).

- **Cohesin Establishment**

The establishment of cohesin during DNA replication requires cohesin to become cohesive, a process yet poorly understood (Sherwood et al., 2010; Skibbens, 2011).

When in G2 or M phase of yeast cells cohesin subunits are expressed they fail in associate with chromosomes, but also fail to establish cohesion, even in the presence of pre-existed one (Hauf et al., 2001; Lengronne et al., 2006; Strom et al., 2007; Strom and Sjogren, 2005). From this is possible to assume that little or no turnover of these cohesins components occurs within the cohesive structure that holds the SC.

Among many players, Eco1 is the key and founding member of a family of cohesin acetyltransferases (CoAT) that acetylate two lysine residues located in the head domain of Smc3 during S phase (Rolef Ben-Shahar et al., 2008; Unal et al., 2008; Zhang et al., 2008). High levels of acetylation are kept until G2 and M phases, decreasing when SC separation occur (Rolef Ben-Shahar et al., 2008), being the deacetylation triggered by this separation. This modification neutralizes the action of Wapl-Pds5, probably through affecting the conformation of the ring, although more research has to be made (Feytout et al., 2011; Gandhi et al., 2006; Heidinger-Pauli et al., 2010b; Kurze et al., 2011; Sutani et al., 2009; Tanaka et al., 2001). Fission yeast seems to present the same mechanistic way of establish cohesin (Tanaka et al., 2001; Vaur et al., 2012)

In human exist two orthologs for the establishment of sister chromatid cohesion N-acetyltransferase 1 (Esco1) and 2 (Esco2) (Hou and Zou, 2005). Nevertheless, Smc3 acetylation is not enough for establishment of cohesion. This may be due to the strong antiestablishment force of the Wapl-Pds5 interaction. Acetylation-dependent binding of sororin is essential to stabilize a fraction of cohesin after DNA replication (Higashi et al., 2012; Lafont et al., 2010; Nishiyama et al., 2010; Schmitz et al., 2007). Pds5 provides a binding surface to the fibroblast growth factor (FGF) motifs present in sororin, which would in this way displace Wapl, thus inhibiting the ability of Wapl to dissociate cohesin from DNA (Nishiyama et al., 2010). After entering in mitosis sororin is phosphorylated, stopping to compete with Wapl.

Cohesin establishment is restricted to S phase with the exception of damage-induced cohesin in yeast. This restriction could be because of the cell cycle CoAT regulation and its interactions with proliferating cell nuclear antigen (PCNA) (Remeseiro and Losada, 2013).

In summary, after cohesion the association between the complex and the chromosomes seem to be more stable. Studies in HeLa cells conclude that most chromosomal cohesin is not stably associated with chromatin and has a mean residence time of less than 25 minutes (min) in G1 and G2 (Gerlich et al., 2006). In G2 cells a different population of cohesin is also observed (one third of the total) whose residence time is much longer. This population may be the complexes that engage in trapping the SC.

- **Prophase Pathway**

In vertebrate cells during mitosis, the prophase pathway is active during prophase and pro-metaphase, where removal of the bulk of cohesins from chromosomes arms happens, whereas cohesin in the centromeric region remain bound until the onset of anaphase (Waizenegger et al., 2000). In animal and most fungal and plant cells, 90% of the cohesin bound to chromosomes dissociates during prophase (Peters et al., 2008) creating a soluble pool of mitotic cohesin that would not be cleaved, being reloaded into chromosomes in telophase.

Dissociation of cohesin during prophase pathway depends on two mitotic kinases: Polo-like kinase 1 (Plk1) and Aurora B (Gimenez-Abian et al., 2004; Lenart et al., 2007; Losada et al., 2002; Sumara et al., 2002; Waizenegger et al., 2000) kinase. Plk1 phosphorylates Scc1/Rad21 and SA1/SA2 subunits of cohesin *in vitro* (Hauf et al., 2005) whereas the substrates for Aurora B kinase are not known yet. The phosphorylation of SA2 is not the only requirement for the removal of cohesin, Wapl and Pds5 also play a critical role for the unloading of cohesins from chromatin. Research suggest that SA2 phosphorylation

makes changes in cohesin that leads to its dissociation by Wapl followed by the opening of a cohesin ring, not dependent on separase.

An important step to know more about Wapl interaction with cohesin was obtained by crystallography structures views of the Wapl, where was seeing that it directly binds to Smc3 ATPase head domain; by this model Smc3 acetylation might stop Wapl's competition with Scc1, thus allowing the exit gate to shut (Chatterjee et al., 2013).

In yeast cohesin remains bound until the onset of anaphase as also the cohesin associated proteins persist on chromatin after prophase (Ciosk et al., 2000). In the beginning of anaphase the bulk of cohesin are removed by the action of separase which cleaves Scc1/Mcd1/Rad21 (Uhlmann et al., 1999).

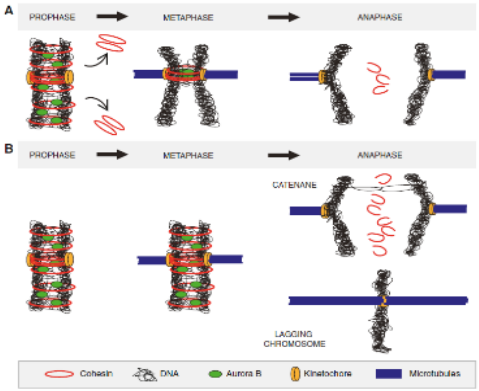


Figure 1.5 Models for the purpose of the Prophase Patway. In a) exemple of a normal path with relocalization of Aurora B to centromeres, removel of cohesin in the prophase patway and decatonation by topoisomerase II at centromeres leading to proper segregation. In b) is a example in the case of no prophase patway existing leading to problems in segregation. Adapted from (Haarhuis et al., 2013).

The need of this phase may be a prerequisite for chromosome condensation once it happens at the same time, however no major condensation problems were observed when this phase is compromised; it is also possible that the prophase pathway contributes to the fidelity of chromosomes segregation as it may be facilitated by the resolution of SC (figure 1.7). This resolution can help with the directionality of the topoisomerase driven reactions towards decatenation, recently some results support this idea from Haarhuis and colleagues once Wapl-depleted human cells undergo anaphase with segregation errors (Haarhuis et al., 2013). The third explanation of the need of this phase could be that the cohesins removed by this way were not destroyed by separase so vertebrate cells finish mitosis with almost unchanged pool of cohesins, which can be reloaded on chromatin contrary to budding yeast that has to resynthesize its kleisin subunit (reviewed in (Peters et al., 2008)).

To address the issue if the exit gate used in interphase is the same use in the prophase pathway (see section1.2.2), Eichinger and colleagues filmed green fluorescent protein (GFP)-marked cohesin in *Drosophila* neuroblasts getting through mitosis. In wild type (wt) flies, the cohesin was readily seen however in Wapl mutant flies or when employing the Smc3-Scc1 fusion construct the cohesin persisted on chromosome arms (Eichinger et al., 2013), the same results was saw in humans (Buheitel and Stemmann, 2013). The chromosomes presented then an increased in cohesion due to the persistence of cohesin, however neither *Drosophila* nor human cells stop/delay from entering in anaphase (Eichinger et al., 2013; Kueng et al., 2006), implying that separase may have no difficulty in cleaving higher amounts of cohesin than normally, along the arms (Murayama and Uhlmann, 2013).

Once the nature of cohesin present in the chromosomes is the same, how just the arm complex are removed but the centromeric and pericentromeric cohesins are not? In these regions there is an accumulation of a member of a class of proteins known as shugoshins, Sgo1 (or Meiotic protein S332 in *Drosophila melanogaster*) (Kerrebrock et al., 1995). When these proteins are depleted, cohesin

dissociates from the centromeric regions at the time of prometaphase (Kitajima et al., 2005; McGuinness et al., 2005; Salic et al., 2004; Tang et al., 2004). So, Sgo1 appears to be the protector of centromeric/pericentromeric cohesin in the prophase pathway from mitotic kinases and Wapl to enable bipolar attachment of sister chromatids to the mitotic spindle. Sgo1 which is recruited by budding uninhibited by benzimidazoles 1 (Bub1)-dependent Histone H2A phosphorylation (Kawashima et al., 2010), physically interacts with a protein called phosphatase 2A (PP2A) and recruits PP2A to the centromeres counteracting sororin phosphorylation, sustaining its ability to counteract Wapl. This complex is able to dephosphorylate the SA2 subunit of the cohesin complex *in vitro* (Kitajima et al., 2006), ensuring that segregation does not start until all chromosomes are bi-oriented. Sgo1 maintains sororin binding to Pds5 and cohesin through PP2A-dependent dephosphorylation. This sororin competes with the well-established cohesion inhibitor Wapl for binding to Pds5, explaining how sororin dephosphorylation by Sgo1-PP2 protects cohesion (Liu et al., 2013) (figures 1.8 and 1.9).

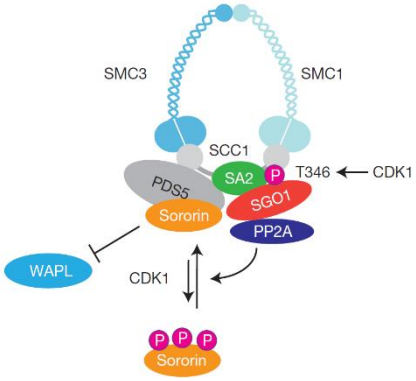


Figure 1.6 Model of cohsein in centromeric positioning during prophase pathway. How Sgo1-PP2A maintains cenromeric cohseion during mitosis in human cells. Adpated from (Liu et al., 2013).

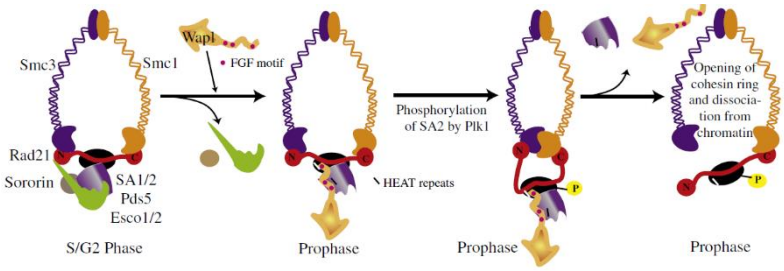


Figure 1.7 Prophase Pathway. The inteactions of Wapl with Pds5 and the disceplement of the interactor of Pds5; SA2 phosphorylation by Plk1 and the interaction between these two complexes leading to open of the ring by Smc3/Scc1. Adapted from (Mehta et al., 2012).

- **Metaphase to Anaphase Transition**

The remaining cohesin at the pericentromeric region in higher eukaryotes as well as the bulk of cohesin in yeast cells is removed from the chromatin during the metaphase to anaphase transition, due to the cleavage of Rad21/Scc1 subunit by separase. Until anaphase onset, separase is physically inhibited by its association to the protein securin. In vertebrate cells, separase is additionally inhibited by Cdk1-mediated phosphorylation and by its binding to Cdk1-associated cyclin B protein (reviews (Huang et al., 2005; Stemmann et al., 2005)) (Stemmann et al., 2001). When the chromosomes are correctly bioriented on the mitotic spindle, the spindle checkpoint (SAC, mentioned in 1.1) is inactivated, resulting on the activation of APC/C by its binding to Cdc20, which was blocked by Mad2 (Musacchio and Salmon, 2007; Nasmyth, 2005). Activated APC^{cdc20} mediates ubiquitin-dependent degradation of securin and cyclin B, which in turn activates separase and inactivates Cdk1. Separase cleaves Scc1/Rad21(Hauf et al., 2001; Uhlmann et al., 1999) at two sites leading to dissociation of cohesin from the chromosomes and consequent separation of the SC (figure 1.10). Rad21 re-accumulates at the end

of the following G1 phase as APC becomes inactive by that time (Mehta et al., 2012). Cohesin's Smc3 protein must be deacetylated in order to be reused in the next cycle. This task is perform by cohesin deacetylates (CoDAC), found in yeast by the name of Hos 1(Beckouet et al., 2010; Borges et al., 2010) and histone deacetylase 8 (HDAC8) in human cells (Deardorff et al., 2012a).

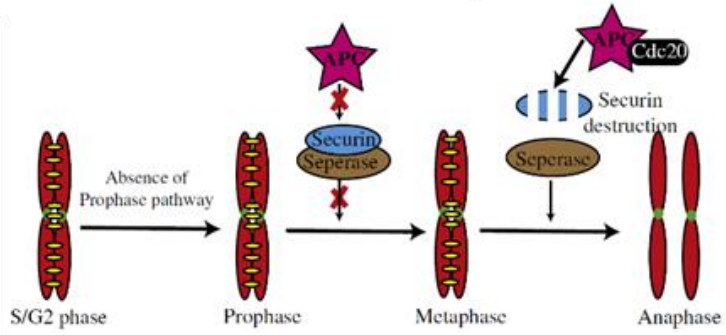


Figure 1.8 Cohesin removal in metazoan. The residual amount of cohesin that stays after the prophase pathway is cleaved in the metaphase to anaphase transition by separase. Adapted from (Mehta et al., 2012).

In summary the cohesin cycle:

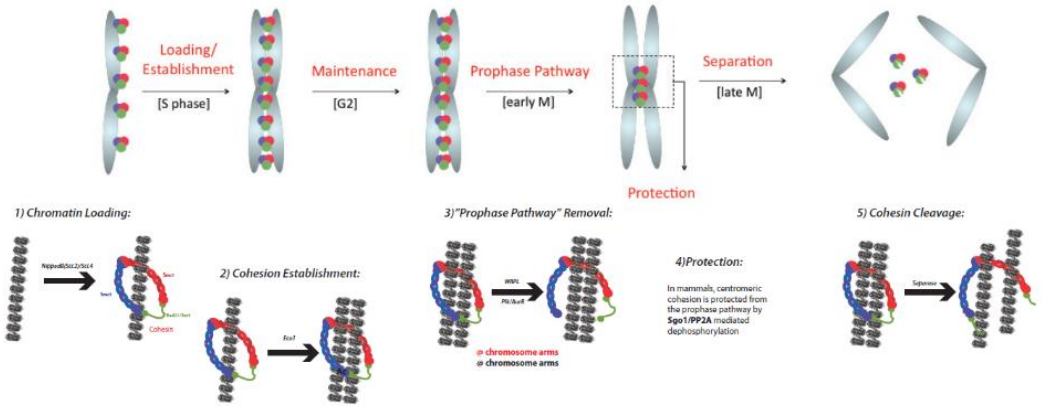


Figure 1.9 Cohesin cycle. Cohesin is established durind the S phase being mantained through the next phases until the prophase patway. Most of the cohesin is removed at this staged being only kept the pericentromeric one. In anaphase, the remaining cohesin is cleaved by separase leading to the dissociation of the sister chromatids for opposing poles.

1.2.2 The entry and exit gate

How SC are entrapped by cohesin rings is still unknown. One possibility is that the ring assembles *de novo* around DNAs, although studies indicating that chromatin-unbound cohesin exists already as a ring (Gruber et al., 2003).

An additional possibility could be that one of the parts of the ring has to open to embrace DNA. Supporting this assumption Gruber and colleagues showed in the yeast *Saccharomyces cerevisiae* that locking the interaction between kleisin's N-

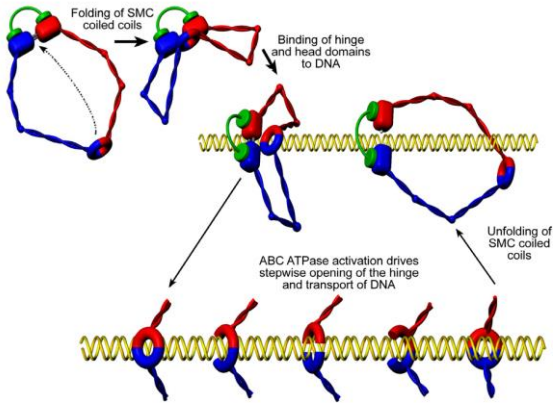


Figure 1.10 Model of how cohesin ring opens. The folding of the Smc1 and Smc3 enables the interactions between head/hinge domains leading to disruption of one ot the two hinge-hinge sites. Adpated from (Gruber et al., 2006)

and C-terminal domains with either Smc1 or Smc3 by grafting a rapamycin-induced dimerization interface, do not affect cohesin's chromatin loading, excluding the Smc/kleisin interface as the entry gate. Smc1 and Smc3 hinge domains locked interfered with the establishment, but not maintenance, of cohesion suggesting that DNA enters by Smc1/3 interface (figure 1.12) (Gruber et al., 2006).

The determination of crystal structure at the hinge domain of Smc1/3 heterodimer in *Mus musculus* demonstrated the existence and importance of a positively charged channel within this region, required for SCC. Additionally, disrupting Smc1/3 interaction at the hinge domain affects Smc3 acetylation and establishment of cohesion. These results suggest that hinges participate in a major conformational change during S phase which possibly promotes hinge opening and acetylation of Smc3 (Kurze et al., 2011).

A recent study in *Drosophila* about the opposite situation (exit by cohesin subunits' interaction) showed with a covalent fusion between Smc3 and Scc1 locking the gate between the two proteins that they bound to chromosomes but exhibited low turnover as it was also observed in Wapl mutants. Nevertheless when the ring was re-opened the Smc3-Scc1 fusion by engineered TEV protease cleavage, cohesin turnover resumed (Eichinger et al., 2013) (figure 1.13). This suggest that DNA exists through a transient gate between Smc3 and Scc1 subunits, corroborating other results obtained in the same group in budding yeast (Chan et al., 2012).

The fact that cohesin has two separate gates makes it even more prone to cohesion decay, as two separate interfaces may lead to cohesin loss if inappropriately regulated.

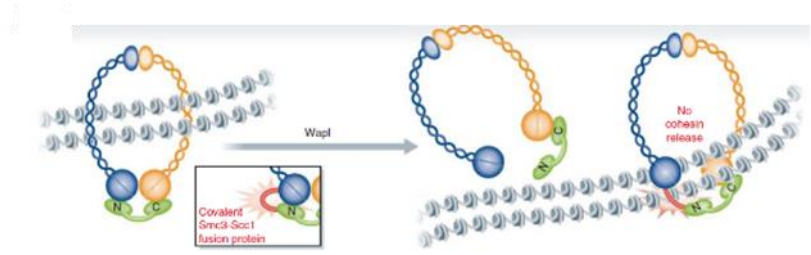


Figure 1.11 Cohesin exit gate model. The experience of Eichinger and co-workers, where a convalent Smc3/Scc1 fusion protein prevents release of cohesin from chromosomes by Wapl in interphase, as well as during prophase patway of cohesin removel (section before). Adapted from (Murayama and Uhlmann, 2013).

1.2.3 Other Cohesin Functions

In addition to promoting sister chromatid cohesion, recent evidence suggest that cohesin is involved in other functions. These novel roles of cohesin will be described below.

- **DNA Replication**

In S phase and G2, cohesin promotes restart of replication forks that stall at regions that are difficult to replicate, such as telomeres, and facilitates repair of double-strand breaks by homologous recombination (Heidinger-Pauli et al., 2010a; Remeseiro et al., 2012). Furthermore, in the absence of cohesin an increased in replication fork collapse may be expected and unrepaired DNA breaks that

could lead to genomic and chromosomal instability upon passage in mitosis. As example, mouse embryonic fibroblasts that lack SA1 show robust centromere cohesion but in telomeres leads to problems in replication promoting missegregation and aneuploidy (Remeseiro et al., 2012).

- **DNA double-strand break (DSB) repair**

Schizosaccharomyces pombe showed the first evidence of the role of cohesin in maintaining the integrity of the genome against DNA damage (Birkenbihl and Subramani, 1992). Since them, several groups have confirmed the role of cohesin in DSB in mitosis (Cortés-Ledesma and Aguilera, 2006; Sjögren and Nasmyth, 2001) and in meiosis (Ellermeier and Smith, 2005).

Cohesin can be loaded and generate SCC in postreplicative phase if there is DNA damage (Strom et al., 2004), which corroborates the thought that the presence of DNA lesions somehow augments Eco1 activity beyond S phase. Curiously, this damage-induced cohesion (DI-cohesion) is generated not only at the site of the damage, but also globally on all the chromosomes in an Eco1-dependent manner (Ünal et al., 2007). The essential job of cohesin in DNA repair is to bring the two SC together in a way that DSB on one sister can be repaired using the unscathed sister as a template for homologous recombination. In response to DSB Scc2 was identified as a factor required for fresh loading of cohesin (Strom et al., 2004).The targets of Eco1 for DI-cohesion seems to be the acetylation of Scc1 and the phosphorylation of Scc1 augments its acetylation (Heidinger-Pauli et al., 2009).

Experiments indicate that cohesin may initiate the process of DSB repair by bringing damage and intact strands in close proximity, but becomes dispensable for the subsequent repair process. Importantly to point out that may be the function of Eco1 as cohesion establishment factor in replicative and postreplicative (DI-cohesion) can differ. Lu and co-workers support this idea by observation in budding yeast that Eco1 mutant fails to provide DSB repair without any perturbation in SCC (Lu et al., 2010).

- **Transcriptional Control**

Cohesin has recently emerged as a key regulator of eukaryotic gene expression, although the mechanism is still poorly understood. In *Drosophila*, a mutant of Nipped B was found to be deficient in activation of homeobox genes (Rollins et al., 1999), but despite this novel interphase role of cohesin as transcription activator, little is known about the precise mechanism by which the complex regulates transcription. Initial studies showed that cohesin persist in the nuclei of most post-mitotic cells, including neurons (Wendt et al., 2008) and that inactivation of cohesins leads to axon pruning defects in postmitotic mushroom body ɣ-neurons in *Drosophila.* This consequences are partially due to a lack of expression of cohesin as a transcriptional regulator of the receptor gene (Pauli et al., 2008; Schuldiner et al., 2008).

The co-localization of cohesin in sites of active genes, as in *Drosophila* and in humans, appears to be related to cohesin's role in transcription, once CTCF is believed to regulate gene expression by insulating interactions of a gene promoter with an enhancer/silencer (Ohlsson et al., 2010). It has been suggested that the regulation of transcription by CTCF is mediated through its ability to recruit cohesins

at the promoters regions. Additionally, Chromatin immunoprecipitation (ChIP-seq) revealed that in mouse embryonic stem cells, cohesin loader (Nipbl), cohesin, and mediator (transcriptional co-activator) co-localize with each other at many sites other than CTCF-binding sites (Kagey et al., 2010). A depletion in one of this factors can lead to changes in gene expression of those whose cis-acting regulatory elements show peaks of cohesin or mediator accumulation.

Different experiences lead to the conclusion that chromosomal looping formed by CTCF can be maintained by cohesin, though cohesin itself is recruited by CTCF(Feeney and Verma-Gaur, 2012; Parelho et al.; Wendt et al., 2008).

There is argued that a higher level of cohesin activity may be required for transcriptional control than is required for SCC [reviewed in (Dorsett, 2011)], seeming that alterations on cohesin or in its loading factor does not affect SCC or chromosome segregation (Gause et al., 2008; Kawauchi et al., 2009; Revenkova et al., 2009; Rollins et al., 2004). To accommodate functional differences (gene expression versus SCC) a dual mode of binding can be considered, forming a strong or weak interactions, by observations in *Drosophila* (Gause et al., 2010). The same group had land support to this idea demonstrating that a pool of cohesin that binds strongly to chromatin is reduced in Nipped B mutants that exhibit altered gene expression, suggesting that strong binding may be essential for regulating transcription (Gause et al., 2010). The same model is also supported in yeast in an *in vitro* system (Onn and Koshland, 2011).

Translation also appears to be influenced by cohesin, although indirectly. In budding yeast and humans cohesin demonstrated to be capable of augmenting translational capacity by increasing transcription of ribosomal ribonucleic acid (rRNA) (Bose et al., 2012).

- **Function at centrosomes**

In budding yeast the centrosome (animal cells) is called spindle pole body (SPB), which during DNA replication is also duplicate to form two sister centrosomes/SPB. Many studies think that cohesin may be involved in the faithful SPB duplication. The first clue appeared when was shown that separase is also required for centriole disengagement and for licensing centrosome duplication (Tsou and Stearns, 2006). Another studies detected the presence of cohesin subunits at the spindle pole (Wong and Blobel, 2008) and centrosomes (Kong et al., 2009). Moreover, siRNA mediated depletion of cohesin subunit Rad21 shown to cause premature separation of paired centrioles (Nakamura et al., 2009).

Centriole disengagement in human cells also happened when exist ectopic activation of separase or depletion of Sgo1, as well as premature SC separation (PSCS) (Schockel et al., 2011). Clarke and colleagues also reported chromosomal cohesin regulators and separase in centrosomal localization of cohesin subunit Rad21 (Gimenez-Abian et al., 2010). A study by Jin et al., showed a probable role of cohesin in SPB cohesion also during yeast meiosis (Jin et al., 2012).

However in *Drosophila* embryos, centriole engagement does not depend on the integrity of the cohesin complex (Oliveira and Nasmyth, 2013), in agreement with other studies where a new substrate for separase was required for the disengagement (Matsuo et al., 2012). Moreover, the disengagement seems to be dependent of the decrease of the Cdk1 activity (Oliveira and Nasmyth, 2013).

In summary, this “potential” role of cohesin is still under debate.

- **Contribution for condensation**

Chromosome condensation is a requirement for faithful chromosome segregation, and is executed by a multi-protein evoluctionarily conserved cohesin-like complex called condensin (Bhalla et al., 2002; Lavoie et al., 2002). In budding and fission yeast changes in cohesin can promote hypo- or hyper-condensation of the chromosomes, although in higher eukaryotes only subtle effects were observed (Ding et al., 2006; Hartman et al., 2000; Losada et al., 2002; Sonoda et al., 2001). Thus, cohesin seem, somehow to influence condensing localization on the chromosomes at least in yeast. A recent study using ChIP followed by hybridization with oligonucleotide tiling arrays showed that DNA binding sites of condensing overlap with sites of occupancy of Scc2/4 complex (cohesin loader) (D'Ambrosio et al., 2008). Seems that at least in budding yeast condensing is loaded at the sites where cohesin is also recruited, however how this interaction happens is poorly understood (Mehta et al., 2013).

- **Meiosis & cohesin**

Chromosomes duplicated during pre-meiotic S phase and two SC are created per chromosome, that is 2x2 SC per pair of homologues chromosomes. The cohesin complex in vertebrate meiocytes contain cohesin core subunits and additional variants of the mitotic ones, which include the SMC1β, two kleisins (REC8, RAD21L) and an additional SA protein, SA3/STAG3, generating cohesin complexes with different subunit composition and possibly separate meiotic functions. In prophase I, the two homologous associate and synapse within the meiosis-specific synaptonemal complex (Jessberger, 2012). The cross keep chiasma links SC and allows genetic exchange, even after late prophase I and metaphase I, being dependent of cohesin to maintain the connection until they resolved. The protection of centromeric cohesion in meiosis I require, as in mitosis, protection from Sgo and PP2A (Ishiguro et al., 2010; Kitajima et al., 2004; Riedel et al., 2006), that protection disappears only in metaphase II to anaphase II transition when the SC separate due to cohesin cleavage and the gametes with one single sister is generated (Kudo et al., 2006).

In 2010, a study in TEV cleavable Rec8 in mice allowed to conclude important facts about the meiosis relationship with cohesin in oocytes. It seems that Scc1 cleavage does not impair meiotic division and the same did not happened when Rec8 is cleaved, generating single sisters; concluding that Scc1 has little or no role during meiotic chromosome segregation. Nevertheless, large amounts of Scc1 were observed suggesting that could be a backup for the first zygotic divisions (Tachibana-Konwalski et al., 2010). Additionally, REC8-type cohesin is necessary and sufficient for arm and centromere cohesion in oocytes (Tachibana-Konwalski et al., 2010). Remarkably, this experiments with TEV protease cleavable assays also gives a firm confirmation that is cohesin that mediated the linkage between sister chromatids in mammalian (Tachibana-Konwalski et al., 2010).

In summary, cohesin in meiosis can be associated with functions as pairing the homologous chromosomes, non-homologous centromere coupling or mono-orientation of sister kinetochores during meiosis I (Mehta et al., 2013).

Oocytes, in particular, can be more prone to problems than spermatocytes once they enter in

meiosis still in the fetus. Meiosis precedes until the end of prophase I and then arrest in a stage known as dictyate arrest. So, female oocytes are quiescent within primordial follicles and only begin to reach full size from puberty on, after hormonal stimulation each month. Then the oocytes continue meiosis I and developed until metaphase II where an oocyte arrest waiting for fertilization that if not happens the oocyte dyes after a few day (Bukovsky et al., 2005; Fair, 2010; Gosden and Lee, 2010; McLaughlin and McIver, 2009; Wassarman, 2002). The arrest time that oocytes can be about four decades in humans and a few years in mice; and for all that time the SC has to be connected for ensuring proper chromosome segregation, which can fail with increasing age (Jessberger, 2012).

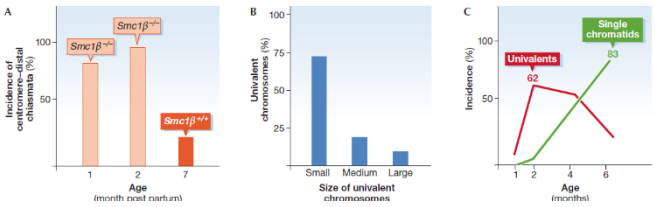
1.2.4 Cohesinopathies

Human syndromes caused by cohesin and cohesin-associated factors mutations resulting in cohesin dysfuntion, are called “cohesinopathies” (Bose and Gerton, 2010; Liu and Krantz, 2008) , being the two classic examples Roberts’ Syndrome (RBS) and Cornelia de Lange Syndrome (CdLS). However, problems like cancer and missegregation of chromosomes in meiosis relative with woman age are consequences of cohesin malfunction.

- **Age-related aneuploidy and cohesin**

As women get older their oocytes become susceptible to chromosome miss-segregation that can lead to aneuploidy.

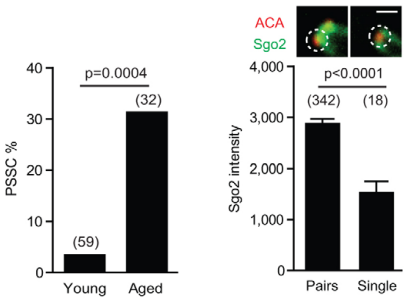
The most frequent of these diseases is Down syndrome which in the majority is caused by missegregation (nondisjunction) of chromosome 21 during meiosis I in oocytes (Gilliland and Hawley, 2005; Hassold and Hunt, 2001). The frequency of this syndrome is 1/1400 births in 20 to 24 year-old women, rising to 1/350 in 35 year-old and 1/25 in 45 years or older women (Yoon et al., 1996). Other trisomes can occurs but with the exception of 13 and 18, usually they are lethal during embryogenesis. As women age, the frequency of nondisjunction in oocytes increases dramatically, where cohesin loss has been proposed to play a crucial role.



Graphic 1.1 Meiosis errors in Smc1β–/–. In a) the distance of chiasmata to more centromere-distal sites; in b) the loss of that chiasmata is bigger in small chromosomes, due to the thought of the movent of the chiasma; and in c) the incidence of univalents and SC. Adpated from (Jessberger, 2012) but based on (Hodges et al., 2005).

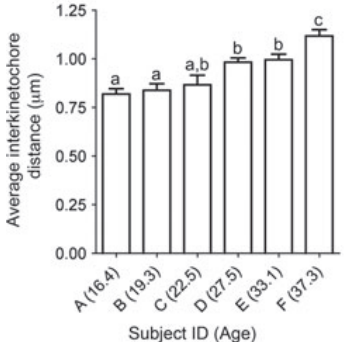
A study where *in vitro* isolated and mature oocytes from mice Smc1β$^{-/-}$ were analysed, single homologues in metaphase I (when they should be together), and this occurrence was aggravated in old mice; furthermore in the animal the chiasmata was lost (Hodges et al., 2005; Revenkova et al., 2004). Additionally, a detailed analysis to these chromosomes seems to suggest that a movement of the chiasmata exist toward more centromere-distal regions that could lead to its lost. This assumption were confirmed and the data is visible on the graphic 1.1; as conclusion cohesin SMC1β can be consider a big factor in age-dependent aneuploidy (Bickel, 2005; Gilliland and Hawley, 2005).

The meiosis-specific kleisin RAD21L does not appear to be involved in cohesin-associated age-dependent aneuploidy. While its absence causes a female age-dependent sterility phenotype, it seems that RAD21L does not function in oocyte meiotic cohesion (Herran et al., 2011). Sgo also diminishes with increasing age of the oocytes and in mice Smc1β$^{-/-}$ (Lister et al., 2010) however is not known if is a cause or a consequence of cohesin's loss.



Graphic 1.2 Loss of chromosome cohesion in aged eggs. The graphic on the left present Prematur separation of sister chromatids (PSSC) in young or aged mice. The right one describes the centromeric Sgo2 intensity at on kinetochore of a pair or from a singe sister, with representative images above. Scale bars: 1 µm. ACA, anti-centromeric antibodies. Adapted from (Yun et al., 2014)

Another studies in rapidly ageing mice strains or very old mice from commonly used strains also appear with higher frequencies aneuploidy eggs than oocytes from young animals as well as an age-dependent loss of REC8 from the centromeres of old oocytes (Chiang et al., 2010; Lister et al., 2010), in agreement with other research (Liu and Keefe, 2008).A technique using real-time kinetochore-tracking approach to study the relative importance of cohesion and congression defects in the segregation of bivalents from aged mice, allowing a level of detail that has not previously been used to study chromosome dynamics in aged eggs (Yun et al., 2014). Live imaging helped discovering that half of oocytes appeared with sister chromatid separation occurring 2 hours after anaphase I, as the metaphase II spindle was assembling. This suggest that errors (weakly attached bivalents) that had origin in meiosis I just manifest themselves in meiosis II (Yun et al., 2014), being premature separation of the dyads the major defect in aged mice eggs (graphic 1.2). This is followed by the information obtained by *in situ* spread with monastrol staining using anti-centromeric antibodies against eggs in metaphase II, where in aged mice presents a large number of PSCS and Sgo2 is lost in single kinetochores from dyads (graphic 1.2). In summary, the measurements show a strong association between an age-related loss in sister chromatid cohesion and reduced Sgo2 in metaphase II eggs (Yun et al., 2014). This coupled with the above information that vulnerability of cohesin in centromeric regions in the metaphase II would be even more increased. Although cohesin proteins are conserved between humans and mouse, it is not known if the deterioration seen in mice is also seen in humans with advanced reproductive age (Garcia-Cruz et al., 2010). Such studies have been hampered by the difficulty in obtaining mature gametes from reproductively young and older women. In 2012, 18 eggs were obtained that follow *in vitro* maturation from a group of different ages (16.4, 19.3, 22.5, 27.5, 33.1, and 37.3 years) where the goal was to access how chromosome cohesion changes with maternal age. For that, the distance between kinetochores of sister chromatids, or the inter-kinetochore distance was measured in eggs using *in situ* chromosome spreading techniques (Duncan et al., 2009). The data show that an absolute increase of 0.28 µm in inter-kinetochore distance between the two age extremes (0.82 ± 0.03 µm in the youngest subject (16.4

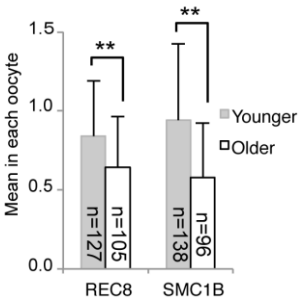


Graphic 1.3 Inter-kinetochore distances increase with age. Uppercase letters correspond to the subject identification and the subject age in years; the different lowercase letters denote significant differences between results. Adapted from (Duncan et al., 2012)

years) to 1.1 ± 0.03 µm in the oldest 37.3 years)(graphic 1.3) (Duncan et al., 2012), this is consistent with previous results in two mouse strains that showed increases of 0.13 and 0.44 µm (Chiang et al., 2010; Merriman et al., 2012). Furthermore, the oldest two subjects also presented chromosome segregation errors, in majority PSCS (Duncan et al., 2012).

Another more recent study also in humans but in this case using all the ovarian tissue to analyse by immunofluorescence signal intensities. A decrease in Rec8 from 34% from younger women to older ones and 38% in Scm1β (graphic 1.4). In this study also mitotic cohesin were analysed however no significant difference was found between young and older women (Tsutsumi et al., 2014).

The data describe in this section reveals how major is the role of cohesin in meiosis, specifically in oocytes where so much more need to be understood about cohesin regulation through time.



Graphic 1.4 Quantification of meiosis-specific cohesins. Cohesin signal intensity means in single oocytes compared between age groups. Women were grouped as younger (≤29-year-old) or older (≥40-year-old) **P,0.01, Student's t-test. Adapted from (Tsutsumi et al., 2014).

Roberts' Syndrome

Also called pseudothalidomide syndrome phocomelia is caused by a mutations of both alleles of ESCO2 (Eco1 in *S. cerevisiae*). RBS is a rare autosomal recessive disorder (Tomkins et al., 1979) where patients present a wide range of clinical phenotypes that include upper and lower limb defects, grow retardation, craniofacial anomalies and mental retardation with limited similarity to the CdLS phenotype (Dorsett, 2007; Vega et al., 2005). The chromosomes of these patients exhibit premature centromere separation and heterochromatin puffing, indicative of SCC defect (Tomkins et al., 1979). In mice and zebrafish knockout of this protein show centromeric cohesion defects and cell cycle aberrations (Monnich et al., 2011; Whelan et al., 2012), once expected since ESCO2 is essential for cohesin establishment.

- **Cornelia de Lange syndrome**

CdLS affects 1:30000 children characterized by facial dysmorphism, hirsutism, upper limb abnormalities, cognitive retardation and growth abnormalities (DeScipio et al., 2005; Liu and Krantz, 2009). More than half of the individuals with this disease present mutation in the gene encoding the cohesin loader Nipbl, on chromosome 5p13 (Krantz et al., 2004; Tonkin et al., 2004). Mutations frameshift or nonsense that leads to Nipbl haploinsufficiency often exhibit more severe phenotypes compared to missense mutations (Gillis et al., 2004).

Mutations on human cohesin subunits Smc1, 3 and Rad21 were also found in a minor subset of

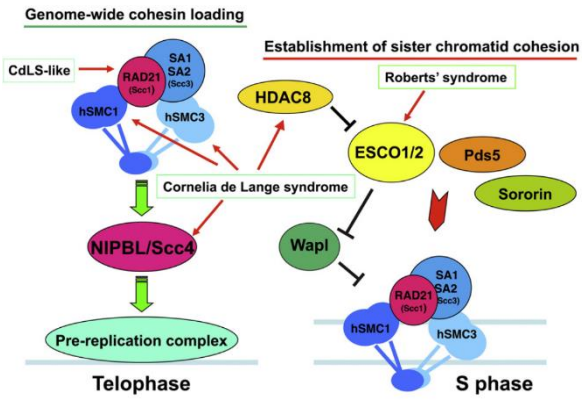


Figure 1.12 Cohesinopathies's localization on cell cycle and its interactions. CdSL appears in majority when there is mutation on the Nibl. RBS in mutations on Esco2. Adapted from (Ball et al., 2014).

clinically milder CdLS cases (~5% and <1%, respectively) (Deardorff et al., 2007; Musio et al., 2006). In the case of mutation in one of the Smc complexes they are missense ones so the patients often present mental retardation as the primary symptom with other less/fewer abnormalities (Deardorff et al., 2007).

Moreover, Rad21 mutations were discover in patients with CdLS-like phenotype (Deardorff et al., 2012b), presenting as phenotype all the classic characteristics but have no mild or no cognitive impairment (Deardorff et al., 2012b). However, these mutations explain about 65% of the CdLS patients while the remaining 35% remains unclear (Ball et al., 2014).

Drosophila and zebrafish mutants with reduced dose of Nipbl or cohesin also display altered gene expression and developmental defects, but no chromosome segregation defects. Seems likely that gene expression, particularly during development, is much more sensitive to cohesin amount/activity than are other functions (Muto et al., 2011).

- **Candidates to the group**

The Warsaw Breakage syndrome (WABS) was first reported in 2010 and consider the new recessive cohesinopathy disorder (van der Lelij et al., 2010). This syndrome displayed microcephaly, pre- and postnatal growth retardation, and abnormal skin pigmentation. This Warsaw's patient presented clinical and cellular features with both RBS and the blood disorder Fanconi anemia. The cytogenetic analysis showed sister chromatid cohesion defects caused by heterochromatin repulsion leading to chromosomes with a 'railroad' appearance, consistent with centromeric cohesion defects and total premature chromatid separation. A search for candidate genes found a link to bi-allelic mutations in the Chlr1/Ddx11 gene (chromosome 12p11) which encodes a protein of the conserved family of Iron–Sulfur (Fe–S) cluster DNA helicases (van der Lelij et al., 2010). This helicase is required for proper sister chromatid cohesion in yeast (Skibbens, 2004) and mammalian cells (Inoue et al., 2007) (Parish et al., 2006).

Facioscapulohumeral dystrophy (FSHD) is the third most common heritable muscular dystrophy in the U.S. It is characterized by progressive wasting of facial, shoulder, and upper arm musculature, which can spread to the abdominal and foot-extensor muscles (Nozawa et al., 2013; Pandya et al., 2008; van der Maarel and Frants, 2005). FSHD can also be considered to be a cohesinopathy, in which D4Z4 (3.3 kb repeat that contains an open reading frame (ORF) for the double-homeobox transcription factor DUX4 retrogene (Gabriels et al., 1999; Geng et al., 2012; Snider et al., 2010) heterochromatin-associated cohesin function is specifically disrupted (Dheur et al., 2011; Zeng et al., 2009). It is speculated that the loss of heterochromatin contributes to the expression of the full-length DUX4 (DUX4fl) in FSHD. However, this has not been explicitly demonstrated (Ball et al., 2014).

- **Cohesin and cancer**

Recent exome sequencing of 4742 human cancer samples across 21 cancer types has identified STAG2 as one of 12 genes that are altered at significant frequencies in at least four tumours (Lawrence et al., 2014). Stag2 seems most common in urothelial bladder cancer ((Balbas-Martinez et al., 2013; Guo et al., 2013; Taylor et al., 2014) although was also found in glioblastoma, Ewing's sarcoma and

melanoma (Solomon et al., 2011). General mutations in Stag2 are often truncating, whereas missense mutations are more frequent in other cohesin genes. The higher mutation rates of STAG2 in most tumours could be explained by the fact that a single hit is sufficient for the loss of SA2 function, and cohesin–SA1 complexes might partially compensate for this loss. Downregulation of SA2 is less detrimental for chromosome segregation than downregulation of SMC1 or SMC3 (Barber et al., 2008). It is unclear how the cells in the tumour with a truncated SMC1 (Guo et al., 2013), which is from a male patient, survive without a functional cohesin complex, once the gene are located on the X chromosome.

As mentioned above, cohesin dysfunction could affect tumorigenesis by increasing genome instability due to faulty DNA replication and/or repair and chromosome missegregation (Duijf and Benezra, 2013). Though aneuploidy and genome instability are detrimental to cell survival, they can also accelerate tumour evolution and adaptability (Holland and Cleveland, 2012).

The role of cohesin in genome organization could also underlie tumour-promoting consequences of cohesin mutation, being the most striking effect the gene expression changes of crucial oncogenes or tumour suppressors. Other options as altering organization of replication factories may slow replication and increase replicative stress (Burrell et al., 2013; Guillou et al., 2010). Moreover reduced cohesion, together with domain decompaction and an increased number of interdomain chromatin contacts (Sofueva et al., 2013) may favour chromosomal translocations.

A study of 1060 patients in myelodysplastic syndrome and acute myeloid leukemia detected somatic cohesin defects in 12% of patients with myeloid malignancies, whereas low expression of these genes was present in an additional 15% of patients. Cross-sectional deep-sequencing analysis for clonal hierarchy demonstrated Stag2, Smc3, and Rad21 mutations to be ancestral in 18%, 18%, and 47% of cases, respectively and each expanded to clonal dominance concordant with disease transformation. Additionally, it seems that cohesin mutations do not contribute to hematopoietic transformation through altered chromosomal instability (Thota et al., 2014).

A recent study identified 11 mutations in the Smc1 gene when the mutational screening was performed in early colorectal adenomas, a precocious step during colorectal cancer development. This was the first report of cohesin gene mutations occurring in precancerous lesion with high frequency. The observation that Smc1 mutations decreases from early adenomas (22.9%) to colorectal cancers (≈5% or less) supports the “hit and run” hypothesis in which cohesin mutations play a role in early stages of tumorigenesis and are not necessary for the maintenance of the malignant phenotype. Moreover they also saw Smc1 mutations cause chromosomal instability and aneuploidy, so the authors suggest that chromosomal instability could be the first determinant of cancer development (Cucco et al., 2014).

In conclusion, cohesin because of its functions can easily lead to problems that cause cancer. The problem in using cohesin as an attractive therapeutic target for treating cancer is for now out of question once cohesin is need for all cells, not just the ones present in tumours. Maybe in the future with the exponential studies about cohesin and its subunits rising, more about its role and behaviour will be known and more about the cancer progression could be discover, helping for its treatment.

1.2.5 Cohesin Decay/Fatigue

Loss of cohesin, although it can happen during mitosis, is by far more studied in meiosis once

cohesin has to be maintained for longer periods (for 30 or 40 years in females) and therefore more prone to fatigue. Although it is still unknown how cohesin can be kept for several decades. Some ideas try to answer this question without much experimental evidence due to the difficulties in studying this process. For example cohesion decay could arise from a gradual decrease of sororin or Sgo. Alternatively, separase could become precociously active or unstable. Alterations on the pH that could affect the interaction cohesin/DNA or oxidative stress could also decrease chromatin-bound cohesin complexes. One hypothesis that could solve part of this problem would be the existence of turnover mechanism that "renews" cohesin complexes. However, the presence of such mechanism has been highly debated. To try to understand that, in 2010, Revenkova and co-workers used a strain of mice carrying a floxed Smc1β locus crossed with GDF9-Cre transgenic strain, which expresses Cre in the primordial follicles immediate after birth. When the gene was floxed before birth and no more expressed, the mice remained fertile. The ovaries and oocytes of the Smc1β-excised animals appeared as wild-type, indicating that there is no need for Smc1β gene expression after mouse oocytes have entered dictyate arrest (Revenkova et al., 2010). This means that cohesin produced before prophase I is sufficient, at least until the above describe effects of cohesion fatigue emerge in very old animals.

Tachibana-Konwalski and colleagues have reached similar conclusion following a different experimental set-up. TEV-cleavable Rec8 or Scc1, after microinjection of TEV into Rec8$^{TEV/TEV}$ or Scc1$^{TEV/TEV}$ oocytes leading to destruction of Rec8- or Scc1-cohesin. The major idea was if non-TEV-cleavable wild-type Rec8 were expressed after the initial establishment of cohesion at DNA replication with TEV cleavable Rec8, means that turnover happens, if after TEV injections they separate is because the only Rec8 present were sensitive to TEV (figure 1.15). By regulating in a developmental and time-dependent manner activation of a conditional Rec8-Myc bacterial artificial chromosome (BAC) transgene, this group show that expression of Rec8 early during meiosis but not during the oocyte growing phase (post-recombination) prevents destruction of bivalents by TEV protease in oocytes whose endogenous genes encode TEV-cleavable Rec8. Based on this it seems that is little, if any, significant turnover of Rec8 using newly synthesized protein during the growing phase of oocytes (Tachibana-Konwalski et al., 2010).

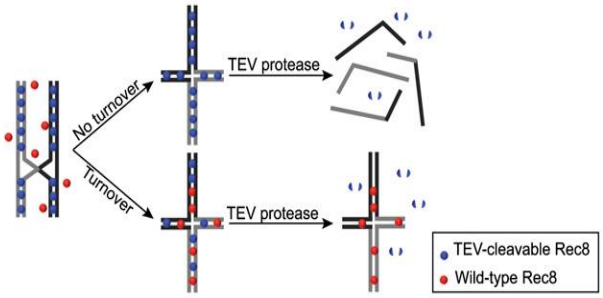


Figure 1.13 The thought behind the experiment. If there is incorporation of cohesin, in this case, Rec8 wt, then when TEV protease is expressed they would not be cleaved. Adapted from (Tachibana-Konwalski et al., 2010).

Both of this studies leads to the conclusion that there is no, or little, turnover of cohesin arrest in oocytes, in mice. However, the decreased in cohesin describe earlier, in humans or mice, seem to occur slowly over time, suggesting that until certain threshold of cohesin's loss, the cohesion is unperturbed.

Murdoch and colleagues tested the hypothesis that partial loss of gene function for two meiosis-specific cohesins, Smc1 β or Rec8 and in both heterozygotes confirm the occurrence of both univalents and single chromatids (Murdoch et al., 2013). Moreover, single-oocyte real-time PCR showed Smc1β mRNA is present in dictyate-arrested mouse oocytes at least up to six months of age, although mRNA

levels were only about 10% of the high levels seen in pachytene (phase of the prophase I) oocytes (Hodges et al., 2005; Jessberger, 2012).

These research supports the fact that an interval is present and how cohesin dosage is important to keep a normal meiosis and mitosis.

In mitosis, Daum and colleagues addressed a different perspective on this subject: the tension, first describe in 1969 (Nicklas and Koch, 1969). A correct attachment of SC to microtubules has to exist for them to resist the pulling forces generated by the mitotic spindle. This tension contributes to the correction of improper kinetochore attachments and is opposed by the cohesin complex that holds the sister chromatids together until the anaphase onset. In this process usually the passage through metaphase is short. However when induced metaphase delay spindle pulling forces can cause asynchronous chromatid separation, a phenomenon called cohesion fatigue (Daum et al., 2011). When this arrest occurs and other chromosome-associated cohesin are compromised, as depletion of Sgo1, cells just delay briefly at metaphase before scattering; the opposite occurs when Wapl is depleted. Treatment of metaphase-arrested cells with chemical Plk1 inhibitors does not block scattering and siRNA-mediated depletion of separase protein does not block cohesion fatigue. Nonetheless, after this phenomenon SC retains cohesin complex (loss of cohesion without cleavage the Scc1/Rad21 subunit). This failure of cohesin in metaphase may be simply due to the strong pulling forces of the kinetochores on the metaphase spindle microtubules that may over time partially rupture the molecular linkages of the cohesin complex beginning at the kinetochores and progressing to the chromosome arms. Another suggesting from the authors was that the pole ward forces acting on the kinetochores may exploit cohesin protein dynamics, locking in momentary releases of the cohesion complex to drive chromatid separation. Many other hypothesis were considered as only a minor fraction of chromosome-associated cohesin is responsible for sister chromatid cohesion at metaphase. If that part was lost may had impair its detection by the authors (Daum et al., 2011). This cohesion fatigue, resulting in unscheduled chromatid separation in cells delayed at metaphase, constitutes a source for chromosome instability (lagging chromosomes, centromere fission or generation of micronuclei) in mitosis, meiosis and carcinogenesis (Daum et al., 2011).

2 GOAL

Sister-chromatid cohesion is pivotal for tension generation as it counteracts the forces exerted by the mitotic spindle. Artificial disruption of sister chromatid cohesion in metaphase-arrested cells leads to the destruction of tension and prompt reactivation of the SAC, within 2 minutes (Oliveira et al., 2010).

The cohesion decay/fatigue discussed in more detail in 1.2.5 can lead to disastrous results as age-related aneuploidy or cancer. So, the goal of this thesis is to estimate how much cohesion decay needs to occur at a given chromosome to surrender to spindle forces and lead to precocious sister chromatids separation (figure 2.1).

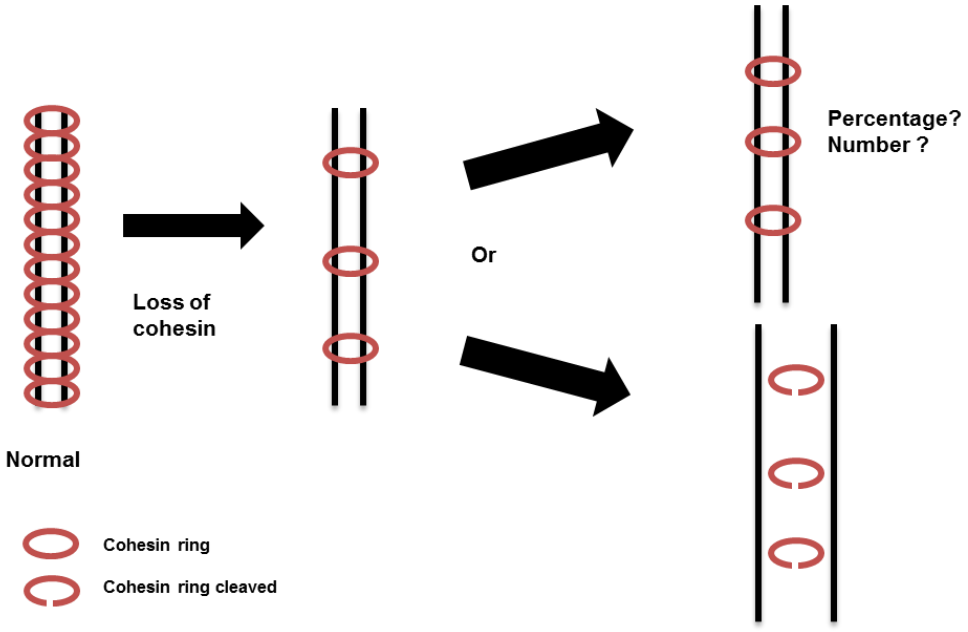


Figure 2.1 Schematics of my goal. If some premature loss of cohesin occurs, which amount would be sufficient to keep the chromosomes together?

This project, when finished, will bring enlightenment about important topics as how SAC reacts with different ratios of cohesin present and how mitosis time varies in early or older embryos after cleavage of determinate ratio.

To answer these questions we will develop experimental tools where we can quantitatively manipulate the levels of cohesin complexes present in mitotic chromosomes and evaluate the consequences.

3 APPROACH

Recently, a system to better understand cohesin and its functions was applied in Drosophila: the TEV system (Pauli et al., 2008), already used in budding yeast *Saccharomyces cerevisiae* (Uhlmann et al., 2000). This system induce sister chromatid separation in *Drosophila* in a rapid, and tissue-/time-specific manner, by cleavage of the kleisin subunit, leading to a rapidly dissociation from chromosomes. The RAD21 gene is located in the third chromosome and Rad21 mutants were created by a P element inserted 4 kb upstream of the transcriptional start of Rad21, which was remobilized by P element Transposase. Four deletions alleles were identified ($Rad21^{ex3}$, $Rad21^{ex8}$, $Rad21^{ex15}$, $Rad21^{ex16}$) and in all four lack exons 1 and 2 which encode the highly conserved N terminus of Rad21 that interacts with the head of Smc3. To rescue Rad21 mutants, transgenic flies that express C-terminally myc-epitope-tagged versions of Rad21 with TEV-cleavage sites were created ($Rad21^{TEV}$). All four versions of $Rad21^{TEV}$ were efficiently cleaved and a $Rad21^{TEV}$ with three TEV sites at position 271 or 550 as well as a version lacking TEV insertions restored full viability and fertility of homozygous with excision alleles when expressed from a tubulin promoter. These TEV sensitive flies can express TEV protease either directly from the heat-shock promoter (hs-TEV) or under the control of the Gal4/Upstream Activation Sequence (UAS) system (figure 3.1). This system was able to promote acute cohesin inactivation in post-mitotic neurons (Pauli et al., 2008), salivary glands (Pauli et al., 2010) and *Drosophila* syncytial embryos (Oliveira et al., 2010).

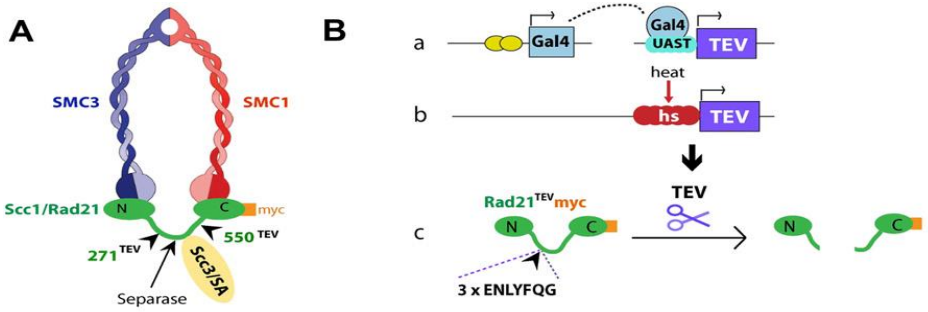


Figure 3.1 TEV-Cleavage system. In a) is presented a scheme of TEV-cleavable Rad21 subunit indicating the TEV-recognition sequences; in Ba) and b) the system controloled by UAS/GAL4 system that allows the expression *in vivo* and the same in c) with the difference that TEV protease is expressed by a HS promoter. Adapted from (Pauli et al., 2008).

Taking advantage of this system, we will try to quantify the amount of cohesin necessary to keep sister chromatids together, as mention above in Goal, and for that different approaches will be tested. First, we will try to express different levels of TEV protease that would lead to different levels of sister chromatid separation; and then, by genetic crosses and recombination (that would give different rations of TEV-cleavable/TEV-resistant complexes) we will titrate different amounts of TEV sensitive cohesin rings bound to chromosomes by western blots and by live imaging of embryos.

4 MATERIAL AND METHODS

4.1 Heat Shock, Chromosome Spreads and Analysis

Flies expressing a HS-TEV protein were submitted to a HS at 37ºC for 45, 20 and 10 minutes. Spreads were performed after 29, 63, 93, 122, 149, 181, 243 and 306 minutes of recovery at room temperature (RT).

The spreads were made with third instar larvae, from which brains were dissected in fresh sodium chloride (NaCl) (VWR Chemicals) at 0,7%. The brains were fixed in a drop of 45% acetic acid for 1 minute and 30 seconds (sec). Then each brain was switched to a drop of 60% acetic acid, in a small siliconized cover slip for 30 sec. After this incubation, a glass slide was placed on the top of the coverslip with the brainand vertical pressure was applied in a way that the brain was smashed. Preparations were droped in liquid nitrogen. After removal from liquid nitrogen, a scalpel was usedto jam off the small cover slip, without sliding. After drying, a 7 µl drop of. Vectashield® Mounting Medium with 4',6-diamidino-2-phenylindole (DAPI) (Vector Laboratories) was used and a small coverslip was putted.. Nail polish was used to seal the preparation that could be stored at 4ºC until imaging.

Images were acquired on a Leica DMIRE2 inverted microscope, equipped with a Hamamatsu C9100 EM-CCD camera, using the a 100x, 1.40 - 0.70NA oil immersion objective, DAPI fluorescence filter sets and DIC optics, controlled with the MicroManager v1.14 software.

4.2 Proteins extracts from brains/ovaries

Five brains/ovaries were dissected in PBS and added to a tube that was on ice and contained 40µl of Radioimmunoprecipitation assay buffer (RIPA). Next, the tissue was squashed and then sonicated at strength7/10 in water, with the samples floating inside a box per 2 minutes, middle power. After this, 40µl of 2x Laemmli Sample Buffer (Sigma-Aldrich®) was added and next the tubes were boiled at 95ºC for 5 minutes. The samples were centrifuge for 5 minutes at 10 000 rotations per minute (rpm), being ready to be loaded on a gel or to be frozen at -20ºC.

4.3 Optimization with TEV buffer

The protocol followed in this trial with TEV buffer (see Appendix 8.4) was the same as mention above with RIPA. Samples were tested with or without sonication.

4.4 Protein extracts for *In Vitro* Cleavage and quantification by Bradford

Mature females (more than 7 days old) were fed with yeast paste for at least 24 hours before the dissection. Between 15-20 ovaries were dissected in phosphate-buffered saline (PBS) 1x (1:10 PBS 10x, recipe in 8.2) and were placed in a tube with 100µl of PBS. The ovaries were squashed and sonicated for 30 sec at strength 4/10 (this is the final protocol for TEV cleavage, check the process of optimization in section 5.3)

A Bradford Assay quantification curve was performed to each experiment. The Bradford solution

(BIO-RAD protein assay) was diluted 1:5 in Milli-Q water and the bovine serum albumin (BSA) (at 10µg/µl) (NZYTech, Lda.) was also diluted in Milli Q water to stay at 1 µg/ul. Different dilutions of BSA were made in Bradford solution and measured using a spectrophotometer SmartSpec™ 3000 (Bio-Rad) at the maximum absorbance frequency of the blue dye which is 595 nm. After obtaining a standard curve, the equation obtained from there allowed to calculate the amount of sample needed to have 40µg per sample. The samples to be cleaved were duplicated (2 tubes), in one TEV protease is added and in other not (check scheme in section 5.3). After 2 hours incubation withTEV, a 4x Laemmli Sample Buffer (Bio-Rad) was add to each tube. The tubes were boiled at 95ºC for 5 minutes, followed by a centrifuging of 5 minutes at 10 000 rpm, being ready to be loaded on a gel or to be frozen at -20ºC.

4.5 Protein electrophoresis and western blotting

The samples were loaded into a polyacrylamide gel (see Appendix 8.1) and run in a Bio-Rad system a 200 volts (V) until all the bands from the protein marker NZYColour Protein Marker II were visible (Nzytech, Lda genes & enzymes). Then, the gel was transferred using a wet system (Bio-Rad) (transfer buffer recipe at Appendix 8.2) at 100V for 1h to a polyvinylidene fluoride (PVDF) membrane (Bio-Rad). The membrane was previously activated with methanol. After transfer, the membrane was incubated in a blocking solution: 5% of powder milk MOLICO® (Nestlé) in PBS-Tween (PBS-T, see Appendix 8.2) (milk-PBS-T), for at least 1h. The primary and secondary antibodies were diluted in the blocking solution. Primary antibody was incubated either one hour at RT or overnight at 4ºC. Washes are needed between the primary and secondary antibody and were perform with PBS-T (3x10min). The secondary antibody (which are conjugated with horseradish peroxidase (HRP)) incubated only for 1h at RT. After secondary antibody incubation, membrane was again washed and kept in PBS until development. Pierce® ECL Western Blotting Substrate (Thermo Scientific) was used to react with secondary antibody and it was incubated on the membrane for 5 min.

X-ray films Amersham Hyperfilm™ ECL (GE Healthcare Life Sciences) were used. After digitalization of the films, Image J software was used to quantify the bands from the films, being the background of each sample subtracted. Calculations were done using Excel.

4.6 Antibodies

The primary antibodies used to perform the immunoblotting were guinea-pig α-Rad21 at a 1:5000 dilution (courtesy of Professor Christian Lehner, Institute of Molecular Life Sciences, Zurich), Monoclonal Anti- mouse α-Tubulin (clone B-5-1-2 ,Sigma-Aldrich®) at a 1:100000 dilution and anti-actin (Abcam) at a 1:5000 dilution.

The secondary antibodies used were anti-guinea pig HRP (GE Healthcare Life Sciences) at 1:20000 and anti-mouse (Jackson ImmunoResearch Inc.) at 1:10000.

4.7 Fly stocks

For a simple interpretation the next table resumes the strains use.

Table 4.1 Strains Stock. Strains used on this project by order of name/number, with corresponding genotype, source and purpose.

Number/Name of the strain	Genotype	Source	Purpose
Wild type	w-		Used as a negative control in western blots.
16	w; UAS-Rad21myc10; Sb /TM3, Ser	Courtesy of Stefan Heidmann	Used for western blot.
202	w; tubpr-Rad21(271-3TEV)myc (3); Sb /TM3, Ser	(Pauli et al., 2008)	Used for western blot.
211	w; +/+; tubpr-Rad21(550-3TEV)myc (4c)	(Pauli et al., 2008)	Used for western blot
269	w; +/+; polyubiq-His-RFP(1.5.4), mw+/TM3, Sb	Courtesy of Jennifer Mummery	Used in live imaging of embryos
363	w; +/+; Rad21(ex15), tubpr-Rad21(550-3TEV) (4c) (rec 7.5)	(Pauli et al., 2008)	Used for western blot.
477	w; hspr-NLSv5TEV/CyOwglacZ ; Rad21ex3/ TM6B ubiGFP	(Pauli et al., 2008)	Used for spreads.
629	w; +/+; Rad21(ex15), polyubiq-His-RFP(1.27.6-187), tubpr-Rad21myc(550-3TEV)(4c)(rec 7.5)(rec Ld)	(Pauli et al., 2008)	Used for western blot and live imaging of embryos.
813	w; If/CyO; Rad21(ex8), tubpr-Rad21(271-3TEV) (8) (rec 1.4) /TM3, Ser	(Pauli et al., 2008)	For recombination.
1353	w; If/CyO; Rad21(ex15), polyubiq-His-RFP, tubpr-Rad21(550-3TEV) /TM3, Ser	(Pauli et al., 2008)	Used for western blot and live imaging of embryos.
1357	w;If/CyO;tubprom-Rad21(wt)-EGFP 2, Rad21 (ex15), polyubiq-His-RFP (rec 2.4)/(TM3,Ser)	(Oliveira et al., 2014)	Used for western blot and live imaging of embryos.
1358	w; tubpr-Rad21(271-3TEV)myc (3)/(CyO); Rad21(ex15), polyubiq-His-RFP, tubpr-Rad21(550-3TEV) /(TM3, Ser)	Cross between 1353x202	Future work.
1359	w; tubpr-Rad21(271-3TEV)myc (3)/(CyO); tubprom-Rad21(wt)-EGFP 2, Rad21 (ex15), polyubiq-His-RFP (rec 2.4)/(TM3, Ser)	Cross between 1357x202	Future work.
1360	w;If/CyO;tubprom-Rad21(wt)-EGFP 2, Rad21 (ex15), polyubiq-His-RFP (rec 2.4), Rad21(ex8), tubpr-Rad21(271-3TEV) (8) (rec	Recombination of 1357 with 813	Future work.

	1.4) / (TM3, Ser)		
1361	w; tubpr-Rad21(271-3TEV)myc (3)/CyO ; tubprom-Rad21(wt)-EGFP 2, Rad21 (ex15), polyubiq-His-RFP (rec 2.4), Rad21(ex8), tubpr-Rad21(271-3TEV) (8) (rec 1.4) / TM3, Ser	Cross between 1360x202	Future work.
1362	w; UAS-Rad21myc10, tubpr-Rad21(271-3TEV)myc (3); Sb /TM3, Ser	Recombination of 16 with 202	Future work.
1363	w; UAS-Rad21myc10 ; Rad21(ex15), polyubiq-His-RFP, tubpr-Rad21(550-3TEV)	Cross between 16x1353	Future work.
1364	w; UAS-Rad21myc10, tubpr-Rad21(271-3TEV)myc (3); Rad21(ex15), polyubiq-His-RFP, tubpr-Rad21(550-3TEV) /(TM3, Ser)	Crosse between 1362x1353	Future work.

4.8 Live imaging of embryos & TEV injections

For microinjection experiments, 2h-3h old embryos were collected and processed according to standard protocols (Sullivan et al., 2000) which requires collection and pre-collection of embryos. After treatment with traditional bleach diluted in water (1:1), halocarbon oil 700 (Sigma-Aldrich®) is putted on the embryos to keep them alive.

Embryos were injected at the posterior pole using a Burleigh PCS-6000 Micromanipulator (Thor Labs) (figure 4.1) and a custom built gas injection system., Pre-pulled Femtotip I injection needles (Eppendorf). TEV protease solution (5µg/µl) was injected in an estimated volume of 5-10% of the total volume of the embryo. Videos were acquired on an Applied Precision Deltavision CORE system, mounted on an Olympus inverted microscope, equipped with Photometrics Cascade II, 1024x1024 EMCCD scientific camera, using the a 20x 0.75NA, mCherry fluorescence filter sets and DIC optics, at 22,5ºC.

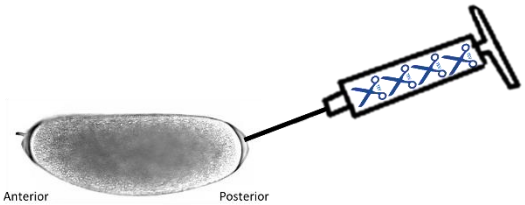


Figure 4.1 Injections scheme.

Each movie was analysed in Image J. From each movie the number of anaphases within an area of 6400 µm^{2}were counted. Anaphase's errors were analysed in each nucleus individually, being the number counted half of the obtained in the metaphase score. The data were analysed in Excel program.

5 RESULTS

5.1 Titration of the amount of TEV protease expressed

The first approach to quantitatively manipulate the levels of cohesin on chromosomes was to vary the levels of the TEV protease expression in a way that different levels of sister chromatid separation could be achieved.

With this in mind, flies with totally cleavable cohesin, which contains only $Rad21^{TEV}$ sensitive cohesins (strain 629, see section 4.7) were crossed with flies with a heat-shock promoter (strain 477) for TEV expression, in a way that the progeny (w; +/HS-TEV; His-RFP, Rad21 (ex15), Rad21 (TEV)/Rad21(ex3)) synthesized TEV protease endogenously when exposed to HS, and thus cleavage of all cohesin in the fly occurred. Analysis of mitotic progression was performed in larval brains, which possess great chromosome morphology to follow during this phase. Chromosome spreads reveal a normal mitosis in wild-type strains, as illustrated in the figure 5.1.

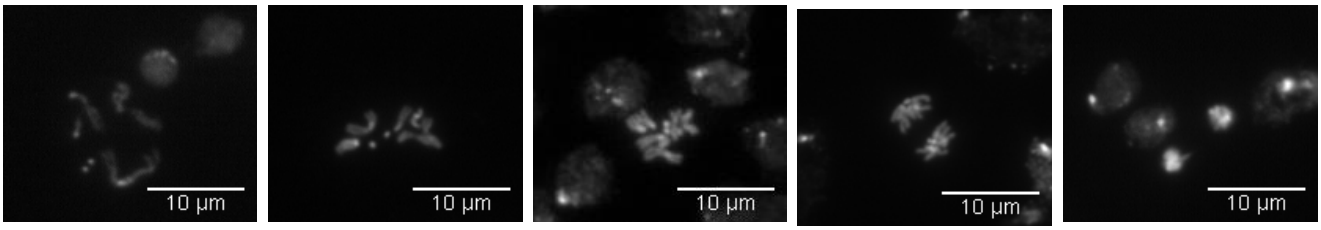


Figure 5.1 Mitotic phases. Prophase, Pro-metaphase, metaphase, anaphase and telephase are represented by order of events in the *Drosophila melanogaster* brain' spreads.

After HS, the TEV protease cleavage induces the appearance of single sisters which were subsequently classified as partial or totally separated as shown in 5.2. Total separation was defined when all the single sisters were scattered as visible in the right side of the figure 5.2. In the cases where only some chromosomes were separated, mostly the fourth chromosome due to its small size. Cells were considered to display partial sister chromatid separation (visible on the left side of the figure 5.2).

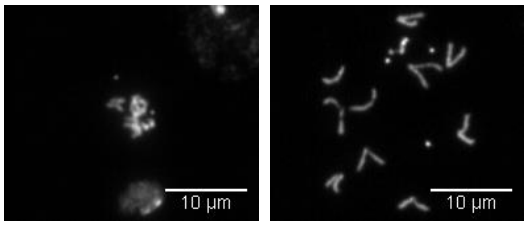
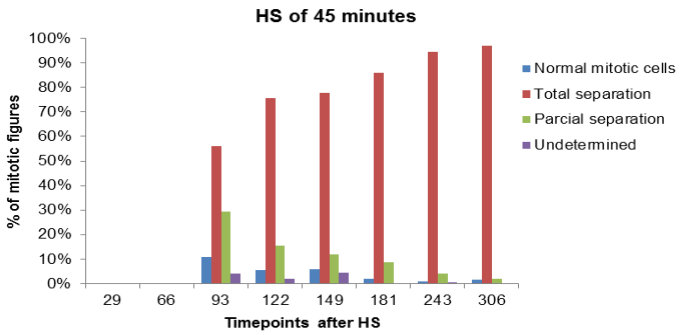
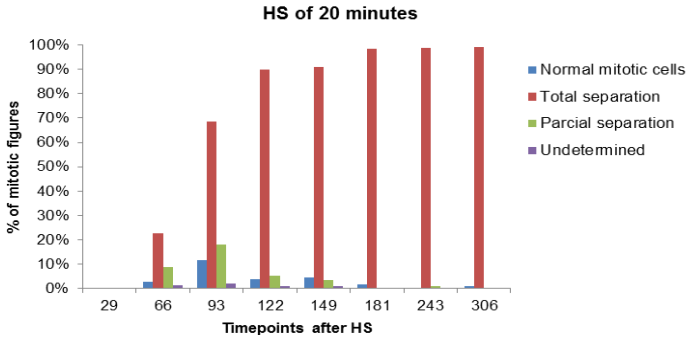


Figure 5.2 Mitotic figures after heat shock. A mitotic figure is classified as partial cleaved when not all single sisters are completelly separated; and considered total cleavage when single sisters can be easily spoted.

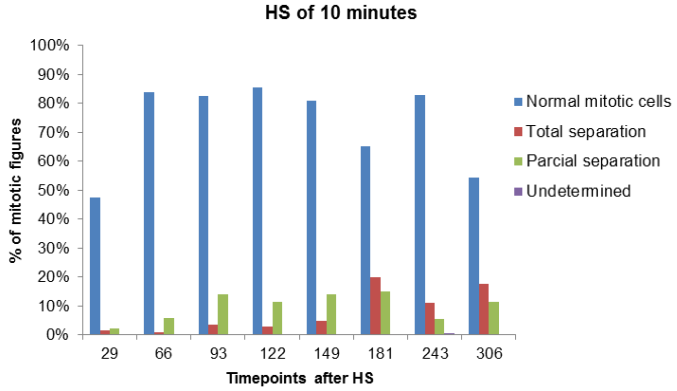
To achieve different levels of TEV expression, the heat shock was performed at 37ºC for variable amounts of time. For each experimental condition a time-course analysis of the chromosome morphology was evaluated, using brain squashes from 3rd instar larval brains. The results are represented in the graphics 5.1, 5.2 and 5.3.



Graphic 5.1 Heat Shock Cleavage of 45 minutes. In general, all mitotic figures were completely cleaved, with only 30% to 10% being partially cleaved.



Graphic 5.2 Heat Shock Cleavage of 20 minutes. In general, all mitotic figures were completely cleaved, with only 20% to less than 10% being partially cleaved.



Graphic 5.1 Heat Shock Cleavage of 10 minutes. In general, all mitotic figures were normal, that means not cleaved. Only about 20% appear totally cleaved and partial even less.

The graphics show that the aim of obtaining partial levels of sister chromatid separation was not achieved, as the images suggest an "all or nothing" event, where the protease expressed is either sufficient to cleave all the cohesin rings (classified on the graphic as "Total separation") or, on the other hand, the amount of TEV expressed barely cuts any ring, leading to normal mitotic figures the majority (classified on the graphic as "Normal mitotic cells"). From these experiments we concluded that using this approach it is very hard to obtain the levels of cohesion molecules needed to keep chromosomes together and alternative approaches must be considered.

5.2 Ratios of Rad21^{TEV} versus Rad21^{wt} in ovaries

Since we failed to successfully provide experimentally different levels of sister chromatid separation with the method described above, the next step was to titrate the amount of TEV sensitive cohesins present in mitotic chromosomes (figure 5.3). To achieve this, genetic backgrounds were established to maintain the number of Rad21^{wt} and increase the copy numbers of Rad21^{TEV}. We expected that this approach should lead to a relative decreased expression level of Rad21^{wt} and consequently, to a progressively lower amount of Rad21^{wt} (TEV-resistant) on mitotic chromosomes.

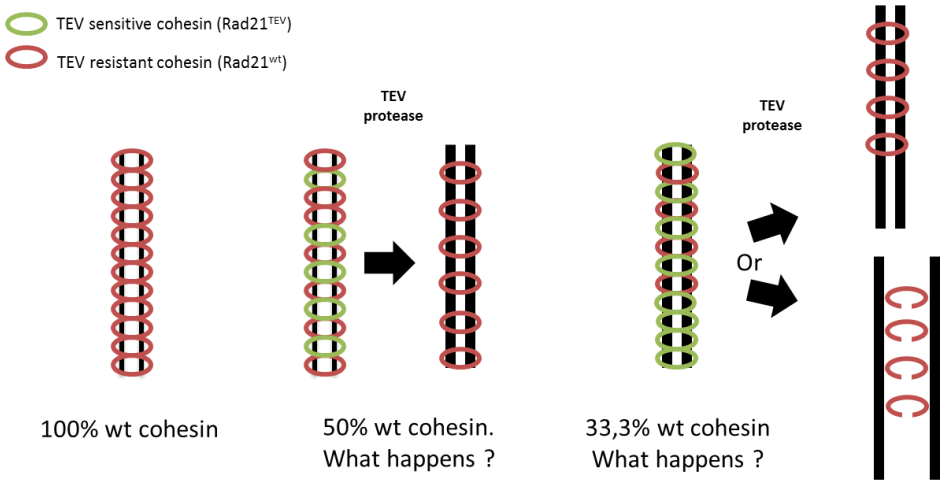


Figure 5.3 Scheme of the goal of this task. Based on the TEV method describe in 1.2.5, the purpose on this task is to increase the levels of TEV sensitive cohesin and by each stept check if that amount is sufficient to keep SC cohesion or not, until the achievement of a percentage where they separete.

To achieve different levels of Rad21^{TEV} versus Rad21^{wt} we used the following strains:

Table 5.1 Strains with different ratios of Rad21. Strains used for increasing the levels of Rad21^{TEV}.

Strain	Genotype	Ratio Rad21^{TEV}: Rad21^{wt}
wt	w-; +/+; gRad21/gRad21	0:2
363	w; +/+; Rad21(ex15), tubpr-Rad21(550-3TEV)	2:0
211	w; +/+; gRad21, tubpr-Rad21(550-3TEV)myc	2:2
363 x wt	W; +/+; Rad21(ex15), tubpr-Rad21(550-3TEV) /gRad21	1:1
363 x 211	w; +/+; Rad21(ex15), tubpr-Rad21(550-3TEV) / gRad21, tubpr-Rad21(550-3TEV)myc	2:1

To evaluate the levels of Rad21 , we performed western blot analysis of fly's ovaries in each of these strains (check section 4.4). The results showed that contrary to what it was expected, the increase of the copy number of Rad21^{TEV} transgenes in the fly did not lead to a proportionally visible increase in the corresponding protein. For example, strain 211 should have 1:1 ratio and we detect very low

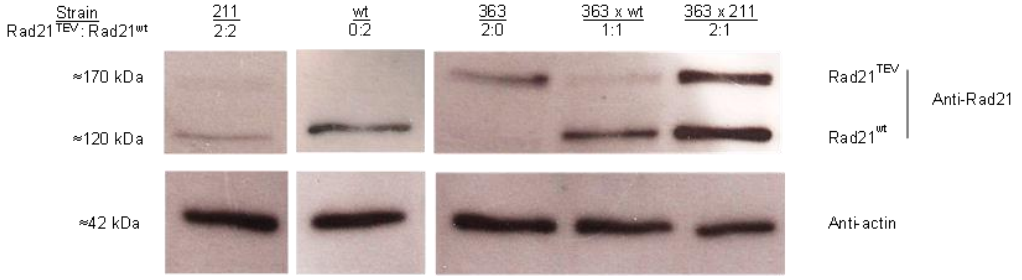


Figure 5.4 Rad21^{TEV} versus Rad21^{wt} in ovaries. Total proteins extracts from ovaries of different strains (see section 4.2) was performed and analysed by western blot. For that antibody against Rad21 subunit was used that stains the endogenous (Rad21^{wt}) and the one with TEV sites (Rad21^{TEV}). Actin was used as loading control.

amounts of Rad21^{TEV} relatively to the endogenous protein. Moreover, even when two copies of Rad21^{TEV} are present (363x211) the levels of Rad21^{TEV} are not higher than Rad21^{wt}. The same was observed in the brains of third instar larvae showing this problem was not tissue specific, visible in figure 5.5.

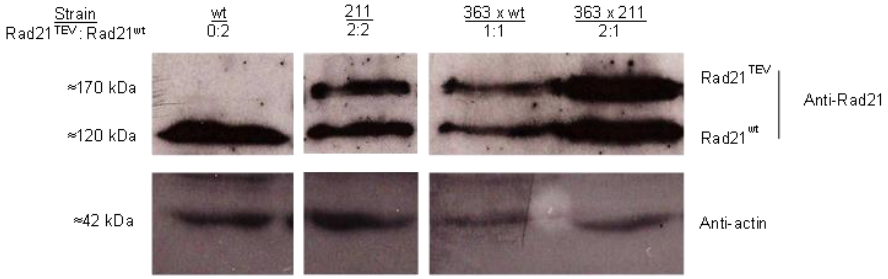


Figure 5.5 Rad21^{TEV} versus Rad21^{wt} in ovaries. Total proteins extracts from brains (see section 4.2). Western blot analyse was performed using an antibody against the Rad21 subunit that stains the endogenous (Rad21^{wt}) and the one with TEV sites (Rad21^{TEV}). Actin was used as loading control.

A possible explanation for this observation is the fact that Rad21^{TEV} gene is regulated by a tubulin promoter and not by Rad21 endogenous one. These differences may lead to protein levels (e.g. by regulation/compensation mechanisms) that overproduce the endogenous version of the protein. Moreover, although the Rad21^{TEV} is fully functional (Pauli et al., 2008), it has a bulky tag at its C-terminus (10xMyc), which may decrease protein stability relatively to untagged endogenous one.

5.3 *In vitro* cleavage of Rad21^{TEV} versus Rad21^{wt} in ovaries

Considering the results above, the strategy had to be changed in order to obtain a more precise manipulation of the relative levels of Rad21^{TEV} and Rad21^{wt} For this purpose, instead of using the strain with Rad21 under the regulation of its endogenous promoter as control (wt), we took advantage of a strain that contains a Rad21 wt tagged with EGFP that is also regulated by a tubulin promoter (strain 1357, Rad21$^{wt\text{-}EGFP}$) (figure 5.6). Using this approach, we expected to obtain different cohesion ratios, as both proteins are under the control of the same promoter and contain a similarly sized tag. This similar tags, however, presented a major challenge to the experiments: the Rad21^{wt} protein appeared in the blot approximately with the same size as the Rad21^{TEV} (figure 5.7).

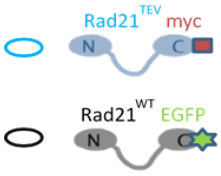


Figure 5.6 Rad21 struture. Rad21^{TEV} has a myc tag with about the same size of Rad21$^{wr\text{-}EGFP}$ due to with EGFP tag, both regulated by a tubulin promoter.

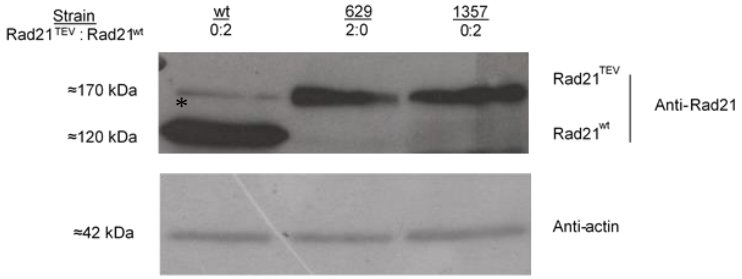


Figure 5.7 Expression of the Rad21$^{TEV\text{-}EGFP}$. The wt used from now on (strain 1357) appeared with an identical size to the Rad21^{TEV} (represented by the 629 strain) making difficult the distintion between them. Total protein extracts from ovaries (see section 4.2). Western blot analysis was performed using an antibody against the Rad21 subunit. Actin was used as loading control. (*) An unspectific band appeared in the wt lane.

To circumvent this problem, an *in vitro* cleavage with TEV protease had to be developed in order to estimate the differences in ratios by western blot. For that the protocol used for the protein extracts showed above (with a denaturating buffer (RIPA)) could not be the same once TEV could also be denaturated, becoming non-functional. The first attentive to do protein extraction was with a TEV buffer (check appendix 8.4) because it seemed to be the more appropriated for permitting TEV protease activity. However, this method failed to extract proteins even after sample sonication.

Next, a simple extraction only with PBS seemed to offer the best conditions for protein extraction and cleavage in the samples, mainly following the RIPA protocol steps, with an additional optimization in the incubation time with TEV protease. No difference was saw between the cleavage during 2 hours incubation and 7 hours (see figure 5.9).

The final optimized protocol included extraction with PBS (figure 5.8), soft sonication of 30 seconds followed by incubation with TEV by 2hours at room temperature.

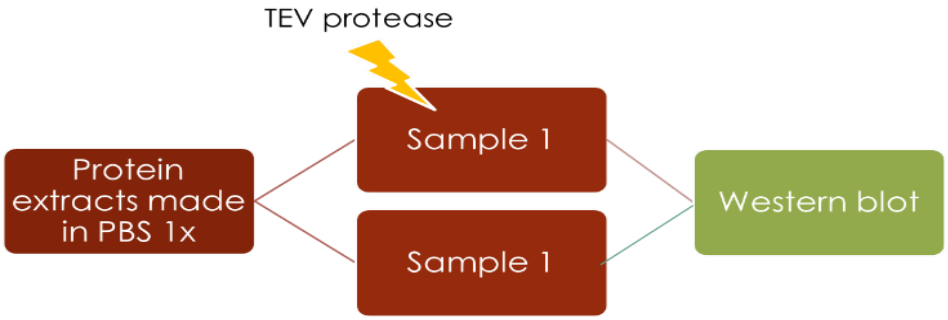


Figure 5.8 *In vitro* extration. A sample was extracted, squashed and sonicated. Then the same amount of that sample was divide in 2 tubes, being in one TEV protease added. The 2 tubes were prepared to a protein eletrophoresis and in the end a western blot was perform.

Figure 5.9 shows the success of the protocol with almost complete cleavage of the 629 strain that solely has Rad21^{TEV} present in the fly (2:0). For more details about the technique design see section 4.4.

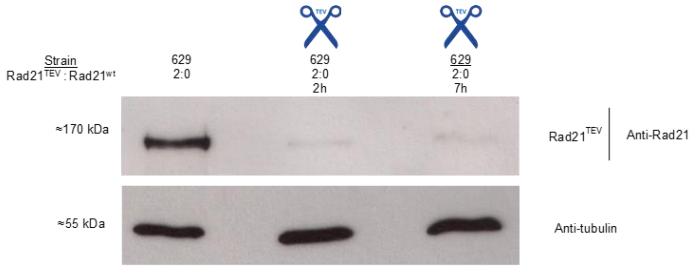


Figure 5.9 Cleavage of the 629 strain. The cohesin cleavage of 629 strain containing only cleavable cohesin (Rad21^{TEV}) was successfully achieved by TEV protease *in vitro*. 2 and 7 hours of incubation with TEV was tested. Rad21 antibody was used to stain the proteins as well as the tubulin one as loading control. ✂ indicates lanes were the extratcs were cleaved. More information check section 4.4.

At this point, a cross between 629 and 1357 strains was made leading to a progeny of 1:1 (Rad21^{TEV} : Rad21$^{wt\text{-}EGFP}$). These protein extracts were cleaved, as shown on the 5.10, were a significant proportion

of non-cleaved protein is visible.

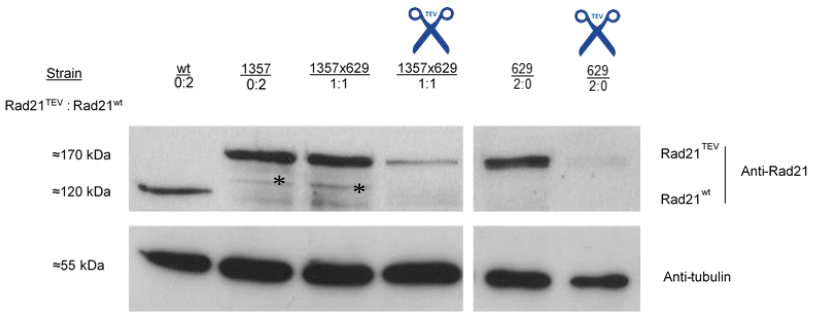
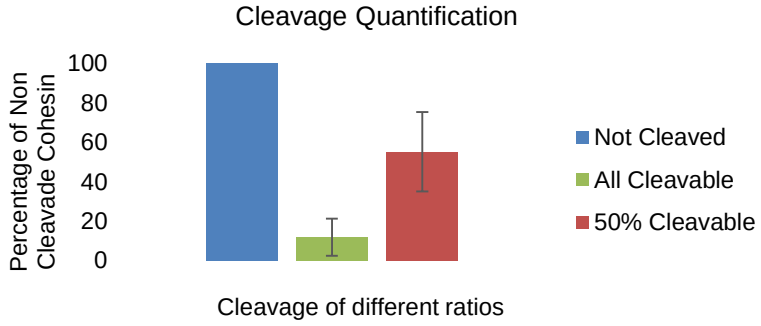


Figure 5.10 Cleavage of the progeny of 629x1357. The cleavage of the strain containing half Rad21^{Tev} was executed in flies originary from the cross between 629 (only Rad21^{Tev}) and 1357 (only Rad21$^{wt\text{-}EGFP}$). This western blot is representative of others made. Rad21 antibody was used to stain the proteins as well as the tubulin one as loading control. ✂ indicates lanes were the extratcs were cleaved. (*) An unspectific band appeared in the wt lane. More information check section 4.4.

The western blots of *in vitro* TEV cleavage were quantified showing that in extracts derived from strains where all cohesins are cleavable, only 11,9% of the protein was detected. This residual uncleaved portion may be due to the western blot technique itself that may require additional optimization, the incubation with TEV that lead to some protein degradation, the availability of the all extract to TEV or from the buffer composition that does not allow the best conditions for the protease to cleave.

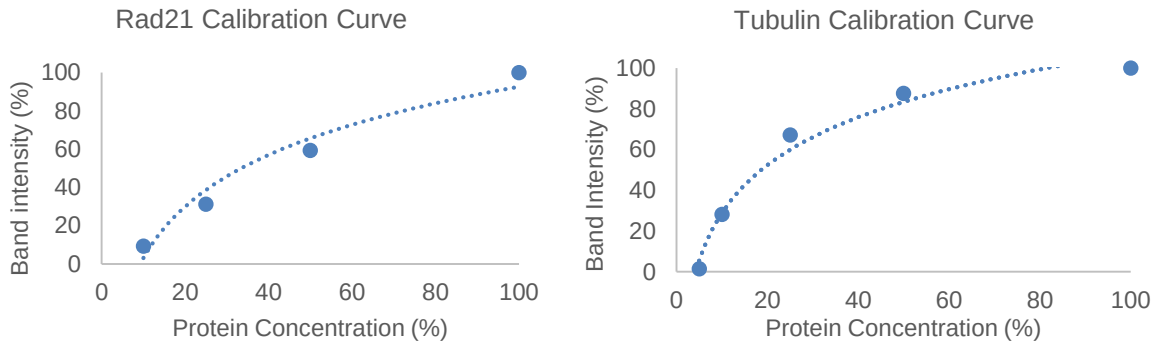
In extracts where 50% of cohesins should be cleaved, 55,2% was found to resist cleavage. If we take into account the residual uncleaved fraction observed in the first experiments we can estimate that 43,3% was the “real” percentage from the 50% cleavable strain (graphic 5.4).



Graphic 5.2 Cleavage quantification. The graphic shows the percentage of the non-cleaved fragments in strains with all cleavable cohesins (629) and with only 50% (1357x629) after being exposed to TEV protease. 11,9% was the remaining amount from the expected total cleavage (green) and from the half cleavable 55,2% (blue). More information check section 4.4. n=5 independent experiments

The quantifications performed above were done under the assumption that the antibody is within the linear range of response. To confirm this notion we have repeated the experiments and in parallel run a titration curve for different percentages of protein extracts, with 6 different serial dilutions (100%, 50%, 25%, 10%, 5%, 2,5% and 1%). As exemplified in the graphic 5.5 this antibody responds almost in linear manner to the amount of protein. However, in future experiments, a similar titration curve will be

made to more accurately estimate the percentages.



Graphic 5.3 Titration curves. In the left is represented the titration curve of Rad21 antibody and in the right its corresponding tubulin control curve.

For the range of protein depletion we were measuring, the lack of a proper normalization based on a titration did not introduce much error. For example, the comparison of the quantification between the amount of not cleaved cohesin without or with the normalization to protein titration showed 64,3% and 60,3%, respectively for the western presented on figure 5.11. Although the difference is not very deep, in future work, this normalization will be applied particularly as in the future, we aim to obtain strains with higher percentages of cleavable cohesion molecules, which in a western blot analysis will present less intense bands in western blot analyses.

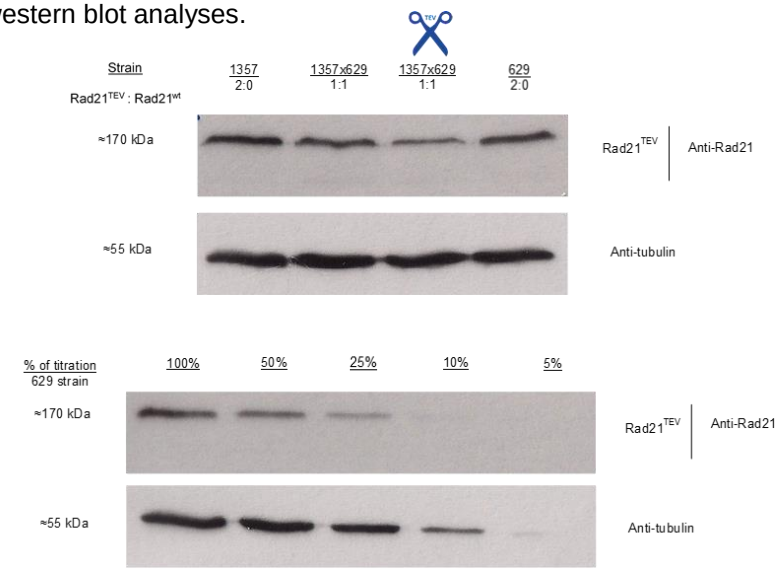


Figure 5.11 Titration Blot. Blots representative how in the future this technique should be controloded. For each blot made with the samples of interest a control blot with consecutive dilutions would be performed. Rad21 antibody was used to stain the proteins as well as the tubulin one as loading control.

5.4 In vivo cleavage of Rad21TEV: embryos injections

After the *in vitro* studies, we decided to address the effect of the 50% loss of cohesive Rad21 molecules *in vivo*. For that, we used Drosophila embryos that were injected with TEV protease. In the

figure 5.12 representative images of an embryo from a strain without TEV cleavable Rad21, that was injected with TEV protease. In this negative control experiment chromosomes aligned and segregated normally, with a short time period from NEBD to anaphase, no checkpoint activation and normal anaphase progression (e.g. no chromosome bridges or unresolved chromosomes) thereafter the time from NEBD to anaphase was short.

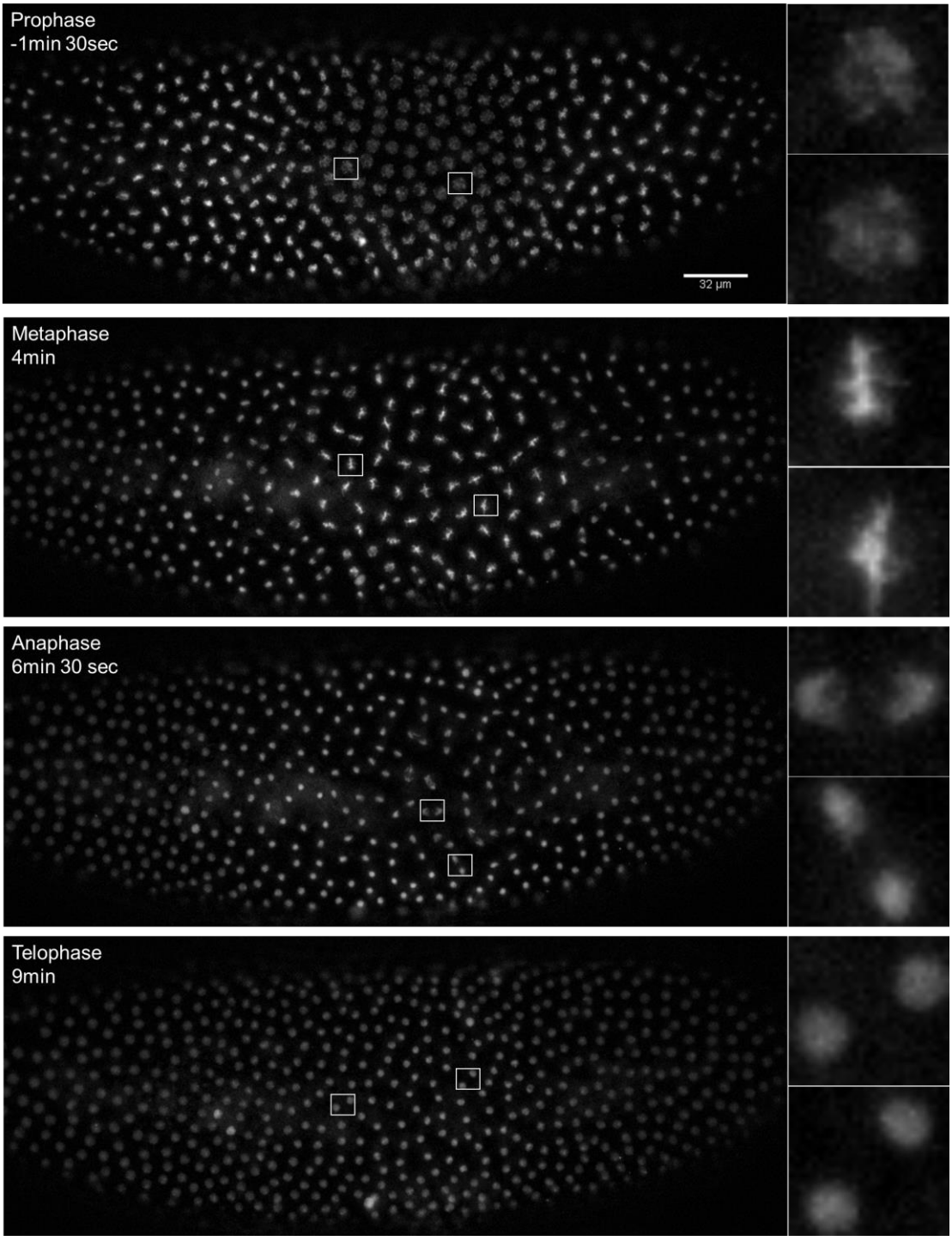


Figure 5.12 Negative control. A normal division after TEV injection is visible in the strain 269 (no cleavable cohesins). No premature separation is visible as well as no delay. Magnifications of each phase are shown in the right side (7x). The time started counting after NEBD.

In the figure 5.13 the positive control is shown. In this case, the strain contained totally cleavable cohesin version (629). When TEV was injected leaded to premature loss of sister chromatid cohesion that induced a mitotic arrest due to SAC activation, once SC were not properly attached. This mitotic arrest make the mitosis longer than the negative control (average of 8 minutes from NEBD until anaphase). When final exit mitosis the chromosomes do it with a major variety of defects in anaphase/telophase as bridges, asymmetric division completely unresolved chromosomes. The results are in agreement with data previously describe in (Oliveira et al., 2010).

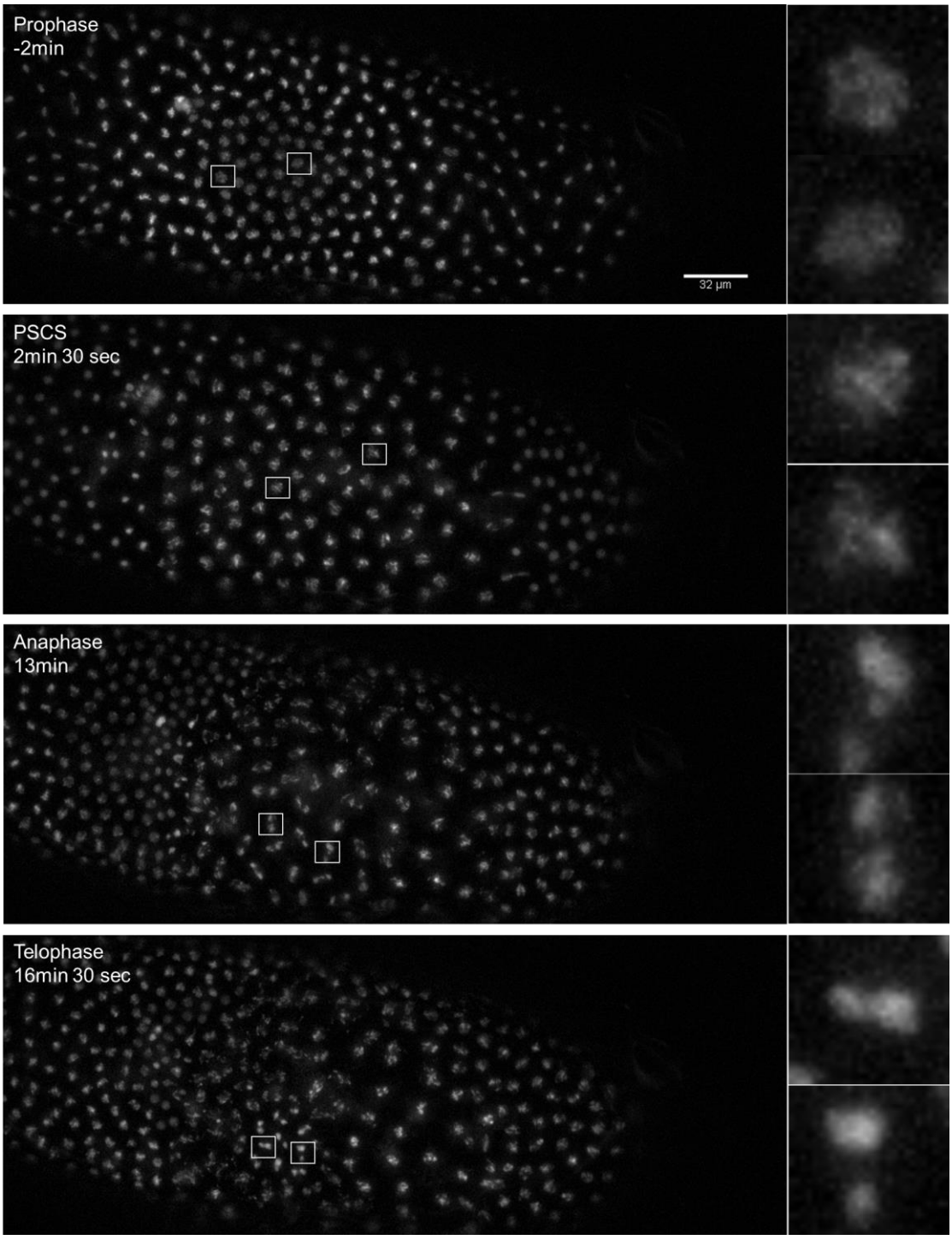


Figure 5.13 Positive control. Strain 629 (all cleavable cohseins) after TEV injection showed several problems in mitosis. A premature sister chromatid separation (PSCS) is visible as well as strong mitotic arrest. Magnifications of different phases are shown in the right (7x). The time started counting after NEBD.

Finally, the same experiment was performed in embryos with 50% of cleavable cohesin. In this case, after TEV injection the embryos showed a shorter mitosis time that the positive control, once no arrest happened because no PSCS was saw, resembling in everything the negative control.

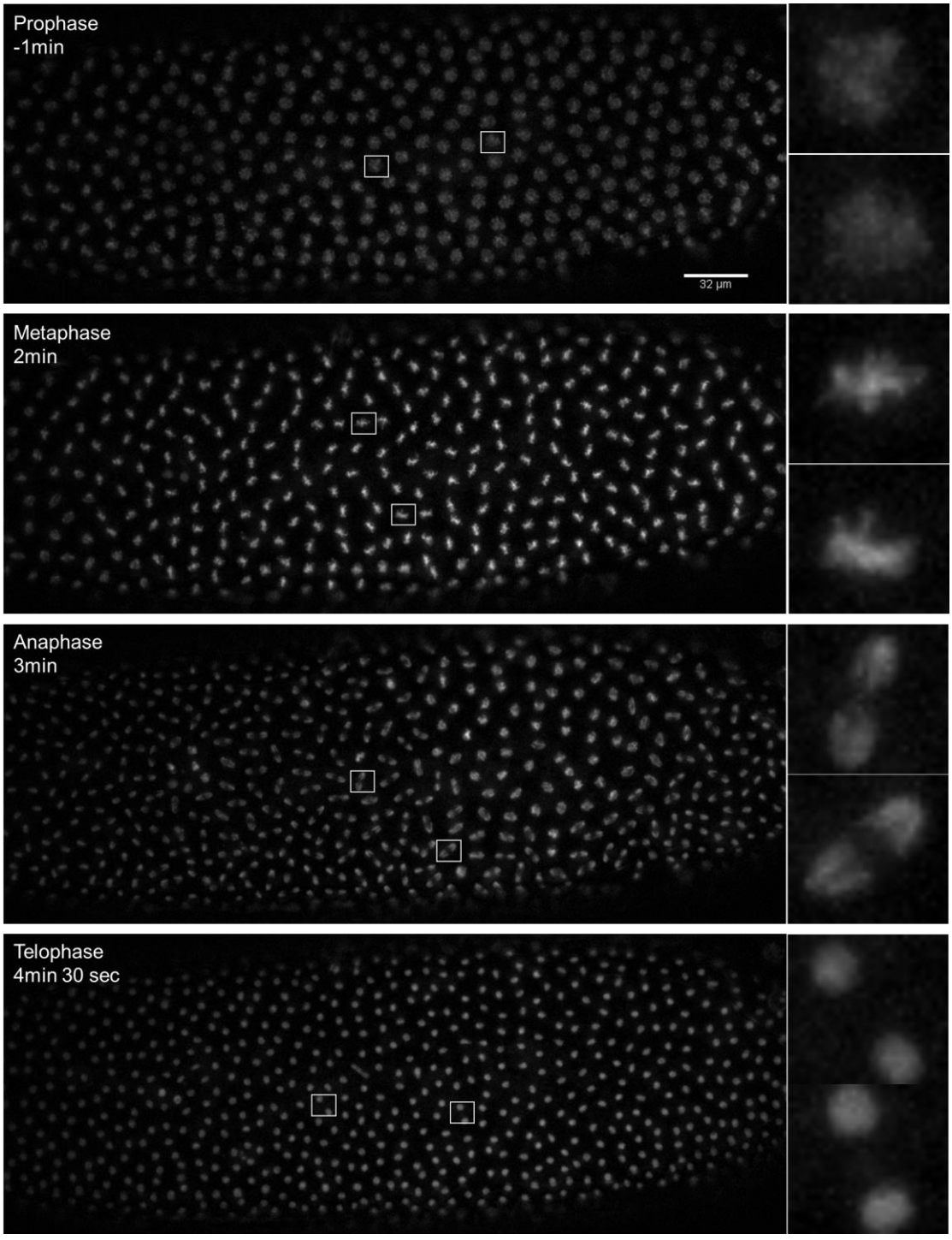
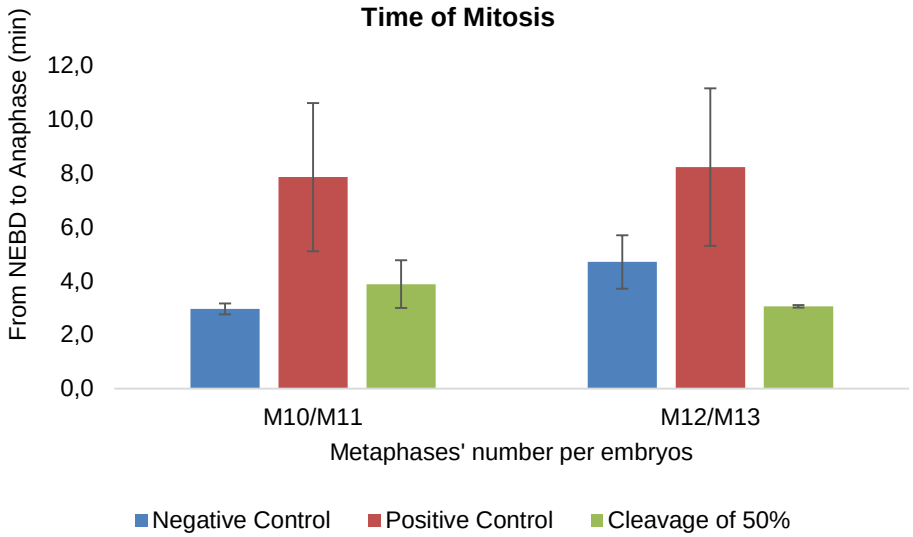


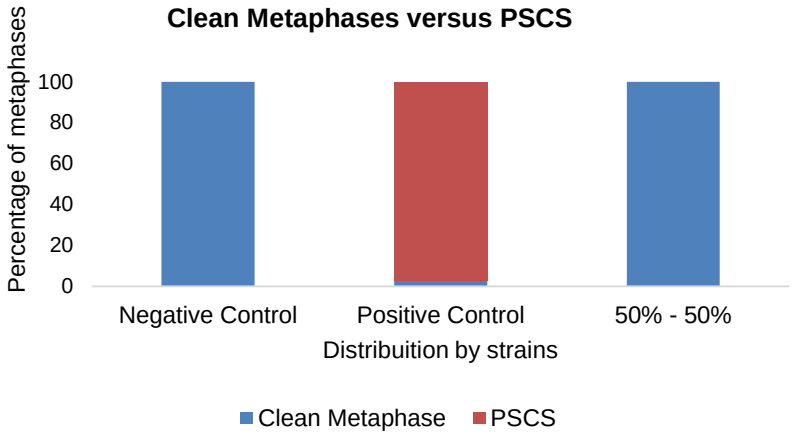
Figure 5.14 50% cleavable strain. The flies obtained from the 1357x629 cross were injected with TEV protease. This embryos presented a normal mitosis as observed in the negative control. In this case, any of the chromosome resolution/problems were observed. Magnifications of each phase is shown in the right.The time started counting after NEBD.

When PSCS happened, as represented in the Figure 5.13, the checkpoint was activated leading to a cycle arrest, followed by anaphase/telophase progression errors as all the chromosomes are not properly aligned due to the loss of cohesion. So, to better understand the observed delay, mitosis time was quantified from the NEBD until the onset of anaphase, for all strains represented above (graphic 5.6). As expected, positive control showed a longer mitosis of 8 minutes. On the contrary, both negative control and the 50% cleavable cohesin strains presented a significantly shorter mitosis duration (4min).



Graphic 5.4 Mitosis' Time. Average mitosis duration in the negative (269 strain), positive (629 strains) and in the 50% cleavable (1357x629 strain), measured from the NEBD until anaphase onset. M represents the number of the mitotic division in which embryos were. n=10 embryos for the negative control and the 50% cleavaded; n=7 for the positive control.

Once TEV protease successfully injected in all three strains, it was reasonable to analyze if any PSCS was present, as we speculated that it could also appear in low rates that would not lead to a delay in mitosis. Normal pared sisters were observed in the negative control and in the 50% cleavable strain (50%-50%) in approximately 100% of the metaphases analysed (figure 5.7).



Graphic 5.5 Clean metaphases versus PSCS. PSCS appeared only in the positive control (629 strain) were all the cohesin was cleaved (red). Negative control (269 strain) and 50%-50% (1357x629 strain) had no PSCS as expected (blue). n= 182 nucleous for negative control, n=211 nucleous for positive and n=211 nucleous for 50%-50%.

TEV injections induced PSCS which leads to many defects in anaphase/telophase and cytokinesis because the chromosomes where not equivalent aligned. These defects were analysed in the different strains being categorized according to the different phenotypes observed when compared to the control ones, in the figure 5.15 (normal metaphase and anaphase and the PSCS):

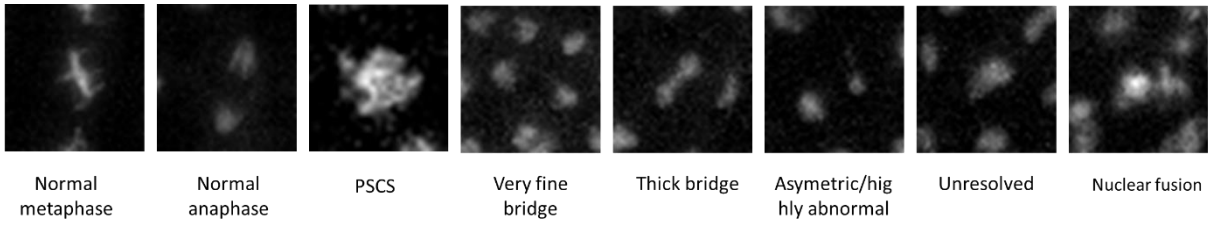
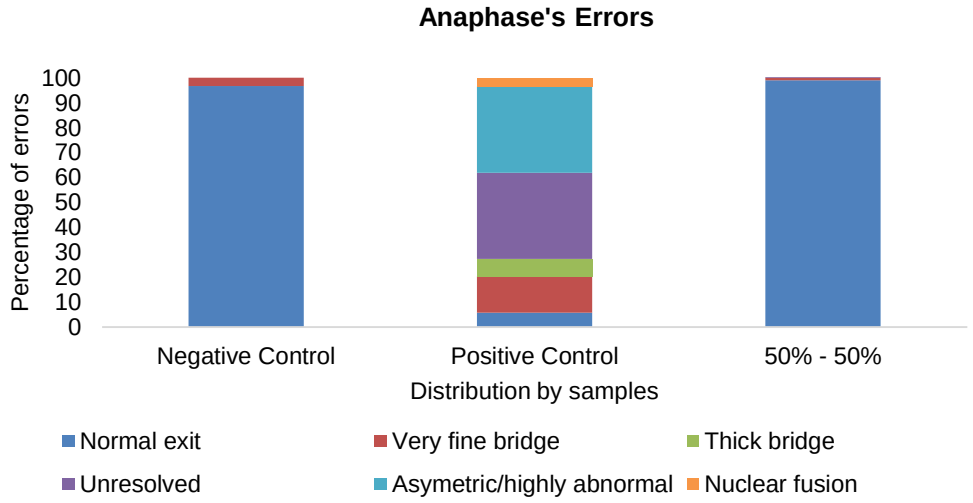


Figure 5.15 Errors' classification. In each embryo of the different strains, the presence of the errors represented in the figures was quantified

These errors were quantified according to the above shown phenotypes in the figure 5.15. The graphic 5.8 illustrates the strong presence of mitotic errors in the positive control and their almost complete absence in negative control and in the 50%-50% strains.



Graphic 5.6 Anaphase's errors. The different errors were counted in the different strains. n= 182 nucleous for negative control, n=211 nucleous for positive and 50%-50%.

5.5 Tools for future studies

The cleavage of 50% of cohesin seems not to impair sister chromatid cohesion, so less amount of $Rad21^{wt}$ has to be tried. For that we have already produced Drosophila strains that will allow us to test different ratios as follows (table 5.2):

Table 5.2 Future ratios. In the future we will increased the number of $Rad21^{TEV}$ copies through crosses leading to ratios of 2:1, 3:1 and 4:1.

Strain	Genotype	Ratio: $Rad21^{TEV}$: $Rad21^{wt}$
1358 x 1357	w; tubpr-Rad21(271-3TEV)myc (3)/CyO; Rad21(ex15), polyubiq-His-RFP,	**2:1**

	tubpr-Rad21(550-3TEV) / tubprom-Rad21(wt)-EGFP 2, Rad21 (ex15), polyubiq-His-RFP (rec 2.4)	
1358 x 1359	w; tubpr-Rad21(271-3TEV)myc (3)/ tubpr-Rad21(271-3TEV)myc (3)/ ; Rad21(ex15), polyubiq-His-RFP, tubpr-Rad21(550-3TEV) / tubprom-Rad21(wt)-EGFP 2, Rad21 (ex15), polyubiq-His-RFP (rec 2.4)	**3:1**
1358 x1361	w; tubpr-Rad21(271-3TEV)myc (3) / tubpr-Rad21(271-3TEV)myc (3) ; tubprom-Rad21(wt)-EGFP 2, Rad21 (ex15), polyubiq-His-RFP (rec 2.4), Rad21(ex8), tubpr-Rad21(271-3TEV) (8) (rec 1.4) / ; Rad21(ex15), polyubiq-His-RFP, tubpr-Rad21(550-3TEV)	**4:1**

If the above ratios (table 5.2) did not allow me to see a premature loss of sister chromatid cohesion, decreasing the number of wild type present in the fly would be the next step. We will take advantage of the leaky expression of Rad21^{UASwt} (with a myc tag) in the II chromosome from the 16 strain (w; UAS-Rad21myc10; gRad21). This strain should expressed extra Rad21 wt when induced by Gal4. However, even in the absence of driver, we detect a significant amount of protein expressed (figure 5.16).

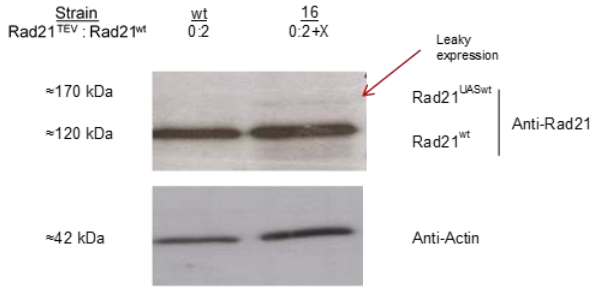


Figure 5.16 16 strain leaky expression. Total protein extracts of ovaries.The quantification of the little leaky expression of Rad21^{UASwt}(16) showed that compared to the endogeneous wt, in the same strain, represents about 11,8% (this unknown amount was represented by X). Western blot analysis was performed using an antibody against the Rad21

A preliminary quantification of this strain reveals that the leaky expression corresponds to 11,8% (Rad21^{UASwt}) comparing to the genomic one (gRad21). Therefore, this leaky expression should, in the future, allow to test further than the 20% resistant cohesin that we will obtain from the cross of 1358x1361, above mention. In this table 5.3 is shown some hypothesis of strains to test.

Table 5.3 Strais accomplished with the 16 strains. This strains will allow us to test small amounts of Rad21^{wt} through the 16's leaky expression.

Strain	Genotype	Ratio: Rad21^{TEV}: Rad21^{wt}
1363	w; UAS-Rad21myc10 ; Rad21(ex15), polyubiq-His-RFP, tubpr-Rad21(550-3TEV)	2:2X
1364	w; UAS-Rad21myc10/tubpr-Rad21(271-3TEV)myc (3) ; Rad21(ex15), polyubiq-His-RFP, tubpr-Rad21(550-3TEV)	4:X

To obtain all the genotypes described above, different *Drosophila* strains had to be established to try to increase the frequency of transgenes ($Rad21^{TEV}$). For that we will take advantage of the short generation time of the fruit fly providing the opportunity to cross many flies at the same time and using genetic recombination to allow in the same chromosome the addition of more alleles. More about recombination in section 8.6.

6 DISCUSSION

Mitosis is the shortest but the most visually stunning phase of the cell cycle, in which over 800 proteins (Sauer et al., 2005) are responsible for proper chromosome segregation of genes. One of the most crucial ones is the cohesin complex, an evolutionarily conserved, three-subunit complex that entraps DNA fibres within its ring-shaped structure.

When chromosome segregation is compromised it can lead to serious problems in meiosis or mitosis, as aneuploidies or cancer. The decay in cohesin complexes seems to play a major role for these consequences, although little is known about how much cohesin can be lost before evident segregation mistakes or if milder losses are able to activate checkpoints that arrest the cell cycle. If these mistakes happen in meiosis, a child life or health can be at risk.

In order to elucidate how much cohesin can hold sister chromatids together, we used *Drosophila melanogaster* as a model animal and its modified cohesin with the TEV system, to bring a quantitative understanding of the process of sister chromatid cohesion, and lift the veil about cohesin decay and how that influences chromosome behaviour and its interaction with the checkpoint mechanisms that control mitosis.

Our results show that *in vivo* cleavage of 50% of cohesin complexes leads to a normal mitosis, without any delay, evident mitotic errors or any precocious loss of cohesion. This is in sharp contrast to the defect observed upon total cleavage of cohesin where drastic mistakes as PSCS and errors of segregation were reported (Oliveira et al., 2010).

So far, not many studies have tried to quantify the amount of cohesin necessary to trap sister DNAs. In budding yeast, using a systematic quantized reductions, strains with 13% and 30% levels of wt Rad21 subunit were made. Heidinger-Pauli and co-works demonstrated that 13% of wt was still enough to keep SCC and chromosome segregation without errors however they verified that other cohesin functions as chromosome condensation, DNA stability and repair were compromised if 30% are left (Heidinger-Pauli et al., 2010a).

The low number that is required for budding yeast's chromosome stability can be related with the fact that there is no prophase pathway in this animal model. Therefore, cohesin complexes will remain all over the chromosome until anaphase onset. By contrast, in metazoans, most of the cohesin complexes is removed from chromosome arms during early stages of mitosis (Nasmyth and Haering, 2009). It was therefore expected that mammalian chromosomes may be more sensitive to cohesion loss as they are held together solely by the remaining complexes at the centromere vicinity.

Therefore, it is important to establish if the percentage obtain by Heidinger-Pauli is substantially different in metazoans or not, and it is still important to know that different levels of cohesin allow different sensitivities to the diverse cohesion functions relatively to that level. In humans, as reported in the section 1.2.4 Cohesinopathies, Tsutsumi's work in oocytes show a significant decrease of 24% for Rec8 and 38% for Smc1β (Tsutsumi et al., 2014) comparing younger with older women. Similar conclusions had already been reported in 2012, although in this case no levels of cohesin proteins have been evaluated and loss of sister chromatid cohesion was estimated by measuring inter-kinetochore distance (Duncan et al., 2012). In the light of the results presented in this thesis, these reported decreases should not impair sister chromatid cohesion! It should be stated, however, that meiotic cells may be more

sensitive to cohesion loss.

If 50% is sufficient to suffice cohesion, why do mitotic chromosomes have more levels of cohesin? A possible explanation is that the increased levels correspond to other functions of cohesin. Indeed, gene expression seems to be more sensitive to cohesin levels that segregation functions (Heidinger-Pauli et al., 2010a; Sofueva et al., 2013).

Another hypothesis is that maybe there is a mechanism of non-deterioration of cohesins (Duncan et al., 2012) stronger in meiosis than in mitosis, once that we report that 50% cleavage do not impair chromosome segregation, rising then, some doubts about the decreased describe above. Furthermore, lacking of more human studies with more subjects and with more younger and older oocytes could give a more precise range of the amount that actually is lost over time.

6.1 Future Perspectives

Lower percentages than 50% are then expected to not hold against the spindle forces leading to PSCS. The next step will be to test flies with 33,3% and 25% of wild-type cohesin, resistant to TEV cleavage. In case of none of these percentages trigger PSCS, then strains with lower expressed amounts of wild-type will be used (see section 5.5). Additionally, a different approach will be tested: an *in vivo* metaphase arrest will be induced by a human ubiquitin-conjugating E2 UbcH10 dominant negative ($UbcH10^{C114S}$, which blocks APC/C substrates) followed by a TEV injection in embryos (Oliveira et al., 2010). The method will be applied in the strains with the different ratios already mentioned above, for a corroboration of the present results and future ones by being phase-specific allowing an additionally better understanding of SAC response, cohesin fatigue and chromosome movement after cleavage. The key advantage of this system is that it keeps chromosomes arrested in metaphase and therefore under spindle forces. It is therefore a more stringent assay to test for maintenance of sister chromatid cohesion.

The experiments described above, once completed, will determine the percentage of necessary cohesins that are sufficient to resist the opposite pulling forces. It will not, however, give the precise number of complexes that sustain cohesion. To estimate that we will use imaging quantification methods to measure the total amount of cohesin present in the chromosomes by quantifying the fluorescence present in the strain 1357 ($Rad21^{wt\text{-}EGFP}$) that is resistant to TEV cleavage and tagged with a fluorescent probe.

6.2 Conclusion

In conclusion, the loss of 50% of cohesin seems to be sufficient to promote functional cohesin to satisfy the SAC activity, without delays or mistakes happening and proper segregation of the sister chromatids. Future experiments will be performed in order to determine the minimal amount of cohesin sufficient to promote sister chromatid cohesion.

7 REFERENCES

Alberts, B., A. Johnson, J. Lewis, M. Raff, K. Roberts, and P. Walter. 2008. Molecular biology of the cell. Garland Science, New York.

Altenburg, E., and H.J. Muller. 1920. The Genetic Basis of Truncate Wing,-an Inconstant and Modifiable Character in Drosophila. Genetics. 5:1-59.

Anderson, D.E., A. Losada, H.P. Erickson, and T. Hirano. 2002. Condensin and cohesin display different arm conformations with characteristic hinge angles. The Journal of cell biology. 156:419-424.

Araye, Q., and K. Sawamura. 2013. Genetic decay of balancer chromosomes in Drosophila melanogaster. Fly. 7:184-186.

Arumugam, P., S. Gruber, K. Tanaka, C.H. Haering, K. Mechtler, and K. Nasmyth. 2003. ATP hydrolysis is required for cohesin's association with chromosomes. Current Biology. 13:1941-1953.

Balbas-Martinez, C., A. Sagrera, E. Carrillo-de-Santa-Pau, J. Earl, M. Marquez, M. Vazquez, E. Lapi, F. Castro-Giner, S. Beltran, M. Bayes, A. Carrato, J.C. Cigudosa, O. Dominguez, M. Gut, J. Herranz, N. Juanpere, M. Kogevinas, X. Langa, E. Lopez-Knowles, J.A. Lorente, J. Lloreta, D.G. Pisano, L. Richart, D. Rico, R.N. Salgado, A. Tardon, S. Chanock, S. Heath, A. Valencia, A. Losada, I. Gut, N. Malats, and F.X. Real. 2013. Recurrent inactivation of STAG2 in bladder cancer is not associated with aneuploidy. Nature genetics. 45:1464-1469.

Ball, A.R., Jr., Y.Y. Chen, and K. Yokomori. 2014. Mechanisms of cohesin-mediated gene regulation and lessons learned from cohesinopathies. Biochimica et biophysica acta. 1839:191-202.

Barber, T.D., K. McManus, K.W. Yuen, M. Reis, G. Parmigiani, D. Shen, I. Barrett, Y. Nouhi, F. Spencer, S. Markowitz, V.E. Velculescu, K.W. Kinzler, B. Vogelstein, C. Lengauer, and P. Hieter. 2008. Chromatid cohesion defects may underlie chromosome instability in human colorectal cancers. Proceedings of the National Academy of Sciences of the United States of America. 105:3443-3448.

Beckouet, F., B. Hu, M.B. Roig, T. Sutani, M. Komata, P. Uluocak, V.L. Katis, K. Shirahige, and K. Nasmyth. 2010. An Smc3 acetylation cycle is essential for establishment of sister chromatid cohesion. Molecular cell. 39:689-699.

Bhalla, N., S. Biggins, and A.W. Murray. 2002. Mutation of YCS4, a budding yeast condensin subunit, affects mitotic and nonmitotic chromosome behavior. Molecular biology of the cell. 13:632-645.

Bickel, S.E. 2005. Aging (not so) gracefully. Nature genetics. 37:1303-1304.

Birkenbihl, R.P., and S. Subramani. 1992. Cloning and characterization of rad21 an essential gene of Schizosaccharomyces pombe involved in DNA double-strand-break repair. Nucleic acids research. 20:6605-6611.

Blat, Y., and N. Kleckner. 1999. Cohesins bind to preferential sites along yeast chromosome III, with differential regulation along arms versus the centric region. Cell. 98:249-259.

Borges, V., C. Lehane, L. Lopez-Serra, H. Flynn, M. Skehel, T. Rolef Ben-Shahar, and F. Uhlmann. 2010. Hos1 deacetylates Smc3 to close the cohesin acetylation cycle. Molecular cell. 39:677-688.

Bose, T., and J.L. Gerton. 2010. Cohesinopathies, gene expression, and chromatin organization. The Journal of cell biology. 189:201-210.

Bose, T., K.K. Lee, S. Lu, B. Xu, B. Harris, B. Slaughter, J. Unruh, A. Garrett, W. McDowell, A. Box, H. Li, A. Peak, S. Ramachandran, C. Seidel, and J.L. Gerton. 2012. Cohesin proteins promote ribosomal RNA production and protein translation in yeast and human cells. PLoS genetics. 8:e1002749.

Buheitel, J., and O. Stemmann. 2013. Prophase pathway-dependent removal of cohesin from human chromosomes requires opening of the Smc3-Scc1 gate. The EMBO journal. 32:666-676.

Bukovsky, A., M.R. Caudle, M. Svetlikova, J. Wimalasena, M.E. Ayala, and R. Dominguez. 2005. Oogenesis in adult mammals, including humans: a review. Endocrine. 26:301-316.

Burrell, R.A., S.E. McClelland, D. Endesfelder, P. Groth, M.C. Weller, N. Shaikh, E. Domingo, N. Kanu, S.M. Dewhurst, E. Gronroos, S.K. Chew, A.J. Rowan, A. Schenk, M. Sheffer, M. Howell, M. Kschischo, A. Behrens, T. Helleday, J. Bartek, I.P. Tomlinson, and C. Swanton. 2013. Replication stress links structural and numerical cancer chromosomal instability. Nature. 494:492-496.

Chan, K.L., M.B. Roig, B. Hu, F. Beckouet, J. Metson, and K. Nasmyth. 2012. Cohesin's DNA exit gate is distinct from its entrance gate and is regulated by acetylation. Cell. 150:961-974.

Chang, C.R., C.S. Wu, Y. Hom, and M.R. Gartenberg. 2005. Targeting of cohesin by transcriptionally silent chromatin. Genes & development. 19:3031-3042.

Chatterjee, A., S. Zakian, X.W. Hu, and M.R. Singleton. 2013. Structural insights into the regulation of cohesion establishment by Wpl1. The EMBO journal. 32:677-687.

Chiang, T., F.E. Duncan, K. Schindler, R.M. Schultz, and M.A. Lampson. 2010. Evidence that weakened

centromere cohesion is a leading cause of age-related aneuploidy in oocytes. Current Biology. 20:1522-1528.
Ciosk, R., M. Shirayama, A. Shevchenko, T. Tanaka, A. Toth, A. Shevchenko, and K. Nasmyth. 2000. Cohesin's binding to chromosomes depends on a separate complex consisting of Scc2 and Scc4 proteins. Molecular cell. 5:243-254.
Cooper, G.M., and R.E. Hausman. 2007. The Cell: A Molecular Approach. ASM Press.
Cortés-Ledesma, F., and A. Aguilera. 2006. Double-strand breaks arising by replication through a nick are repaired by cohesin-dependent sister-chromatid exchange.
Cucco, F., A. Servadio, V. Gatti, P. Bianchi, L. Mannini, A. Prodosmo, E. De Vitis, G. Basso, A. Friuli, L. Laghi, S. Soddu, G. Fontanini, and A. Musio. 2014. Mutant cohesin drives chromosomal instability in early colorectal adenomas. Human molecular genetics.
D'Ambrosio, C., C.K. Schmidt, Y. Katou, G. Kelly, T. Itoh, K. Shirahige, and F. Uhlmann. 2008. Identification of cis-acting sites for condensin loading onto budding yeast chromosomes. Genes & development. 22:2215-2227.
Daum, J.R., T.A. Potapova, S. Sivakumar, J.J. Daniel, J.N. Flynn, S. Rankin, and G.J. Gorbsky. 2011. Cohesion fatigue induces chromatid separation in cells delayed at metaphase. Current Biology. 21:1018-1024.
Deardorff, M.A., M. Bando, R. Nakato, E. Watrin, T. Itoh, M. Minamino, K. Saitoh, M. Komata, Y. Katou, D. Clark, K.E. Cole, E. De Baere, C. Decroos, N. Di Donato, S. Ernst, L.J. Francey, Y. Gyftodimou, K. Hirashima, M. Hullings, Y. Ishikawa, C. Jaulin, M. Kaur, T. Kiyono, P.M. Lombardi, L. Magnaghi-Jaulin, G.R. Mortier, N. Nozaki, M.B. Petersen, H. Seimiya, V.M. Siu, Y. Suzuki, K. Takagaki, J.J. Wilde, P.J. Willems, C. Prigent, G. Gillessen-Kaesbach, D.W. Christianson, F.J. Kaiser, L.G. Jackson, T. Hirota, I.D. Krantz, and K. Shirahige. 2012a. HDAC8 mutations in Cornelia de Lange syndrome affect the cohesin acetylation cycle. Nature. 489:313-317.
Deardorff, M.A., M. Kaur, D. Yaeger, A. Rampuria, S. Korolev, J. Pie, C. Gil-Rodriguez, M. Arnedo, B. Loeys, A.D. Kline, M. Wilson, K. Lillquist, V. Siu, F.J. Ramos, A. Musio, L.S. Jackson, D. Dorsett, and I.D. Krantz. 2007. Mutations in cohesin complex members SMC3 and SMC1A cause a mild variant of cornelia de Lange syndrome with predominant mental retardation. American journal of human genetics. 80:485-494.
Deardorff, M.A., J.J. Wilde, M. Albrecht, E. Dickinson, S. Tennstedt, D. Braunholz, M. Monnich, Y. Yan, W. Xu, M.C. Gil-Rodriguez, D. Clark, H. Hakonarson, S. Halbach, L.D. Michelis, A. Rampuria, E. Rossier, S. Spranger, L. Van Maldergem, S.A. Lynch, G. Gillessen-Kaesbach, H.J. Ludecke, R.G. Ramsay, M.J. McKay, I.D. Krantz, H. Xu, J.A. Horsfield, and F.J. Kaiser. 2012b. RAD21 mutations cause a human cohesinopathy. American journal of human genetics. 90:1014-1027.
den Elzen, N., and J. Pines. 2001. Cyclin A is destroyed in prometaphase and can delay chromosome alignment and anaphase. The Journal of cell biology. 153:121-136.
Denison, S.H., E. Kafer, and G.S. May. 1993. Mutation in the bimD gene of Aspergillus nidulans confers a conditional mitotic block and sensitivity to DNA damaging agents. Genetics. 134:1085-1096.
DeScipio, C., M. Kaur, D. Yaeger, J.W. Innis, N.B. Spinner, L.G. Jackson, and I.D. Krantz. 2005. Chromosome rearrangements in cornelia de Lange syndrome (CdLS): report of a der(3)t(3;12)(p25.3;p13.3) in two half sibs with features of CdLS and review of reported CdLS cases with chromosome rearrangements. American journal of medical genetics. Part A. 137a:276-282.
Dheur, S., S.J. Saupe, S. Genier, S. Vazquez, and J.P. Javerzat. 2011. Role for cohesin in the formation of a heterochromatic domain at fission yeast subtelomeres. Molecular and cellular biology. 31:1088-1097.
Ding, D.Q., N. Sakurai, Y. Katou, T. Itoh, K. Shirahige, T. Haraguchi, and Y. Hiraoka. 2006. Meiotic cohesins modulate chromosome compaction during meiotic prophase in fission yeast. The Journal of cell biology. 174:499-508.
Dorsett, D. 2007. Roles of the sister chromatid cohesion apparatus in gene expression, development, and human syndromes. Chromosoma. 116:1-13.
Dorsett, D. 2011. Cohesin: genomic insights into controlling gene transcription and development. Current Opinion in Genetics & Development. 21:199-206.
Duijf, P.H., and R. Benezra. 2013. The cancer biology of whole-chromosome instability. Oncogene. 32:4727-4736.
Duncan, F.E., T. Chiang, R.M. Schultz, and M.A. Lampson. 2009. Evidence that a defective spindle assembly checkpoint is not the primary cause of maternal age-associated aneuploidy in mouse eggs. Biology of reproduction. 81:768-776.
Duncan, F.E., J.E. Hornick, M.A. Lampson, R.M. Schultz, L.D. Shea, and T.K. Woodruff. 2012. Chromosome cohesion decreases in human eggs with advanced maternal age. Aging cell. 11:1121-1124.
Eichinger, C.S., A. Kurze, R.A. Oliveira, and K. Nasmyth. 2013. Disengaging the Smc3/kleisin interface releases

cohesin from Drosophila chromosomes during interphase and mitosis. The EMBO journal. 32:656-665.
Ellermeier, C., and G.R. Smith. 2005. Cohesins are required for meiotic DNA breakage and recombination in Schizosaccharomyces pombe. Proceedings of the National Academy of Sciences of the United States of America. 102:10952-10957.
Fair, T. 2010. Mammalian oocyte development: checkpoints for competence. Reproduction, fertility, and development. 22:13-20.
Feeney, A.J., and J. Verma-Gaur. 2012. CTCF-cohesin complex: architect of chromatin structure regulates V(D)J rearrangement. Cell Res. 22:280-282.
Feytout, A., S. Vaur, S. Genier, S. Vazquez, and J.P. Javerzat. 2011. Psm3 acetylation on conserved lysine residues is dispensable for viability in fission yeast but contributes to Eso1-mediated sister chromatid cohesion by antagonizing Wpl1. Molecular and cellular biology. 31:1771-1786.
Flemming, W. 1882. Zellsubstanz, Kern und Zelltheilung. Vogel.
Furuno, N., N. den Elzen, and J. Pines. 1999. Human cyclin A is required for mitosis until mid prophase. The Journal of cell biology. 147:295-306.
Gabriels, J., M.C. Beckers, H. Ding, A. De Vriese, S. Plaisance, S.M. van der Maarel, G.W. Padberg, R.R. Frants, J.E. Hewitt, D. Collen, and A. Belayew. 1999. Nucleotide sequence of the partially deleted D4Z4 locus in a patient with FSHD identifies a putative gene within each 3.3 kb element. Gene. 236:25-32.
Gandhi, R., P.J. Gillespie, and T. Hirano. 2006. Human Wapl is a cohesin-binding protein that promotes sister-chromatid resolution in mitotic prophase. Current Biology. 16:2406-2417.
Garcia-Cruz, R., M.A. Brieno, I. Roig, M. Grossmann, E. Velilla, A. Pujol, L. Cabero, A. Pessarrodona, J.L. Barbero, and M. Garcia Caldes. 2010. Dynamics of cohesin proteins REC8, STAG3, SMC1 beta and SMC3 are consistent with a role in sister chromatid cohesion during meiosis in human oocytes. Human reproduction. 25:2316-2327.
Gause, M., Z. Misulovin, A. Bilyeu, and D. Dorsett. 2010. Dosage-sensitive regulation of cohesin chromosome binding and dynamics by Nipped-B, Pds5, and Wapl. Molecular and cellular biology. 30:4940-4951.
Gause, M., H. Webber, Z. Misulovin, G. Haller, R. Rollins, J. Eissenberg, S. Bickel, and D. Dorsett. 2008. Functional links between Drosophila Nipped-B and cohesin in somatic and meiotic cells. Chromosoma. 117:51-66.
Geng, L.N., Z. Yao, L. Snider, A.P. Fong, J.N. Cech, J.M. Young, S.M. van der Maarel, W.L. Ruzzo, R.C. Gentleman, R. Tawil, and S.J. Tapscott. 2012. DUX4 activates germline genes, retroelements, and immune mediators: implications for facioscapulohumeral dystrophy. Developmental cell. 22:38-51.
Gerlich, D., B. Koch, F. Dupeux, J.M. Peters, and J. Ellenberg. 2006. Live-cell imaging reveals a stable cohesin-chromatin interaction after but not before DNA replication. Current biology. 16:1571-1578.
Gilliland, W.D., and R.S. Hawley. 2005. Cohesin and the maternal age effect. Cell. 123:371-373.
Gillis, L.A., J. McCallum, M. Kaur, C. DeScipio, D. Yaeger, A. Mariani, A.D. Kline, H.H. Li, M. Devoto, L.G. Jackson, and I.D. Krantz. 2004. NIPBL mutational analysis in 120 individuals with Cornelia de Lange syndrome and evaluation of genotype-phenotype correlations. American journal of human genetics. 75:610-623.
Gimenez-Abian, J.F., L.A. Diaz-Martinez, N.A. Beauchene, W.S. Hsu, H.J. Tsai, and D.J. Clarke. 2010. Determinants of Rad21 localization at the centrosome in human cells. Cell cycle. 9:1759-1763.
Gimenez-Abian, J.F., I. Sumara, T. Hirota, S. Hauf, D. Gerlich, C. de la Torre, J. Ellenberg, and J.M. Peters. 2004. Regulation of sister chromatid cohesion between chromosome arms. Current biology : CB. 14:1187-1193.
Glynn, E.F., P.C. Megee, H.G. Yu, C. Mistrot, E. Unal, D.E. Koshland, J.L. DeRisi, and J.L. Gerton. 2004. Genome-wide mapping of the cohesin complex in the yeast Saccharomyces cerevisiae. PLoS biology. 2:E259.
Gong, D., J.R. Pomerening, J.W. Myers, C. Gustavsson, J.T. Jones, A.T. Hahn, T. Meyer, and J.E. Ferrell, Jr. 2007. Cyclin A2 regulates nuclear-envelope breakdown and the nuclear accumulation of cyclin B1. Current biology : CB. 17:85-91.
Gosden, R., and B. Lee. 2010. Portrait of an oocyte: our obscure origin. The Journal of clinical investigation. 120:973-983.
Gruber, S., P. Arumugam, Y. Katou, D. Kuglitsch, W. Helmhart, K. Shirahige, and K. Nasmyth. 2006. Evidence that loading of cohesin onto chromosomes involves opening of its SMC hinge. Cell. 127:523-537.
Gruber, S., C.H. Haering, and K. Nasmyth. 2003. Chromosomal cohesin forms a ring. Cell. 112:765-777.
Guacci, V., D. Koshland, and A. Strunnikov. 1997. A direct link between sister chromatid cohesion and chromosome condensation revealed through the analysis of MCD1 in S. cerevisiae. Cell. 91:47-57.
Guillou, E., A. Ibarra, V. Coulon, J. Casado-Vela, D. Rico, I. Casal, E. Schwob, A. Losada, and J. Mendez. 2010. Cohesin organizes chromatin loops at DNA replication factories. Genes & development. 24:2812-2822.
Guo, G., X. Sun, C. Chen, S. Wu, P. Huang, Z. Li, M. Dean, Y. Huang, W. Jia, Q. Zhou, A. Tang, Z. Yang, X. Li,

P. Song, X. Zhao, R. Ye, S. Zhang, Z. Lin, M. Qi, S. Wan, L. Xie, F. Fan, M.L. Nickerson, X. Zou, X. Hu, L. Xing, Z. Lv, H. Mei, S. Gao, C. Liang, Z. Gao, J. Lu, Y. Yu, C. Liu, L. Li, X. Fang, Z. Jiang, J. Yang, C. Li, X. Zhao, J. Chen, F. Zhang, Y. Lai, Z. Lin, F. Zhou, H. Chen, H.C. Chan, S. Tsang, D. Theodorescu, Y. Li, X. Zhang, J. Wang, H. Yang, Y. Gui, J. Wang, and Z. Cai. 2013. Whole-genome and whole-exome sequencing of bladder cancer identifies frequent alterations in genes involved in sister chromatid cohesion and segregation. Nature genetics. 45:1459-1463.

Haarhuis, J.H., A.M. Elbatsh, B. van den Broek, D. Camps, H. Erkan, K. Jalink, R.H. Medema, and B.D. Rowland. 2013. WAPL-mediated removal of cohesin protects against segregation errors and aneuploidy. Current biology. 23:2071-2077.

Haering, C.H., A.M. Farcas, P. Arumugam, J. Metson, and K. Nasmyth. 2008. The cohesin ring concatenates sister DNA molecules. Nature. 454:297-301.

Haering, C.H., J. Lowe, A. Hochwagen, and K. Nasmyth. 2002. Molecular architecture of SMC proteins and the yeast cohesin complex. Molecular cell. 9:773-788.

Haering, C.H., D. Schoffnegger, T. Nishino, W. Helmhart, K. Nasmyth, and J. Lowe. 2004. Structure and stability of cohesin's Smc1-kleisin interaction. Molecular cell. 15:951-964.

Hartman, T., K. Stead, D. Koshland, and V. Guacci. 2000. Pds5p is an essential chromosomal protein required for both sister chromatid cohesion and condensation in Saccharomyces cerevisiae. The Journal of cell biology. 151:613-626.

Hassold, T., and P. Hunt. 2001. To err (meiotically) is human: the genesis of human aneuploidy. Nature reviews. Genetics. 2:280-291.

Hauf, S., E. Roitinger, B. Koch, C.M. Dittrich, K. Mechtler, and J.M. Peters. 2005. Dissociation of cohesin from chromosome arms and loss of arm cohesion during early mitosis depends on phosphorylation of SA2. PLoS biology. 3:69.

Hauf, S., I.C. Waizenegger, and J.M. Peters. 2001. Cohesin cleavage by separase required for anaphase and cytokinesis in human cells. Science. 293:1320-1323.

Heidinger-Pauli, J.M., O. Mert, C. Davenport, V. Guacci, and D. Koshland. 2010a. Systematic reduction of cohesin differentially affects chromosome segregation, condensation, and DNA repair. Current biology. 20:957-963.

Heidinger-Pauli, J.M., I. Onn, and D. Koshland. 2010b. Genetic evidence that the acetylation of the Smc3p subunit of cohesin modulates its ATP-bound state to promote cohesion establishment in Saccharomyces cerevisiae. Genetics. 185:1249-1256.

Heidinger-Pauli, J.M., E. Unal, and D. Koshland. 2009. Distinct targets of the Eco1 acetyltransferase modulate cohesion in S phase and in response to DNA damage. Molecular cell. 34:311-321.

Herran, Y., C. Gutierrez-Caballero, M. Sanchez-Martin, T. Hernandez, A. Viera, J.L. Barbero, E. de Alava, D.G. de Rooij, J.A. Suja, E. Llano, and A.M. Pendas. 2011. The cohesin subunit RAD21L functions in meiotic synapsis and exhibits sexual dimorphism in fertility. The EMBO journal. 30:3091-3105.

Higashi, T.L., M. Ikeda, H. Tanaka, T. Nakagawa, M. Bando, K. Shirahige, Y. Kubota, H. Takisawa, H. Masukata, and T.S. Takahashi. 2012. The prereplication complex recruits XEco2 to chromatin to promote cohesin acetylation in Xenopus egg extracts. Current biology. 22:977-988.

Hirano, M., and T. Hirano. 2002. Hinge-mediated dimerization of SMC protein is essential for its dynamic interaction with DNA. The EMBO journal. 21:5733-5744.

Hodges, C.A., E. Revenkova, R. Jessberger, T.J. Hassold, and P.A. Hunt. 2005. SMC1beta-deficient female mice provide evidence that cohesins are a missing link in age-related nondisjunction. Nature genetics. 37:1351-1355.

Holland, A.J., and D.W. Cleveland. 2012. Losing balance: the origin and impact of aneuploidy in cancer. EMBO reports. 13:501-514.

Hou, F., and H. Zou. 2005. Two human orthologues of Eco1/Ctf7 acetyltransferases are both required for proper sister-chromatid cohesion. Molecular biology of the cell. 16:3908-3918.

Hu, B., T. Itoh, A. Mishra, Y. Katoh, K.L. Chan, W. Upcher, C. Godlee, M.B. Roig, K. Shirahige, and K. Nasmyth. 2011. ATP hydrolysis is required for relocating cohesin from sites occupied by its Scc2/4 loading complex. Current biology : CB. 21:12-24.

Huang, C.E., M. Milutinovich, and D. Koshland. 2005. Rings, bracelet or snaps: fashionable alternatives for Smc complexes. Philosophical transactions of the Royal Society of London. Series B, Biological sciences. 360:537-542.

Inoue, A., T. Li, S.K. Roby, M.B. Valentine, M. Inoue, K. Boyd, V.J. Kidd, and J.M. Lahti. 2007. Loss of ChlR1 helicase in mouse causes lethality due to the accumulation of aneuploid cells generated by cohesion defects and placental malformation. Cell cycle. 6:1646-1654.

Ishiguro, T., K. Tanaka, T. Sakuno, and Y. Watanabe. 2010. Shugoshin-PP2A counteracts casein-kinase-1-

dependent cleavage of Rec8 by separase. Nature cell biology. 12:500-506.
Ivanov, D., and K. Nasmyth. 2005. A topological interaction between cohesin rings and a circular minichromosome. Cell. 122:849-860.
Jessberger, R. 2012. Age-related aneuploidy through cohesion exhaustion. EMBO reports. 13:539-546.
Jin, H., M. Avey, and H.G. Yu. 2012. Is cohesin required for spindle-pole-body/centrosome cohesion? Communicative & integrative biology. 5:26-29.
Jones, R. 2012. Mitosis explained. Version 9 September 2014. http://www.howitworksdaily.com/mitosis-explained/ in How it works, http://www.howitworksdaily.com/
Kagey, M.H., J.J. Newman, S. Bilodeau, Y. Zhan, D.A. Orlando, N.L. van Berkum, C.C. Ebmeier, J. Goossens, P.B. Rahl, S.S. Levine, D.J. Taatjes, J. Dekker, and R.A. Young. 2010. Mediator and cohesin connect gene expression and chromatin architecture. Nature. 467:430-435.
Kawashima, S.A., Y. Yamagishi, T. Honda, K. Ishiguro, and Y. Watanabe. 2010. Phosphorylation of H2A by Bub1 prevents chromosomal instability through localizing shugoshin. Science. 327:172-177.
Kawauchi, S., A.L. Calof, R. Santos, M.E. Lopez-Burks, C.M. Young, M.P. Hoang, A. Chua, T. Lao, M.S. Lechner, J.A. Daniel, A. Nussenzweig, L. Kitzes, K. Yokomori, B. Hallgrimsson, and A.D. Lander. 2009. Multiple organ system defects and transcriptional dysregulation in the Nipbl(+/-) mouse, a model of Cornelia de Lange Syndrome. PLoS genetics. 5:e1000650.
Kerrebrock, A.W., D.P. Moore, J.S. Wu, and T.L. Orr-Weaver. 1995. Mei-S332, a Drosophila protein required for sister-chromatid cohesion, can localize to meiotic centromere regions. Cell. 83:247-256.
Kitajima, T.S., S. Hauf, M. Ohsugi, T. Yamamoto, and Y. Watanabe. 2005. Human Bub1 defines the persistent cohesion site along the mitotic chromosome by affecting Shugoshin localization. Current biology : CB. 15:353-359.
Kitajima, T.S., S.A. Kawashima, and Y. Watanabe. 2004. The conserved kinetochore protein shugoshin protects centromeric cohesion during meiosis. Nature. 427:510-517.
Kitajima, T.S., T. Sakuno, K. Ishiguro, S. Iemura, T. Natsume, S.A. Kawashima, and Y. Watanabe. 2006. Shugoshin collaborates with protein phosphatase 2A to protect cohesin. Nature. 441:46-52.
Kogut, I., J. Wang, V. Guacci, R.K. Mistry, and P.C. Megee. 2009. The Scc2/Scc4 cohesin loader determines the distribution of cohesin on budding yeast chromosomes. Genes & development. 23:2345-2357.
Kong, X., A.R. Ball, Jr., E. Sonoda, J. Feng, S. Takeda, T. Fukagawa, T.J. Yen, and K. Yokomori. 2009. Cohesin associates with spindle poles in a mitosis-specific manner and functions in spindle assembly in vertebrate cells. Molecular biology of the cell. 20:1289-1301.
Krantz, I.D., J. McCallum, C. DeScipio, M. Kaur, L.A. Gillis, D. Yaeger, L. Jukofsky, N. Wasserman, A. Bottani, C.A. Morris, M.J. Nowaczyk, H. Toriello, M.J. Bamshad, J.C. Carey, E. Rappaport, S. Kawauchi, A.D. Lander, A.L. Calof, H.H. Li, M. Devoto, and L.G. Jackson. 2004. Cornelia de Lange syndrome is caused by mutations in NIPBL, the human homolog of Drosophila melanogaster Nipped-B. Nature genetics. 36:631-635.
Kudo, N.R., K. Wassmann, M. Anger, M. Schuh, K.G. Wirth, H. Xu, W. Helmhart, H. Kudo, M. McKay, B. Maro, J. Ellenberg, P. de Boer, and K. Nasmyth. 2006. Resolution of chiasmata in oocytes requires separase-mediated proteolysis. Cell. 126:135-146.
Kueng, S., B. Hegemann, B.H. Peters, J.J. Lipp, A. Schleiffer, K. Mechtler, and J.M. Peters. 2006. Wapl controls the dynamic association of cohesin with chromatin. Cell. 127:955-967.
Kurze, A., K.A. Michie, S.E. Dixon, A. Mishra, T. Itoh, S. Khalid, L. Strmecki, K. Shirahige, C.H. Haering, J. Lowe, and K. Nasmyth. 2011. A positively charged channel within the Smc1/Smc3 hinge required for sister chromatid cohesion. The EMBO journal. 30:364-378.
Lafont, A.L., J. Song, and S. Rankin. 2010. Sororin cooperates with the acetyltransferase Eco2 to ensure DNA replication-dependent sister chromatid cohesion. Proceedings of the National Academy of Sciences of the United States of America. 107:20364-20369.
Laloraya, S., V. Guacci, and D. Koshland. 2000. Chromosomal addresses of the cohesin component Mcd1p. The Journal of cell biology. 151:1047-1056.
Lavoie, B.D., E. Hogan, and D. Koshland. 2002. In vivo dissection of the chromosome condensation machinery: reversibility of condensation distinguishes contributions of condensin and cohesin. The Journal of cell biology. 156:805-815.
Lawrence, M.S., P. Stojanov, C.H. Mermel, J.T. Robinson, L.A. Garraway, T.R. Golub, M. Meyerson, S.B. Gabriel, E.S. Lander, and G. Getz. 2014. Discovery and saturation analysis of cancer genes across 21 tumour types. Nature. 505:495-501.
Lenart, P., M. Petronczki, M. Steegmaier, B. Di Fiore, J.J. Lipp, M. Hoffmann, W.J. Rettig, N. Kraut, and J.M. Peters. 2007. The small-molecule inhibitor BI 2536 reveals novel insights into mitotic roles of polo-like kinase 1. Current biology : CB. 17:304-315.

Lengronne, A., Y. Katou, S. Mori, S. Yokobayashi, G.P. Kelly, T. Itoh, Y. Watanabe, K. Shirahige, and F. Uhlmann. 2004. Cohesin relocation from sites of chromosomal loading to places of convergent transcription. Nature. 430:573-578.

Lengronne, A., J. McIntyre, Y. Katou, Y. Kanoh, K.P. Hopfner, K. Shirahige, and F. Uhlmann. 2006. Establishment of sister chromatid cohesion at the S. cerevisiae replication fork. Molecular cell. 23:787-799.

Lister, L.M., A. Kouznetsova, L.A. Hyslop, D. Kalleas, S.L. Pace, J.C. Barel, A. Nathan, V. Floros, C. Adelfalk, Y. Watanabe, R. Jessberger, T.B. Kirkwood, C. Hoog, and M. Herbert. 2010. Age-related meiotic segregation errors in mammalian oocytes are preceded by depletion of cohesin and Sgo2. Current biology : CB. 20:1511-1521.

Liu, H., S. Rankin, and H. Yu. 2013. Phosphorylation-enabled binding of SGO1-PP2A to cohesin protects sororin and centromeric cohesion during mitosis. Nature cell biology. 15:40-49.

Liu, J., and I.D. Krantz. 2008. Cohesin and human disease. Annual review of genomics and human genetics. 9:303-320.

Liu, J., and I.D. Krantz. 2009. Cornelia de Lange syndrome, cohesin, and beyond. Clinical genetics. 76:303-314.

Liu, L., and D.L. Keefe. 2008. Defective cohesin is associated with age-dependent misaligned chromosomes in oocytes. Reproductive biomedicine online. 16:103-112.

Losada, A., M. Hirano, and T. Hirano. 2002. Cohesin release is required for sister chromatid resolution, but not for condensin-mediated compaction, at the onset of mitosis. Genes & development. 16:3004-3016.

Losada, A., T. Yokochi, R. Kobayashi, and T. Hirano. 2000. Identification and characterization of SA/Scc3p subunits in the Xenopus and human cohesin complexes. The Journal of cell biology. 150:405-416.

Lu, S., M. Goering, S. Gard, B. Xiong, A.J. McNairn, S.L. Jaspersen, and J.L. Gerton. 2010. Eco1 is important for DNA damage repair in S. cerevisiae. Cell cycle. 9:3315-3327.

Matsuo, K., K. Ohsumi, M. Iwabuchi, T. Kawamata, Y. Ono, and M. Takahashi. 2012. Kendrin is a novel substrate for separase involved in the licensing of centriole duplication. Current biology : CB. 22:915-921.

McGuinness, B.E., T. Hirota, N.R. Kudo, J.M. Peters, and K. Nasmyth. 2005. Shugoshin prevents dissociation of cohesin from centromeres during mitosis in vertebrate cells. PLoS biology. 3:e86.

McLaughlin, E.A., and S.C. McIver. 2009. Awakening the oocyte: controlling primordial follicle development. Reproduction (Cambridge, England). 137:1-11.

Megee, P.C., C. Mistrot, V. Guacci, and D. Koshland. 1999. The centromeric sister chromatid cohesion site directs Mcd1p binding to adjacent sequences. Molecular cell. 4:445-450.

Mehta, G.D., R. Kumar, S. Srivastava, and S.K. Ghosh. 2013. Cohesin: functions beyond sister chromatid cohesion. FEBS letters. 587:2299-2312.

Mehta, G.D., S.M. Rizvi, and S.K. Ghosh. 2012. Cohesin: a guardian of genome integrity. Biochimica et biophysica acta. 1823:1324-1342.

Melby, T.E., C.N. Ciampaglio, G. Briscoe, and H.P. Erickson. 1998. The symmetrical structure of structural maintenance of chromosomes (SMC) and MukB proteins: long, antiparallel coiled coils, folded at a flexible hinge. The Journal of cell biology. 142:1595-1604.

Merriman, J.A., P.C. Jennings, E.A. McLaughlin, and K.T. Jones. 2012. Effect of aging on superovulation efficiency, aneuploidy rates, and sister chromatid cohesion in mice aged up to 15 months. Biology of reproduction. 86:49.

Michaelis, C., R. Ciosk, and K. Nasmyth. 1997. Cohesins: chromosomal proteins that prevent premature separation of sister chromatids. Cell. 91:35-45.

Misulovin, Z., Y.B. Schwartz, X.Y. Li, T.G. Kahn, M. Gause, S. MacArthur, J.C. Fay, M.B. Eisen, V. Pirrotta, M.D. Biggin, and D. Dorsett. 2008. Association of cohesin and Nipped-B with transcriptionally active regions of the Drosophila melanogaster genome. Chromosoma. 117:89-102.

Monnich, M., Z. Kuriger, C.G. Print, and J.A. Horsfield. 2011. A zebrafish model of Roberts syndrome reveals that Esco2 depletion interferes with development by disrupting the cell cycle. PloS one. 6:e20051.

Morgan, D.O. 2007. The Cell Cycle Principles of Control. New Science Press,, London.

Muller, H.J. 1918. Genetic Variability, Twin Hybrids and Constant Hybrids, in a Case of Balanced Lethal Factors. Genetics. 3:422-499.

Murayama, Y., and F. Uhlmann. 2013. Chromosome segregation: how to open cohesin without cutting the ring? The EMBO journal. 32:614-616.

Murdoch, B., N. Owen, M. Stevense, H. Smith, S. Nagaoka, T. Hassold, M. McKay, H. Xu, J. Fu, E. Revenkova, R. Jessberger, and P. Hunt. 2013. Altered cohesin gene dosage affects Mammalian meiotic chromosome structure and behavior. PLoS genetics. 9:e1003241.

Musacchio, A., and E.D. Salmon. 2007. The spindle-assembly checkpoint in space and time. Nature reviews. Molecular cell biology. 8:379-393.

Musio, A., A. Selicorni, M.L. Focarelli, C. Gervasini, D. Milani, S. Russo, P. Vezzoni, and L. Larizza. 2006. X-linked Cornelia de Lange syndrome owing to SMC1L1 mutations. Nature genetics. 38:528-530.
Nakamura, A., H. Arai, and N. Fujita. 2009. Centrosomal Aki1 and cohesin function in separase-regulated centriole disengagement. The Journal of cell biology. 187:607-614.
Nasmyth, K. 2005. How do so few control so many? Cell. 120:739-746.
Nasmyth, K., and C.H. Haering. 2009. Cohesin: its roles and mechanisms. Annual review of genetics. 43:525-558.
Nicklas, R.B., and C.A. Koch. 1969. Chromosome micromanipulation. 3. Spindle fiber tension and the reorientation of mal-oriented chromosomes. The Journal of cell biology. 43:40-50.
Nishiyama, T., R. Ladurner, J. Schmitz, E. Kreidl, A. Schleiffer, V. Bhaskara, M. Bando, K. Shirahige, A.A. Hyman, K. Mechtler, and J.M. Peters. 2010. Sororin mediates sister chromatid cohesion by antagonizing Wapl. Cell. 143:737-749.
Nozawa, R.S., K. Nagao, K.T. Igami, S. Shibata, N. Shirai, N. Nozaki, T. Sado, H. Kimura, and C. Obuse. 2013. Human inactive X chromosome is compacted through a PRC2-independent SMCHD1-HBiX1 pathway. Nature structural & molecular biology. 20:566-573.
Ocampo-Hafalla, M.T., and F. Uhlmann. 2011. Cohesin loading and sliding. Journal of cell science. 124:685-691.
Ohlsson, R., M. Bartkuhn, and R. Renkawitz. 2010. CTCF shapes chromatin by multiple mechanisms: the impact of 20 years of CTCF research on understanding the workings of chromatin. Chromosoma. 119:351-360.
Oliveira, R.A., R.S. Hamilton, A. Pauli, I. Davis, and K. Nasmyth. 2010. Cohesin cleavage and Cdk inhibition trigger formation of daughter nuclei. Nature cell biology. 12:185-192.
Oliveira, R.A., S. Kotadia, A. Tavares, M. Mirkovic, K. Bowlin, C.S. Eichinger, K. Nasmyth, and W. Sullivan. 2014. Centromere-independent accumulation of cohesin at ectopic heterochromatin sites induces chromosome stretching during anaphase. PLoS biology. 12.
Oliveira, R.A., and K. Nasmyth. 2013. Cohesin cleavage is insufficient for centriole disengagement in Drosophila. Current biology : CB. 23:R601-603.
Onn, I., and D. Koshland. 2011. In vitro assembly of physiological cohesin/DNA complexes. Proceedings of the National Academy of Sciences of the United States of America. 108:12198-12205.
Pandya, S., W.M. King, and R. Tawil. 2008. Facioscapulohumeral dystrophy. Physical therapy. 88:105-113.
Panizza, S., T. Tanaka, A. Hochwagen, F. Eisenhaber, and K. Nasmyth. 2000. Pds5 cooperates with cohesin in maintaining sister chromatid cohesion. Current biology : CB. 10:1557-1564.
Parelho, V., S. Hadjur, M. Spivakov, M. Leleu, S. Sauer, H.C. Gregson, A. Jarmuz, C. Canzonetta, Z. Webster, T. Nesterova, B.S. Cobb, K. Yokomori, N. Dillon, L. Aragon, A.G. Fisher, and M. Merkenschlager. 2008. Cohesins Functionally Associate with CTCF on Mammalian Chromosome Arms. Cell. 132:422-433.
Parish, J.L., J. Rosa, X. Wang, J.M. Lahti, S.J. Doxsey, and E.J. Androphy. 2006. The DNA helicase ChlR1 is required for sister chromatid cohesion in mammalian cells. Journal of cell science. 119:4857-4865.
Pauli, A., F. Althoff, R.A. Oliveira, S. Heidmann, O. Schuldiner, C.F. Lehner, B.J. Dickson, and K. Nasmyth. 2008. Cell-type-specific TEV protease cleavage reveals cohesin functions in Drosophila neurons. Developmental cell. 14:239-251.
Pauli, A., J.G. van Bemmel, R.A. Oliveira, T. Itoh, K. Shirahige, B. van Steensel, and K. Nasmyth. 2010. A direct role for cohesin in gene regulation and ecdysone response in Drosophila salivary glands. Current biology : CB. 20:1787-1798.
Peters, J.M., A. Tedeschi, and J. Schmitz. 2008. The cohesin complex and its roles in chromosome biology. Genes & development. 22:3089-3114.
Rankin, S., N.G. Ayad, and M.W. Kirschner. 2005. Sororin, a substrate of the anaphase-promoting complex, is required for sister chromatid cohesion in vertebrates. Molecular cell. 18:185-200.
Remeseiro, S., A. Cuadrado, M. Carretero, P. Martinez, W.C. Drosopoulos, M. Canamero, C.L. Schildkraut, M.A. Blasco, and A. Losada. 2012. Cohesin-SA1 deficiency drives aneuploidy and tumourigenesis in mice due to impaired replication of telomeres. The EMBO journal. 31:2076-2089.
Remeseiro, S., and A. Losada. 2013. Cohesin, a chromatin engagement ring. Current opinion in cell biology. 25:63-71.
Revenkova, E., M. Eijpe, C. Heyting, C.A. Hodges, P.A. Hunt, B. Liebe, H. Scherthan, and R. Jessberger. 2004. Cohesin SMC1 beta is required for meiotic chromosome dynamics, sister chromatid cohesion and DNA recombination. Nature cell biology. 6:555-562.
Revenkova, E., M.L. Focarelli, L. Susani, M. Paulis, M.T. Bassi, L. Mannini, A. Frattini, D. Delia, I. Krantz, P. Vezzoni, R. Jessberger, and A. Musio. 2009. Cornelia de Lange syndrome mutations in SMC1A or SMC3 affect binding to DNA. Human molecular genetics. 18:418-427.
Revenkova, E., K. Herrmann, C. Adelfalk, and R. Jessberger. 2010. Oocyte cohesin expression restricted to predictyate stages provides full fertility and prevents aneuploidy. Current biology : CB. 20:1529-1533.
Riedel, C.G., V.L. Katis, Y. Katou, S. Mori, T. Itoh, W. Helmhart, M. Galova, M. Petronczki, J. Gregan, B. Cetin,

I. Mudrak, E. Ogris, K. Mechtler, L. Pelletier, F. Buchholz, K. Shirahige, and K. Nasmyth. 2006. Protein phosphatase 2A protects centromeric sister chromatid cohesion during meiosis I. Nature. 441:53-61.
Rolef Ben-Shahar, T., S. Heeger, C. Lehane, P. East, H. Flynn, M. Skehel, and F. Uhlmann. 2008. Eco1-dependent cohesin acetylation during establishment of sister chromatid cohesion. Science. 321:563-566.
Rollins, R.A., M. Korom, N. Aulner, A. Martens, and D. Dorsett. 2004. Drosophila nipped-B protein supports sister chromatid cohesion and opposes the stromalin/Scc3 cohesion factor to facilitate long-range activation of the cut gene. Molecular and cellular biology. 24:3100-3111.
Rollins, R.A., P. Morcillo, and D. Dorsett. 1999. Nipped-B, a Drosophila homologue of chromosomal adherins, participates in activation by remote enhancers in the cut and Ultrabithorax genes. Genetics. 152:577-593.
Rowland, B.D., M.B. Roig, T. Nishino, A. Kurze, P. Uluocak, A. Mishra, F. Beckouet, P. Underwood, J. Metson, R. Imre, K. Mechtler, V.L. Katis, and K. Nasmyth. 2009. Building sister chromatid cohesion: smc3 acetylation counteracts an antiestablishment activity. Molecular cell. 33:763-774.
Salic, A., J.C. Waters, and T.J. Mitchison. 2004. Vertebrate shugoshin links sister centromere cohesion and kinetochore microtubule stability in mitosis. Cell. 118:567-578.
Sauer, G., R. Korner, A. Hanisch, A. Ries, E.A. Nigg, and H.H. Sillje. 2005. Proteome analysis of the human mitotic spindle. Molecular & cellular proteomics : MCP. 4:35-43.
Schmitz, J., E. Watrin, P. Lenart, K. Mechtler, and J.M. Peters. 2007. Sororin is required for stable binding of cohesin to chromatin and for sister chromatid cohesion in interphase. Current biology : CB. 17:630-636.
Schneider, A. 1873. Untersuchungen über Plathelminthen. J. Ricker, Giessen.
Schockel, L., M. Mockel, B. Mayer, D. Boos, and O. Stemmann. 2011. Cleavage of cohesin rings coordinates the separation of centrioles and chromatids. Nature cell biology. 13:966-972.
Schuldiner, O., D. Berdnik, J.M. Levy, J.S. Wu, D. Luginbuhl, A.C. Gontang, and L. Luo. 2008. piggyBac-Based Mosaic Screen Identifies a Postmitotic Function for Cohesin in Regulating Developmental Axon Pruning. Developmental cell. 14:227-238.
Sherwood, R., T.S. Takahashi, and P.V. Jallepalli. 2010. Sister acts: coordinating DNA replication and cohesion establishment. Genes & development. 24:2723-2731.
Sjögren, C., and K. Nasmyth. 2001. Sister chromatid cohesion is required for postreplicative double-strand break repair in Saccharomyces cerevisiae. Current Biology. 11:991-995.
Skibbens, R.V. 2004. Chl1p, a DNA helicase-like protein in budding yeast, functions in sister-chromatid cohesion. Genetics. 166:33-42.
Skibbens, R.V. 2011. Sticking a fork in cohesin--it's not done yet! Trends in genetics : TIG. 27:499-506.
Skibbens, R.V., L.B. Corson, D. Koshland, and P. Hieter. 1999. Ctf7p is essential for sister chromatid cohesion and links mitotic chromosome structure to the DNA replication machinery. Genes & development. 13:307-319.
Snider, L., L.N. Geng, R.J. Lemmers, M. Kyba, C.B. Ware, A.M. Nelson, R. Tawil, G.N. Filippova, S.M. van der Maarel, S.J. Tapscott, and D.G. Miller. 2010. Facioscapulohumeral dystrophy: incomplete suppression of a retrotransposed gene. PLoS genetics. 6:e1001181.
Sofueva, S., E. Yaffe, W.C. Chan, D. Georgopoulou, M. Vietri Rudan, H. Mira-Bontenbal, S.M. Pollard, G.P. Schroth, A. Tanay, and S. Hadjur. 2013. Cohesin-mediated interactions organize chromosomal domain architecture. The EMBO journal. 32:3119-3129.
Solomon, D.A., T. Kim, L.A. Diaz-Martinez, J. Fair, A.G. Elkahloun, B.T. Harris, J.A. Toretsky, S.A. Rosenberg, N. Shukla, M. Ladanyi, Y. Samuels, C.D. James, H. Yu, J.S. Kim, and T. Waldman. 2011. Mutational inactivation of STAG2 causes aneuploidy in human cancer. Science. 333:1039-1043.
Sonoda, E., T. Matsusaka, C. Morrison, P. Vagnarelli, O. Hoshi, T. Ushiki, K. Nojima, T. Fukagawa, I.C. Waizenegger, J.M. Peters, W.C. Earnshaw, and S. Takeda. 2001. Scc1/Rad21/Mcd1 is required for sister chromatid cohesion and kinetochore function in vertebrate cells. Developmental cell. 1:759-770.
Stemmann, O., D. Boos, and I.H. Gorr. 2005. Rephrasing anaphase: separase FEARs shugoshin. Chromosoma. 113:409-417.
Stemmann, O., H. Zou, S.A. Gerber, S.P. Gygi, and M.W. Kirschner. 2001. Dual inhibition of sister chromatid separation at metaphase. Cell. 107:715-726.
Strom, L., C. Karlsson, H.B. Lindroos, S. Wedahl, Y. Katou, K. Shirahige, and C. Sjogren. 2007. Postreplicative formation of cohesion is required for repair and induced by a single DNA break. Science. 317:242-245.
Strom, L., H.B. Lindroos, K. Shirahige, and C. Sjogren. 2004. Postreplicative recruitment of cohesin to double-strand breaks is required for DNA repair. Molecular cell. 16:1003-1015.
Strom, L., and C. Sjogren. 2005. DNA damage-induced cohesion. Cell cycle. 4:536-539.
Strunnikov, A.V., V.L. Larionov, and D. Koshland. 1993. SMC1: an essential yeast gene encoding a putative head-rod-tail protein is required for nuclear division and defines a new ubiquitous protein family. The Journal of cell biology. 123:1635-1648.

Sturtevant, A.H. 1919. Inherited linkage variations in the second chromosome. Carnegie Inst., Washington.
Sullivan, M., and D.O. Morgan. 2007. Finishing mitosis, one step at a time. Nature reviews. Molecular cell biology. 8:894-903.
Sumara, I., E. Vorlaufer, C. Gieffers, B.H. Peters, and J.M. Peters. 2000. Characterization of vertebrate cohesin complexes and their regulation in prophase. The Journal of cell biology. 151:749-762.
Sumara, I., E. Vorlaufer, P.T. Stukenberg, O. Kelm, N. Redemann, E.A. Nigg, and J.M. Peters. 2002. The dissociation of cohesin from chromosomes in prophase is regulated by Polo-like kinase. Molecular cell. 9:515-525.
Sutani, T., T. Kawaguchi, R. Kanno, T. Itoh, and K. Shirahige. 2009. Budding yeast Wpl1(Rad61)-Pds5 complex counteracts sister chromatid cohesion-establishing reaction. Current biology : CB. 19:492-497.
Tachibana-Konwalski, K., J. Godwin, L. van der Weyden, L. Champion, N.R. Kudo, D.J. Adams, and K. Nasmyth. 2010. Rec8-containing cohesin maintains bivalents without turnover during the growing phase of mouse oocytes. Genes & development. 24:2505-2516.
Tanaka, K., Z. Hao, M. Kai, and H. Okayama. 2001. Establishment and maintenance of sister chromatid cohesion in fission yeast by a unique mechanism. The EMBO journal. 20:5779-5790.
Tanaka, T., M.P. Cosma, K. Wirth, and K. Nasmyth. 1999. Identification of cohesin association sites at centromeres and along chromosome arms. Cell. 98:847-858.
Tang, Z., Y. Sun, S.E. Harley, H. Zou, and H. Yu. 2004. Human Bub1 protects centromeric sister-chromatid cohesion through Shugoshin during mitosis. Proceedings of the National Academy of Sciences of the United States of America. 101:18012-18017.
Taylor, C.F., F.M. Platt, C.D. Hurst, H.H. Thygesen, and M.A. Knowles. 2014. Frequent inactivating mutations of STAG2 in bladder cancer are associated with low tumour grade and stage and inversely related to chromosomal copy number changes. Human molecular genetics. 23:1964-1974.
Thota, S., A.D. Viny, H. Makishima, B. Spitzer, T. Radivoyevitch, B. Przychodzen, M.A. Sekeres, R.L. Levine, and J.P. Maciejewski. 2014. Genetic alterations of the cohesin complex genes in myeloid malignancies. Blood.
Tomkins, D., A. Hunter, and M. Roberts. 1979. Cytogenetic findings in Roberts-SC phocomelia syndrome(s). American journal of medical genetics. 4:17-26.
Tonkin, E.T., T.J. Wang, S. Lisgo, M.J. Bamshad, and T. Strachan. 2004. NIPBL, encoding a homolog of fungal Scc2-type sister chromatid cohesion proteins and fly Nipped-B, is mutated in Cornelia de Lange syndrome. Nature genetics. 36:636-641.
Toth, A., R. Ciosk, F. Uhlmann, M. Galova, A. Schleiffer, and K. Nasmyth. 1999. Yeast cohesin complex requires a conserved protein, Eco1p(Ctf7), to establish cohesion between sister chromatids during DNA replication. Genes & development. 13:320-333.
Tsou, M.F., and T. Stearns. 2006. Mechanism limiting centrosome duplication to once per cell cycle. Nature. 442:947-951.
Tsutsumi, M., R. Fujiwara, H. Nishizawa, M. Ito, H. Kogo, H. Inagaki, T. Ohye, T. Kato, T. Fujii, and H. Kurahashi. 2014. Age-related decrease of meiotic cohesins in human oocytes. PloS one. 9:e96710.
Uhlmann, F., F. Lottspeich, and K. Nasmyth. 1999. Sister-chromatid separation at anaphase onset is promoted by cleavage of the cohesin subunit Scc1. Nature. 400:37-42.
Uhlmann, F., and K. Nasmyth. 1998. Cohesion between sister chromatids must be established during DNA replication. Current biology : CB. 8:1095-1101.
Uhlmann, F., D. Wernic, M.A. Poupart, E.V. Koonin, and K. Nasmyth. 2000. Cleavage of cohesin by the CD clan protease separin triggers anaphase in yeast. Cell. 103:375-386.
Unal, E., J.M. Heidinger-Pauli, W. Kim, V. Guacci, I. Onn, S.P. Gygi, and D.E. Koshland. 2008. A molecular determinant for the establishment of sister chromatid cohesion. Science. 321:566-569.
Ünal, E., J.M. Heidinger-Pauli, and D. Koshland. 2007. DNA Double-Strand Breaks Trigger Genome-Wide Sister-Chromatid Cohesion Through Eco1 (Ctf7). Science. 317:245-248.
van der Lelij, P., K.H. Chrzanowska, B.C. Godthelp, M.A. Rooimans, A.B. Oostra, M. Stumm, M.Z. Zdzienicka, H. Joenje, and J.P. de Winter. 2010. Warsaw breakage syndrome, a cohesinopathy associated with mutations in the XPD helicase family member DDX11/ChlR1. American journal of human genetics. 86:262-266.
van der Maarel, S.M., and R.R. Frants. 2005. The D4Z4 repeat-mediated pathogenesis of facioscapulohumeral muscular dystrophy. American journal of human genetics. 76:375-386.
van Heemst, D., F. James, S. Poggeler, V. Berteaux-Lecellier, and D. Zickler. 1999. Spo76p is a conserved chromosome morphogenesis protein that links the mitotic and meiotic programs. Cell. 98:261-271.
Vaur, S., A. Feytout, S. Vazquez, and J.P. Javerzat. 2012. Pds5 promotes cohesin acetylation and stable cohesin-chromosome interaction. EMBO reports. 13:645-652.

Vega, H., Q. Waisfisz, M. Gordillo, N. Sakai, I. Yanagihara, M. Yamada, D. van Gosliga, H. Kayserili, C. Xu, K. Ozono, E.W. Jabs, K. Inui, and H. Joenje. 2005. Roberts syndrome is caused by mutations in ESCO2, a human homolog of yeast ECO1 that is essential for the establishment of sister chromatid cohesion. Nature genetics. 37:468-470.

Waizenegger, I.C., S. Hauf, A. Meinke, and J.M. Peters. 2000. Two distinct pathways remove mammalian cohesin from chromosome arms in prophase and from centromeres in anaphase. Cell. 103:399-410.

Wassarman, P.M. 2002. Channels of communication in the ovary. Nature cell biology. 4 Suppl:s7-9.

Weitzer, S., C. Lehane, and F. Uhlmann. 2003. A model for ATP hydrolysis-dependent binding of cohesin to DNA. Current biology : CB. 13:1930-1940.

Wendt, K.S., K. Yoshida, T. Itoh, M. Bando, B. Koch, E. Schirghuber, S. Tsutsumi, G. Nagae, K. Ishihara, T. Mishiro, K. Yahata, F. Imamoto, H. Aburatani, M. Nakao, N. Imamoto, K. Maeshima, K. Shirahige, and J.M. Peters. 2008. Cohesin mediates transcriptional insulation by CCCTC-binding factor. Nature. 451:796-801.

Whelan, G., E. Kreidl, G. Wutz, A. Egner, J.M. Peters, and G. Eichele. 2012. Cohesin acetyltransferase Esco2 is a cell viability factor and is required for cohesion in pericentric heterochromatin. The EMBO journal. 31:71-82.

Wong, R.W., and G. Blobel. 2008. Cohesin subunit SMC1 associates with mitotic microtubules at the spindle pole. Proceedings of the National Academy of Sciences of the United States of America. 105:15441-15445.

Yoon, P.W., S.B. Freeman, S.L. Sherman, L.F. Taft, Y. Gu, D. Pettay, W.D. Flanders, M.J. Khoury, and T.J. Hassold. 1996. Advanced maternal age and the risk of Down syndrome characterized by the meiotic stage of chromosomal error: a population-based study. American journal of human genetics. 58:628-633.

Yun, Y., S.I. Lane, and K.T. Jones. 2014. Premature dyad separation in meiosis II is the major segregation error with maternal age in mouse oocytes. Development. 141:199-208.

Zeng, W., J.C. de Greef, Y.Y. Chen, R. Chien, X. Kong, H.C. Gregson, S.T. Winokur, A. Pyle, K.D. Robertson, J.A. Schmiesing, V.E. Kimonis, J. Balog, R.R. Frants, A.R. Ball, Jr., L.F. Lock, P.J. Donovan, S.M. van der Maarel, and K. Yokomori. 2009. Specific loss of histone H3 lysine 9 trimethylation and HP1gamma/cohesin binding at D4Z4 repeats is associated with facioscapulohumeral dystrophy (FSHD). PLoS genetics. 5:e1000559.

Zhang, J., X. Shi, Y. Li, B.J. Kim, J. Jia, Z. Huang, T. Yang, X. Fu, S.Y. Jung, Y. Wang, P. Zhang, S.T. Kim, X. Pan, and J. Qin. 2008. Acetylation of Smc3 by Eco1 is required for S phase sister chromatid cohesion in both human and yeast. Molecular cell. 31:143-151.

8 APPENDIXES

8.1 Protein gels

Table 8.1: Protein Gels' recipe. Resolving gels were made at 10% acrylamide and stacking at 4%.

RESOLVING GEL	10%	STACKING GEL	4%
Protogel (30%)	3.3 ml	**Protogel (30%)**	650 ul
Tris pH 8.8 (1M)	3.75 ml	**Tris pH 6.8 (1M)**	625 ul
MILIQ WATER	2.85 ml	**MiliQ water**	3.675 ml
SDS (10%)	100 ul	**SDS (10%)**	50 ul
APS (10%)	50 ul	**APS (10%)**	25 ul
TEMED	5 ul	**TEMED**	5 ul
TOTAL (2 GELS)	**10 ml**	**Total (2 gels)**	**5 ml**

8.2Buffer solutions

- **10X Running Buffer**

30,3g Tris

143g glycine

10g SDS

Fill with H_2O up to 1L, autoclave and store at room temperature.

- **10X Transfer Buffer**

116,3g tris

58,65g glycine

Fill with H_2O up to 2L, autoclave and store at room temperature.

- **10X PBS at pH 7.4**

80g NaCl

2g KCl

14,4g Na_2HPO4

2,4g KH_2PO_4

Adjust pH to 7.4; H_2O up to 1L and autoclave and store at room temperature.

- **100x PBS-Tween**

50mL Tween20

950mL PBS 1x

- **1x PBS-Tween**

10mL from 100x PBS-Tween

100mL from 10x PBS

Fill with H_2O until 1L.

8.3 RIPA lysis buffer

RIPA lysis buffer (10mM Tris pH =7,5; 150 mM NaCl, 0,1% SDS, 1% Triton-X, 1% Deoxycholate, 5mM EDTA and 1 Protease Inhibitor Cocktail EDTA-free tablet (Roche) per 10 ml of buffer.

8.4 TEV buffer

50mM TRIS
0,5 mM EDTA
1mM DTT

8.5Cohesin subunits in different species

Table 8.2 Cohesin subunits and its regulatory factors. Nomenclature of cohesin and its associted proteins through different species.

Cohesin subunits and its regulatory factors

	S. cerevisiae	*S. pombe*	*D. melanogaster*	*X. laevis*	*H. sapiens / M. musculus*
SMC	Smc1 Smc3	Psm1 Psm3	Smc1 Smc3	Smc1 Smc3	Smc1α Smc1β Smc3
α•Kleisin	Scc1/Mcd1 Rec8	Rad21 Rec8	Rad21 C(2)M	Rad21	Scc1/Rad21 Rad21L, Rec8
α•Kleisin interacting subunits	Scc3	Psc3 Rec11	SA	SA1, SA2	SA1/Stag1, SA2/Stag2 SA3/Stag3
Regulatory factors	Pds5 Rad61/Wapl -	Pds5 Wapl -	Pds5 Wapl Dalmatian	Pds5A, Pds5B Wapl Sororin	Pds5A, Pds5B/APRIN Wapl/Wapal Sororin
Cohesin loading complex	Scc4	Mis4 Ssl3	Nipped-B Scc4	Scc2 Scc4	Nipbl/Scc2 Mau2/Scc4
Cohesin Acetyl Transferases (CoATs)	Eco1/Ctf7	Eso1	Deco, San	Esco1, Esco2	Esco1, Esco2
Cohesin Deacetylases (CoDACs)	Hos1	-	-	-	HDAC8

Proteins in red correspond to meiotic isoforms.

8.6 Recombination example

Genetic recombination is the process by which two DNA molecules exchange genetic information, producing a new recombination of alleles. In *Drosophila,* recombination happens only in females.

Furthermore, fly genetics often makes use of multiple inversions present on a balancer chromosome (called balancers) that suppress recombination over most of their length (Altenburg and Muller, 1920; Muller, 1918). Chromosomal inversions suppress recombination in regions where homologous chromosomes are not collinear (Sturtevant, 1919). Moreover, balancers generally carry at least one dominant visible marker and are lethal in homozygotes, causing them to be maintained in a heterozygous state (Araye and Sawamura, 2013).

We wanted to put in the some chromosome two copies of Rad21, one not resistant (1357, $Rad21^{wt\text{-}EGFP}$) and one sensitive (813, $Rad21^{TEV}$) to TEV, in the III chromosome. Red triangles represents 1357 and 813 black ones. The circles represent a genetic marker and a balancer with other genetic marker, but for the simplification of the scheme both are represented with the same figure.

Males and females were crossed from the two strains of interest (step 1). Virgin females originated from this cross are then crossed with male flies that just contain balancer and marker chromosomes (circle) (step 2). Genetic recombination takes place during oogenesis of these females. As a result, each gamete results from a single recombination event. Given that each transgene carries the white gene (red eye colour) in addition to the desired insert, recombinants can be selected based on eye colour. The progeny will present different colour ranges depending on the recombination that occurs. The desired chromosome contains two insertions (and hence two white genes) and therefore the darkest eyed males will be the ones to be selected (step 2).

That selected male is then crossed with multiple virgins females in order to give a progeny where the selection of flies carrying the recombinant chromosome and a balancer (step 3) can be made. This last cross forms a stable stock given that the presence of the balancer ensures the recombination will not be lost.

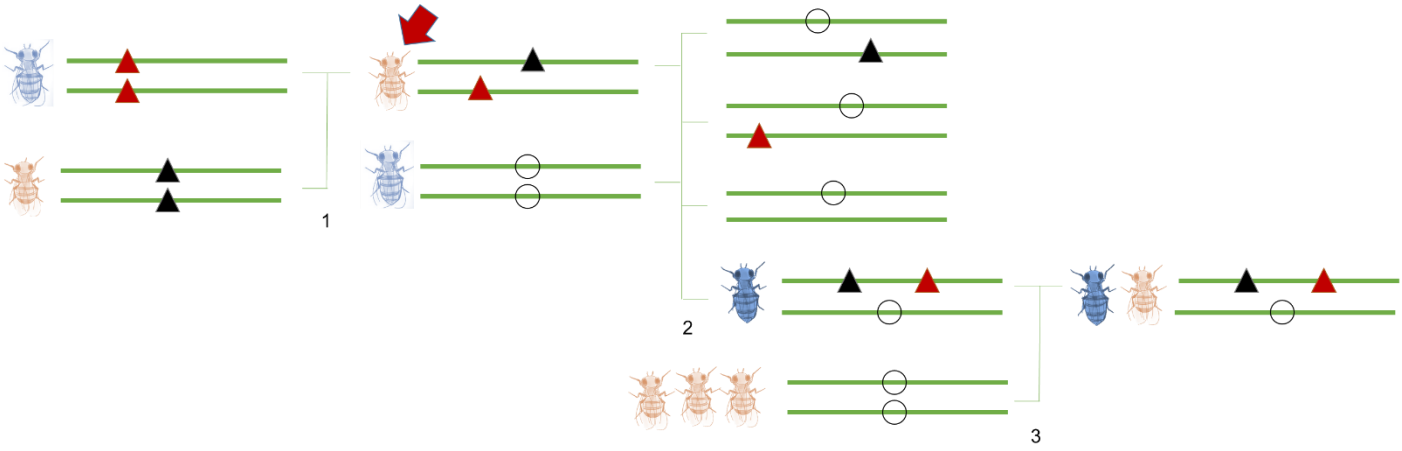


Figure 8.1 Recombination scheme. Recombination will occur in the female with the arrow , that by multiple selections (eye colour here represented as the darker blue male) will give rise to a stable stock in the end.