# Maintenance of metaphase chromosome architecture by condensin I

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*Cover:* Handmade knitted metaphase chromosomes accompanied by an interphase chromatin represented by entangled yarn threads. The background contains a microscopy image of a live mitotic *Drosophila* embryo; chromatin is marked by histone-RFP (orange) and centromeres by CID-EGFP (cyan). Knitting pattern of chromosomes can be found in Appendix 11.
*Graphics authors: Ewa Piskadło and Raquel A. Oliveira*

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***List of publications:***
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***Declaration:*** This dissertation is a result of my own research carried out between July 2013 and July 2017 in the laboratory of Dr. Raquel A. Oliveira, Instituto Gulbenkian de Ciência in Oeiras, Portugal, within the PhD Programme in Integrative Biomedical Sciences (edition 2013).

***Declaração:*** Esta dissertação é o resultado do meu próprio trabalho desenvolvido entre Julho 2013 e Juhlo 2017 no laboratório do Doutora Raquel A. Oliveira, Instituto Gulbenkian de Ciência em Oeiras, Portugal, no âmbito do Programa de Doutoramento em Integrative Biomedical Sciences (edição 2013).

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## Summary

FAITHFUL segregation of the genome into two daughter cells is one of the most fundamental events for every living organism. In each round of the cell cycle, cells need to orchestrate a sequence of complex steps to replicate their genetic material, pack it neatly into mitotic chromosomes and perform their precise separation when all the prerequisites are met. One of the most fascinating questions in biology is to understand the internal organization of mitotic chromosomes. Even though mitotic chromosomes were first described around 140 years ago, how exactly interphase DNA molecules are packed to become mitotic chromosomes is still a mystery. Despite the lack of precise details about chromosome condensation mechanisms, it is believed that in the heart of this process lies a group of protein complexes called condensins. The mechanism by which condensins are able to enforce or guide the condensation process is yet unknown. In this thesis, we will present our advances in understanding condensin's function in maintaining mitotic chromosome compaction and internal architecture.

Condensin's role in mitosis was extensively studied using mutants for its subunits or by slow depletion approaches. Those methods were nonetheless not precise or fast enough to permit accurate studies of condensin's role in maintaining chromosome's structure. In the search for an acute tool that would allow decisive studies of fast processes, such as specific stages of mitosis, we have developed a *Drosophila melanogaster* system for condensin I inactivation based on Tobacco Etch Virus (TEV) protease cleavage. The steps performed to build this system are the subject of Chapter 2. We show that it is possible to inactivate condensin I in the context of a developing organism with great efficiency and time control.

Having generated a tool to study condensin I with an unprecedented temporal resolution, we have endeavored to explore condensin I's role in the maintenance of metaphase chromosome architecture, as described in Chapter 3. Based on our data we propose that condensin I works in collaboration with topoisomerase II constantly throughout mitosis to ensure a correct amount of links between DNA molecules. Removing functional condensin I breaks this balance resulting in an increased number of erroneous entanglements introduced *de novo* by topoisomerase II. Such entanglements increase chromatin density leading to hyper-

compaction of chromosomes during metaphase. At the end, we discuss those results in the context of proposed models of condensin I actions, especially its cooperation with topoisomerase II, and in the broader context of chromatin dynamics.

Mitotic chromosomes are constantly subjected to various forces acting on them. Chapter 4 contains preliminary data showing that soon after the destruction of the mitotic spindle, scattered chromatids rapidly congress back together. These findings suggest that other factors besides the mitotic spindle can arrange the location of the chromosomes. We hypothesize that such inwards forces may influence the surfaces of chromosomes, which can exacerbate the overcompaction phenotype described in Chapter 3, observed after inactivation of condensin I. We further speculate what factors could cause the observed phenomena.

In this thesis, we explore condensin I's role in mitosis using a novel system for condensin I inactivation in *Drosophila* embryos. We propose that condensin I governs the physical properties of chromosomes and their internal structure by imposing control over the amount of inter-DNA intertwines throughout mitosis. We thus uncover a fascinating highly controlled dynamics of the chromosome catenation state and provide new knowledge valuable for the full understanding of mitotic chromosome condensation and architecture.

# Sumário

A correcta segregação do genoma recém-duplicado para ambas as células filhas é um evento fudamental para qualquer organismo vivo. Em cada ciclo celular, as células têm de coordenar uma sequência bem estabelecida de eventos complexos que lhes permitem realizar a replicação do DNA, a compactação do mesmo em cromosomas mitóticos e a sua separação precisa. Cada um destes eventos é supervisionado por mecanismos de controlo que apenas permitem a passagem ao próximo passo após todos os pré-requisitos terem sido garantidos.

Uma das questões mais fascinantes em biologia é comprender a organização interna dos cromossomas mitóticos. De facto, os cromossomas mitóticos foram descritos há cerca de 140 anos, mas o mecanismo exacto que permite ás células compactarem o seu material genético em cromossomas durante a mitose continua a ser desconhecido. Existe um grupo de proteínas que é fundamental para este processo, as condensinas, no entanto o exacto modo de acção destas proteínas na promoção e/ou manutenção da condensação do material genético está ainda por esclarecer. Nesta dissertação serão descritos os avanços que fizemos para a compreensão do papel da condensina I na manutenção da estrutura interna e compactação dos cromosomas em mitose. Tradicionalmente, os estudos da função da condensina foram feitos recorrendo a mutantes para as diferentes subunidades da proteína ou por deplecção da mesma por períodos longos, contudo esta abordagem não permite ter resolução temporal suficiente para investigar de forma precisa o papel da condensina. Para colmatar esta limitação, desevolvemos um sistema de inactivação da condensina I baseado na clivagem da proteína pela protease TEV (Tobacco Etch Virus). Este sistema, cujo desenvolvimento e implementação constam no Capítulo 2, tem a vantagem de permitir inactivar rapidamente a condensina I nos diferentes momentos da mitose. A resolução temporal sem precendentes, é assim decisiva para compreender a função da proteína em processos que são naturalmente rápidos.

Para além da rapidez na inactivação da condensina, demonstramos ainda que o sistema funciona no contexto de desenvolvimento dum organismo com grande eficiência. Após validação do nosso sistema, utilizámos esta abordagem para explorar a função da condensina na manutenção da arquitectura dos cromosomas

em mitose, tal como descrito no Capítulo 3. Com base nos nossos resultados, propomos que a condensina I actua em conjunto com a topoisomerase II de forma constante durante a mitose para assegurar a quantidade certa de ligações entre as moléculas de DNA. Ao remover funcionalmente a condensina I, verifica-se um desequilíbrio entre as duas proteínas o que resulta no aumento dos emaranhados no DNA introduzidos de novo pela topoisomerase II. Estas ligações promovem o aumento da densidade da cromatina o que provoca uma hipercondensação dos cromossomas durante a metafase. No final, discutimos estes resultados no contexto dos modelos propostos para o mecanismo de acção da condensina I., especificamente no que diz respeito á sua cooperação com a topoisomerase II e no contexto mais amplo da dinâmica da cromatina.

Os cromosomas mitóticos estão constantemente sujeitos á acção de várias forças. No Capítulo 4 constam resultados perliminares que mostram que logo após a destruição do fuso mitótico, as cromátides mitóticas, já em anafase, voltam a convergir para o centro da célula. Esta observação sugere que a localização dos cromosomas/cromátides em mitose é determinada por outros factores para além do fuso mitótico. Perante isto, formulámos a hipótese que estas forças em direcção ao centro influenciam a superfície dos cromossomas o que pode exacerbar o fenótipo de hiper-compactação observada após inactivação da condensina I, descrito no Capítulo 3. Adicionalmente, especulamos sobre outros factores que estão potencialmente envolvidos neste fenómeno.

Em conclusão, neste trabalho exploramos o papel da condensina I em mitose usando para isso um sistema novo, rápido e eficaz para a inactivação da proteína em embriões de Drosophila. Pela interpretação dos nossos resultados propomos que a condensina I regula as propriedades físicas e estrutura interna dos cromosomas através do controlo da quantidade de emaranhados nas moléculas de DNA durante a mitose. Este estudo permitiu desvendar que a dinâmica do estado de catenação dos cromossomas é um processo altamente controlado e fascinante. Contribuímos assim com novo e fundamental conhecimento para a compreensão da condensação e arquitetura dos cromossomas em mitose.

# Contents

# Chapter 1

# General introduction

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## 1.1 Cell cycle and mitosis

### 1.1.1 Cell cycle

The cell cycle is a highly conserved and ordered process. It allows the creation of a genetically identical copy of a cell and is the basis of cell multiplication, growth, and differentiation into specialized units. Most simply, cell cycle can be divided into two parts – mitosis, when genome segregation takes place, and interphase, which is typically the longest part of cell cycle. During interphase, cell's functions are focused on growth, metabolizing nutrients and producing all the necessary proteins needed to sustain life. In multicellular organisms, cells in interphase perform their specialized functions in the context of the whole organism. The progression through all the stages of cell cycle is under control of protein regulators, mostly cyclins and cyclin-dependent kinases that synchronize the processes and help to perform quality control over the events, activating checkpoint mechanisms in case of disruptions to prevent faulty division. Interphase period comprises few consecutive phases that are focused on cell vitality functions and preparing the genome for the subsequent division. Just after the previous division cells enter into G1 phase (or G0, if they halt their proliferation permanently or temporarily) to intensively grow, rearrange organelles, increase transcription and translation in preparation of next stages. Once the quality conditions are met, cells enter the S phase. Then each molecule of DNA is copied exactly once via a semiconservative mechanism and cell cycle moves to G2 phase. In this phase cell resumes intensive metabolic activity and growth and mitochondria need to supply enough energy for mitotic division. G2 phase is followed by entry to mitotic division and creation of two daughter cells, each starting its own new cell cycle.

### 1.1.2 Mitosis

Mitosis has been first described in the 19th century and has captivated generations of scientists ever since. This fascinating process comprises the assembly of interphase chromatin into individual chromosomes and subsequently the equal separation of the genetic material between two daughter cells. Mitosis is undoubtedly an extremely complex operation that needs to be precisely conducted and controlled under the penalty of dismantling genome integrity. Mitosis can be divided into few stages. The first one – called prophase – is when chromatin begins to condense and rearrange to form compacted, rod-shaped chromosomes. At the same time sister chromatids begin their resolution into separate units (Nagasaka et al. 2016). Around the nucleus microtubules are reorganized to form a network of microtubules originating from two centrosomes (or microtubule organizing centres) that move towards opposite poles of the cell to form the mitotic spindle. Later in prophase the nuclear envelope formed around eukaryotic nucleus is disintegrated in a process known as nuclear envelope breakdown (NEBD). Chromosomes are then captured and bioriented by microtubules from the opposite poles in a process called 'search and capture' (Heald and Khodjakov 2015). In the next stage, metaphase, chromosomes reach almost the full condensation and are captured by the microtubules and the correct, bioriented attachments are stabilized (Sarangapani and Asbury 2014). This causes all chromosomes of the cell to be aligned on the so called metaphase plate, which is a plane equidistant to both centrosomes. Such arrangement is able to trigger anaphase stage, in which chromosomes reach their maximal compaction and when sister chromatids separate, allowing microtubules to segregate DNA molecules to the opposite poles (Kamenz and Hauf 2017). In the final stage of mitosis, telophase, nuclear envelope is reformed around two freshly separated sets of chromosomes reconstituting nuclei and cytokinesis is triggered to separate the mother cell into two entities (Hetzer 2010).

## 1.2 Architecture of mitotic chromosomes

Mitotic chromosomes are striking structures in a cell and were of the first described already in the 19th century. Mitotic chromosome assembly, although

poorly understood at the molecular level, fulfils three major tasks essential for faithful chromosome segregation: Firstly, it ensures chromosome compaction, making cell division feasible within the cell space. Secondly, it provides chromosomes with the right mechanical properties (e.g. bendiness and rigidity) to facilitate their drastic movements during mitosis. Lastly, it ensures the resolution of the topological constrains that exist between the two sister DNA molecules, as well as between neighbouring chromosomes (chromosome individualization), a key requisite for efficient chromosome partitioning. Despite the utmost importance of chromosome condensation for the fidelity of mitosis, the molecular mechanisms that drive this process remain very unclear.

### 1.2.1 Morphology of mitotic chromosomes

Chromatin is a structure composed of DNA and various proteins and RNAs interacting with it. To ensure that DNA molecules fit inside of a human interphase nucleus, they need to be compacted 200-1000 times compared to their stretched, linear length (Lawrence et al. 1990). Importantly the condensation in interphase cannot be too restrictive to permit access to transcriptional, replication and regulatory sites, allowing cell to perform its genetic program. The first level of compaction is wrapping the DNA around histones to form nucleosomes. Histones are extremely conserved proteins and they have many variants (Biterge and Schneider 2014). Some of them, so called core histones H2A, H2B, H3, and H4, form an octamers around which DNA is wrapped 1.67 times in a left-handed turn. Regions of DNA between octamers and bound to H1 histone to stabilize the nucleosome structure. This basic nucleosome strand can be further compacter to reach desired compaction. Modifications of histone post-translational modifications helps regulating local compaction of the chromatin (Bowman and Poirier 2015; Wilkins et al. 2014; Kruitwagen et al. 2015). Also specific histones mark certain regions of chromatin to change properties of chromatin, such as CENP-A binding to centromeres to allow kinetochore assembly.

To ensure that cell division is feasible within the cell space, vertebrate cells compact their DNA around 2-3 times more than in interphase, as estimated by chromatin volume measurements (Martin and Cardoso 2010; Mora-Bermudez et al. 2007) and Förster resonance energy transfer (FRET)-based assays between

histones (Llères et al. 2009). Spatial compaction, however, is not the only important outcome of condensation. The structural reorganization during condensation leads to the separation of the identical sister chromatids from each other (known as sister chromatid resolution). Several topological constrains arise throughout interphase (most notably during DNA replication) that result in the entanglement of the two DNA molecules. The resolution of such intertwines (i.e., individualization) is crucial for efficient and faithful chromosome segregation during mitosis. Condensation of chromatin into sturdy chromosomes is also necessary to establish proper physical properties. Chromosomes must be stiff, resilient and elastic enough to withstand forces coming from pulling microtubules and cytoplasmic drags during mitosis to prevent damage and breaks caused by external tensions.

Centromeres, morphologically visible as constrictions in the X-shaped chromosomes, are specialized regions of chromosomes to which sister chromatids are connected until anaphase in majority of animals. They also allow the assembly of the kinetochore, a proteinaceous structure to which microtubules are binding during mitosis and are crucial for successful chromosome segregation. Centromeres are enriched in $\alpha$-sequences and a specific variants of histone, CENP-A, a variant of core histone H3 (Schalch and Steiner 2017). Most importantly, centromeres constitute a chromatin scaffold on which kinetochores assemble in order to anchor spindle microtubules to chromosomes (Nagpal and Fukagawa 2016). Kinetochores are complex structures that comprise of multiple proteins of various functions, such as structural (i.e. CENP-B), motor (i.e. dynein) or checkpoint proteins (ie. Mad2, BubR1) (Nagpal and Fukagawa 2016). The main function of kinetochores is to ensure polarity of the division and ensuring biorientation of chromosomes before segregation and transmitting dragging forces to chromatids once separation occurs.

### 1.2.2 Models of mitotic chromosome folding

Over the past decades detailed characterization of metaphase chromosomes, using different cytological approaches, has led to the proposal of several models for mitotic chromosome assembly (Figure 1.1).

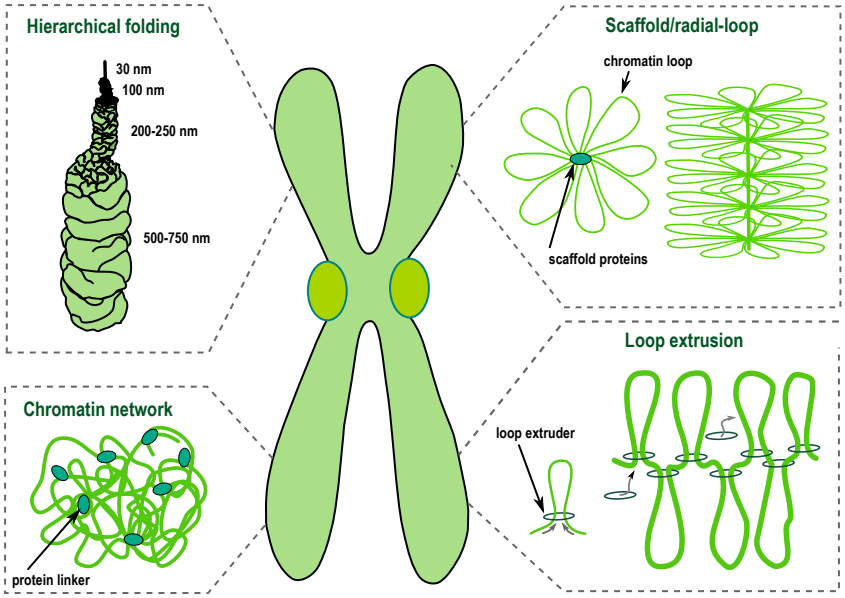



**Fig. 1.1. Schematic represenation of several possible models of mitotic chromosome folding.**

### Hierarchical folding

Classical views on chromosome organization postulate that mitotic chromosomes result from chromatin fibre folding. DuPraw suggested that fibre folding occurs randomly, transversely and longitudinally, with no intermediate levels of compaction (DuPraw 1966). However, mitotic chromosomes fold into a reproducible structure in every mitosis, at least to some extent. Mitotic chromosomes acquire a reproducible length and display an invariable signature pattern of bands after staining with specific dyes, such as Giemsa. Moreover, specific DNA sequences occupy a reproducible position along the longitudinal and transverse axes of the chromosome (Baumgartner et al. 1991). Although some degree of randomness was observed within chromosomal domains (Dietzel and Belmont 2001; Strukov and Belmont 2009), chromosome assembly cannot be explained as a purely random process.

Alternatively, it has been suggested that metaphase chromosomes result from helical coiling events (helical-coiling model). The nucleo-histone fibre is proposed to be coiled up into a helix which is hierarchically wound up into larger

helices to achieve the compactness of the mitotic chromosome (Sedat and Manuelidis 1978; Belmont 1987). This model has been widely accepted as lower levels of chromatin organization were long postulated to result from hierarchical folding: wrapping of DNA around nucleosomes forms an 11-nm bead-on-a-string structure that coils up into a 30nm fibre. However, the existence of 30 nm fibre *in vivo* is yet to be confirmed and has been recently highly debated(Maeshima et al. 2011; Joti et al. 2012; Razin and Gavrilov 2014). A strong argument against existence of an ordered hierarchical architecture of mitotic chromosomes was recently presented using a ChromEMT method. This approach merges electron microscopy tomography imaging and special labelling enhancing the DNA contrast, combined with mild treatment to preserve native structure of chromatin, in contrast to standard electron microscopy assays (Ou et al. 2017). High resolution imaging of human epithelial cells using this method failed to uncover any signs of discrete higher-order chromatin fibres. The only motif found was unordered flexible chains of various length and 5- to 24 nm in diameter, that are packed to different density depending of the cell cycle stage, with the highest packing density reached in mitosis.

### Scaffold model

Using EM studies, Paulson and Laemmli (Paulson and Laemmli 1977) brought a novel view on chromosome organization. Upon histone removal, chromosomes revealed a scaffold or core that has the shape of intact chromosomes, surrounded by loops of chromatin attached to this central core (Adolph et al. 1977; Earnshaw 1983). These and subsequent studies lead to the consolidation of the scaffold/radial-loop model which argues that radial DNA loops extend out from a protein element or scaffold positioned along the central axis of the chromatid. However, this model has been challenged by studies that evaluate the components for chromosome continuity (see below). Moreover, major components of the chromosome scaffold were shown to display a highly dynamic association with mitotic chromatin (Gerlich et al. 2006; Oliveira, Heidmann, et al. 2007; Christensen et al. 2002; Tavormina et al. 2002), arguing against the existence of a stiff schaffold anchoring DNA loops.

### Chromatin network model

Analysis of the biophysical properties of mitotic chromosome has challenged the idea that the continuity of mitotic chromosomes depends on its proteinaceous core, in contrast to what the chromosome scaffold would predict. Taking advantage of the highly elastic behavior displayed by mitotic chromosomes, *in vitro* elasticity measurements revealed that the elastic response of mitotic chromosomes is lost after DNA digestion (Poirier and Marko 2002). Mild protease treatment, in contrast, does not impair a reversible elastic response, despite a progressively reduced force constant (Poirier and Marko 2002; Pope et al. 2006). This led to the proposal of the chromatin-network model in which chromatin itself is proposed to be the mechanical contiguous component of the mitotic chromosome.

### Loop extrusion

Loop extrusion is a relatively new model of how mitotic chromosomes can compact and be organized. It can be viewed as a specific variant of chromatin network model in some aspects and it has rapidly gained a great recognition in the chromosome field. The general idea is that instead of chromosome loops being anchored to a stiff scaffold in the chromosomal axis, the loops are generated by constant, dynamic pulling of DNA through a specialized ring-like motor proteins that cause organization and compaction at the same time. This model first emerged in 1990 as 'DNA reeling mechanism' to explain proposed existence of loop-based organization of chromosomes (Riggs 1990). Later, the idea was raised by several researchers who pointed at SMC complexes (namely cohesin and condensin) to be possible loop extruding factors (Nasmyth 2001; Alipour and Marko 2012). In this model mitotic chromosomes would be loop-based structures. In contrast to a standard loop/scaffold model, loop extrusion-based condensation does not require any stiff scaffold, and the loops would be very dynamic, regulated by loop extrusion protein factors. The loop extrusion was shown in polymer dynamics models to be sufficient to explain mitotic chromosome compaction and individualization of even a mammalian-sized chromosomes (Goloborodko, Marko, et al. 2016; Goloborodko, Imakaev, et al. 2016; Naumova et al. 2013; Alipour and Marko 2012; Fudenberg et al. 2016).

#### Other models of chromosome folding

More recent ideas for the internal folding of chromosomes have that mitotic chromosomes are arranged into stacks of 6nm layers (Daban 2015). Those layers would be perpendicular to the chromosome axis and contain around 1 Mb of consequent DNA. Such arrangement of chromosomes has the advantage of explaining properties of G-bands and geometry of chromosome translocations in a better way than other models.

Despite the differential contributions for chromatin/protein components within the chromosome organization, these models might not be mutually exclusive and stacks, coils and radial extruded loops may co-exist within a less ordered structure.

## 1.3 Factors shaping mitotic chromosomes

Despite the several unknowns on the precise molecular details of chromosome assembly, some key components are believed to be crucial for chromosome organization.

### 1.3.1 Condensins

Condensins are a conserved group of multi-subunit proteins fulfilling many roles in chromatin organization throughout the cell cycle, but their most prominent function is to ensure efficient chromosome segregation (reviewed in Hirano 2012, Piazza, Haering, et al. 2013, and Hirano 2016). They were first isolated from *Xenopus* eggs extract and it was suggested that this protein complex is required for proper chromosome condensation *in vitro* (Hirano and Mitchison 1994; Hirano, Kobayashi, et al. 1997). However, subsequent studies have challenged the view for condensin's requirement in chromosome condensation, as chromosomes do condense to a certain degree upon condensin's inactivation in several *in vivo* studies (Hudson et al. 2003; Gerlich et al. 2006; Steffensen et al. 2001; Oliveira, Coelho, et al. 2005; Hagstrom et al. 2002; Lavoie, Hogan, et al. 2002). In addition to chromosome compaction, several studies revealed other roles for condensin in mitotic chromosome organization: maintenance of chromosomal structural integrity (Gerlich et al. 2006; Oliveira, Coelho, et al. 2005; Ribeiro

et al. 2009) and resolution of topological DNA entanglements (Oliveira, Coelho, et al. 2005; Ribeiro et al. 2009; Steffensen et al. 2001; Hagstrom et al. 2002; Hudson et al. 2003). Condensins' function in mitosis and beyond it are discussed in more details later in this Chapter.

### 1.3.2 Topoisomerase II

Topoisomerase II can introduce several changes in the topology of DNA molecules by driving both supercoiling and relaxing the supercoils, and also the catenation and decatenation of DNA molecules (Schoeffler and Berger 2005). Although some of these reactions can be brought about by topoisomerase I, only topoisomerase II can promote the resolution of catenated sister-DNA molecules. Topoisomerase II is able decatenate intertwined DNAs by transiently cutting both strands of a DNA molecule, which are then resealed after passage through another DNA duplex. It is therefore essential for sister chromatid resolution and their efficient separation at the end of mitosis. Topoisomerase II is also a major component of the chromosome scaffold (Earnshaw 1985) and it has long been debatable whether or not this enzyme is promoting chromosome compaction in addition (or in parallel) to sister chromatid resolution (see more detailed discussion in Chapter 3 and Chapter 5).

### 1.3.3 Kif4

Kif4 is a motor protein able to bind to mitotic chromosomes. Studies in vertebrate cells reveal that Kif4 contributes to the establishment of a correct morphology and structure of chromosomes (Mazumdar et al. 2004; Samejima et al. 2012).It is proposed to cooperate or work alongside condensin in shortening the lateral axis of chromosomes, possibly by creating loops of chromatin (Samejima et al. 2012), although little is known about the molecular mechanisms in this process. Kif4 was also reported to play an important role in mouse meiosis segregation (Camlin et al. 2017), suggesting that Kif4 assist in both kinds of cell division. Interestingly, it was recently observed that condensin I is associating with Kif4 in human cell extracts (Takahashi et al. 2016). This binding, as well as Kif4 motor activity, are necessary for precise axial localization of condensin I to mitotic chromosome axis and granting mitosis chromosomes their biophysical

properties. These findings highlight the tight cooperation between condensin I and Kif4 in establishing mitotic chromosomes.

### 1.3.4 Histone modifications

During mitosis and concomitantly with chromosome condensation, the landscape of histone modifications is altered. Histone H1, the linker histone, is hyper-phosphorylated during mitosis (Fischer and Laemmli 1980; Boggs et al. 2000) and it was initially thought to directly participate in condensation. However, subsequent studies suggest that histone H1 phosphorylation is not necessary for condensation (Guo et al. 1995; Shen et al. 1995) but nevertheless changes the overall chromatin structure (Maresca et al. 2005; Fan et al. 2005). Another key mitotic histone modification is phosphorylation of serine 10 residue of histone 3 (H3 S10), by the mitotic kinase Aurora B (Hendzel et al. 1997). The role for this modification in chromosome condensation has also been controversial (Van Hooser et al. 1998; Wei et al. 1999; Hsu et al. 2000) although recent evidence propose that it drives recruitment of deacetylase Hst2 which, in turn, induces deacetylation of lysine 16 of histone 4. This change in the properties of histone 4 tail promotes interaction with histones H2A and H2B from other nucleosomes (Wilkins et al. 2014), thereby shortening the distance between neighbouring nucleosomes. This would thus support that histone modifications can alone promote the condensation of chromosomes. It should be noted that several histones and histone modifications were also described to be a chromosomal 'receptor' for condensin binding (Ball et al. 2002; Kim et al. 2009; Liu et al. 2010; Tada et al. 2011). Thus, some histone modifications may not be a direct contributor for chromosome compaction but rather a facilitator, by promoting the binding of specialized proteins that model DNA topology.

## 1.4 Condensins

### 1.4.1 SMC complexes family

The name SMC is an abbreviation of Structural Maintenance of Chromosomes and the name reflects well on the major common task of those complexes. These

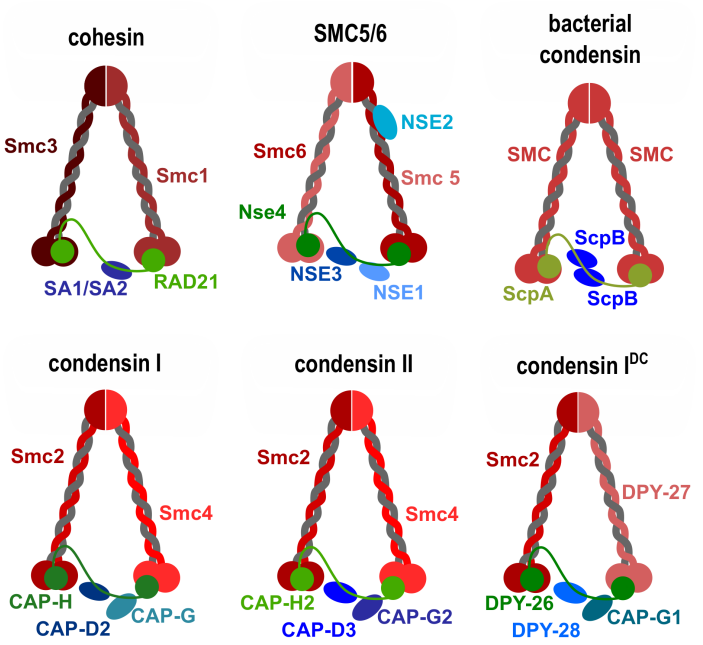
well-conserved proteins are necessary for various aspects of chromatin architecture thought the cell cycle, including (but not limited to) chromosome condensation, sister chromatid cohesion, regulation of interphase chromatin interactions and DNA damage repair.

### Cohesin

The canonical role of cohesin in proliferating cells is holding together two strands of identical replicated DNA after replication. This assures even distribution of DNA to the daughter cells in anaphase by allowing chromosome biorientation by mitotic spindle towards the opposite cell poles. Cohesin forms a ring large enough to fit two strands of naked DNA, therefore it can encircle two DNA molecules and bind them together (Haering, Löwe, et al. 2002; Gruber et al. 2003; Ivanov and Nasmyth 2005; Haering, Farcas, et al. 2008). During replication cohesin is loaded onto freshly replicated DNA to establish cohesion. Replicated DNA is kept tightly together by cohesin along all the chromosome length. In yeast, cohesin is kept this way until the very beginning of anaphase, at which point cohesin is rapidly removed to allow segregation of sister chromatids (Uhlmann, Lottspeich, et al. 1999). In higher eukaryotes, however, cohesin is removed from the arms of chromosomes in prophase by regulated opening of the cohesin ring that allows DNA to release DNA from its topological embrace, but the cohesin is kept around centromeric region (Haarhuis et al. 2014; Mirkovic and Oliveira 2017). This allows mitotic chromosomes to establish its well-known X-shaped morphology in the next stage of mitosis, metaphase, with sister chromatids separated along their arms and connected mostly around centromeric region. The rest of cohesin is released once anaphase is initiated, by rapid proteolytic cleavage of cohesin's kleisin subunit by protein named separase to facilitate segregation of the DNA (Uhlmann, Wernic, et al. 2000; Hauf et al. 2001; Oliveira, Hamilton, et al. 2010).

Besides its role in segregation fiedelity, cohesin is implicated in regulation of genome in interphase. For example, cohesin contributes to gene regulation by changing long-range DNA contacts in *cis* (Hadjur et al. 2009; Nativio et al. 2009; Zuin et al. 2014; Sofueva et al. 2013) and is proposed to act as a major factor for higher order organization of interphase nucleus (reviewed in Barrington et al.

a




b

| | condensin complex | subunits | | | | |
|---|---|---|---|---|---|---|
| | | SMC | | kleisin | regulatory (Kite/Hawk) | |
| Vertebrate | condensin I | SMC2 (CAP-E) | SMC4 (CAP-C) | CAP-H | CAP-G | CAP-D2 |
| | condensin II | | | CAP-H2 | CAP-G2 | CAP-D3 |
| *Drosophila melanogaster* | condensin I | SMC2 | SMC4 | Barren (CAP-H) | CAP-G | CAP-D2 |
| | condensin II | | | CAP-H2 | —— | CAP-D3 |
| *C. elegans* | condensin I | | SMC4 | DPY-26 | CAPG-1 | DPY-28 |
| | condensin II | Mix1 | SMC4 | KLE-2 | CAPG-2 | HCP-6 |
| | condensin I^DC | | DPY-27 | DPY-26 | CAPG-1 | DPY-28 |
| *S. pombe* | condensin | Cut14 | Cut3 | Cnd2 | Cnd1 | Cnd3 |
| *S. cerevisiae* | condensin | SMC2 | SMC4 | Brn1 | Ycs4 | Ycg1/Ycg5 |
| *B. subtilis* | SMC/ScpAB | SMC/BsSMC | | ScpA | ScpB | |
| *E. coli* | MukBEF | MukB | | MukF | MukE | |

**Fig. 1.2. Structural Maintenance of Chromosomes complexes.** **(a)** Cartoon representation of main SMC complexes. **(b)** Table of subunit composition of condensin complexes in various organisms.

2017).

### SMC5/6

SMC5/6 complex, still unnamed in contrast to cohesin and condensin, has been mostly studied for its role in DNA repair. Most likely SMC5/6 is involved in homologous recombination needed for resolving replication products and for DNA damage repair. Mutations in SMC5/6's subunits lead to hypersensitivity when challenged with agents causing DNA damage or replication forks stalling, such as UV light, ionizing radiation or hydroxyurea (Lehmann et al. 1995; De Piccoli et al. 2006; Ampatzidou et al. 2006; Zhao and Blobel 2005; McDonald et al. 2003). Besides facilitating DNA repair, SMC5/6 complex is proposed to also play a role in maintaining sister chromatin cohesion in yeast and chicken and human cells (Gallego-Paez et al. 2014; Stephan et al. 2011; Almedawar et al. 2012).

### Eukaryotic condensins

Condensins, as their name suggests, were believed to be a major driver for mitotic chromosome condensation (Hirano, Kobayashi, et al. 1997; Freeman et al. 2000). Since obtaining its name the role of condensin in promoting efficient compaction of chromosomes has remained rather controversial (Bhat et al. 1996; Steffensen et al. 2001; Hagstrom et al. 2002; Hudson et al. 2003; Oliveira, Coelho, et al. 2005). Along with its precise role, the mechanisms of action of condensins on mitotic chromatin still remain enigmatic. Eukaryotic condensins are a small group of protein complexes that are quite conserved and necessary to support live in nearly all known eukaryotes. Almost all eukaryotes possess condensin I, and majority also have condensin II (Hirano 2012), that vary in their non-SMC subunit composition (Figure 1.2b).

Besides the mitotic roles, which will be described in more details later in this Chapter, condensins were also found to influence interphase organization of chromatin. Condensin II, thanks to its association to chromatin during interphase, plays several roles outside mitosis in several organisms. In *Drosophila* condensin II was shown to antagonize transvection (Hartl et al. 2008), which is a process of influencing transcriptional activity of certain alleles by the action of

the corresponding allele on the homologous chromosome. Such activity could be explained by condensin II's ability to restrict *trans* interactions between homologous chromatids. Probably through the same ability of disrupting long-range interactions in trans, condensin II was also implicated in dispersing polytene chromosomes in *Drosophila's* ovarian nurse cells. Polytene chromosomes have multiple copies of chromosomes that align with their homologs creating massive chromosomes. During oogenesis in *Drosophila* polytene chromosomes must be dispersed, and mutations of condensin II is preventing unpairing and polytene chromosomes cannot be disassembled (Hartl et al. 2008). Condensin II has also an influence of gene transcription during interphase in *C. elegans*, *Drosophila*, mouse, and human cells (Kranz et al. 2013; Dowen et al. 2013; Longworth et al. 2012; Wallace et al. 2015; Yuen et al. 2017). Also yeast were shown to control their transcription via condensin complex, such as clustering tRNA genes (Haeusler et al. 2008; D'Ambrosio et al. 2008). Moreover, condensin is required in order to compartmentalize chromosomes in interphase into discrete chromosome territories, deciding on interphase chromatin architecture in *Drosophila* (condensin II), *C. elegans* (condensin $I^{DC}$), and fission yeast (Bauer, Hartl, et al. 2012; Lau et al. 2014; Iwasaki et al. 2016) .

Besides condensin I and II, a third eukaryotic condensin variant was identified in *C. elegans*, named condensin $I^{DC}$ after 'dosage compensation', which accurately describes its main function (Csankovszki et al. 2009). Condensin $I^{DC}$ differs from condensin I only by replacing SMC4 by it's another version, called DPY-27. In contrast to cohesin and the other condensins that work globally, condensin $I^{DC}$ is associating to X chromosome only in order to equalizing transcription of X chromosome in hermaphrodites.

### Prokaryotic SMCs

Three families of SMC complexes were identified in many bacteria and archaea up to date, MukBEF, SMC-ScpAB, and MksBEF, and they play a vital role in chromosome segregation. The prokaryotic organisms proven to be a great tool in SMC complexes research. Thanks to small size of their genome and simple manipulation on SMC proteins they provided important insights for understanding the mechanistic processes governing SMCs.

MukBEF is the first ever described SMC complex, and it can be encountered in enterobacteria and some γ-proteobacteria (Niki, Jaffé, et al. 1991; Hiraga et al. 2000). Mutating MukBEF complex in *E. coli* results in chromosome condensation problems, segregation anomalies, as judged by anucleated cells and sharp decrease in colony viability (Niki, Jaffé, et al. 1991; Yamanaka et al. 1996; Wang, Mordukhova, et al. 2006), suggesting that MukBEF serves a similar role in mitosis as eukaryotic condensin.

Similar problems in genome division were observed when the second group of prokaryotic SMC complexes, named SMC-ScpAB. SMC-ScpAB are found in many bacteria and archaea not possessing MukBEF complexes. When SMC-ScpAB were mutated in bacteria normally expressing SMC-ScpAB, namely *B. subtilis* and *C. crescentus*, it give rise to chromosome compaction and segregation defects (Britton et al. 1998; Wang, Tang, et al. 2014), confirming their condensin-like role. SMC-ScpAB bears much closer similarity to condensin and cohesin of eukaryotes than other prokaryotic SMC groups (Cobbe and Heck 2004). Recent publication uncovered a mechanism by which SMC-ScpAB is able to condense and segregate DNA in *B. subtilis* by loop extrusion mechanism. It proposes that SMC complex is loaded on the circular chromosome on the *parS* site by ParB protein (Wang, Brandão, et al. 2017) followed by translocation of the prokaryotic condensin via active loop extrusion to travel through the circular genome, juxtaposing the arms of the chromosome by multiple sliding SMC rings, leading to compaction (Wang, Brandão, et al. 2017).

The third SMC prokaryotic complex was found through bioinformatics analysis which identified novel proteins resembling MukBEF, therefore newly discovered complexes family was named MksBEF (MukBEF-like SMC proteins) (Petrushenko et al. 2011). This protein is not highly conserved and it can be found in large variety of proteobacteria, and can also be present in one organism together with other MksBEF, MukBEF or SMC-ScpAB (Petrushenko et al. 2011). TThe exact roles MksBEF complex are not yet fully explored.

### 1.4.2 Architecture of SMC complexes

SMC protein family are a group of complexes built on a similar structural plan (Figure 1.2a). The core of the complex are SMC protein dimers. Each SMC sub-

unit protein is 1000-1500 amino acid long and has three distinct parts. Firstly, a head of SMC, which is a globular domain containing ABC-type ATPase including Walker A/B motifs responsible for enzymatic abilities of SMC complexes. On the opposite side of the protein there is a hinge domain that is responsible for proper folding of the protein, interacting with its partner during dimerization, and other functionalities of the holocomplex. Those two parts are connected by a ~50nm coiled coil. In eukaryotes they are always heterodimers, namely SMC1-SMC3 for cohesin, SMC2-SMC4 for condensins, or SMC5-SMC6, while in prokaryotes SMCs (SMC, MukB, and MksB) subunits form homodimers.

Two SMC proteins are directly interacting by their hinges and the heads of SMCs are connected by another protein, called kleisin after Greek word 'closure' (κλείσιμο or kleisimo). Prokaryotic kleisins include ScpA from SMC/ScpAB, MukF from MukBEF and MksF from MksBEF complexes. Analysis of kleisin/SMC interphases showed that N-terminus of kleisin is binding to the lower part of coiled coil of first SMC subunit via its helix-turn-helix motif (Onn et al. 2007; Bürmann et al. 2013; Gligoris et al. 2014), and opposite end of kleisin is connecting to the bottom part of globular ATPase head of the other SMC protein via its winged-helix domain (Bürmann et al. 2013; Haering, Schoffnegger, et al. 2004; Onn et al. 2007). Kleisin subunit is connecting two SMCs to form a closed ring-like structure that is believed to be a key feature in organizing chromatin, as it allows topological entrapment of DNA inside of the SMC complex ring.

Peripheral subunits are believed to modulate the behavior of a given SMC complex. Those subunits bind to the kleisin and can belong to either Kite or Hawk group of proteins (Palecek and Gruber 2015; Wells et al. 2017). Prokaryotic SMC complexes and eukaryotic SMC5/6 contain peripheral subunits belonging to Kite family, eukaryotic cohesin and condensins use Hawk proteins. In general, all those subunits are important to support function of the holocomplex. In particular Hawk subunits of eukaryotic condensin and cohesin were shown to be crucial for regulation of their respective complexes. Pds5 and Scc3, regulatory subunits of cohesin, play a major role in regulating cohesin's ability to encircle DNA. In case of eukaryotic condensins it was shown that Hawks subunits are necessary to support condensin function in yeast, *Xenopus* and human cells (Lavoie, Hogan, et al. 2002; Piazza, Rutkowska, et al. 2014; Kinoshita et al.

2015; Bhalla et al. 2002; Sutani et al. 1999), probably due to the elastic nature of HEAT repeat motifs that are able to regulate dynamics of binding to DNA and influence rate of ATP hydrolysis of the complex depending on its environment (Forwood et al. 2010; Kinoshita et al. 2015).

### 1.4.3 Discovery of condensins

The first gene encoding a protein belonging to the SMC family was described in *E. coli*. A mutation of *mukB* gene caused generation of anucleated bacteria (Niki, Jaffé, et al. 1991). Soon after that a genetic screen in buddying yeast led to discovery of SMC1 (stability of minichromosomes) protein that was crucial for chromosome segregation, as mutation of *smc1-1* gene lead to large increase in minichromosome nondisjunction rate (Strunnikov AV, Larionov VL 1993). The same study predicted that SMC1 gene is conserved in evolution both in prokaryotes and eukaryotes, and its protein product represents a novel protein family. This followed by fission yeast studies describing SMC2 (*cut14*) and SMC4 (*cut3*) subunits if condensin that proved to be necessary for chromosomes segregation and condensation (Saka et al. 1994).

Parallel studies of mitotic structure of human cells revealed that when mitotic chromosomes are stripped of histones in particular condition, the protein scaffold is holding radial DNA loops, keeping the general shape of the chromosomes (Adolph et al. 1977; Earnshaw 1983). The subsequent analysis identified ScII (SMC2), closely related to SMC1 just discovered in yeast, to be the major component of such scaffold (Saitoh et al. 1994).

At the approximately the same time biochemical analysis of *Xenopus* egg extracts uncovered that sperm chromosome condensation in this system requires not only histones, but also a set of other proteins associating to the chromatin. Those proteins were identified to be topoisomerase II and XCAP-C and XCAP-E, later known generally as SMC4 and SMC2. These two proteins were proposed to form a heterodimer and due their sequence were qualified to belong to the SMC family (Hirano and Mitchison 1994). Further analysis of *Xenopus* egg extracts revealed that this mysterious complex was not a heterodimer, but rather a pentamer containing XCAP-C, XCAP-E, XCAP-D2, XCAP-G, and XCAP-H (which was described just a year before in *Drosophila melanogaster* to be neces-

sary for chromosome segregation fidelity (Bhat et al. 1996)) This freshly defined complex was named condensin, as it was believed to be a main driver of chromosome condensation (Hirano, Kobayashi, et al. 1997). Later it was shown that there are multiple versions of condensins. Besides the canonical condensin complex described by Hirano's group (Hirano, Kobayashi, et al. 1997), condensin I, some organisms were shown to possess different variants of condensin. Almost a decade after identifying condensin I, condensin II was described to exist besides condensin I in HeLa cells, which shared SMC2 and SMC4 subunits, but had its own regulatory subunits and displayed a significantly different behavior in the cell (Ono et al. 2003). Another different form of condensin was identified in *Caenorhabditis elegans*, which besides condensin I and II also has a unique condensin $I^{DC}$ that plays an important role in dosage compensation (Csankovszki et al. 2009).

### 1.4.4 Enzymatic activity of condensins

The exact reactions of condensin complex in chromosomal context and how its enzymatic activity affects chromosome condensation is not clearly understood. *In vitro* studies have brought some clues of what are the basic reactions performed by condensin complexes. These studies, described below, shed some light on possible modes of action, helping to build and test models of condensins loading and action.

Condensin was first shown to able to introduce positive supercoiling in circular DNA plasmids in presence of ATP and topoisomerase I (Kimura and Hirano 1997). Supercoiling is only possible when all the subunits of condensin are present (Kimura and Hirano 2000), so this process requires the whole intact complex, in contrast to some other condensin's enzymatic activities. Condensin's positive supercoiling activity is tightly regulated in a cell cycle dependent manner, as condensins from *Xenopus* extracts, human cells and yeast require phosphorylation by Cyclin-dependent kinase 1 (Cdk1) and Polo kinase to accelerate their supercoiling activity (Kimura 1998; Kimura, Cuvier, et al. 2001; St-Pierre et al. 2009). Interestingly, condensins are able to change the global topology of DNA, introducing vast amount of positive supercoil(Kimura, Rybenkov, et al. 1999; Stray et al. 2005). Mechanistic insight of condensin-mediated supercoiling

*in vivo* and how it influences mitotic chromosomes are nonetheless still missing.

The next reaction, closely related to positive supercoiling, is decatenation, which means disentangling two topologically linked fragments of DNA. Although the only enzyme in eukaryotes that is able to change catenation state in such way is topoisomerase II, condensin has been implicated in aiding in this process indirectly. Condensin's ability to introduce positive supercoiling in catenated substrates would be driving topoisomerase II's activity towards decatenation of entangled DNA, which was shown *in vivo* in yeast minichromosomes (Baxter, Sen, et al. 2011; Charbin et al. 2014; Sen et al. 2016), which is thought to be crucial for chromosome condensation and segregation.

Another of enzymatic reaction of condensin observed *in vitro* is an ability to reanneal separated strands of double-stranded DNA (Sakai et al. 2003). Renaturation of single-stranded DNA does not require the whole complex. Instead, SMC2-SMC4 heterodimer alone was shown to be more efficient in strand annealing than the entire condensin (Sakai et al. 2003). It may be explained by a high affinity of hinge domain to bind to single-stranded DNA (Hirano and Hirano 2006; Griese et al. 2010; Akai et al. 2011; Niki and Yano 2016), which may underlie the condensin's loading process, explaining why the dimer association to single-stranded DNA is particularly high. It was also proposed that, thanks to its reannealing activity, condensin might work *in vivo* as a 'mitotic cleanser', facilitating removing unwanted proteins or transcripts from the unwounded (single-stranded) fragments of DNA and reforming double-stranded DNA for mitotic process (Niki and Yano 2016). However, there are no direct proofs for this hypothesis.

Recently condensin was shown to be able to translocate along DNA molecules in an ATP-hydrolysis dependent manner (Terekawa et al. 2017). This ability is one of necessary qualification needed to qualify as a hypothetical loop extruder in the loop extrusion model of chromatin organization. Loop extrusion-like process by prokaryotic condensin SMC-ScpA *in vivo* was described in *B. subtilis*. SMC complex is loaded onto a single site, parS, and is subsequently traveling the chromosome by actively enlarging the loop as it travels towards the opposite end of the circular DNA (Wang, Brandão, et al. 2017). Crystallography data of prokaryotic SMC complex suggests that SMC might perform loop extrusion by

capture-merging cycle thanks to its ability to switch between open and closed state upon ATP hydrolysis (Diebold-Durand et al. 2017).

### 1.4.5 Spatial and temporal localization and ratio of condensin I and II

There are two main condensin complexes in animal cells and they differ significantly in their localization throughout the cell cycle. Condensin I in interphase is restricted to cytoplasm and only allowed to enter the nucleus in early mitosis, and in contrast, condensin II is bound to chromatin both in interphase and mitosis (Hirota et al. 2004; Ono 2004). In mitosis both condensins are accumulated in the longitudinal chromatids' axes, but do not tend to overlap perfectly (Ono et al. 2003). Temporal studies showed that condensin II is first to localize to the axes, and condensin I binds slightly later. Those observations appear to support the hypothesis of two step compaction of mitotic chromosomes, where two subsequent folding actions are required for condensation (Hirano 2005; Poirier and Marko 2002; Naumova et al. 2013). In such model condensins would be good candidates to drive various modes of compaction – condensin II, already present in nucleus since interphase, could induce the first changes, followed by condensin I binding and its action as a second step. *In vivo* studies seem to support this idea. Chicken cells depleted of either condensin I or condensin II show different phenotypes of disruption of mitotic chromosomes, therefore their function in generating and organizing chromosomes are not redundant (Green et al. 2012). The authors of this publication, based on microscopy and other data, propose that condensin II is responsible for axial stacking of DNA loop and their long range and more stable interaction, followed by condensin I introducing frequent, dynamic, short range loops for higher order organization. The mode of binding to chromatin is quite different between condensin I and II. Condensin I is very dynamic, associating and dissociating from the mitotic chromosomes with recovery time after photobleaching of very few minutes for HeLa cell and *Drosophila* embryos (Gerlich et al. 2006; Oliveira, Heidmann, et al. 2007), while condensin II is much more stably bound to chromatin, with very weak recovery after photobleaching (Gerlich et al. 2006).

In yeast condensin II was not found and the only condensin in yeast resem-

bles more condensin I in its function. Interestingly, condensin in fission yeast *S. pombe* is excluded from nucleus during interphase and only binds to chromatin in mitosis (Sutani et al. 1999), closely resembling mammalian condensin I, while in buddying yeast *S. cerevisiae* condensin localizes to the chromatin regardless of cell cycle stage (Freeman et al. 2000), suggesting that condensins in various organisms can be fine-tuned to perform slightly different roles.

Interestingly, even if a given organism does express both condensin I and condensin II, their relative proportion and importance is varying between the species. In *Xenopus* egg extracts the ratio of condensin I and II is about 1:5, 1:10 is found in chicken cells, and 1:1 in HeLa cells (Ono et al. 2003; Shintomi and Hirano 2011; Ohta et al. 2010). Whether the relative abundance plays a role in shaping the chromosomes was addressed in *Xenopus* eggs extracts and in chicken cells. It was shown that condensin I and II are not redundant and that depletion of one of the condensins (changing the ratio to 1:0 or 0:1) leads to different phenotypes – depleting condensin I makes chromosomes shorter and wider, while removing condensin II is leading to too long and thin chromosomes (Ono et al. 2003; Hirota et al. 2004; Green et al. 2012). A more precise tool to assess the importance of precise controlled ratio was developed in *Xenopus* extracts. Rather than depleting completely one complex, it allowed changing condensin I to condensin II ratio from 1:5 to 1:1 causing a change in the morphology of chromosomes to become shorter and thicker than the control situation, showing that the ratio between the two complexes indeed is important, not only binary matter of their presence or absence (Shintomi and Hirano 2011).

Interestingly, in case of *Drosophila* functions of condensin I and II are even more separated. Mutating condensin II subunits CAP-H2 and CAP-D3 produce viable flies, although with male sterility problems (Savvidou et al. 2005; Hartl et al. 2008), whereas removing condensin I subunits is embryonic lethal, suggesting that development is strongly biased for condensin I, and condensin II is more important for germline development and interphase functions (Hartl et al. 2008; Hirano 2012)).

### 1.4.6 Condensins in chromosome compaction

Condensin was proclaimed to contribute to mitotic condensation process since the first experiments in cell-free extracts of *Xenopus laevis* eggs, where it was shown to be necessary to trigger formation of chromosome-like structures from decondensed chromatin (Hirano, Kobayashi, et al. 1997). Further exploring the *Xenopus* extract system in more detail confirmed the need of condensin in establishing chromosome condensation, by removing particular subunits of condensin and observing failure in obtaining chromosomes (Hirano and Mitchison 1994; Hirano, Kobayashi, et al. 1997). More recently, the necessity of condensin in *Xenopus* system was confirmed by another *in vitro* study that pinpointed just six purified factors that are needed to condense egg chromosomes, and one of them was condensin I primed by Cdk1 phosphorylation (Shintomi, Takahashi, et al. 2015). Also in some other systems chromosome condensation was disrupted once cells were deprived of condensin. For example in yeast *S. cerevisiae* and *S. pombe* loss of condensin leads to condensation defects (Freeman et al. 2000; Sutani et al. 1999; Saka et al. 1994; Petrova et al. 2013; Lavoie, Tuffo, et al. 2000; Kruitwagen et al. 2015). In particular quantitative microscopy analysis proved that condensin is responsible for long range compaction in buddying yeast (Kruitwagen et al. 2015), supporting a direct role of condensin in imposing compaction. In addition increasing amounts of condensin II in interphase cells in *Drosophila* leads to overcondensation of their chromatin (Buster et al. 2013) that could imply that condensins have an intrinsic ability to generate chromatin compaction. At the same time *in vitro* studies of naked DNA stretched by magnetic tweezers allowed to observe directly that condensin is able to compact the DNA. This approach revealed that purified condensin complexes isolated from *E. coli*, *S. cerevisiae*, and *X. laevis* can induce shortening of DNA molecules in an ATP-dependent manner (Strick et al. 2004; Cui et al. 2008; Eeftens et al. 2017). However it is not certain how closely this artificial model can be translated onto histone-based chromatin *in vivo*, especially that histones may constitute a barrier for condensin loading onto DNA (Toselli Mollereau et al. 2016) and likely change DNA bending properties.

Whether condensin really induced compaction per se has been questioned since depletion of condensin in some organisms leads to very mild phenotype

in chromosome condensation, with much more severe problems observed in segregation efficiency. For example *C. elegans* embryos with SMC-4 silenced by RNAi can reach high levels of compaction of chromosomes in mitosis, but their morphology is faulty (Hagstrom 2002). Also chromosomes of chicken cells depleted of condensins display a relatively normal morphology, but they are very sensitive to external factors that shows that they lack internal structural integrity (Hudson et al. 2003). In *Drosophila* chromosomes did not exhibit major condensation problems in mutants of condensin's subunits CAP-H/Barren (Bhat et al. 1996; Oliveira, Coelho, et al. 2005; Coelho et al. 2003), SMC4 (Steffensen et al. 2001), or CAP-G (Dej et al. 2004). Instead, centromere stiffness is impaired, suggesting underlying disruption of chromosome architecture (Oliveira, Coelho, et al. 2005). In contrast, newer studies studies in DT40 chicken cells, but this time with more precise conditional SMC2 subunit knockout, showed that condensin-depleted chromosomes reached only 60% of compaction level of their wild type counterparts (Vagnarelli et al. 2006), exposing a stronger condensation phenotype. These defects are accompanied by a strong impairment of stiffness of centromeric regions of chromosomes established in such cells (Ribeiro et al. 2009), as in case of *Drosophila* mutants. Metaphase chromosomes in HeLa cells depletion of condensin I subunits does not affect metaphase compaction level, but it clearly impairs mechanical stability of centromeres, as they experience excessive stretching, unable to resist spindle forces (Gerlich et al. 2006). Mouse oocytes require condensin II (and condensin I, to a smaller extent) both for condensation of meiotic chromosomes and to confer their rigidity (Houlard et al. 2015).

The parsimonious conclusion of those depletion/inactivation experiments is that condensin's main responsibility is to organize internal structure of mitotic chromosomes rather than inducing compaction *per se*. Absence of condensin is therefore probably affecting the inner architecture of chromosomes, which in turn may lead to mechanical and compaction problems, such as condensation issues, wrong morphology and severely diminished resistance to perturbations. Mechanical disruption in centromeric region in response to condensin removal is particularly evident, as distance between centromeres or kinetochores in metaphase is clearly increased (Oliveira, Coelho, et al. 2005; Gerlich et al. 2006; Ribeiro et al. 2009; Samoshkin et al. 2009). This can be explained by the

fact that centromeres, being attached to kinetochore components, are subjected directly to strong forces and it is easier to compromise this region comparing to chromosome arms. It is proposed that condensin-made loops are crucial for creating specific spring-like structure of centromeres and ensuring proper physical properties to allow withstanding spindle forces, and achieving bioorientation by responding to kinetochore attachment state (Stephens et al. 2013; Lawrimore et al. 2015).

How could condensins impose chromatin compaction and/or organization? One of probable solutions was that condensin is able to cause bringing together two distant regions of a DNA molecule and create a loop by supercoiling and/or topological entrapment (Cuylen, Metz, et al. 2011; Cuylen and Haering 2011; Baxter and Aragón 2012; Samejima et al. 2012).Since the compaction would happen only within a single DNA molecule, it also facilitates individualization of sister chromatids in mitosis, separating the molecule from its sister and other DNAs. Some speculated that efficient compaction via looping would require oligomerization or other kind of condensin complexes clustering in order to congregate DNA loops and further promote their spatial compaction (Swedlow and Hirano 2003; Hirano and Hirano 2006; Strick et al. 2004). Such cooperative behavior also explains the localization of condensin inside the chromosomal axis in metaphase chromosomes to hold the loops. The oligomerization may be a strategy of some bacterial SMC complexes (Cui et al. 2008; Matoba et al. 2005), but was not proven so far in eukaryotic organisms.

As described before, condensin is able to introduce positive supercoiling into a naked DNA, and generating globally great amount of supercoiled structures. This activity was proposed as a possible way to achieve chromosome compaction by condensin (Bazett-Jones et al. 2002). Surprisingly, very recent data using DNA of different topology controlled by magnetic tweezers showed that yeast condensin isolated from *S. cerevisiae* is still able to compact nicked DNA, in which it is not possible to create positive supercoils since one strand is broken (Eeftens et al. 2017). That results might be interpreted as that condensin's action is not dependent of on introducing new supercoils and condensin is rather stabilizing already existing topological structures.

Newer ideas of condensin-based chromosome organization are assuming that

condensin may be a loop extrusion factor. Condensin would be able to bind to a single site of the DNA and progressively extrude DNA to create a loop. Multiple condensins extruding loops along the chromosome in theory would be enough to drive efficient condensation alone, as loop extruding factors are able to lead to chromosome-like compaction, condensation kinetics and segregation in several biophysical models (Alipour and Marko 2012; Goloborodko, Marko, et al. 2016; Goloborodko, Imakaev, et al. 2016). One most compelling feature of the loop extrusion model for mitotic condensation is that condensin can only create loops within a single DNA molecule as the starting point is a single point on a chromosome. This allows to avoid creating erroneous links between sister chromatids or different chromosomes, that would lead to segregation problems, especially in anaphase. In alternative models, evoking that condensin produces loops via binding to two distinct sites and joining them together, this was a major caveat, as it would be difficult to explain how condensin would always create links between the same chromatid in a crowded nucleus. In addition, the loop extrusion model can explain accumulation of condensin molecules within the axis of chromosomes, with DNA loops spread around it (Alipour and Marko 2012; Goloborodko, Marko, et al. 2016) without a need of complex oligomerization, as loops can only be extruded as long as loop extrusion factor doesn't encounter some barrier, for example another condensin extruding its loop. This would cause condensins stacking together or relatively close to each other at the central axis.

### 1.4.7 Condensins in sister chromatids resolution

Another important aspect of condensin's function in mitosis is resolving sister chromatids before anaphase onset. The recurring phenotype of removing condensins from cells or organisms was severe problems in genome segregation. Those issues ranged from lack of chromatids resolution before anaphase to anaphase bridges and lagging chromosomes upon anaphase onset (Saka et al. 1994; Bhat et al. 1996; Sutani et al. 1999; Steffensen et al. 2001; Hagstrom et al. 2002; Oliveira, Coelho, et al. 2005; Ono 2004). Replicated chromosomes are topologically entangled during interphase mostly during the nature of replication forks action. Those links between DNA molecules need to be eliminated before

anaphase. The enzyme able to resolve the links is topoisomerase II, which can cut double helix from one of the entangled DNA molecules, pass it through the other molecule and re-join the cut region to fix the cut. The opposite reaction, topological linking of two DNAs, is also performed by topoisomerase II and for a long time researchers could not explain why topoisomerase II behaves *in vivo* like a Maxwell's demon, selectively performing mostly only one of two possible reaction. This phenomena can only be explained if topoisomerase II has some inbuilt feature allowing it work this unusual way, or alternatively that there is another factor biasing topoisomerase II reaction strongly towards decatenation. Condensin is proposed to aid decatenation performed by topoisomerase II, as compromising condensin results in problems in chromosome segregation (Steffensen et al. 2001; Hagstrom 2002; Hudson et al. 2003; Oliveira, Coelho, et al. 2005; Gerlich et al. 2006; Ribeiro et al. 2009). Possible scenarios of interplay between condensin I and topoisomerase II are discussed in Chapter 1.6.

How exactly can condensin influence decatenation was never completely understood. A recent model for solving directionality biasing is based on condensin's ability to introduce supercoiling. Incidentally, positively supercoiled plasmids were shown to be a preferred substrate for topoisomerase II decatenation reaction (Baxter, Sen, et al. 2011). Therefore condensin is proposed to change the topological landscape of genome by generating positively supercoiled loops, creating a favorable substrate for decatenation rather than prompting new catenations (Baxter, Sen, et al. 2011; Baxter and Aragón 2012). Another way through which condensin might be biasing topoisomerase's reaction towards decatenation is physically separating freshly catenated DNAs far apart from each other, to make the reverse linking process energetically unfavorable for topoisomerase II (Cuylen and Haering 2011). Along the same lines of reasoning, condensin has a probable role in inducing loops within a single chromatid, therefore increasing probability of intrachromatid interactions and decreasing contacts between different chromatids. This may decrease the likelihood of creating erroneous connections between different DNA molecules and promoting intra-chromatid entanglements, which probably are important for chromosome compaction and mechanical properties.

Condensin was also linked to cohesin removal process, as well essential

for efficient segregation in anaphase. Several sources point that condensin removal leads to persistence of cohesin on chromosomes arms, indicated by preventing chromatids resolution in HeLa cells (Hirota et al. 2004) or impairment of anaphase segregation in mitotic and meiotic division in yeast (Renshaw et al. 2010; Yu and Koshland 2005). This opens a possibility that condensin is facilitating removal of cohesin rings entrapping sister chromatids. Likely scenario by which condensin is abolishing cohesion is by shaping chromosomes to induce forces and topology favorable to cause breaks in cohesin rings or otherwise forcing their removal once anaphase process is starting and chromatids begin to separate (Cuylen and Haering 2011).

How condensin can impose condensation and resolution and what (if any) is the relationship between those two processes is a great priority in understanding a bigger picture of mitotic chromosome internal organization and dynamics.

### 1.4.8 Regulation of condensins

The first level of regulating condensins' activity is their spatial distribution during the cell cycle. Condensin I in majority of eukaryotes and condensin in *S. pombe* in interphase is physically separated from its target, chromatin, by being limited to the cytoplasm and not gaining the access to the chromatin until early stages of mitosis. Condensin in *S. cerevisiae* has an access to the chromatin throughout the entire cell cycle, but to limit its activity the level of one of the Hawk subunits, Yscg1 (CAP-G), is downregulated (Doughty et al. 2016). Limiting activity of condensin via decreasing the level of the proteins was also shown in *Drosophila*, where CAP-H2 subunit of condensin II is degraded by $SCF^{slimb}$-dependent ubiquitination and preventing this degradation causes overcompaction of interphase chromatin (Buster et al. 2013).

Also condensin's subunits themselves may be able to change dynamics of the whole complex. Recent studies of *Xenopus* cell-free extracts suggest that Hawk subunits of condensin I (CAP-G and CAP-D2) have an opposite function in chromosome maintenance and their balanced action is crucial for regulating the shape of chromosomes. This proposal is based on the findings that pre-established chromosomes exposed to condensin complexes missing CAP-G or CAP-D2 subunits give rise to the opposite morphologies of chromosomes, respectively creating

thin, messy chromosomes with elongated axis or fuzzy chromosomes with completely destabilized axis (Kinoshita et al. 2015).

Another level of control of condensins are based on cell-cycle specific regulators. Majority of condensins' subunits were identified to have multiple phosphorylation sites (Nousiainen et al. 2006; St-Pierre et al. 2009; Bazile et al. 2010) that influence condensin's localization and function, as indicated by changes of pattern in phosphorylation of condensin in human cells in different phases of cell cycle (Takemoto 2003). Several kinases and phosphatases were shown to change phosphorylation state of condensin in variety of different organisms, such as PLK, CDK, Aurora B and casein kinase 2, PP2A and Cdc14 (as reviewed extensively in Hirano 2012; Piazza, Haering, et al. 2013). In short, common pattern in vertebrate for condensin phospho-regulation is inducing low activity in interphase due to low CDK activity and phosphorylation by CK2. At the beginning of mitosis condensin I is granted access to chromatin and is activated by dephosphorylation of CK2 sites and phosphorylation of CDK in a cyclin B-dependent manner, in addition to phosphorylation by PLK and Aurora B. Condensin II is activated a bit earlier than condensin I by cyclin A-dependent CDK phosphorylation. After mitosis, condensin I is subjected to dephosphorylation by mitotic phosphatases and condensin II by PP2A to again decrease their activity.

## 1.5 Topoisomerase II in mitosis

Topoisomerase II is a homodimer that performs a unique role in living cells. Besides the ability to change supercoiling state of DNA that it shares with other types of topoisomerases, only topoisomerase II can untangle topologically intertwined DNA molecules (catenations), as well as enzymatically introduce new entanglements. These reactions are accomplished through a strand-passing activity, in which one double-stranded DNA segment passes through a transient double-strand break in another DNA molecule.This catenation/decatenation activity is crucial to maintain a proper topological state of both interphase and mitotic chromosomes. Although the role of topoisomerase II is not limited to mitosis (Pommier et al. 2016), faulty action of this enzyme is highly evident during cell division.

### 1.5.1 Topoisomerase II and sister chromatid resolution

Two replicated DNA molecules, identical sister chromatids, are extensively topologically entangled with each other mainly as a consequence of semi-conservative replication process (Branzei and Foiani 2010). One of the very well established roles of topoisomerase II is the resolution of these catenations between DNA molecules. Timely resolution of catenates is especially important during mitosis to ensure the physical individualization of sister DNA molecules (and also neighbouring chromosomes), that need to be distributed between the two daughter cells. Failures in disentangling two copies of DNA in cell division may result in serious damage to the DNA, with drastic consequences to the cell. Consequently, cells lacking topoisomerase II undergo a faulty anaphase with extensive chromatin bridges (Uemura et al. 1987; Clarke, Johnson, et al. 1993; Oliveira, Hamilton, et al. 2010). Most of the catenations linking DNA molecules are resolved during replication or before mitotic entry. Indeed, measurements of the frequency of catenated circular minichromosomes, throughout the cell cycle, revealed that the majority of DNA entanglements are rapidly removed by topoisomerase II before mitosis (Charbin et al. 2014).

Prophase is a crucial time for chromatin compaction and chromatids resolution. Analysis of the kinetics of sister chromatid resolution has been recently studied in great detail. It was revealed that the vast majority of mitotic entanglements between sister chromatids are resolved until the end of prophase allowing to clearly individualize two separate chromatids axes in late prophase and this process requires topoisomerase II activity (Liang et al. 2015; Nagasaka et al. 2016). Interestingly, this topoisomerase II- dependent individualization of sister chromatids starts already in early prophase and coincides in time with chromosome condensation (Nagasaka et al. 2016).

Although the bulk of catenation between sister chromatids is resolved in prophase, some catenations persist into metaphase (or even later), especially in the centromeric region. Accordingly, topoisomerase II's preferred localization in metaphase are centromeres (Sumner 1996; Díaz-Martínez et al. 2006). The distribution of topoisomerase II on mammalian chromosomes is probably reflecting a high level of entanglements in this region and thus aiding in their resolution before the anaphase onset.

Importantly, recent findings provide a critical change in our understanding of chromosome resolution by highlighting the reversibility of this process. Overexpression of topoisomerase II was shown to be sufficient to introduce catenations in metaphase-arrested minichromosomes in yeast, providing the DNA molecules are close to each other (Sen et al. 2016). These results highlight that previously separated DNA molecules are able to re-intertwine as a consequence of topoisomerase II's action. This implies that during metaphase, catenations are not only resolved, but they can arise *de novo*. The amount of catenations during metaphase, therefore, results from a net effect of this bidirectional process. Tight regulation of topoisomerase II activity is thus required to ensure that chromosomes display enough entanglements to ensure the right compaction and mechanical stiffness (discussed below), which is still compatible with their efficient resolution in late anaphase.

### 1.5.2 Topoisomerase II and chromosome compaction

The idea that topoisomerase II could be involved in chromosome compaction stems from classical studies that revealed that this enzyme is one of the most abundant non-histone proteins found on mitotic chromosomes. Early research on chromosome structure showed that after histone extraction, chromosomes on electron microscopy images take shape of loops of DNA attached to a dense scaffold (Paulson and Laemmli 1977; Adolph et al. 1977; Earnshaw 1983). Analysis of composition of the observed scaffold revealed that the major components were topoisomerase II and condensin I (Gasser et al. 1986; Earnshaw 1985). This discovery led to the proposal of the scaffold/radial-loops model for chromosome folding. As mentioned in Chapter 1.2.2, the scaffold model is highly debated. Temporary binding to the axis and lack of stable association to chromatin was shown *in vivo* and is arguing against topoisomerase II forming a highly stable stiff scaffold for DNA loops. Instead, the enzymatic action of topoisomerase II may underlie mitotic chromosome assembly (discussed below).

The extent to which topoisomerase II contributes to chromosome compaction has been difficult to establish as various research done in different model systems presents conflicting results. Studies using topoisomerase II inhibitors invariably report that in addition to severe chromosome segregation defects, chromosome

compaction is also impaired (Chen et al. 1984; Buchenau et al. 1993; Andoh et al. 1993; Anderson and Roberge 1996).

Genetic studies in *S. cerevisiae* failed to detect significant changes in chromosome compaction in mutants for topoisomerase II (Lavoie, Hogan, et al. 2002). These studies were based on FISH measurements of the rDNA locus and thus may reflect a particular organization of these chromosomal regions. By contrast, direct measurements of the distance between two distal chromosomal sites support that topoisomerase II is required for linear condensation in budding yeast (Vas et al. 2007). Similar studies in *S. pombe* further support the role of topoisomerase II in chromosome compaction (Uemura et al. 1987; Petrova et al. 2013).

In metazoans, cells lacking topoisomerase II display abnormal chromosome morphology, particularly along their longitudinal axis. However, the extent of these defects is highly variable across various studies ranging from very mild defects or delayed kinetics to severe morphological alterations. These include studies in plants (Roca et al. 1994), *C. elegans* (Ladouceur et al. 2017), *D. melanogaster* (Chang et al. 2003; Somma et al. 2008; Mengoli et al. 2014), chicken cells (Samejima et al. 2012; Johnson et al. 2009), and human cells (Carpenter and Porter 2004; Gonzalez et al. 2011; Sakaguchi and Kikuchi 2004). Studies *in vitro* where sperm chromatin is incubated with *X. laevis* mitotic extracts has also provided a valuable tool to dissect the mechanisms of mitotic chromosome assembly. Topoisomerase II was shown to be absolutely required for the condensation of interphase nuclei into discrete chromosomes in these *in vitro* systems (Uemura et al. 1987; Hirano T 1993). More recently, topoisomerase II was shown to be one of six factors necessary to reconstitute phenotypical condensation of interphase *Xenopus* sperm chromatin, *in vitro* (Shintomi, Takahashi, et al. 2015).

How could topoisomerase mediate shortening of the chromosomal axis remains unknown. It is not clear whether or not the effect on chromosome compaction results from topoisomerase catalytic activity or, alternatively, a non-enzymatic role of this protein. Classical studies highlight the high abundance of topoisomerase II, estimated to be three copies per 70,000-base loop (Gasser et al. 1986), which argued for a more structural role. However, accumulating evidence does support that topoisomerase's role in chromosome compaction involves its enzy-

matic catenation activity (Christensen et al. 2002; Farr et al. 2014).

If compaction is indeed dependent on catalytic activities of toposimerase II, how can catenation/decatenation reactions dictate the state of chromosome compaction, particularly along the longitudinal axis? A potential explanation is that the presence of extensive catenations linking sister DNA molecules could alone preclude the assembly and compaction of mitotic chromosomes. Alternatively, maintenance of chromosome morphology may require a more active role of topoisomerase II throughout mitosis. A possible model is that topoisomerase II is introducing self-entanglements in the DNA molecules and thereby promote shortening of axial length (Kawamura et al. 2010; Bauer, Marie, et al. 2012).

### 1.5.3 Topoisomerase II and biophysical properties of chromosomes

Another important aspect of creating mitotic chromosomes is to ensure the right mechanical properties of chromatin to sustain DNA integrity when chromosomes are subjected to the pulling and pushing forces imposed by the mitotic spindle, cytoplasmic drag, and other factors. The regulation of the topological entanglements within a chromatin network provides a great means for changing physical properties of chromosomes, such as stiffness, elasticity, bending rigidity, physical dimensions. Thus, topoisomerase II is believed to contribute to mitotic chromosome structure also by modulating the biophysical properties of chromosomes. This idea was first raised after observations that topoisomerase II is able to decrease elastic stiffness of isolated mitotic chromosomes (Kawamura et al. 2010). These experiments led to the proposal that the amount of imposed by self-entanglements within the chromosomes would influence the biophysical properties of mitotic chromosomes. As topoisomerase II is able to both entangle and disentangle DNA, it would provide a great way to modulate stiffness and elasticity of chromosomes throughout mitosis. This idea is further supported by microfluidics approaches, in which manipulation of topoisomerase II activity lead to drastic changes of shape of protease-treated mammalian chromosomes, presumably by changing the density of catenations in DNA network within individual chromatids (Bauer, Marie, et al. 2012).

In addition yeast mutants of topoisomerase II suffer from problems in tension-dependent checkpoints in mitosis (Warsi et al. 2008), suggesting that lack of

topoisomerase II may impair stiffness of centromeric region. In agreement, several studies report that topoisomerase II removal triggers a metaphase arrest that delays anaphase onset (Andrews et al. 2006; Skoufias et al. 2004). It has been argued that such delay reflects the presence of a 'topology checkpoint' (Clarke, Vas, et al. 2006). It is nevertheless conceivable that a compromised structure on the pericentromeric chromatin may alone perturb microtubule-kinetochore attachments and thereby trigger the Spindle Assembly Checkpoint by conventional means.

## 1.6 Interplay between condensin I and topoisomerase II

As outlined above, topoisomerase II is actively engaged into shaping mitotic chromosomes throughout the process of nuclear division. It promotes the disentanglement of sister DNA molecules, required for efficient chromosome resolution. In parallel, this enzyme contributes to the compaction of individual chromatids, possibly by introducing self-entanglements. This dual function raises a strong directionality problem. How can topoisomerase efficiently remove catenations in *trans* and thereby resolve sister DNA intertwines, concomitantly with introducing entanglements in *cis* to compact/confer rigidity to mitotic chromatin? In other words, how does topoisomerase II distinguish between strands from the same DNA molecule from heterologous strands?

Condensin complexes, in addition to the proposed structural role, were shown to facilitate sister chromatids separation, as lack of condensin in multiple organisms lead to impaired segregation in anaphase (Bhat et al. 1996; Charbin et al. 2014; Oliveira, Coelho, et al. 2005; Steffensen et al. 2001; Hagstrom et al. 2002). The exact mechanism of how condensin is aiding at resolving DNA is not fully understood. Unlike topoisomerase II, condensin complexes cannot (de)catenate the DNA molecules. Thus, it is highly probable that condensin is cooperating with topoisomerase II (directly or indirectly) to achieve this goal. Various mechanisms have been hypothesized to establish how condensin could aid in sister chromatid resolution although several conflicting observations preclude a define answer. While some studies propose that condensin directly enhances topoisomerase II enzymatic activity (Bhat et al. 1996; Coelho et al. 2003), others failed to detect a similar activation, suggesting that condensin promotes sister chro-

matid resolution by other means than activation of topoisomerase II's catalytic activity (Cuvier and Hirano 2003; Charbin et al. 2014). Depletion of condensins leads to topoisomerase II's delocalization from the chromosome axis (Coelho et al. 2003) and topoisomerase II recruitment to chromosome arms during anaphase was shown to be a condensin-dependent process (Leonard et al. 2015). However, topoisomerase II is still able to bind to metaphase chromosomes in the absence of condensin (Bhat et al. 1996; Coelho et al. 2003; Samejima et al. 2012), implying that condensin does not dictate chromatin targeting of topoisomerase II.

Condensin seems to modulate sister chromatid resolution independently of topoisomerase II activation and/or chromatin targeting, suggesting that the interplay between those two maybe more on a functional basis. Accordingly, it has long been speculated that condensin could somehow provide directionality for topoisomerase II reactions. Recent studies now provide evidence in support of this directionality model in which condensin emerges as a critical complex to favor sister chromatid resolution rather than their re-intertwine (Baxter, Sen, et al. 2011; Sen et al. 2016). These results imply that condensin is not modulating topoisomerase II's catalytic properties, but it is imposing a strong bias towards decatenation, absolutely necessary to drive decatenation needed for sister disentanglement and to prevent topoisomerase II from introducing excessive *de novo* entanglements.

In contrast to the cooperative action for condensin and topoisomerase II in sister chromatid resolution, the interplay of these two proteins in chromatin compaction is far much less understood. Phenotypic analysis suggest they have opposing/distinct roles: condensins were proposed to drive lateral compaction, while topoisomerase II to induce axial compaction (Samejima et al. 2012; Shintomi and Hirano 2011). Interestingly, a contact probability model could also explain these distinct function. In wild-type chromosomes the presence of condensin may not only instruct topoisomerase II to avoid re-catenation in *trans*, but it may also promote and regulate the extent of self-entanglements, and consequently chromatin compaction.

Multiple aspects of mitotic chromosome assembly and maintenance are still unknown. The list of open questions include the influence of condensin I in establishing and maintaining architecture of chromosomes. Does condensin play

any role once chromosomes are established? Is condensin actively compact pre-established chromosomes? Does condensin's cooperation with topoisomerase II conveys any significance beyond initial sister resolution in prophase? In this thesis I will aim to provide answers to those crucial questions. Clarifying the exact mechanism of condensin action will bring us closer to fully understanding the true internal organization of mitotic chromosomes, one of the most enigmatic structures in biology.

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# CHAPTER 2

# Development of an acute system for condensin I inactivation

ABSTRACT CHAPTER 2

In biology we often gain a great amount of insights by removing an object of interest and inferring its function by observing the arising phenotypes. Condensins have been studied by this approach, using various methods to induce removal of condensin, such as mutations abolishing the functionality of the gene or inducing gene silencing. Such tools brought many valuable insights to the field, but carry vast limitations, such as slow and incomplete removal. This leads to accumulative phenotypes and does not allow to study condensin's removal effects during particular parts of mitosis. To overcome those limitations we have designed and developed a TEV protease-based system that would allow precise, efficient and time-controlled inactivation of Barren, one of condensin I's subunits, in *Drosophila melanogaster* embryos. After modifying genomic Barren gene to contain TEV protease cleavage sites we created *Drosophila* lines containing only TEV-cleavable Barren with no wild type version of this protein. We have further proved that the Barren$^{TEV}$ protein can be readily cut *in vivo* and *in vitro*, which results in removal of the condensin I complex from the mitotic chromosomes. Inactivating Barren subunit in mitotic embryos prior to mitosis leads to very severe in chromosome segregation and structure of centromeric regions, compliant with previous slow depletion or mutation studies of condensin I. We have thus generated a novel, efficient tool to compromise condensin I.

## Author contribution:

*All the experiments presented in this chapter were performed and analyzed by Ewa Piskadlo. The experiments were designed by Ewa Piskadlo and Raquel A. Oliveira. Alexandra Tavares prepared and purified proteins used in some of the experiments.*

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## 2.1 Introduction

Chromosome condensation is a complex process composed of sister chromatids resolution, individualization, compaction, and acquiring mechanical properties, such as stiffness. Condensins gained its very suggestive name due to initial data hinting that condensins are a major factor driving mitotic chromosome condensation or compaction (Hirano and Mitchison 1994). Since those initial experiments more and more evidences accumulated that condensins might play lesser role in compaction and is instead responsible for internal organization (Hagstrom et al. 2002; Hudson et al. 2003; Kimura and Hirano 1997; Lavoie et al. 2002; Oliveira, Coelho, et al. 2005; Ribeiro et al. 2009; Steffensen et al. 2001; Houlard et al. 2015). In addition condensins were found to be involved in resolution of sister chromatids in initial stages of mitosis (Gerlich et al. 2006; Hagstrom et al. 2002; Hirano and Hirano 2006; Hudson et al. 2003; Oliveira, Coelho, et al. 2005; Ribeiro et al. 2009; Steffensen et al. 2001) and in many interphase functions (reviewed in Piazza, Haering, et al. 2013 and Rana and Bosco 2017).

What is the true role of condensins in mitosis is rather difficult to assess. Very common methods for the analysis of the role of a particular a biological factor rely on removing it from the system followed by the study of cellular response. In the case of condensins (and other mitotic proteins) this approach is limited by the fast nature of cell division. Removal of a protein of interest by such methods, such as RNA interference or genetic depletion, is usually a long process, often taking hours or even days while mitosis time scale is often less than one hour. As a consequence once a cell reaches maximal level of protein depletion, it will most probably have gone through several divisions with lowered levels of the proteins, likely already causing abnormalities and problems in cell cycle. Therefore slow

depletion methods give us insight into cumulative state of continuous stress of divisions without the protein of interest, making the analysis of the effect quite difficult. In addition, RNA interference-based approaches often do not remove all of the targeted protein, leaving a few percent of unaffected pool. This is particularly problematic if the role of the protein of interest is enzymatic, as even a small fraction of intact protein can be quite robust to partially perform its function, clouding the analysis of protein's intended depletion. Nonetheless, over the years RNAi experiments shed a light on condensin complexes function in cell cycle.

To overcome limitations of slow depletion methods and to gain more precise insight into condensin I's function, a new strategy of very rapid, complete inactivation was needed to allow study condensin I inactivation phenotypes well within one cell cycle, providing much higher temporal resolution. Ultimately, the goal was to rapidly inactivate or remove condensin I specifically from metaphase chromosomes, rather than decreasing pool of condensin long before mitosis.

*Drosophila melanogaster* is a perfect model for studying the role of condensin I in mitosis due to its multiple, synchronized nuclei, a large degree of chromosome compaction in mitosis, and convenience for genetic manipulation to add new elements to the system. In addition, early embryos enable a direct supply of the desired substances into the cytoplasm via injections, such as drugs or mRNAs. This provides a great timing precision of administering substances during the experiments and therefore improves accuracy and reproducibility of the results and simplifies their interpretation.

Acute condensin I inactivation approach will permit a direct assessment of condensin I role in metaphase chromosomes, allowing studying purely the structural maintenance role condensin I in metaphase. This approach, thus, excludes potential artifacts in structure that arose from faulty assembly of chromosomes in earlier stages of interphase and mitosis. This will also avoid any cumulative, indirect effects coming from few rounds of cell cycle divisions with compromised condensin, as in slow, conventional depletion methods.

To rapidly inactivate condensin I in *Drosophila melanogaster* cells we adopted a strategy of cleaving one of its subunits using Tobacco Etch Virus (TEV) protease. A very similar approach was previously applied with great success to

inactivate SMC complexes, for example cohesin in yeast (Uhlmann et al. 2000) and fruit flies (Pauli et al. 2008; Oliveira, Hamilton, et al. 2010; Mirkovic et al. 2015) or condensin complexes in yeast and mouse oocytes (Cuylen et al. 2011; Houlard et al. 2015). TEV protease is a specific, efficient protease which is cleaving ENLYFQ/S sequence (where / is the cleaved bond) (reviewed in Cesaratto et al. 2016). Fast cleavage of kleisin subunit of condensin I (called Barren in *Drosophila*) by TEV protease was predicted to cause irreversible opening of the ring–like structure of condensin I, hence inactivating its biological activity. The Barren subunit was chosen to be a target for TEV protease cleavage, because it is the only subunit creating the ring that is specific to condensin I (two others, SMC2 and SMC4, are also part of condensin II subunit) and contains an unstructured linker. Moreover, it was recently shown that changing the length of prokaryotic SMC proteins compromises functionality of the complex (Bürmann et al. 2017). Alas, an exact structure of Barren is not solved and the exact interphase between Barren and the other four subunits of condensin I complex were not known at the time of designing TEV–cleavable Barren. For this reason, we have chosen four sites to insert TEV protease cleavage sites, in case some of them would not be functional due to structural incompatibility, and tested their performance as a tool for condensin I inactivation.

In this thesis we will describe developing and testing the TEV protein based inactivation of condensin I in *Drosophila melanogaster*. We will demonstrate the methodology behind creating fly strains carrying cleavable condensin I at endogenous levels and tests performed to assess the efficiency of the system. The results prove that TEV-cleavable Barren flies are fully viable and condensin I can be acutely cleaved and removed from chromosomes of *Drosophila* syncytial embryos. The phenotypes obtained with the acute system of *in vivo* condensin I cleavage by TEV is consistent with data from another methodology, but is much more precise and will allow expending the analysis of condensin I function specifically on mitotic chromosomes.

## 2.2 Results

### 2.2.1 Selecting TEV protease cleavage sites for Barren subunit of condensin I

The first challenge in designing a TEV-cleavable form of Barren was finding a right site to insert the sequence recognized by TEV protease. In this case we have used three consecutive repeats of sequence ENLYFQS as a target. To find good candidate sites to insert this sequence into Barren protein three main parameters were taken into account: *1.* the less conserved in evolution the site is, the less it is crucial for protein function *2.* the region of TEV insertion site should not have a defined secondary structure, minimizing risk of compromising Barren's function, *3.* TEV site insertion area should be exposed to the outside of the Barren protein to facilitate TEV protease recognition and access to the TEV sites in order to cut Barren protein

To identify conserved and non-conserved regions in the Barren sequence, multiple alignment of Barren/CAP-H proteins was performed. Condensin subunits are generally quite conserved (Hirano 2012), and therefore we could analyze
Barren/CAP-H sequences from various organisms, from yeast to human, to increase confidence in the final result. The organisms' sources together with their sequence identifiers are listed in Materials and Methods section. The analyses resulted in defining relatively more conserved regions in the sequence (Figure 2.1, not all analyzed sequences are shown). It is worth to note that the most conservation is observed close to N- and C- proximal ends, where Barren is engaged in interacting with SMC2 and SMC4 respectively. There are also multiple conserved regions and residues in the linker region, which may serve as docking site for HEAT subunits of condensin I (CAP-G and CAP-D2) or serve some yet undescribed role.

The structure of Barren subunit is not available, therefore predicting software were used to estimate its secondary structure regions and surface accessibility. Freely available web software IUPred was used to perform secondary structure (Dosztányi et al. 2005) (Figure 2.2a) and NetSurfP 1.0 for surface accessibility (Petersen et al. 2009) (Figure 2.2b), both based solely on *Drosophila* Barren's

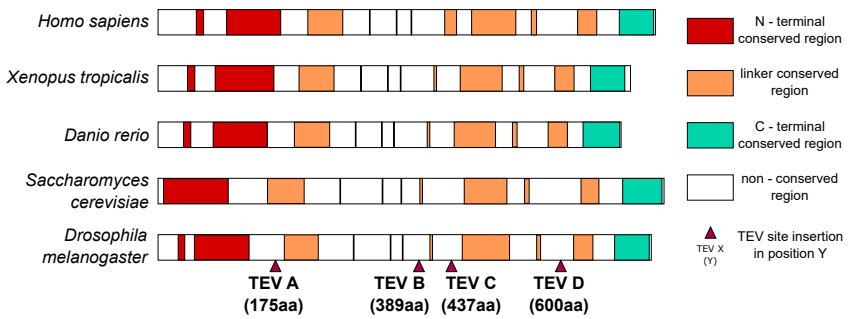


**Fig. 2.1. Conserved regions of Barren subunit of condensin I. (a)** Multiple sequence alignment of Barren/CapH sequence showing higly conserved and less conserved regions and sites selected to insert TEV cleavage sites expressed in amino acid residue. Not all sequences used for building the alignment have been visualized, full details available in Materials and Methods section.

amino acid sequence *FBpp0080881*. Using the predictions regions with high structural disorder (meaning low probability of secondary structure) and high relative surface accessibility (good exposure to the environment) the following four candidate sites were chosen: 175aa (Barren$^{\text{TEV A}}$), 389aa (Barren$^{\text{TEV B}}$), 437aa (Barren$^{\text{TEV C}}$), and 600aa (Barren$^{\text{TEV D}}$) (Figure 2.1 and Figure 2.2).

### 2.2.2 Cloning TEV sites into Barren

The starting point of creating Barren-cleavable flies was a plasmid encoding a genomic region of the Barren gene (2L:20,058,197..20,061,861), together with Barren's endogenous promoter, cloned in the pBlueScriptSK(-) plasmid (kindly provided by Beat Suter, Institute of Cell Biology, University of Bern (Masrouha et al. 2003). The entire region is shown in Figure 2.3 a. The presence of the original regulatory elements was a huge advantage, as it would facilitate expression of TEV-sensitive Barren in fly tissues at the levels very close to the endogenous levels, reducing the risk of mortality or experimental artifacts due to under- or overexpression of the protein. The genomic region was modified to encode the cleavage sequence in the desired sites and myc tags for easier detection (Figure 2.3 b). A scheme of plasmid modifications can be found in Appendix 3. At the end the entire modified genomic region was transferred to pCaSpeR4 plasmids in order to enable subsequent *Drosophila* transformation.

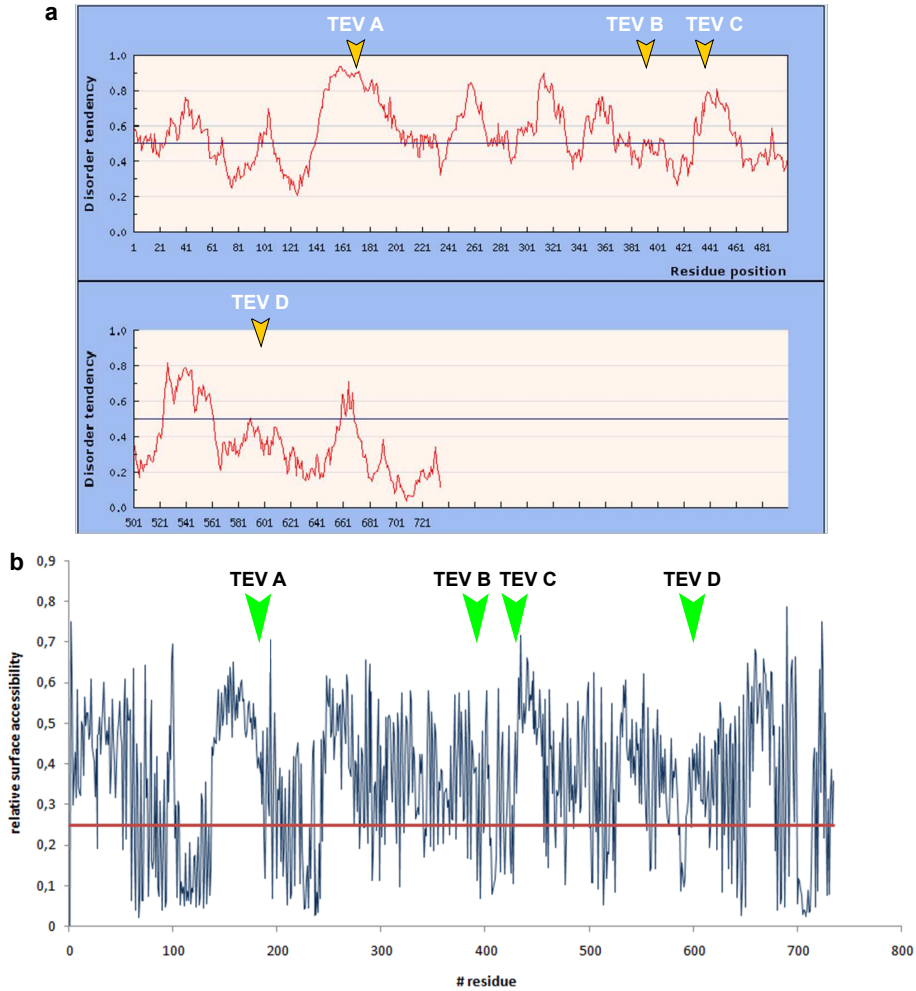


**Fig. 2.2. Predictions of Barren subunit structure and surface accessibility** Prediction of unstructured regions of *Drosophila* Barren subunit (*FBpp0080881*). The horizontal line at 0.5 disorder tendency signifies the threshold value – residues below this value are likely involved in forming secondary structures, above it residues are likely unstructured. Areas in which TEV cleavage sites were chosen are marked with arrowheads. **(b)** Predicted surface accessibility of *Drosophila* Barren subunit based on sequence *FBpp0080881*. Residues above the threshold (red horizontal line) are probable to be exposed to the environment. Green arrowheads point to residues selected for TEV sites insertion.

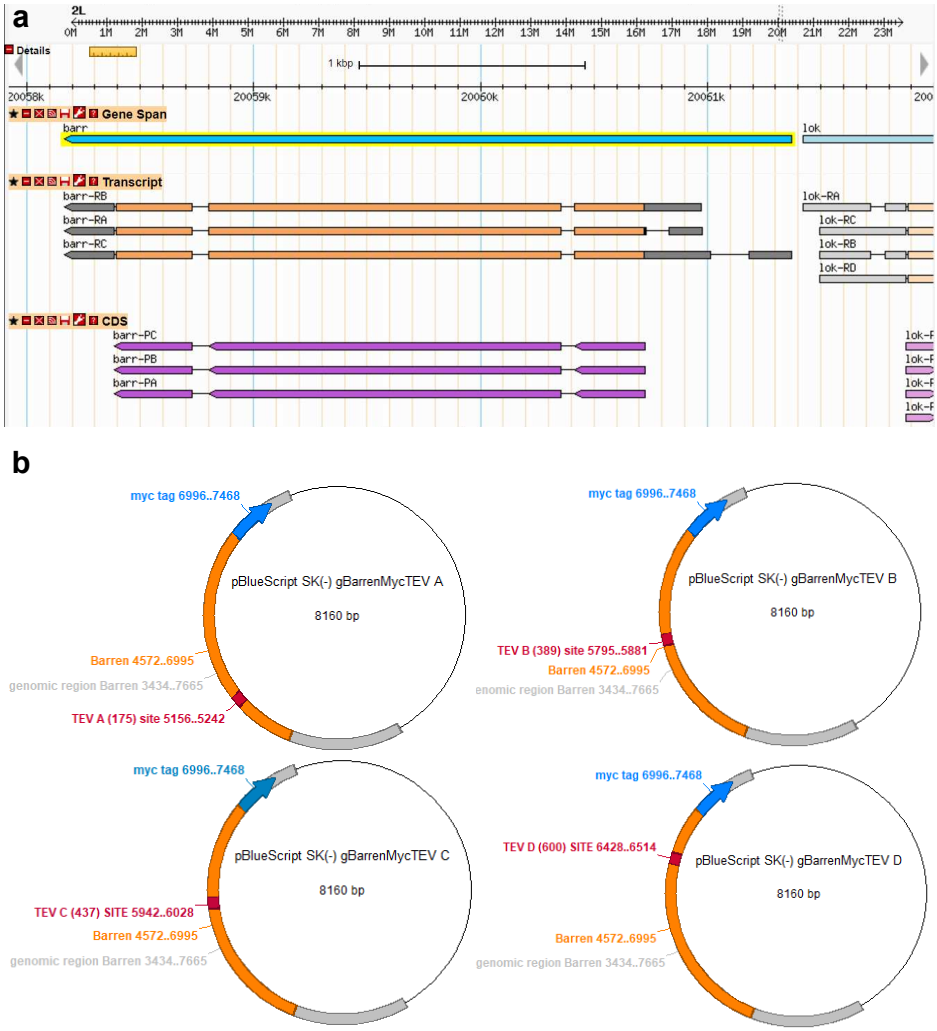


**Fig. 2.3. Genomic region of Barren used for creating Barren$^{\text{TEV A-D}}$ ectopic constructs.** **(a)** FlyBase map of genomic region of Barren 2L:20,058,197..20,061,861 which was used to construct Barren$^{\text{TEV}}$ plasmids. Gene span, mapped transcripts and CDS (coding DNA sequence) is presented. **(b)** Maps of final pBlueScript SK(-) plasmids encoding genomic Barren modified to contain myc tags and TEV cleavage sites. Plasmids are marked for genomic region 2L:20,058,197..20,061,861 (grey), Barren gene (orange), 10 repeats of myc tag (blue) and three repeats of TEV cleavage sites (red).

### 2.2.3 Transient expression of Barren$^{\text{TEV A-D}}$ constructs in DL2 cells

Generated TEV-cleavable Barren constructs were tested *in vivo*, to check whether constructs are correctly translated, localize to mitotic chromosomes similarly to Barren$^{\text{wt}}$, and can be efficiently cleaved by TEV protease. To perform those experiments, *Drosophila* DL2 cells transfected with Barren$^{\text{TEV A-D}}$ constructs were used. In short, cells were transiently transfected with pCaSpeR4 plasmids encoding Barren$^{\text{TEV}}$Myc10, Barren$^{\text{wt}}$Myc10, or empty plasmid, using Effectene Transfection Kit. In those experiments the empty pCaSpeR4 was used as a negative control for translation, localization and cleavage by TEV protease, while Barren$^{\text{wt}}$Myc10 served as a positive control for translation and localization to chromosomes, and as a negative control to TEV protease cleavage. If constructs were also analyzed for TEV cleavage, cells were co-transfected with pRmHa-3 TEV protease, and protease expression was induced by addition of $CuSO_4$ into medium 24 hours prior to harvesting the cells.

Western blot analysis of transfected DL2 cells show that all TEV-cleavable Barren constructs and wild type Barren are expressing a full-length protein in DL2 cells (Figure 2.4a). Co-expressing TEV protease in those cells allows to efficiently cut TEV sites in all the cleavable constructs, as indicated by disappearance of full-length protein detected with myc tag antibody (Figure 2.4a). The cleavage fragment can only be seen in case of Barren$^{\text{TEV A}}$, while we observe no cleaved, myc-tagged protein fragment in the remaining TEV-cleavable Barren constructs. It is possible that the cleaved fragment of TEV A containing myc tag is large and stable enough to survive for longer periods in the cell, while other, shorter cleavage fragments with myc tag are less stable and are being degraded much faster, preventing their detection with the assay used in this study.

To evaluate whether Barren$^{\text{TEV A-D}}$ constructs are still able to perform their function after introduction of the TEV protease recognition sites and introduction of a myc tag, we performed localization studies in DL2 cells. The assumption was that if a construct can localize correctly to mitotic chromosomes the same way as wild-type Barren, there is a good chance that the modified protein is functional, while lack of association to chromosomes in mitosis would predict that the protein is functionally compromised, probably unable to support condensin's function.

The DL2 cells transiently expressing Barren$^{\text{TEV A-D}}$Myc10 or Barren$^{\text{wt}}$Myc10 constructs were enriched in metaphase population by colchicine arrest, fixed, stained for presence of the myc tag and Ser10 phosphorylated histone H3 (mitosis marker) by immunofluorescence and imaged in a wide field microscope (Figure 2.4b). All exogenously expressed Barren constructs could be detected in the cytoplasm. Not all cells were expressing the constructs due to transfection method's efficiency significantly lower than 100%. Nonetheless, if cells were mitotic, the signal from myc tag was enriched in the chromosomes region, suggesting the correct localization of all Barren$^{\text{TEV A-D}}$ constructs. Together, those results suggested that all Barren$^{\text{TEV A-D}}$ constructs are promising candidates to replace non-cleavable Barren in *Drosophila*.

### 2.2.4 Generating *Drosophila melanogaster* strains surviving only on TEV-cleavable version of Barren subunit

Once Barren$^{\text{TEV A-D}}$ constructs were tested in cell culture and proved promising, *Drosophila* strains expressing the constructs were created. The strains were generated by random P-element insertion of a given construct into random sites on chromosomes, supported by pCaSpeR4 vector. Flies were selected for presence of Barren$^{\text{TEV A-D}}$ constructs based on eye color (white gene selection) followed by adding a balancer to the strain to stabilize the insertion in the line and map the insertion chromosome.

We obtained a total of 16 lines where the insertions were mapped to the various chromosomes (Appendix 1). To obtain fly strains that solely contain TEV-cleavable versions of Barren protein, we combined these novel strains with genetic backgrounds that lack the endogenous protein. These experiments serve the double goal of obtaining the desired strains but also to test the functionality of our engineered proteins. Strains carrying the TEV-cleavable version of Barren were thus probed if they can support viability in Barren null mutant background. The mutant allele *barr*$^{L305}$ was previously described as embryonic lethal null allele for Barren (Bhat et al. 1996).

Fly strains encoding Barren$^{\text{TEV A-D}}$ proteins were then crossed with strains carrying deletions of endogenous Barren$^{\text{wt}}$ in order to obtain strains expressing TEV-cleavable version of Barren only. For this we used both *barr*$^{L305}$ allele and

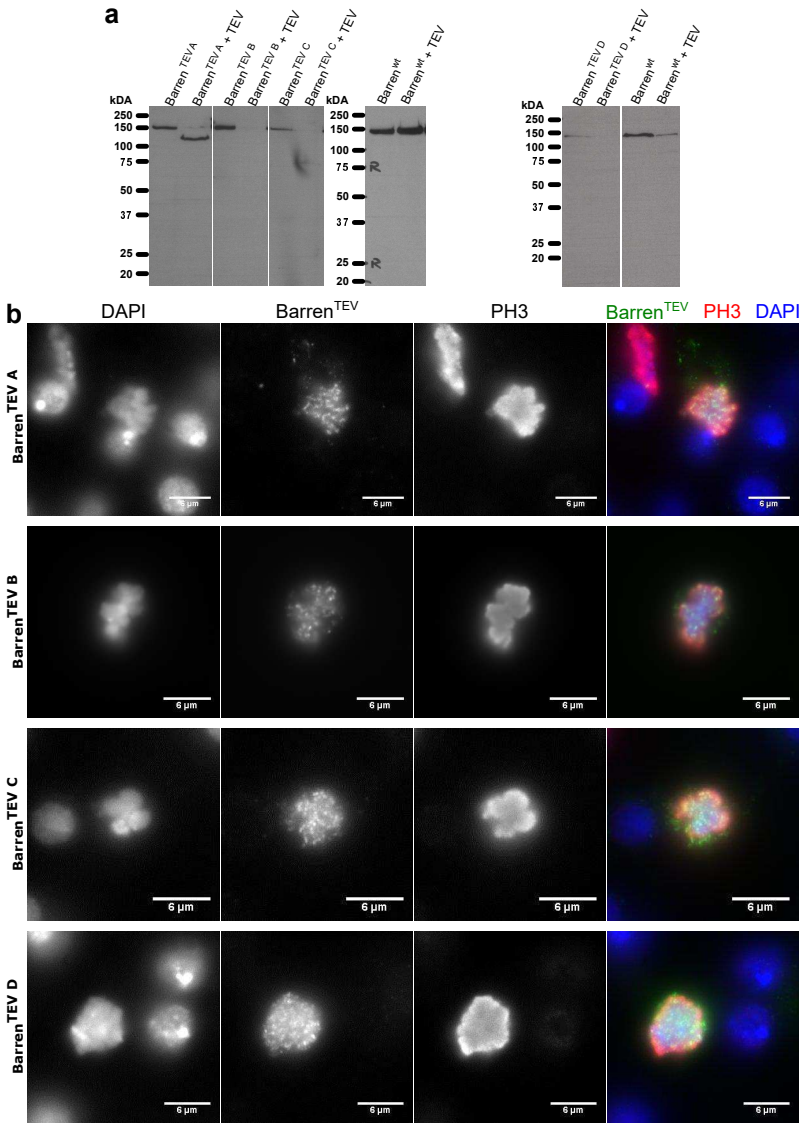


**Fig. 2.4. Transient expression and cleavage of Barren$^{\text{TEV A-D}}$ constructs in DL2 cells.** **(a)** Western blot of DL2 cells transiently expressing Barren$^{\text{TEV}}$ constructs using myc tag antibody. Parallel samples were induced to co-express TEV protease. **(b)** Representative immunofluorescence images of DL2 cells expressing Barren$^{\text{TEV}}$ constructs. Fixed cells were probed for presence of Barren$^{\text{TEV}}$ constructs with myc tag antibody, for mitotic chromosomes with antibody against Ser10 phosphorylated H3 histone and for DNA with DAPI. Scale bars are 6µm.

a deficiency that carries a deletion for that chromosomal region Df(2L)Exel7077 excision (Blommington #7850, RRID:BDSC_7850). The resulting flies ($^{barrL305}$ / Df(2L)Excel7077 ; Barren$^{TEV}$Myc10 / +) have the sequence of functional endogenous Barren removed from the second chromosome. As null-background of Barren is not viable in *Drosophila*, the only way to sustain development in the created flies is a rescue by correct expression of a functional, artificial TEV-sensitive Barren. Simplified scheme of crosses performed to obtain strains is presented in Figure 2.5a.

All the constructs in Barren-null background were able to support viability of flies in a Barren null background, although flies with Barren$^{TEV\ B}$ strain were particularly weak and resistant to create a stable line. The flies were tested by Western blot to confirm the presence of Barren$^{TEV}$ protein and absence of Barren$^{wt}$ protein using a polyclonal antibody (Bhat et al. 1996) (Figure 2.5b). It is also worth to underline that the levels of Barren$^{TEV}$ proteins are roughly the same as the Barren$^{wt}$.

In conclusion, these experiments demonstrate the full functionality of the engineered proteins and enabled the establishment of fly lines that solely contain TEV-cleavable versions of the protein.

### 2.2.5 Testing efficiency of Barren$^{TEVA-D}$ proteins cleavage *in vitro* and *in vivo*.

TEV-cleavable Barren subunits expressed in flies were tested for efficiency of cleavage and condensin complex behavior upon adding TEV protease. Kinetics of Barren$^{TEV\ A-D}$ proteins cleavage was evaluated *in vitro* using *Drosophila* ovarian extracts. Embryos, our target tissue, depends on maternal deposition of syncytial divisions of proteins and mRNAs for syncytial divisions, which takes place during oocyte maturation in the ovaries. As ovaries are easier to obtain in large number, we have used this tissue as a good proxy for protein composition of early embryos. In short, dissected ovaries were homogenized and treated with an excess of TEV protease. The samples were taken every few minutes to assess levels of uncut and cut fraction of Barren$^{TEV}$ protein by Western blot (Figure 2.6a-d). Flies expressing Rad21$^{TEV}$Myc kleisin subunit of cohesin were used as a positive control for TEV protease cleavage (Figure 2.6e), and TEV-cleavable

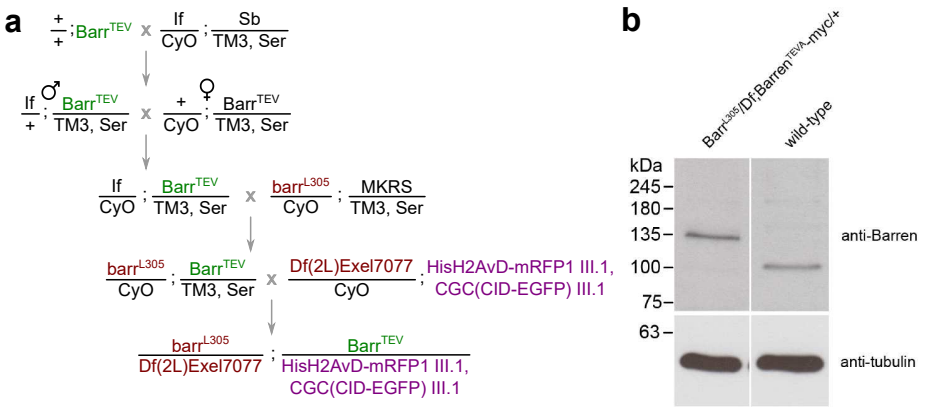


**Fig. 2.5. Generating *Drosophila melanogaster* strains surviving only on TEV – cleavable version of Barren subunit.** **(a)** Scheme of fly crosses performed to generate strain in endogenous Barren-null background, surviving solely on Barren$^{TEV}$, also expressing histone His2Av–mRFP1 and centromere marker CID-EGFP. **(b)** Western blot analysis of protein extract of *Drosophila* ovaries was probed for presence of Barren subunit of condensin I by using a polyclonal anti-Barren antibody. Strains compared in this assay were wild-type flies and a strain in null background of endogenous Barren and carrying Barren$^{TEV\ A}$ insertion. Tubulin was used as a loading control.

constructs treated with TEV protease buffer were a negative control for cleavage and protein stability (Figure 2.6a-e). The experiment was repeated independently three times. To estimate cleavage efficiency, the non-cleaved to cleaved protein fraction was quantified and pooled together to create cleavage kinetics curves for Barren$^{TEV\ A\text{-}D}$ proteins (Figure 2.6f). From all four TEV-cleavable versions of Barren, Barren$^{TEV\ C}$ proved to be cleaved most efficiently by TEV protease, although the difference between the constructs is not very large. Therefore based on the fast kinetics of TEV cleavage and the viability of fly stocks carrying cleavable construct only, flies expressing Barren$^{TEV\ A}$ were selected to be the strain of choice and from now on Barren$^{TEV\ A}$ strain will be referred to as Barren$^{TEV}$.

Behavior of condensin I complex following Barren cleavage was monitored by live imaging in *Drosophila* embryo in 4D. The Barren$^{TEV}$ protein from default *Drosophila* strain is tagged with myc tag, therefore undetectable in live microscopy. To follow Barren localization upon cleavage, embryos of Barren$^{TEV}$ strain were injected with mRNA encoding Barren$^{TEV\ A}$-EGFP to visualize the

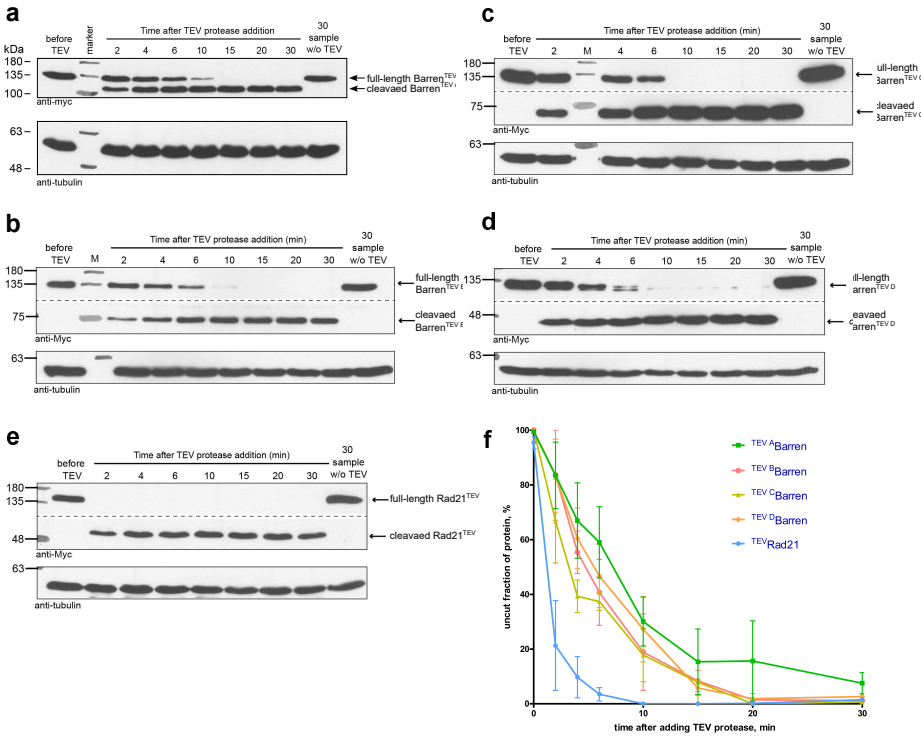


**Fig. 2.6. Testing efficiency of BarrenTEVA-D proteins cleavage *in vitro*.** **(a-e)** Western blot analysis of *in vitro* cleavage of different versions of myc-tagged Barren$^{\text{TEV A}}$ **(a)**, Barren$^{\text{TEV B}}$ **(b)**, Barren$^{\text{TEV C}}$ **(c)**, Barren$^{\text{TEV D}}$ **(d)**, or Rad21$^{\text{TEV}}$ **(e)**. Extracts were prepared from ovaries of flies expressing TEV-cleavable Barren/Rad21 and incubated with TEV protease for the indicated time points. The presence of full-length and cleaved Barren was monitored by western blot using myc antibodies. Tubulin was used as loading control. **(f)** Quantification of data Western blot presented in **(a-e)** combined together with two other independent repeats of *in vitro* cleavage and Western blot.

protein of interest. Once the embryos expressed Barren$^{\text{TEV A}}$-EGFP they were injected with UbcH10$^{\text{C114S}}$ mutant in order to cause metaphase arrest. TEV protease was injected into arrested embryos and time-lapse imaging recorded localization of Barren after its cleavage (Figure 2.7a). Results of these experiments show that Barren is losing its localization from mitotic chromosomes *in vivo* within just few minutes of supplying TEV protease. The fluorescent signal from Barren$^{\text{TEV}}$-EGFP on chromosomes is getting below our detection level within around 10 minutes.

Cleavage timing from *in vivo* embryo experiment are approximately matching expected time of cleavage *in vitro* from ovarian extracts: the vast majority of protein was cut around 10 min after TEV addition, and the total cleavage was observed after around 15 minutes (Figure 2.6a), We may therefore assume that *in vivo*, in embryos, removal of Barren$^{TEV}$ by TEV protease is obtained in the organism context within around 10-15 minutes.

To ensure that the entire condensin I complex, not only the cut Barren, is removed from chromosomes upon TEV-induced Barren cleavage, we traced localization of SMC2-EGFP following TEV protease addition. In order to perform this experiment, a strain expressing Barren$^{TEV}$ (in Barren$^{wt}$-null background) and SMC2-EGFP was created. The embryos were arrested in metaphase by colchicine injection and were subsequently injected with TEV protease or protease buffer and followed by time-lapse imaging. The SMC2 subunit of condensin complexes is removed after TEV protease addition and its fluorescence signal is very significantly reduced (Figure 2.7b). Those results imply that upon Barren cleavage, the entire condensin I complex is removed from mitotic chromosomes, providing higher level of confidence that Barren cleavage can efficiently compromise functionality of the entire condensin I complex.

### 2.2.6 Inactivation of condensin I prior to mitosis.

Next, we confronted our fast depletion system of condensin I based on TEV protease depletion with slow depletion methods used in the literature (i.e. RNAi or conditional knock-out) (Coelho et al. 2003; Hagstrom et al. 2002; Oliveira, Coelho, et al. 2005; Vagnarelli et al. 2006) to evaluate whether the phenotype of condensin I removal in interphase will be the same in both approaches.

To analyze the result of acute inactivation of condensin I in interphase and how it effects the following mitosis, we used embryos of Barren$^{TEV}$ strain expressing histone-RFP to mark chromatin and a centromere histone variant CID (human CENP A homolog) marked with EGFP. Embryos undergoing the 11th – 13th syncytial division were injected with TEV protease in very early interphase and followed by time-lapse imaging by wide field microscopy through the entire interphase and the consequent mitosis. We observe that nuclei of the embryo with TEV-cleaved Barren significantly condense prior to metaphase, but do not

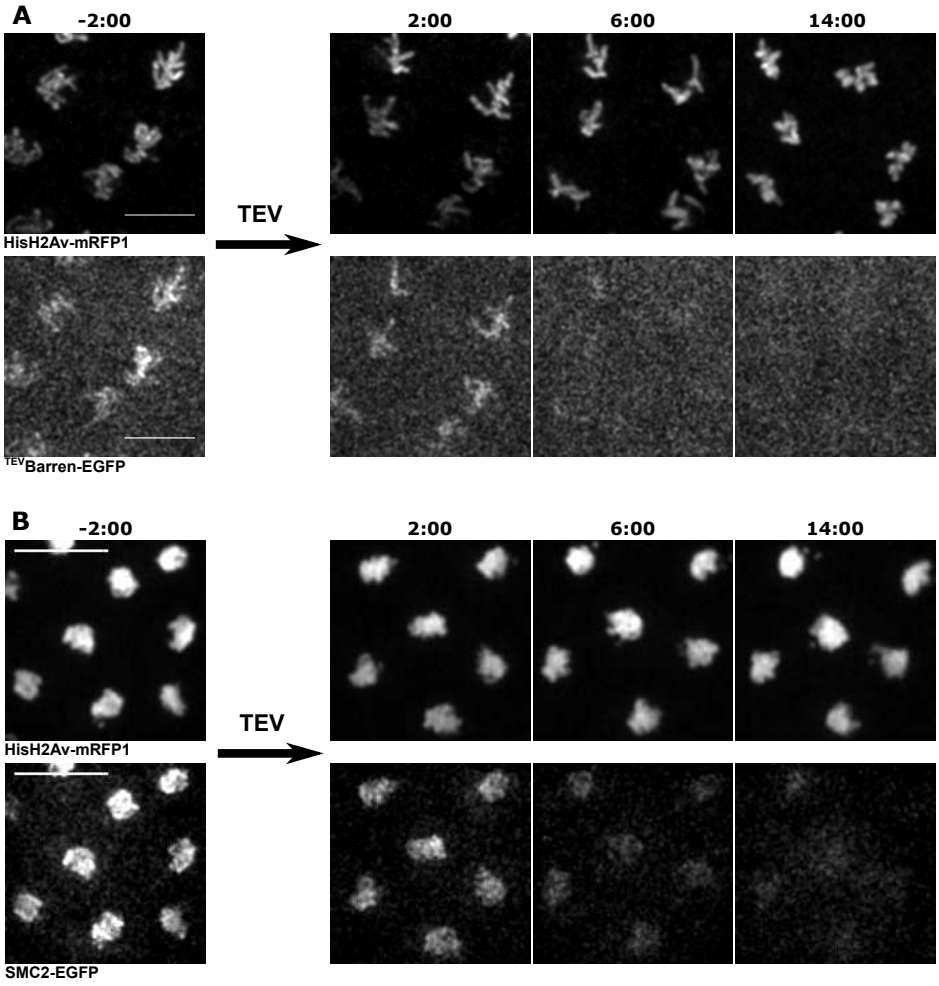


**Fig. 2.7. Visualizing behavior of condensin subunits after Barren$^{TEV}$ *in vivo*.** **(a)** Early embryos (0-30 min old) expressing HisH2AvD-mRFP1 were injected with mRNA coding for Barren$^{TEV}$-EGFP.. Embryos were aged for 1h-1h30m to allow for protein expression. Embryos were injected with 12 mg/ml UbcH10$^{C114S}$ protein to arrest in metaphase and subsequently with 13 mg/ml TEV protease. **(b)** Embryos expressing SMC2-EGFP subunit of condensins and HisH2AvD-mRFP1 in Barren$^{TEV}$ background were arrested in metaphase by 2mM colchicine injection. Once nuclei reached metaphase, TEV protease was injected. (a-b) Images depict the same region before and after TEV injection; times (minutes:seconds) are relative to the time of injection; scale bar is 10 μm.

reach proper alignment of centromeres (Figure 2.8 and Movie 1). Even more striking, when nuclei try to undergo anaphase, chromatids are not resolved at all, as implied by massive amount of anaphase bridges (Figure 2.8 and Movie 2), while the control embryos injected with TEV protease buffer segregate towards the opposite poles without obstacles. The phenotypes of lack of sister chromatids segregation and centromere impairment in condensin I depletion were previously described in the literature with slow depletion methods and are in compliance with the results obtained by TEV protease mediated condensin I inactivation (Gerlich et al. 2006; Hagstrom et al. 2002; Hirano and Hirano 2006; Oliveira, Coelho, et al. 2005; Ribeiro et al. 2009; Steffensen et al. 2001).

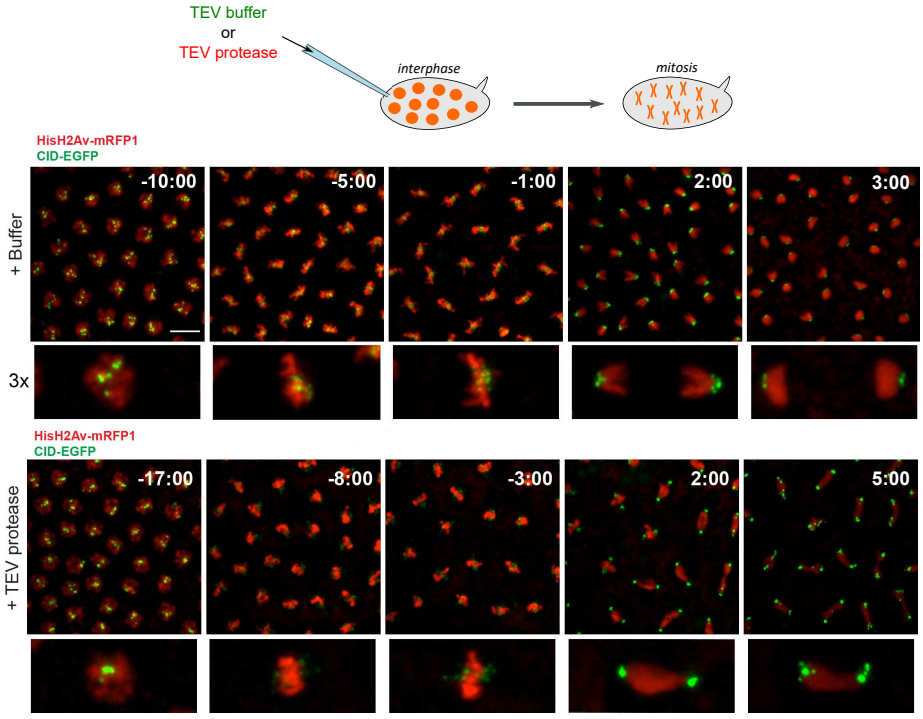


**Fig. 2.8. Condensin I inactivation prior to mitotic entry.** **(a)** Embryos surviving solely on Barren$^{TEV}$ were injected with buffer or 13 mg/ml TEV protease ~ 10-15 minutes before mitosis; Embryos also express His2Av–mRFP1 (red) and CID-EGFP (green); scale bars, 10 µm. Bottom rows show higher magnifications (~ 3x) of a single nuclear division. Times (minutes:seconds) are relative to the time of anaphase onset.

## 2.3 Discussion

This chapter describes the development and testing of a novel tool able to acutely inactivate condensin I complex in *Drosophila melanogaster*. The major drive to create the novel system was evading the ambiguous nature of results obtained by slow depletion methodologies, to allow deeper and more definitive answers towards condensin's role in mitosis. We aimed to study the effect of condensin I on maintenance of structure of mitotic chromosomes that were previously established in the presence of condensin I and not perturbed in any way. This approach would provide a unique opportunity to disentangle condensin's role in sister chromatids resolution from its structural role.

To analyze the effect of condensin I's removal from normally pre-established chromosomes, rapid inactivation of condensin I is crucial. Chromosomes in *Drosophila* embryos can be arrested in mitosis only for limited amount of time (usually around 30 – 40 min) before they suffer strong adverse effects due to the arrest, such as overcondensation and nuclei fusion. Therefore removal of protein from chromosomes has to be as fast as possible to not only allow complete inactivation of the protein of interest, but also to provide time frame long enough to expose arising phenotype and its kinetics.

Our strategy of condensin I inactivation was based on breaking the closed-ring structure of the complex by cleaving one of its subunits. The rationale behind this strategy was based on the fact that condensin I, like other SMC complexes, needs the closed-ring structure to perform its function (Gruber et al. 2003; Gligoris et al. 2014; Wilhelm et al. 2015). TEV-mediated system of cleavage was previously used to inactivate cohesin complex in *Drosophila melanogaster*, providing confidence for successful outcome of the new tool. Similarly to yeast system of TEV-cleavable condensin (Cuylen et al. 2011) we have targeted kleisin CAP-H subunit of condensin I, named Barren in *Drosophila*. We predicted that, as in the yeast model, cutting one of the protein of the ring–forming complex will cause the topologically closed structure to open, deeming the whole condensin I complex to lose its functionality.

We have successfully developed a tool that allows an acute, efficient inactivation of condensin I complex in *Drosophila melanogaster* embryo. In this

system Barren subunit of condensin I was modified to include three consecutive cleavage sites recognized by an exogenous TEV protease. Unfortunately, at the time of designing the TEV protease cleavage sites into Barren little data of its structure or exact interaction interphases with other subunits were known. More recent data were able to map interaction sites between Barren and the other non-SMC subunits by crosslinking those protein in *S. cerevisiae* (Piazza, Rutkowska, et al. 2014). The sites of strong interactions between Barren and Ycs4 and Ycg1 subunits are shown on Figure 2.9. The sites we have chosen do not seem to be inserted in any of those interaction hubs, providing that those exact sites are conserved in evolution. In addition, Figure 2.9 illustrates location of TEV sequences used in yeast (Cuylen et al. 2011) for comparison of the TEV sites locations. The site TEV A (175) in *Drosophila* appears to be in a similar location as yeast's TEV (141), TEV B (437) is rather close to yeast's TEV (363), and TEV D (600) is placed in a comparable position as TEV (622) in yeast. None of constructs we created has a similar location of TEV cleavage site used mouse system TEV (273) (Houlard et al. 2015).

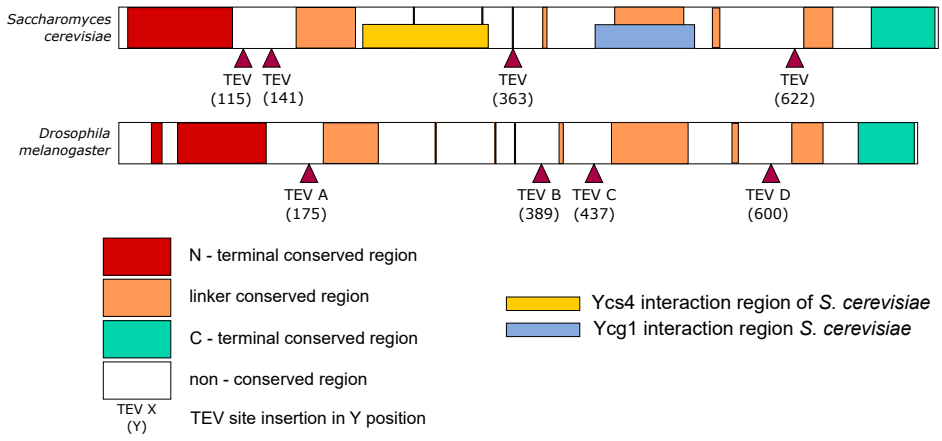


**Fig. 2.9. Known interactions between Barren and other non-SMC subunits in yeast shown in the multiple sequence alignment.** The interactions between Ycs4 or Ycg1 and Barren in *S. cerevisiae* was shown to be strongest for the Barren fragments 224-340aa and 439-531aa for Ycs4 and Ycg1 respectively, as published in Piazza, Rutkowska, et al. 2014. The sites of TEV sequence insertions that are able to rescue Barren function and be cleaved by TEV protease are marked for *Drosophila melanogaster* (this publication), and *S. cerevisiae* (Cuylen et al. 2011).

Measurements of cleavage kinetics by TEV protease proved that it is possible to cut *in vitro* the engineered Barren proteins isolated from flies. Comparing to cleavage of Rad21, kleisin subunit of cohesin, Barren is cut more slowly. In our *in vitro* cleavage system of ovarian protein extracts Rad21 is completely cleaved within 2 – 4 minutes after protease addition, whereas complete cleavage of Barren takes around after 15 – 20 minutes, and is similar between all TEV–sensitive versions of Barren. The source of such a large difference of cleavage efficiency is not known. In sonicated protein extract used in our assay both Barren and Rad21 should be mostly in the solution, therefore the difference cannot be explained by various location or binding on the chromosomes. One of possible reasons for divergent cleavage of kleisins might be the spatial structure of the proteins. It can be speculated that Barren is very differently folded, leading to burying the TEV recognition sequence deeper into protein, decreasing efficiency of the protease to recognize and/or cleave the protein. Alternatively, The TEV recognition sites in Barren may be close to the interphase of interactions between Barren and other subunits of condensin I, also leading to decreased exposure to TEV protease. Nonetheless, even if TEV protease cleaves Barren slower than expected based on previous Rad21 data, the time window of 10 – 15 min to inactivate condensin I is still reasonable and much faster than alternative methods. Moreover, the TEV-dependent cleavage allows to inactivate the entire population of Barren, decreasing any possible analyses misinterpretations that could arise from incomplete cleavage of protein of interest.

Live imaging of EGFP-tagged Barren$^{TEV}$ allowed to confirm removal of cut protein from the mitotic chromosomes. Presence of SMC2 subunit of condensin I is also decreasing to almost undetectable level upon Barren inactivation. This not only confirms that the entire complex is losing functionality, but also shows that levels of condensin II in *Drosophila* embryo are very low in mitosis. This observation supports the notion of condensin I being the major mitotic version of condensin, as predicted based on much larger severity of condensin I mutations comparing to condensin II in *Drosophila*. Namely, condensin II mutants do not show signs of mitotic defects and can produce viable, yet sterile, progeny (Savvidou et al. 2005; Hartl et al. 2008). On the other hand depletion of condensin I complex alone (Oliveira, Coelho, et al. 2005) or both condensin complexes at

the same time (Steffensen et al. 2001; Coelho et al. 2003), leads to the same phenotype in mitosis, enforcing the notion that condensin I is a major mitotic condensin complex in *Drosophila*. Additionally, one of subunits of condensin II, CAP-G2, was not found in *Drosophila* even on the genomic level (Ono et al. 2003), suggesting major changes to the complex comparing to other organisms. It is conceivable that condensin I is partially redundant in its function in mitosis, allowing for the development, but due to its lack of access to the nucleus outside of mitosis it cannot rescue all effects of condensin II depletion. Whether condensin II indeed plays a role in mitosis, despite its low levels, or its role is limited to interphase and germ line development is yet to be determined.

Some of the best described phenotypes due to condensin I depletion were structural abnormalities, especially in the centromeric region, and lack of sister chromatids resolution. Even though the main advantages of the TEV protease-based condensin I inactivation system is to be able to inactivate condensin I specifically in metaphase, inactivation of condensin I in interphase was a very important step in developing the system. We meant to test whether the results of acute inactivation of condensin I in interphase in single round of cell division will lead to the same phenotypes obtained by slow, often incomplete depletion methods. Using TEV–mediated condensin I inactivation in early interphase and following cell cycle progression, very similar phenotype of lack of condensin I was observed, namely lack of proper centromere alignment and severe problems in sister chromatids resolution. Other published works in which showed mostly thin anaphase bridges as the result of persistent catenation in *C. elegans* (Hagstrom et al. 2002) or HeLa cells (Gerlich et al. 2006). Even other *Drosophila* tissues, such as S2 cells or neuroblasts (Steffensen et al. 2001) show lesser failure in segregation than our system, which produces much more severe failure of very thick bridges with complete lack of segregation. This is most likely due to two factors: firstly, efficient and complete removal of functional protein, and secondly due to rapid divisions in early embryonic stages. Direct injections of TEV protease to cytoplasm allows faster impairing condensin I than majority of alternative methods and therefore the effect on chromatin segregation is elevated. The suspected reason for the massive amounts of unresolved catenations we observed is extremely fast replication characteristic for fast dividing *Drosophila* embryos.

The entire fruit fly genome in early syncytial division can be replicated in as little as 4 minutes (Blumenthal et al. 1974). To cope with such limited time for replication, much higher number of origin of replication sites are fired (Sasaki et al. 1999; Kermi et al. 2017). This may lead to high amount of converging replication forks, therefore tremendous number of catenations between freshly replicated DNA molecules. Those multiple intertwines must be efficiently removed before the fast approaching anaphase. In addition, checkpoints are muted in early *Drosophila* embryogenesis, which likely renders embryos even more susceptible to segregation errors. Therefore thanks to the properties of early fly development perturbations in chromatids resolution are exacerbated and more readily detectable comparing to other systems.

To summarize, the results of this chapter reinforce the proposed notion that condensin I is involved in both mechanical reinforcement of chromosomes as well in removing entanglements between sister chromatids DNA. Moreover, the TEV cleavage system proofs to be highly suitable to assess the role of condensin I in metaphase chromosomes, which will be explored in Chapter 3.

## 2.4 Materials and Methods

### 2.4.1 Generation of recombinant plasmids

Recombinant plasmids were created using standard molecular biology techniques. In short, PCR reaction products or plasmids were digested with desired restriction enzymes according to manuals. Digested PCR products were purified directly using QIAquick Gel Extraction Kit (Qiagen), and the linearized plasmids were run in 0.8% agarose gel with ethidium bromide, the selected band of appropriate size was cut and purified using the QIAquick Gel Extraction Kit. The vector was dephosphorylated using 5 units of Antarctic Phosphatase (New England Biolabs) and ligated with the insert at 1:3 vector to insert molar ratio. Ligation was performed using 5 units of T4 DNA Ligase (Thermo Scientific) per 20µl of final reaction mix volume, containing a Rapid Ligation Buffer. The ligation mix was incubated for 5 min in room temperature. DH5$\alpha$competent bacterial cells were transformed with 5 µl of the obtained ligation reaction and grown

overnight at 37°C on standard LB-agarose medium containing appropriate selective antibiotics. Single colonies were amplified in a few-milliliter scale liquid cultures in LB containing selective antibiotics in 37°C, 300 RPM shaking for 10 – 16 hours. The plasmid DNA of single clones were isolated using ZR Plasmid Miniprep Classic Kit (Zymo Research). Isolated plasmid DNA was tested for correct sequence and insert orientation by sequencing and restriction enzyme digestion followed by electrophoresis in 0.8% agarose gel with RedSafe (INtRON Biotechnology).

### 2.4.2 Constructing Baren$^{TEV}$EGFP plasmids

cDNA was amplified from a cDNA clone (RE15383, Drosophila Genomics Resource Center) using the primers 5' ACCATGGCTAGCACTCTGCCCCGCTTAGAAACTCCG 3' and 5'ATTCTAGAATCCAACACCTGGCGAATTTGAAAG TCC3'. This amplified DNA was cloned into pRNA-EGFP to make a C-terminal fusions of Barren. Subsequently, TEV sites were introduced as follows: an AvrII site was introduced at the desired location by site directed mutagenesis using the following primers: for TEV in position 175 (A) forward 5' GCAAAAGAAGCACCTAGGGCACCGAAGCCCAAACGG 3' and reverse 5' CCGTTTGGGC7 TTCGGTGCCCTAGGTGCTTCTTTTG 3', for TEV in position 389 (B) forward 5' CGGCTACGTAAGCAGCCTAGGACAGAGTTCATCGAGG 3' and reverse 5' CGGCTACGTAAGCAGCCTAGGACAGAGTTCATCGAGG 3', for TEV in position 437 (C) forward 5' GCACTTTCTCGCAGCCTAGGACCAAT GGACAGGTG 3' and reverse 5' CACCTGTCCATTGGTCCTAGGCTGCGAGAAAGTGC 3', and for TEV in position 600 (D) forward 5' GATTGGCCTCACCCAGCCTAGGATGAACGCCACTTGC 3' and reverse 5' GCAAGTGGC GTTCATCCTAGGCTGGGTGAGGCCAATC 3'. 3×TEV recognition sites, flanked by SpeI and AvrII sites, were cloned into AvrII-cut plasmid. Insert orientation was determined by sequencing. The maps of the obtained sequences are presented in Appendix 2.

### 2.4.3 Constructing genomic Barren$^{TEV}$Myc plasmids

To obtain a transgenic flies carrying TEV-cleavable version of Barren gene, pCaspeR4 plasmids compatible with P-element integration were constructed to

carry genomic Barren sequence regulatory elements, 10 repeats of myc tag and three consecutive TEV protease recognition sites inside the coding sequence. A construct carrying a ~4.7 kb Barren genomic region was used as a starting point (kindly provided by Beat Suter, Institute of Cell Biology, University of Bern). This region contains the regulatory sequences and was previously shown to restore Barren function (Masrouha et al. 2003) Firstly, genomic Barren sequence with ten repeats of myc tag at C terminus in pBlueScript SK(-) vector was subjected to site-directed mutagenesis to introduce AflII and AscI sites, using following primers: AflII forward - 5' GCGCATTTCAGCTGGCTTAAGTGCACGAACTCTTACG 3', AflII reverse – 5' CGTAAGAGTTCGTGCACTTAAGCC AGCTGAAATGCGC 3', forward AscI – 5' GCACCGAGTTCGAGGGCGCGC CGTCGCAGGTG 3', reverse AscI – 5' CACCTGCGACGGCGCGC CCTCGAACTCGGTGC 3'. The plasmid was then cut with AscI and AflII restriction enzymes and fragment of size 6.6 kbp was used as a vector. This vector was ligated to PCR product of primers amplifying middle part of cDNA sequence from pRNA Barren-EGFP with 3×TEV sites in various positions, depending of a template (position 175 (A), or 389 (B), or 437 (C), or 600 (D)). Primers used for the amplification were the same as ones used for AflII and AscI sites site-directed mutagenesis and AlfII and AscI sites at the ends: forward AflII– 5' GCGCATTTCAGCTGGCTTAAGTGCACGAACTCTTACG 3', reverse AscI – 5' CACCTGCGACGGCGCGCCCTCGAACTCGGTGC 3'. The maps of the obtained sequences are presented in Appendix 3.

### 2.4.4 Fly strains

To destroy condensin by TEV protease-mediated cleavage, strains carrying solely TEV-sensitive Barren versions were produced. Each variant of genomic Barren with different TEV sites was cloned into pCaSpeR4 vector used for fly transformation. Transgenic flies were produced by P-element integration (BestGene). Transgenes were placed in a $barr^{L305}$ background, a Barren null allele (Bhat et al. 1996) over a deficiency for the corresponding genomic region (Df(2L)Exel7077, stock #7850 from Bloomington stock center). To destroy cohesin by TEV-protease we used strains carrying $Rad21^{TEV}$, previously described (Pauli et al. 2008; Oliveira, Hamilton, et al. 2010). Fly strains also expressed His2AvD-mRFP1 or polyu-

biquitin His2B-RFP, to monitor DNA and CID-EGFP to monitor centromeres (Schuh et al. 2007). A list with detailed genotypes can be found in Table 2.1.

**Table 2.1.** Fly strains used in this thesis.

| CHR # * | Genotype | Reference |
|---|---|---|
| 1418 | BarrL305/CyO | Bhat et al. 1996 |
| 1421 | Df(2L)Exel7077/CyO | Blommington #7850 |
| 1513 | w;; Barr$^{175\text{ - }3TEV}$-myc10 III.5 | Piskadlo et al. 2017 |
| 1509 | w; Barr$^{175\text{ - }3TEV}$-myc10 II.1 | Piskadlo et al. 2017 |
| 1522 | w;; Barr$^{389\text{ - }3TEV}$-myc10 III.2 | Piskadlo et al. 2017 |
| 1514 | w;; Barr$^{437\text{ - }3TEV}$-myc10 III.1 | Piskadlo et al. 2017 |
| 1520 | w;; Barr$^{600\text{ - }3TEV}$-myc10 III.3 | Piskadlo et al. 2017 |
| 1525 | w;; Barr$^{wt}$-myc10 III.1 | Piskadlo et al. 2017 |
| 1560 | w; *barr*$^{L305}$/ Df(2L)Exel7077 ; Barr$^{175\text{ - }3TEV}$-myc10 III.5 | Piskadlo et al. 2017 |
| 820 | w;; HisH2AvD-mRFP1 III.1, CGC(CID-EGFP) III.1 | Schuh et al. 2007 |
| 1564 | Df(2L)Exel7077 / CyO ; HisH2AvD-mRFP1 III.1, CGC(CID-EGFP) III.1 | Piskadlo et al. 2017 |
| - | w; *barr*$^{L305}$/ Df(2L)Exel7077; Barr$^{175\text{ - }3TEV}$-myc10 III.5/HisH2AvD-mRFP1 III.1,CGC(CID-EGFP) III.1 | Piskadlo et al. 2017 |
| 1646 | w; textitbarr$^{L305}$, Barr$^{175\text{ - }3TEV}$-myc10 II.1; +/+ | Piskadlo et al. 2017 |
| 1649 | w*;; M[w+mc=gSMC2h-EGFP.attB]ZH-96E | Herzog et al. 2013 |
| - | w; *barr*$^{L305}$, Barr$^{175\text{ - }3TEV}$-myc10 II.1/Df(2L)Exel7077; M[w+mc=gSMC2h-EGFP.attB]ZH-96E/HisH2AvD-mRFP1 III.1,CGC(CID-EGFP) III.1 | Piskadlo et al. 2017 |

* Number of the laboratory's internal fly database.

### 2.4.5 Western blotting

For DL2 cells, the density of cell cultures were determined and $5 \times 10^5$ cells per one Western blot sample were spun down for 5 minutes, 500g. The supernatant medium was removed, cells were washed once in PBS and 20 µl PBS was added to resuspend the cells. 20 µl of 2× concentrated Laemmli sample buffer (Sigma Aldrich) was added and the solution was incubated in 95–100°C for 5 min. Samples were stored in -20°C until use.

*Drosophila* ovaries sample preparation is described in details below. Samples were loaded on a 10% SDS-gel for electrophoresis, transferred onto a PVDF membrane. Western-blot analysis was performed according to standard protocols using the following antibodies: anti myc-tag (1:200, 9E11, Santa Cruz Biotechnology), anti-$\alpha$-tubulin (1:50.000, DM1A, Sigma) and anti-Barren (1:3000, kindly

provided by Hugo Bellen (Bhat et al. 1996).

Ovaries were dissected from females and homogenized in PBS. Extracts were sonicated for 2 min in a water bath (power 5- Sonicator XL2020, Misonix). After centrifugation for 10 min at 15 000 rpm at 4°C, the supernatant was removed and adjusted to a final concentration of 2μg/μl. For cleavage experiments, 80 μl of extract were incubated with 2 μg of TEV protease. At the indicated time points, 10μl of the reaction were diluted with sample buffer, boiled and stored at -20°C.

### 2.4.6 Transient transfection of DL2 cells

Transient transfections were performed with Effectene Transfection Reagent kit (Quiagen). The cells were seeded in 1.6 ml at $8\times10^5$ cells/ml density in Schneider's medium (Gibco) with 10% fetal bovine serum (Gibco) and Penicillin - Streptomycin mix (Sigma-Aldrich) in 6-well plates and were grown for 24 hours in 25°C. After that time the Effectene Transfection Reagent mix containing 400 ng of DNA was added, as instructed manufacturer's protocol. Cells were incubated for around 48 hours in normal conditions. If plasmids needed to be induced by copper, filter-sterilized $CuSO_4$ (Sigma) solution was added to final concentration 1 mM 24 hours prior to harvesting cells. To arrest cells in metaphase colchicine (Sigma Aldrich) was added to medium at final concentration 0.03 mM 4 hours prior to harvesting.

### 2.4.7 Immunofluorescence of DL2 cells

Cell culture was enriched in metaphase cells by adding colchicine (Sigma Aldrich) to the medium to final concentration 30 μM and incubated for around 3 hours in optimal growth conditions. After that the density of cells in the culture was measured in a counting chamber and the cells in medium were transferred to a fresh wells with 18 mm ø coverslip coated with concanavalin A (Sigma) in a cell density 3.2 x $10^6$ cells per coverslip, and let to settle in room temperature for 30 min, to allow adhesion to the coverslip. After that the excess medium was aspirated and cells were fixed for 8 minutes in solution of 3.7% formaldehyde (Sigma Aldrich) and 0.5% Triton X-100 (Sigma Aldrich) in PBS, followed by washing the cells twice in PBS with 0.1% TritonX-100 (PBST) and once in PBS,

5 minutes for each washing step. The cells were blocked in blocking solution (5% goat serum in PBST) for 30 minutes in room temperature and washed three times with PBST, 5min each wash. Cells were then incubated overnight in 4°C with solution of primary antibodies in blocking solution. After three washed with PBST, coverslips were incubated in a secondary antibodies in blocking solution for 2 hours in room temperature, in a dark chamber. After three washes in PBST, the coverslips were mounted on slides with VECTASHIELD Mounting Medium with DAPI (Vector). Antibodies used were anti-my tag (1:100, 9E11, Santa Cruz Biotechnology) and anti-Ser10 phosphorylated H3 (1:500, sc-8656, Santa Cruz Biotechnology).

### 2.4.8 Microscopy

Aligned embryos on coverslips were covered with Series 700 halocarbon oil (Halocarbon Products Corporation). Imaging of embryos after mRNA injection was performed with a spinning disc Revolution XD microscope (Andor) at 22°C. Stacks of around 20 frames 1 μm were taken at indicated times using a 100×1.4 oil immersion objective (Nikon) and iXon+ 512 EMCCD camera (Andor). Time-lapse microscopy was performed with an inverted wide-field DeltaVision microscope (Applied Precision Inc.) at 18 – 20 °C in a temperature-controlled room. One stack of ~ 20 frames (0.8 μm apart) was acquired every 1 or 2 minutes using a 100×1.4 oil immersion objective (Olympus) and an EMCCD camera (Roper Cascade 1024). Wide-field images were restored by deconvolution with the Huygens v15.10/16.10 deconvolution software using a calculated point-spread function (Scientific Volume Imaging). Movies were assembled using FIJI software (Schindelin et al. 2012) and selected stills were processed with Photoshop CS6 (Adobe).

### 2.4.9 Microinjections

Microinjection experiments were performed as previously described (Oliveira, Hamilton, et al. 2010). 1 – 1.5 h old embryos (or 0 – 30 min for mRNA injections) were collected and processed according to standard protocols and embryos were injected at the posterior pole (up to three sequential injections) using a Burleigh Thorlabs Micromanipulator, a Femtojet microinjection system (Eppen-

dorf), and pre-pulled Femtotip I needles (Eppendorf). Embryos were injected with buffer, drugs or proteins purified from *E. coli* at the following concentrations: TEV buffer (20 mM Tris-HCl at pH 8.0, 1 mM EDTA, 50 mM NaCl, and 2 mM DTT), 13 mg/ml TEV protease in TEV buffer, 12 mg/ml UbcH10$^{C114S}$, 2mM colchicine (Sigma-Aldrich).

### 2.4.10 Protein purification

Purified TEV protease was described previously (Haering et al. 2008). Purification of UbcH10$^{wt}$ and UbcH10$^{C114S}$ was performed from BL21 cells as previously described (Oliveira, Hamilton, et al. 2010), with minor modifications, as follows. Bacterial cells were grown for 16 hours at 37°C, 225rpm. This preculture was used to inoculate fresh LB media and cells were allowed to grow until 0.8/1 ODs. Cultures were then induced with 1mM IPTG and after 4h of induction at 37°C, 225rpm, cells were harvested. Pellets were resuspended in Lysis Buffer (20mM Tris-HCL pH7.5, 0.5M NaCl, 5mM Imidazole with protease inhibitors) and sonicated 5× on ice in 30 sec cycles (power 5, Sonicator XL2020, Misonix). The soluble fraction of the extracts was then incubated in TALON Metal Affinity Resin (Takara), according to manufacturer's instructions. After several washes with Lysis Buffer, the resin coated with the protein was packed into a Poly-Prep Chromatography Column (Biorad). Proteins were eluted in the same buffer with 300mM imidazole. For buffer exchange, purified UbcH10$^{wt}$ and UbcH10$^{C114S}$ proteins were dialyzed overnight, at 40C, in a Slide-a-Lyzer 7KDa Dialysis cassettes (Thermo Scientific). Final storage buffer was 20mM Tris-HCL pH7.5, 0.3M NaCl). The purified proteins were concentrated in a Vivaspin 6 Centrifugal Concentrator MWCO 10 000KDa (GE Healthcare).

### 2.4.11 mRNA expression of Barren$^{TEV}$Myc in *Drosophila* embryos

Barren$^{TEV\ A}$-EGFP was cloned into a pRNA plasmid and mRNA was synthesized by *in vitro* transcription with the mMessage mMachine T3 kit (Ambion), followed by purification with RNeasy kit (Qiagen), and elution in RNase-free water. To probe for the efficiency of Barren$^{TEV}$ removal, 0 – 30 min old embryos surviving only on Barren$^{TEV}$-Myc were injected with Barren$^{TEV}$-EGFP mRNA in pure water at ~ 2.2 mg ml-1. Embryos were left to develop at 22°C for 1,5 -

2h, to allow for protein translation, before the subsequent injections.

### 2.4.12 Multi-sequence alignment of Barren subunit of condensin I and basic structure prediction

The multiple sequence alignment was constructed using sequences of CAPH (Barren) protein from species listed in Table 2.2, representing various clades of organisms. The alignment was generated using MAFFT software (Katoh et al. 2002). Following parameters were applied: G-INS-i strategy (very slow; recommended for <200 sequences with global homology), scoring matrix BLOSUM62. Visualization of multiple alignment was done in Jalview software (Waterhouse et al. 2009), which helped to visualize conserved regions of Barren sequence.

To predict unstructured regions of Barren in *Drosophila melanogaster* IUPred (Dosztányi et al. 2005) software was used to estimate disorder tendency of the protein along residues from its amino acid sequence. The threshold of disorder tendency was set to -0.5. Surface accessibility of the same sequence was assessed by NetSurfP 1.0 (Petersen et al. 2009) with the default settings and threshold set to 25%.

**Table 2.2.** List of sequences used to build multi-sequence alignment of Barren/Cap-H subunits.

| Organism | Identifier number (Ensembl) of CAPH sequence |
|---|---|
| *Homo sapiens* | ENSP00000240423 |
| *Macaca mulatta* | ENSMMUP00000030631 |
| *Canis familiaris* | ENSCAFP00000009828 |
| *Ornithorhynchus anatinus* | ENSOANP00000004313 |
| *Gallus gallus* | ENSGALP00000039960 |
| *Xenopus tropicalis* | ENSXETP00000054771 |
| *Danio rerio* | ENSDARP00000082118 |
| *Petromyzon marinus* | ENSPMAP00000008021 |
| *Ciona intestinalis* | ENSCINP00000013216 |
| *Drosophila melanogaster* | FBpp0080881 |
| *Drosophila yakuba* | FBpp0257623 |
| *Drosophila virilis* | FBpp0232310 |
| *Saccharomyces cerevisiae* | YBL097W |

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# CHAPTER 3

# Metaphase chromosome structure is dynamically maintained by condensin I-directed DNA (de)catenation

Abstract Chapter 3

Mitotic chromosome assembly remains a big mystery in biology. Condensin complexes are pivotal for chromosome architecture yet how they shape mitotic chromatin remains unknown. Using acute inactivation approaches and live-cell imaging in *Drosophila* embryos, we dissect the role of condensin I in the maintenance of mitotic chromosome structure with unprecedented temporal resolution. Removal of condensin I from pre-established chromosomes results in rapid disassembly of centromeric regions while most chromatin mass undergoes hyper-compaction. This is accompanied by drastic changes in the degree of sister chromatid intertwines. While wild-type metaphase chromosomes display residual levels of catenations, upon timely removal of condensin I, chromosomes present high levels of *de novo* topoisomerase II-dependent re-entanglements, and complete failure in chromosome segregation. Topoisomerase II is thus capable of re-intertwining previously separated DNA molecules and condensin I continuously required to counteract this erroneous activity. We propose that maintenance of chromosome resolution is a highly dynamic bidirectional process.

## Author contribution:

*All the experiments presented in this chapter were performed by Ewa Piskadlo. The experiments were designed by Ewa Piskadlo and Raquel Oliveira. Raquel A. Oliveira has performed chromatid movement analysis presented Figure 3.9. and part of histone profile analysis presented on Figure 3.12, Ewa Piskadlo analyzed the remainder of the data. Alexandra Tavares prepared and purified proteins used in the experiments.*

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## 3.1 Introduction

Mitotic chromosome assembly, although poorly understood at the molecular level (Piskadlo and Oliveira 2016), fulfils three major tasks essential for faithful chromosome segregation: Firstly, it ensures chromosome compaction making cell division feasible within the cell space. Secondly, it provides chromosomes with the right mechanical properties (e.g. bendiness and rigidity) to facilitate their drastic movements during mitosis. Lastly, it ensures the resolution of the topological constrains that exist between the two sister DNA molecules, as well as between neighbouring chromosomes (chromosome individualization), a key requisite for efficient chromosome partitioning. At the heart of these structural changes are the condensin complexes. Condensin complexes, one of the most abundant non-histone complexes on mitotic chromosomes (Ono et al. 2003; Hirano, Kobayashi, et al. 1997; Cuylen et al. 2011), are composed of two Structural Maintenance of Chromosome (SMC) proteins (SMC2 and SMC4) bridged by a kleisin subunit (Barren/Cap-H for condensin I and Barren2/Cap-H2 for condensin II). Despite extensive research over the last years, how condensins contribute to chromosome morphology is not completely understood. Biochemical and phenotypic analysis of condensin depletion suggest several possible activities for these complexes, including the resolution of DNA entanglements (Steffensen et al. 2001; Hagstrom et al. 2002; Hudson et al. 2003; Oliveira, Coelho, et al. 2005; Gerlich et al. 2006; Hirano and Hirano 2006; Ribeiro et al. 2009) and structural integrity by conferring chromosome rigidity (Oliveira, Coelho, et al. 2005; Gerlich et al. 2006; Ribeiro et al. 2009; Houlard et al. 2015). Whether or not these complexes also promote chromatin compaction remains controversial (Hagstrom et al. 2002; Hirano and Hirano 2006; Hirano, Kobayashi, et al. 1997; Hudson et al. 2003; Kimura and Hirano 1997; Lavoie et al. 2002; Oliveira,

Coelho, et al. 2005; Steffensen et al. 2001). The multiple phenotypes observed on mitotic chromosomes upon depletion of condensin complexes raise the possibility that these complexes may have distinct roles at different times of mitosis. Additionally, several lines of evidence support that these complexes also influence interphase chromosome structure (Cobbe et al. 2006). Thus, it is difficult, if not impossible to interpret the results when condensins are depleted prior to mitotic entry using conventional depletion approaches.

To circumvent this limitation, we make use of the tools developed in Chapter 2 and adopt a 'reverse and acute' approach to dissect the role of condensin I in the maintenance of chromosome organization. We find that inactivation of one condensin I specifically during metaphase leads to over-compaction at the majority of chromosomal regions. We further demonstrate that upon condensin I cleavage previously separated sister DNA molecules undergo topoisomerase II-dependent re-intertwining and complete failure in chromosome segregation.

## 3.2 Results

### 3.2.1 Centromere impairment following condensin I removal.

To test the role of condensin I in the maintenance of chromosome architecture we allowed mitotic chromosomes to assemble without any perturbation on their structure and subsequently disrupted condensin I during the metaphase-arrest. Embryos were arrested in metaphase, with a functional mitotic spindle, by the use of a catalytically dead dominant-negative form of the E2 ubiquitin ligase necessary for anaphase onset (UbcH10$^{C114S}$) (Rape et al. 2006; Oliveira, Hamilton, et al. 2010). Arrested embryos were subsequently injected with TEV protease to destroy condensin I (scheme of the experiment is depicted on Figure 3.1). Given the known role of condensin I in the rigidity of pericentromeric region of chromosomes (Oliveira, Coelho, et al. 2005; Gerlich et al. 2006; Ribeiro et al. 2009), we first tested the effect of TEV protease injection at those chromosomal sites. Whereas injection of buffer causes no change in the distance between centromeres (Figure 3.2 a,c, and Movie 3), TEV protease injection in strains containing solely TEV-cleavable Barren results in rapid separation of centromeres, that appear to stretch towards the poles, leaving behind the majority of the chromatin

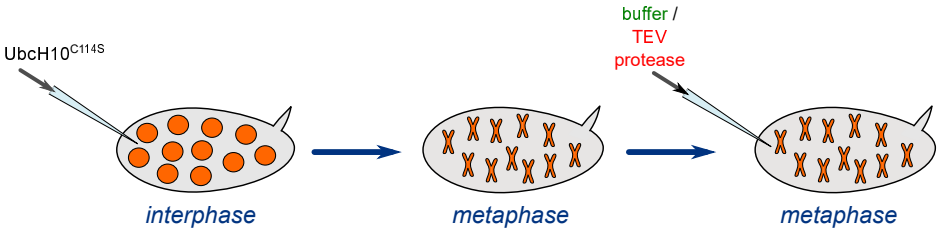


**Fig. 3.1. Schematic representation of the experimental layout.**

mass (Figure 3.2b,c, and Movie 4). These findings imply that condensin I is not only required for the establishment of a rigid structure at the pericentromeric domains prior to metaphase, but also for the maintenance of such organization.

### 3.2.2 Measuring chromosome compaction in absence of condensin I.

After describing the drastic disorganisation of the centromeric regions, we asked whether non-centromeric chromatin follows similar effects. Softening the chromosome at the centromeres most likely indicates disassembling the loops forming the chromosomes. One may predict that if functional condensin is removed, DNA loops may be abolished not only at the centromeres, but also along the arms of the chromosomes. This lack of constrain of chromatin could lead to a general decompaction of the chromosomes.

We defined chromosome compaction by degree of chromatin density, inferred from the signal of fluorescently labelled histone H2Av-mRFP1. To quantify the changes in chromosome compaction upon condensin inactivation, we used quantitative imaging analysis to monitor the mean voxel intensity, volume and surface area of each metaphase plate, over time, in 3D (Figure 3.3). Surprisingly, we found that injection of TEV protease in strains surviving only on Barren$^{TEV}$ leads to an overall over-compaction of the entire chromatin mass, as evidenced by an increase in the mean voxel intensity and a decrease in both the surface area and the volume of the metaphase plate (Figure 3.3c).

To exclude a possibility that TEV protease itself is imposing overcompaction via any unexpected secondary effect, we have tested influence of TEV protease on chromosome compaction in embryos expressing Barren$^{wt}$. Injection of the

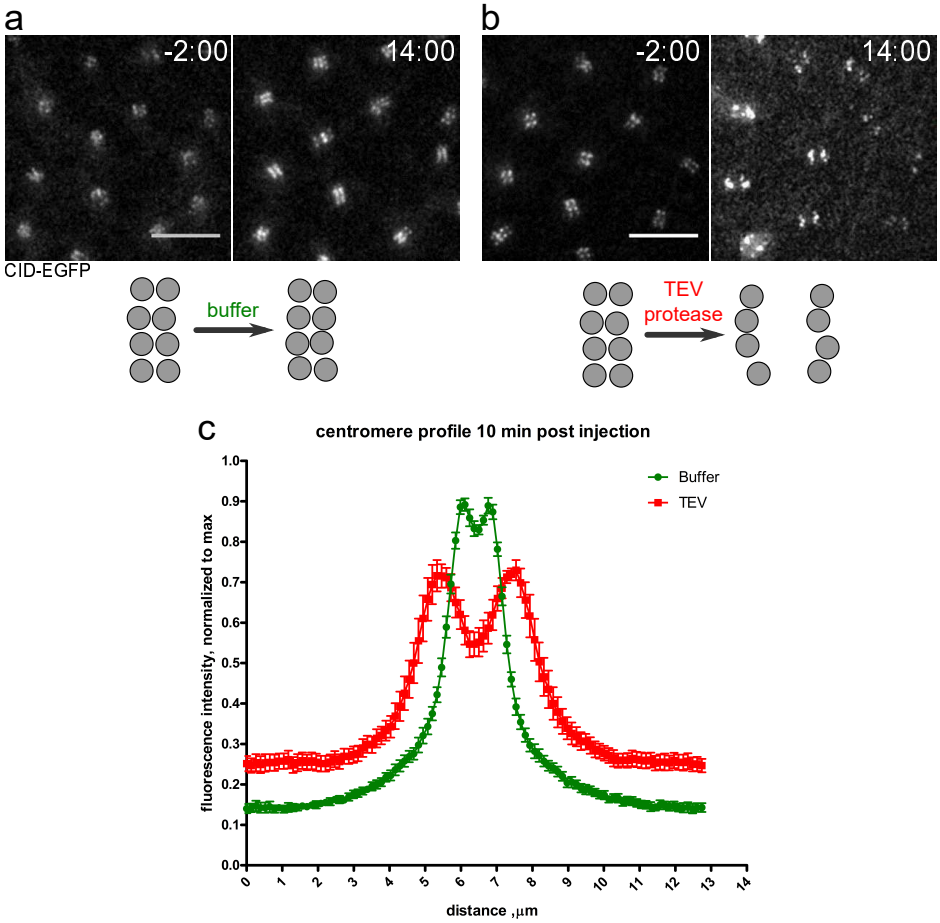


**Fig. 3.2. Centromere impairment following condensin I removal.** Embryos expressing solely Barren$^{TEV}$ were injected with 12 mg/ml of a dominant-negative form of the human E2 ubiquitin-conjugating enzyme (UbcH10$^{C114S}$) to induce a metaphase arrest. Embryos were subsequently injected with TEV protease buffer **(a)** or 13 mg/ml TEV protease **(b)**; Images depict embryos 2 minutes before the second injection and 14 minutes after. Embryos also express His2A–mRFP1 (not shown) and CID-EGFP (grey); scale bars, 10 µm. Insets show higher magnifications (2.5×) of a single metaphase. Times (minutes:seconds) are relative to the time of the second injection. **(c)** Quantitative analysis of centromere positioning 10 minutes after the second injection, as above; graph shows average ± SEM of individual embryos (n≥7 embryos for each experimental condition); for each embryo, a minimum of 8 metaphases was measured

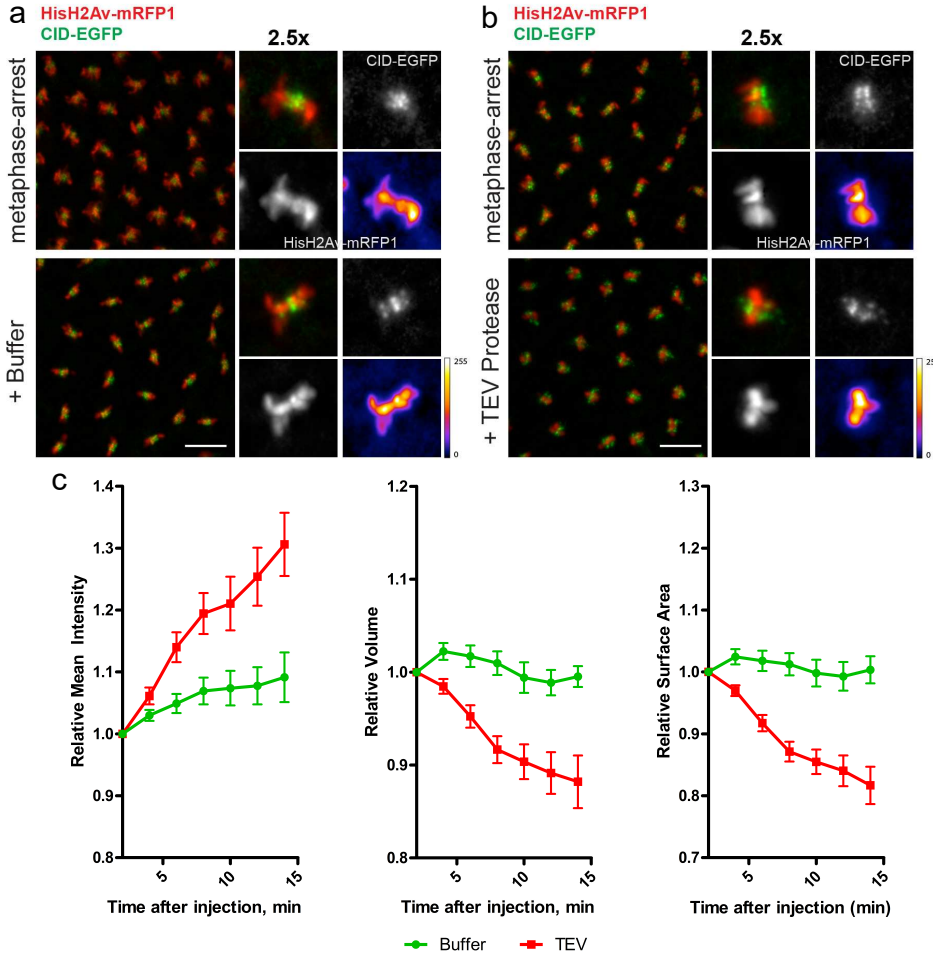


**Fig. 3.3. Condensin I inactivation in pre-assembled chromosomes leads to hyper-compaction of mitotic chromosomes.** Embryos expressing solely Barren$^{TEV}$ were injected with 12 mg/ml of UbcH10$^{C114S}$ to induce a metaphase arrest. Embryos were subsequently injected with buffer **(a)**, 13 mg/ml TEV protease **(b)**. Images depict embryos before the second injection and 14 minutes after. Embryos also express His2A–mRFP1 (red) and CID-EGFP (green); scale bars, 10 µm. Insets show higher magnifications (2.5×) of a single metaphase. Times (minutes:seconds) are relative to the time of the second injection. (c) Quantifications of mean voxel intensity, volume and surface area of the entire metaphase plate quantified in 3D, over time, and normalized to the time of the second injection. Graphs represent the average ± SEM of individual embryos (n≥10 embryos for each experimental condition); for each embryo, a minimum of 8 metaphases was quantified.

protease in strains that do not contain TEV-cleavage sites within any protein does not result in any evident change in chromosome compaction relative to buffer control (Figure 3.4), implying that chromosome over-compaction is specific of condensin I inactivation.

In order to explore changes in chromatin morphology and localization, we have optimized electron microscopy imaging of mitotic-arrested embryos as an additional tool. Embryos, temporarily immobilized to coverslips by low-melting point agarose, are arrested in metaphase by UbcH10$^{C114S}$ and injected with TEV protease or buffer. Around 20 minutes after the second injection embryos were processed for electron microscopy imaging. Sections of a few embryos we managed to obtain contain visible chromosomes. The images we acquired so far show no great difference in density of chromatin between tested conditions (Figure 3.5). Nonetheless, more embryos need to be imaged and more detailed analysis must be performed. Obtaining larger number of embryos processed for electron microscopy is quite time-consuming and technically challenging, as embryos pierced with needles are extremely delicate. The majority become heavily damaged during manipulations following injection, especially while unsticking them from the agarose, cleaning from the excess of the halocarbon oil, and loading onto freezing chamber. Despite technical difficulties, further electron microscopy analysis can yield useful insights of chromosome morphology after condensin I inactivation in the future.

### 3.2.3 Simultaneous inactivation of condensin I and topoisomerase II abolishes chromosome overcompaction.

Topoisomerase II has been previously implicated in chromosome compaction although its role in the process remains controversial (Piskadlo and Oliveira 2016). We have questioned how topoisomerase II inactivation will affect compaction of the condensin I-depleted chromosomes.

Topoisomerase II is an important target in cancer therapy and many catalytic inhibitors were developed (Larsen et al. 2003). We have chosen a few inhibitors of various modes of action – ICRF-193, aclarubucin, suramin, novobiocin, and merbarone. The mechanism of inhibition imposed by ICRF-193 is based on clamping the topoisomerase II onto DNA, trapping the enzyme to its binding site

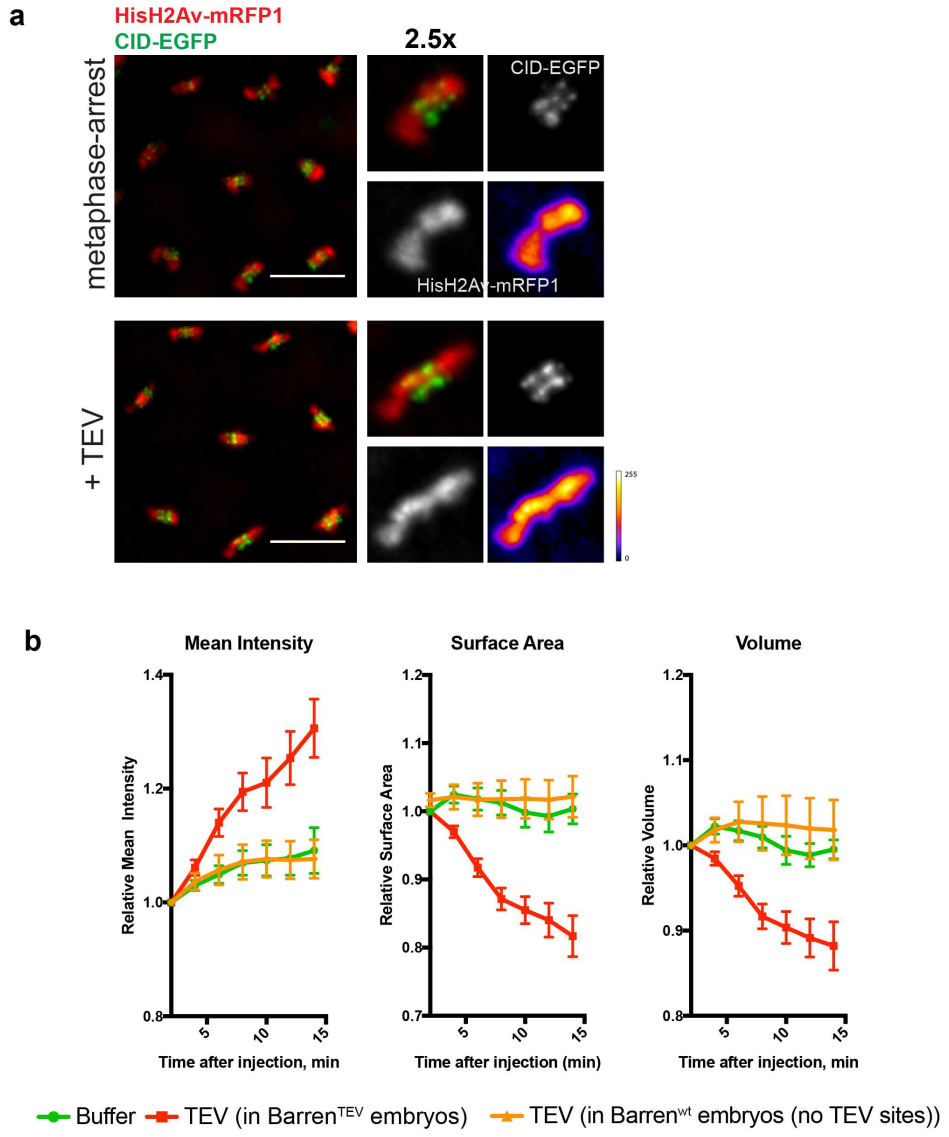


**Fig. 3.4. Chromosome condensation induced by TEV-protease depends on TEV cleavage sites present in Barren$^{TEV}$. (a)** Representative images from embryos that do not contain TEV-cleavage sites in Barren. Embryos were injected with UbcH10$^{C114S}$ to induce a metaphase arrest and subsequently injected with 13 mg/ml TEV protease. Embryos also express HisH2Av-RFP1 (red) and CID-EGFP (green); scale bar is 10 µm. (b) Quantifications of mean voxel intensity, volume and surface area of the entire metaphase plate quantified in 3D, over time, and normalized to the time of the second injection. Graphs represent the average ± SEM of individual embryos (n≥10 embryos for each experimental condition); for each embryo, a minimum of 8 metaphases was quantified.

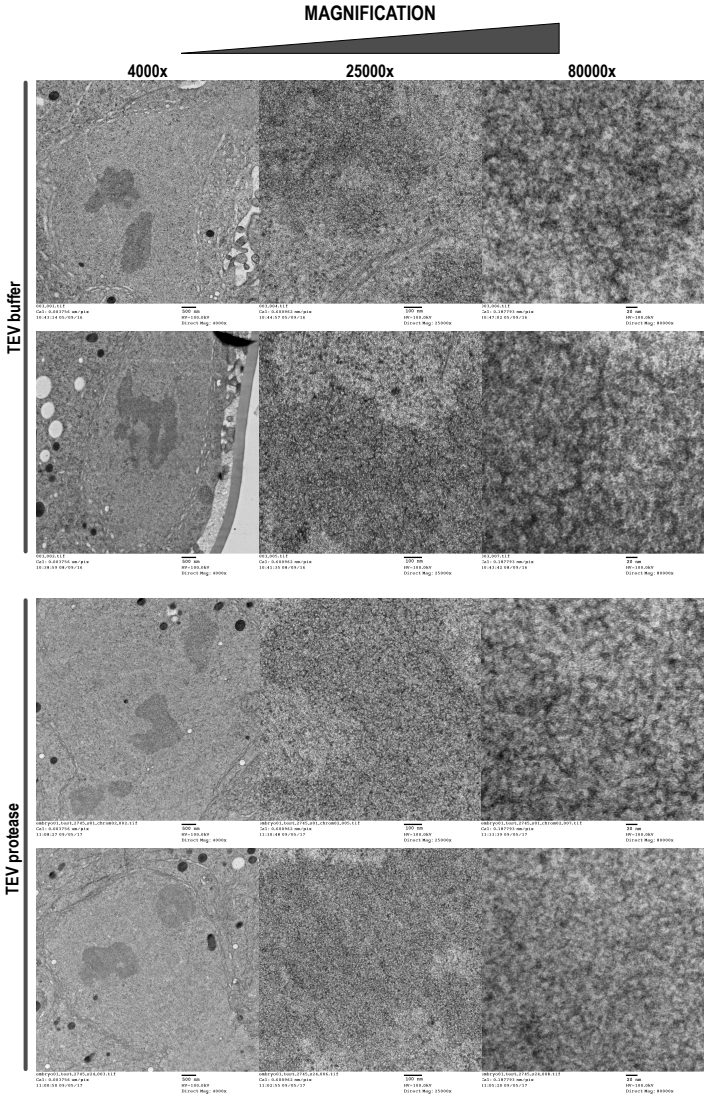


**Fig. 3.5. Electron microscopy images of embryos treated with TEV protease or buffer.** Embryos surviving only on BarrenTEV were arrested in metaphase by injection of 12 mg/ml UbcH10$^{C114S}$. In metaphase the embryos were injected with either buffer or 13 mg/ml TEV protease. After 15-20 minutes of incubation embryos were frozen under high pressure and subjected to processing for transmission electron microscopy. Two independent embryos per each condition are presented under various magnifications (4000, 25000, and 80000x).

(Roca et al. 1994). Aclarubicin and suramin prevent binding of topoisomerase II to DNA (Sørensen et al. 1992; Bojanowski et al. 1992). Novobiocin in turn can block the ATP binding site of topoisomerase II (Gormley et al. 1996), blocking its catalytic cycle before cutting one of the DNA strands. Merbarone's activity is due to inhibiting DNA cleavage by topoisomerase II (Fortune and Osheroff 1998).

The ability to block topoisomerase II activity by a given inhibitor in our system was assessed by injecting a drug in the interphase embryo and observing anaphase bridges. As topoisomerase II is necessary for sister chromatids segregation and maintaining chromosome structure, inactivating it would give rise to severe anaphase bridges. Embryos injected with ICRF-193 in interphase suffer from major anaphase bridges and problems with condensation (Figure 3.6b). Unfortunately, none of the other tested inhibitors was able to produce anaphase bridges in our *Drosophila* experimental setup (Figure 3.6c-f) and resembled control embryos injected with TEV buffer that does not raise segregation defects (Figure 3.6a).

Another approach tested to block topoisomerase II was using antibodies raised against *Drosophila* topoisomerase II (kindly provided by Paul Fisher). The serum containing antibodies was injected in interphase and resulted in lack of separation of chromatids and lack of segregation in anaphase (although in some nuclei spindle is able to stretch some centromeres more towards the poles), and chromosomes decondense after the failed anaphase (tetraploid nuclei shown in Figure 3.6g). Nonetheless, blocking the topoisomerase II with an antibody is much less convenient and controlled approach than using small molecule drugs. For this reason, we decided to use ICRF-193 as a primary tool for topoisomerase II inhibition.

We used ICRF-193 to inhibit topoisomerase II in UbcH10$^{C114S}$ metaphase-arrested embryos (according to scheme in Figure 3.1). In contrast to condensin I inactivation, inhibition of topoisomerase II leads to rapid de-compaction of chromosomes (Figure 3.7, and Movie 5). Also centromeres do not separate comparing to buffer control, suggesting that rigidity of centromeres is not compromised (Figure 3.8b).

To ensure that the decompaction of chromatin observed in ICRF-193 treated

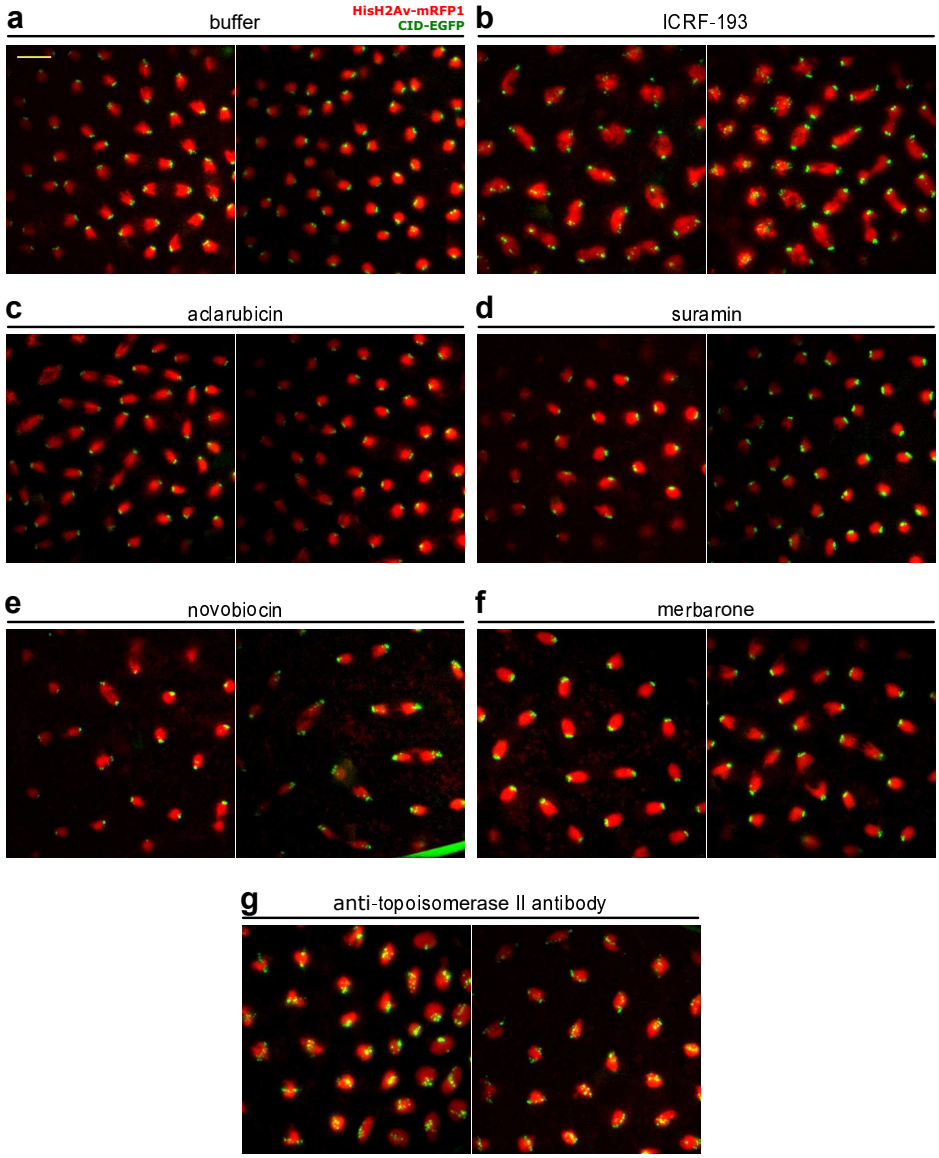


**Fig. 3.6. Testing inhibitors of topoisomerase II in *Drosophila* embryo. (a-g)**: Representative images of interphase embryos expressing HisH2Av-RFP1 (red) and CID-EGFP (green) injected with buffer, indicated drugs, or anti-topoisomerase II antibodies and followed by live imaging in 3D, in time; scale bar is 10 μm. Anaphase figures are presented for two independent embryos. Concentrations of drugs used were as followed: ICRF-193 280 μM; aclarubicin 1mg/ml; suramin 2mM; novobiocin 5mM; merbarone 300 μM.

chromosomes is due to inactivating topoisomerase II rather than secondary disruption of chromatin architecture caused by trapping this enzyme on chromatin, we wished to compare the results with a different methods of topoisomerase II inactivation. We have repeated the experiment using antibodies against *Drosophila* topoisomerase II. Antibodies injection into UbcH10-arrested embryos caused initial rise in chromosome compaction, followed by rapid decompaction (Figure 3.8b). The tendency for decompaction after a while appears to be in agreement with the ICRF-193 data (Figure 3.8a,b) and suggests that blocking topoisomerase II may indeed be necessary to actively maintain compaction and shape of chromosomes. Nonetheless, early overcompaction in response to the antibody cannot be easily explained and might be a sign of extensive disruption of chromatin, not even necessarily invoked by topoisomerase II inactivation per se, but other components of the injected serum. The fluctuating response over time in these experiments does not allow a conclusive response.

Although we cannot exclude that chromosome decompaction may be exacerbated by the fact that ICRF-193 traps topoisomerase II onto chromatin, our results support that topoisomerase II may contribute to chromosome compaction in metaphase, consistent with previous observations (Samejima et al. 2012), possibly by promoting self-entanglements within the same chromatid (Kawamura et al. 2010).

Our data so far confirms that condensin I and topoisomerase II both impact metaphase compaction maintenance, although in opposite directions. To further explore how those two components may contribute to chromosome compaction, we have assessed how topoisomerase II inhibition influences condensin I-depleted chromosomes. We have combined inactivation of both topoisomerase II and condensin I by injecting mixture of ICRF-193 and TEV protease into metaphase arrested embryos in Barren$^{TEV}$ background, according to scheme in Figure 3.1. This resulted in chromosome decompaction similar to topoisomerase II inhibition alone (Figure 3.8a,c and Movie 6). This finding implies that chromatin hyper-compaction observed upon loss of condensin I depends on topoisomerase II activity. At the same time centromeres are rapidly disrupted, to the same degree as in condensin I-inactivated embryos, implying softening of centromere region (Figure 3.8b). Functional interplay between condensin

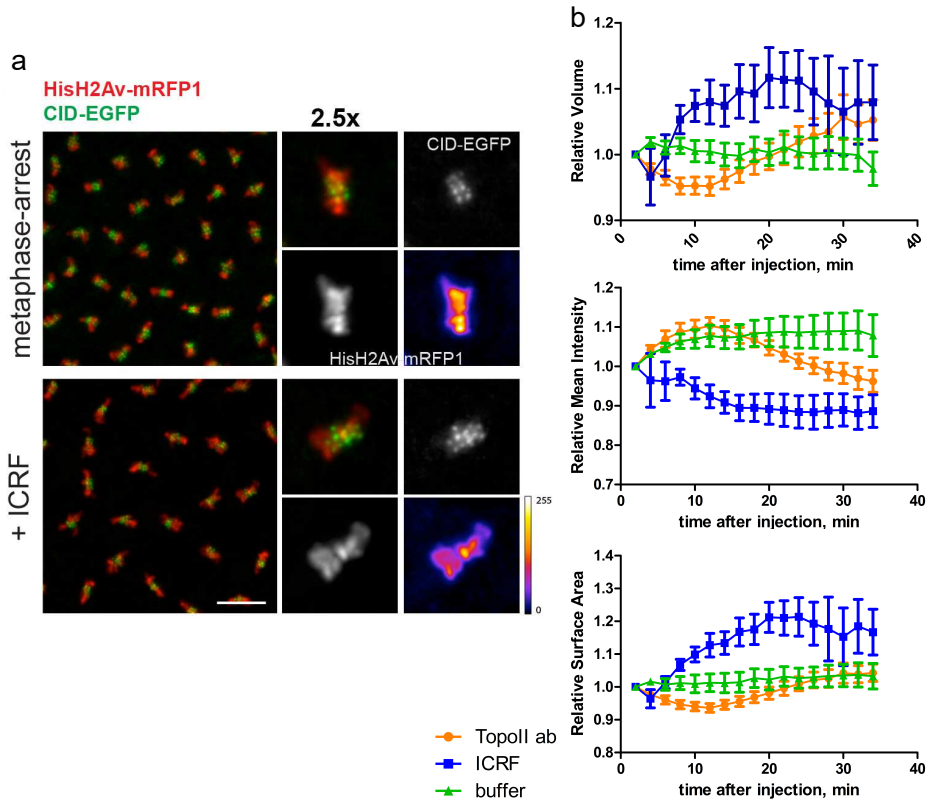


**Fig. 3.7. Inactivation of toposimerase II leads to chromosome decompaction.** Embryos expressing solely Barren$^{TEV}$ were injected with 12 mg/ml of UbcH10$^{C114S}$ to induce a metaphase arrest. Embryos were subsequently injected with 280 μM ICRF-193 or anti-topoisomerase II antibody **(a)**. Images depict embryos before the second injection and 14 minutes after. Embryos also express His2A–mRFP1 (red) and CID-EGFP (green); scale bars, 10 μm. Insets show higher magnifications (2.5×) of a single metaphase. Times (minutes:seconds) are relative to the time of the second injection. **(c)** Quantifications of mean voxel intensity, volume and surface area of the entire metaphase plate quantified in 3D, over time, and normalized to the time of the second injection. Graphs represent the average ± SEM of individual embryos (n≥10 embryos for each experimental condition); for each embryo, a minimum of 8 metaphases was quantified.

I and topoisomerase II was previously anticipated and it seem to be a key to understanding condensin I depletion-dependent overcompaction. Therefore we turned our focus towards investigating how those two complex may influence compaction state of chromosomes in metaphase.

### 3.2.4 Does condensin I inactivation lead to re-entanglement of DNA *in vivo*?

The unexpected finding that condensin I inactivation promotes further chromosome compaction, together with the observation that topoisomerase II inhibition reverts this hyper-compaction phenotype, lead us to hypothesize that the observed increase in compaction stems from re-entanglements of DNA strands, which would consequently lead to an increase in chromatin density. Enzymatically, topoisomerase II can promote both the decatenation and the concatenation of DNA strands. Efficient chromosome segregation requires that topoisomerase II I is strongly biased towards decatenation prior to anaphase onset but it is conceivable that topoisomerase II can additionally drive the concatenation of native metaphase chromosomes, *in vivo*.

To test whether condensin I removal leads to re-catenation of previously separated sisters, we tested several predictions that arise from this hypothesis: First, the hyper-compaction observed in metaphase, if derived from sister-chromatid re-intertwining, should be dependent on the proximity between DNA molecules. The physical separation of sister chromatids will increase the distance between these two molecules, placing them too far apart, and consequently decreasing the likelihood of their re-entanglement, as recently proposed (Sen et al. 2016). Secondly, re-intertwining in late metaphase should lead to severe segregation failures. And lastly, DNA entanglements and the consequent segregation defects should depend on topoisomerase II activity. All these hypothesis are tested in the following sections.

### 3.2.5 Testing the proximity effect.

To evaluate the effect of sister chromatid proximity in chromosome condensation upon condensin inactivation we combined TEV-mediated cleavage of condensin I and cohesin by the use of strains carrying TEV-sensitive Rad21 (cohesin) and

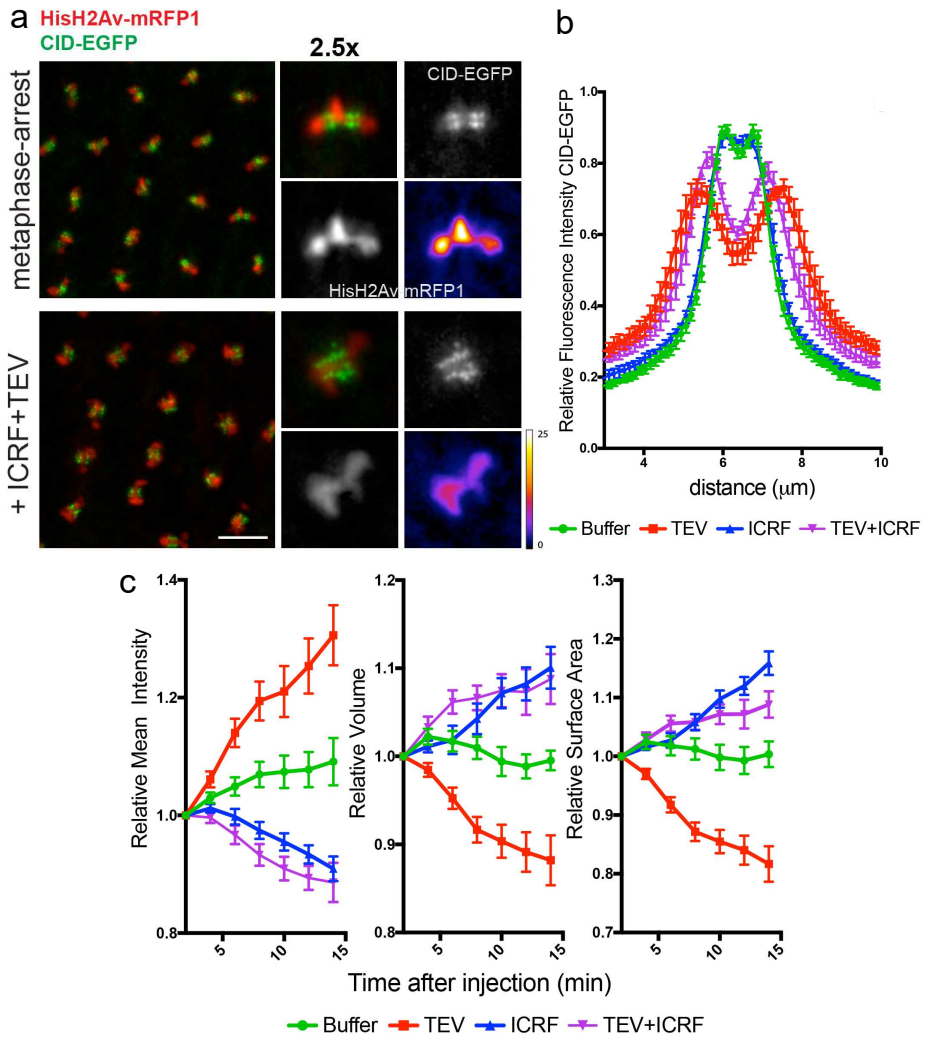


**Fig. 3.8. Simultaneous inactivation of condensin I and toposimerase II abolishes chromosome overcompaction and drived decompaction.** Embryos expressing solely Barren$^{TEV}$ were injected with 12 mg/ml of UbcH10$^{C114S}$ to induce a metaphase arrest. Embryos were subsequently injected with buffer, 13 mg/ml TEV protease, 280 μM ICRF-193 or a mixture containing 13 mg/ml TEV protease and 280 μM ICRF-193; **(a)** Images depict embryos before the second injection and 14 minutes after TEV and ICRF-193 mix injection. Embryos also express His2A–mRFP1 (red) and Cid-EGFP (green); scale bars, 10 μm. Insets show higher magnifications (2.5×) of a single metaphase. Times (minutes:seconds) are relative to the time of the second injection. *Legend continued on the next page*

**Fig. 3.8.** **(b)** Quantitative analysis of centromere positioning 10 minutes after the second injection, as above; graph shows average ± SEM of individual embryos (n≥7 embryos for each experimental condition); for each embryo, a minimum of 8 metaphases was measured; **(c)** quantifications of mean voxel intensity, volume and surface area of the entire metaphase plate quantified in 3D, over time, and normalized to the time of the second injection. Graphs represent the average ± SEM of individual embryos (n≥10 embryos for each experimental condition); for each embryo, a minimum of 8 metaphases was quantified.

Barren (condensin) proteins. We took advantage of the fact that Rad21$^{TEV}$ cleavage is more efficient than Barren$^{TEV}$ (Figure 2.6), which allowed the analysis of changes in chromosome architecture upon condensin inactivation after artificial separation of sister chromatids. Upon TEV protease injection, pole-ward chromosome segregation is initiated within 2 – 5 minutes and with similar kinetics in both strains (Figure 3.9a).

After the initial pole-ward chromatid movement, isolated chromatids shuffle between the poles, consistent with previous observations (Oliveira, Hamilton, et al. 2010). To quantify the degree of movement, we used a displacement-quantification method that infers chromosome movement by the overlap between chromosome positions on consecutive frames (Mirkovic et al. 2015). Cohesin cleavage alone leads to strong shuffling of isolated single chromatids, as previously described. However, concomitant inactivation of condensin and cohesin results in much slower chromatid movements, with chromatids accumulating in the middle of the segregation plane (Figure 3.9b,c). Condensin I is thus important for efficient movement of isolated chromatids. This may be due abnormal centromere/kinetochore structure and/or to a possible role for condensin in the error-correction process, as recently proposed (Verzijlbergen et al. 2014; Peplowska et al. 2014).

The reduced chromosome movement observed upon condensin I inactivation leads to considerable differences in chromatid positioning in both experimental set-ups. Thus, we restricted our chromosome morphology/compaction analysis to measurements of isolated single sisters, as quantifying the entire chromatin mass would include many confounding variables. Measurements of isolated single chromatids were performed at 20 min after injections and normalized to the values at 5 minutes after protease injection (once complete separation has oc-

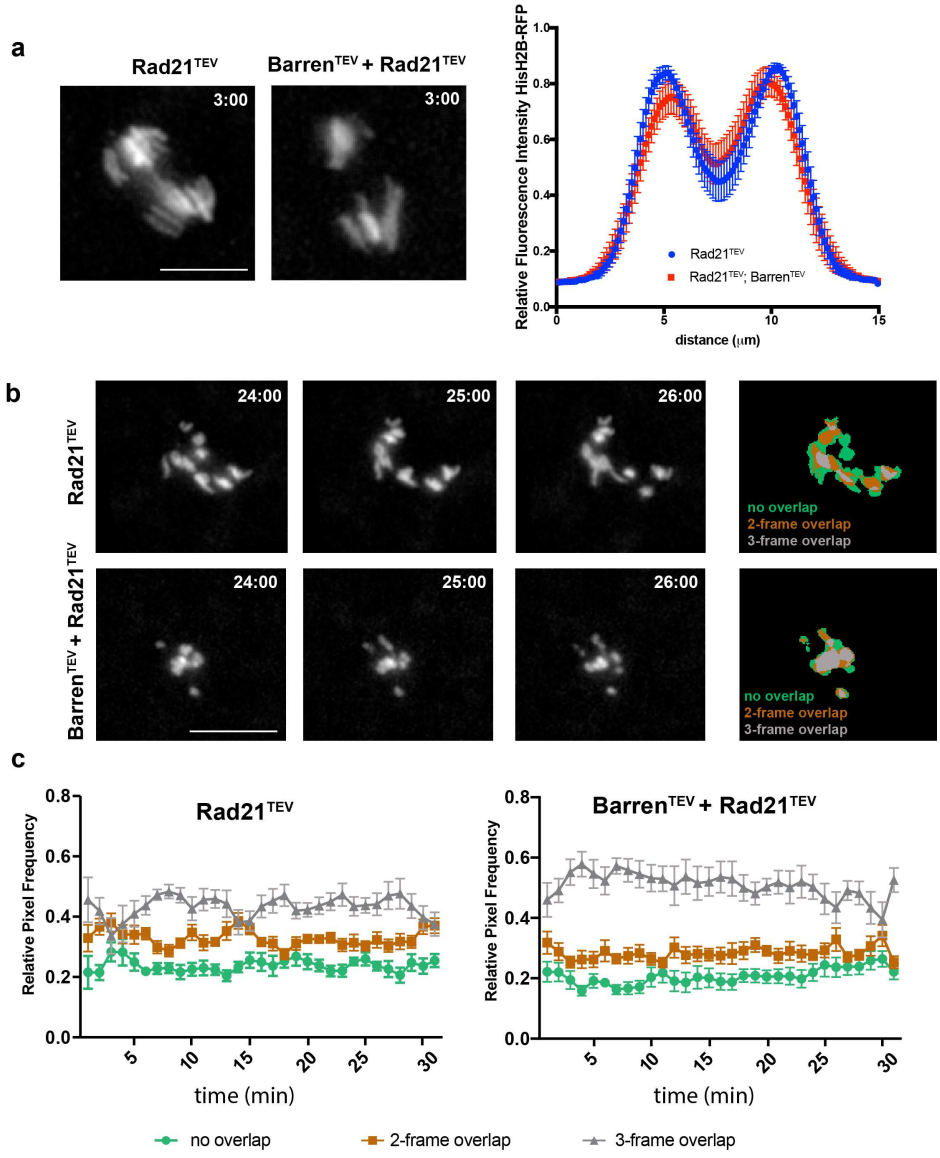


**Fig. 3.9. Condensin I inactivation in separated sister chromatids reduces their movement.** **(a)** Representative images of the initial separation after TEV-mediated cleavage of Rad21$^{TEV}$ and Rad21$^{TEV}$ + Barren$^{TEV}$. Graph plots the relative distribution of HisH2B-RFP at the maximal state of sister chromatid separation triggered by TEV-mediated cleavage of Rad21$^{TEV}$, in strains that contain solely Rad21$^{TEV}$ or both Rad21$^{TEV}$ and Barren$^{TEV}$. A 15 μm line was used to measure plot profiles along the segregation plane, measured 3-5 minutes after TEV protease injection. Graphs plot the average ± SEM of individual embryos (n≥7embryos for each experimental condition). For each embryo, between 8 and 12 anaphases were analysed. *Legend continued on the next page*

**Fig. 3.9.** **(b)** Example of chromosome movement analysis; left panel represents average of the binary images of three consecutive frames, used to estimate chromosome displacements: blue, non-overlapping pixels; green, two- out of three-frame overlap; grey, three-frame overlap. Scale bar is 10 μm. **(c)** Frequency of overlapping pixels to estimate chromosome displacement (as in b), over time, after TEV protease injection.

curred but no significant changes in chromosome structure was yet observed). Chromatids considerably change their shape, becoming thicker and shorter (Figure 3.10a-c, Movie 7, and Movie 8), as previously described (Ono et al. 2003; Green et al. 2012). To directly measure the degree of compaction of these isolated sisters, we measured their mean voxel intensity. This analysis revealed that despite the significant changes in chromatid organization, there is no overall change in the mean voxel intensity of single chromatids (Figure 3.10d), indicating that shape changes are not accompanied by an overall increase in chromatin compaction. We therefore conclude that condensin I inactivation does not promote further chromosome compaction if sister chromatids are physically apart, in contrast to the effect observed in metaphase-arrested chromosomes (Figure 3.3). These results support that over-compaction observed in metaphase chromosomes may arise from sister chromatid re-intertwining, consistent with previous observations using yeast circular mini-chromosomes (Sen et al. 2016). It is conceivable that condensin I inactivation also promotes abnormal re-entanglement in *cis* (between distal regions of the same chromatid). The shape changes observed upon condensin inactivation from isolated sisters (shorter and thicker chromatids) could indeed be explained by an excess of concatenation within the same DNA molecule, leading to the shortening of the longitudinal axis. However, our compaction measurements indicate that such changes, if occur, do not lead to detectable increase in chromatin density.

### 3.2.6 Assessing levels of *de novo* catenations in condensin I-inactivated embryos.

Next we sought to evaluate chromosome segregation defects, which serve as an indirect read-out for the amount of DNA catenations bridging DNA molecules. To monitor segregation defects when condensin I or topoisomerase II are inacti-

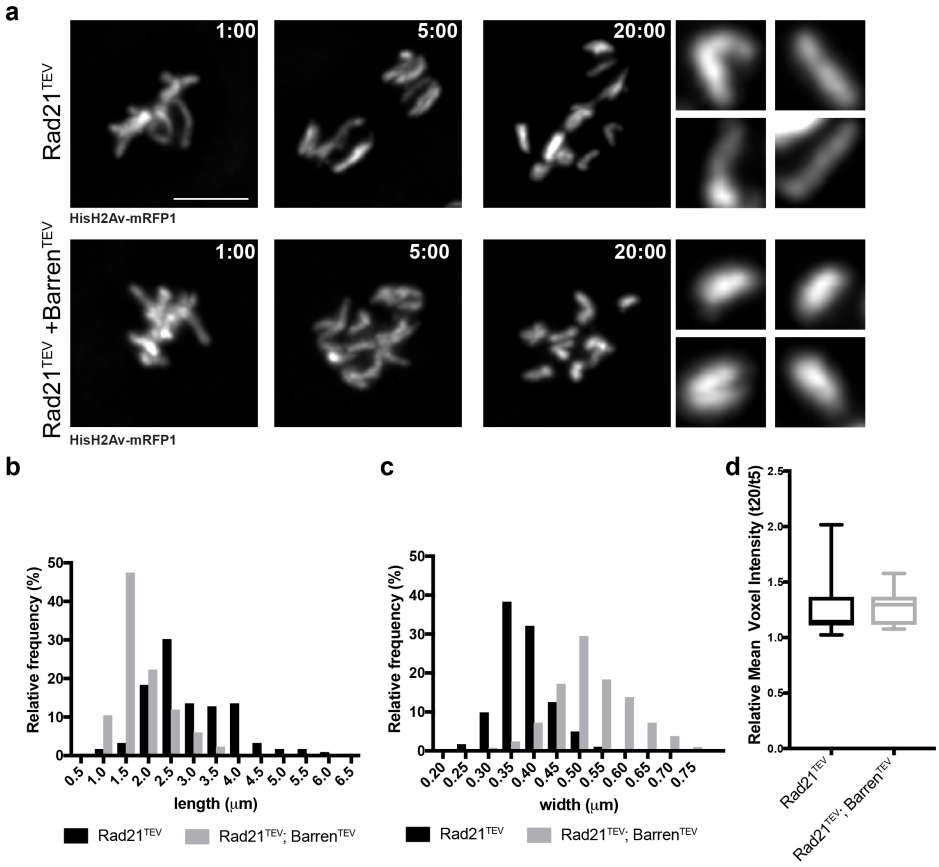


**Fig. 3.10. Chromosome over-compaction depends on sister-chromatid proximity.** **(a)** Stills from metaphase-arrested embryos after injection of TEV protease in strains surviving solely on Rad21$^{TEV}$ (cohesin cleavage) or Rad21$^{TEV}$ + Barren$^{TEV}$ (cohesin and condensin cleavage); embryos also express HisH2B-RFP; scale bars, 5 μm. Insets show higher magnifications (3×) of single chromatids 20 min after TEV injection. Times (minutes:seconds) are relative to the time of TEV injection. **(b-c)** Relative frequency of sister chromatid length **(b)** and width **(c)** at 20 minutes after TEV injections (n≥120 single chromatids from 7 independent embryos for each experimental condition). (d) Mean voxel intensity of isolated single chromatids 20 minutes after TEV injections, normalized to mean voxel intensity 5 minutes past injection. (n≥10 embryos for each experimental condition).

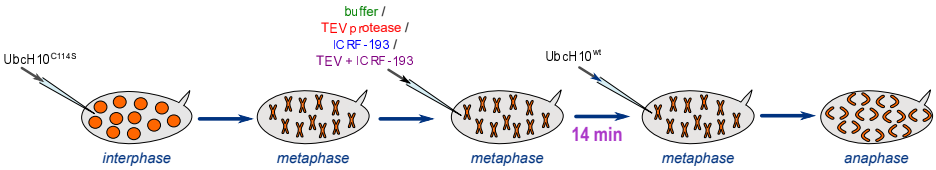


**Fig. 3.11. Schematic represenation of experimental setup of time-controlled metaphse arrest followed by inducing anaphase entry.**

vated specifically in metaphase, we developed conditions in which unperturbed chromosomes would be transiently arrested in metaphase by the dominant negative UbcH10$^{C114S}$, for ~3-5 minutes, and subsequently injected with the respective perturbing factor in metaphase. Embryos were subsequently injected with a wild-type version of UbcH10 around 14 minutes later, which causes anaphase onset and mitotic exit in about 4-8 minutes (experimental setup depicted on Figure 3.11). We monitored the segregation efficiency during anaphase by quantitative analysis of the profile of Histone H2AvD-mRFP (to visualize chromatin separation) and CID-EGFP (to infer centromere segregation) along the segregation plane (Figure 3.12). In this assay, injection of buffer causes virtually no defects in the segregation of sister chromatids (Figure 3.12b, Figure 3.13, and Movie 9).

Inactivation of topoisomerase II under these conditions leads mostly to the appearance of fine chromatid bridges (Figure 3.12c, and Movie 10). These residual bridges are insufficient to delay centromere separation (11,01±2,03 μm upon ICRF-193 treatment compared to 10,72±1,69 μm in buffer-injected embryos; Figure 3.12c). The extent of chromatin bridges observed upon metaphase-specific inactivation of topoisomerase II is considerably lower when compared to experiments where this enzyme is inhibited prior to mitotic entry (Figure 3.13). These findings imply that in metaphase-arrested chromosomes the amount of unresolved catenations is residual. In contrast, inactivation of condensin I during metaphase leads to complete impairment of the segregation process, as revealed by the high frequency of 'fused' chromatin masses, with the chromosomes remaining in the centre of the segregation plane, and a significant decrease in the distance between segregating centromeres (6,08±0,92 μm) (Figure 3.12d, and Movie 11). The degree of segregation defects observed in these metaphase-inactivation experiments, is even higher than the defects observed upon con-

densin inactivation prior to mitotic entry (Figure 3.13). The severity of segregation impairment upon metaphase-specific condensin I inactivation indicates that in the absence of this complex previously resolved sister DNA molecules undergo re-catenation.

To directly test this hypothesis, we asessed whether or not *de novo* chromatin intertwines take place upon condensin inactivation, as the formation of these new links should depend on topoisomerase II activity. If topoisomerase II-dependent re-catenation occurs upon condensin I inactivation, one would expect that the combination of topoisomerase II and condensin I inactivation should reduce the amount of chromatin trapped in the middle of the segregation plane. On the contrary, if segregation defects result from pre-existing catenations, combined inhibition of condensin I and topoisomerase II should increase, or at least maintain, the density of chromosome bridges and segregation impairment.

To address this issue, we used the same experimental layout as above but induced concomitant inactivation of condensin I and topoisomerase II during the metaphase arrest. These experiments reveal a partial rescue of the retained chromatin mass, inferred by HisH2Av-mRFP1 profile (Figure 3.12e, and Movie 12). Statistical analysis of histone profiles revealed that condensin I inactivation profile significantly differs from profile of simultaneous condensin I and topoisomerase II inactivation at the distances approximately 2.77–5.2μm and 10.66–11.7μm (marked with blue horizontal lines on the graph from Figure 3.12e). This suggests that chromosomes after simultaneous condensin II and topoisomerase II depletion are less intertwines than in case of condensin I-inactivation alone. It is worth no note that chromosome positioning may not be linearly correlated with the amount of linkages bridging the two sister chromatids and thus the reduction on chromosome intertwines may be even more pronounced than inferred by histone profiles. In accordance with this notion, the efficiency of chromosome separation inferred by the position of centromeres returns to levels indistinguishable from wild-type (Figure 3.12a, and Movie 12). Thus, concomitant inactivation of condensin I and topoisomerase II significantly reverts the defects associated with condensin I removal. We therefore conclude that the segregation defects observed upon metaphase-specific condensin I inactivation are caused by *de novo* topoisomerase II-dependent re-intertwining of previously separated

sister chromatids.

## 3.3 Discussion

The role of condensin complexes in chromosome compaction has been extensively debated. Here we provide evidence that temporally controlled inactivation of condensin I, specifically during metaphase, causes an increase in the overall levels of chromosome compaction in non-centromeric regions. These findings strongly argue that condensin I is required to maintain chromosomal architecture but not to sustain their compacted state. Studies using similar inactivation techniques in mouse oocytes have proposed that condensins confirm longitudinal rigidity, as chromosomes disassemble upon condensin inactivation (particularly condensin II) (Houlard et al. 2015). At first sight, these findings may be perceived as in sharp contrast to our current observations. It should nevertheless be noted that meiotic chromosomes are under very different force-balance than their mitotic counterparts. In particular, spindle attachment on meiotic bivalents imposes stretching along the longitudinal axis of chromosomes, similarly to what we report here for the pericentromeric region in mitotic chromosomes. Our results now demonstrate that when chromosomes are not subjected to significant additional forces, condensin I inactivation results in an overall chromatin over-compaction rather than chromosome de-condensation. This force-dependent phenotype may explain several inconsistencies in prior analysis on condensins depletion that as sample preparation, chromosome state, presence/absence of microtubules, or even the cell division type (mitosis vs meiosis) may play a major role in chromosome morphology. In contrast to condensin I inactivation, topoisomerase II inhibition leads to rapid chromosome decompaction. These finding are consistent with the idea that metaphase chromosome structure is organized as a chromatin network resultant from self-entanglements of DNA strands, as initially proposed by biophysical studies on isolated chromosomes (Kawamura et al. 2010). Restricting/favouring chromosome entanglements may thus dictate the state of chromosome compaction.

Condensin has been previously proposed to interplay with topoisomerase II, both for chromosome compaction and sister chromatid resolution. The exact

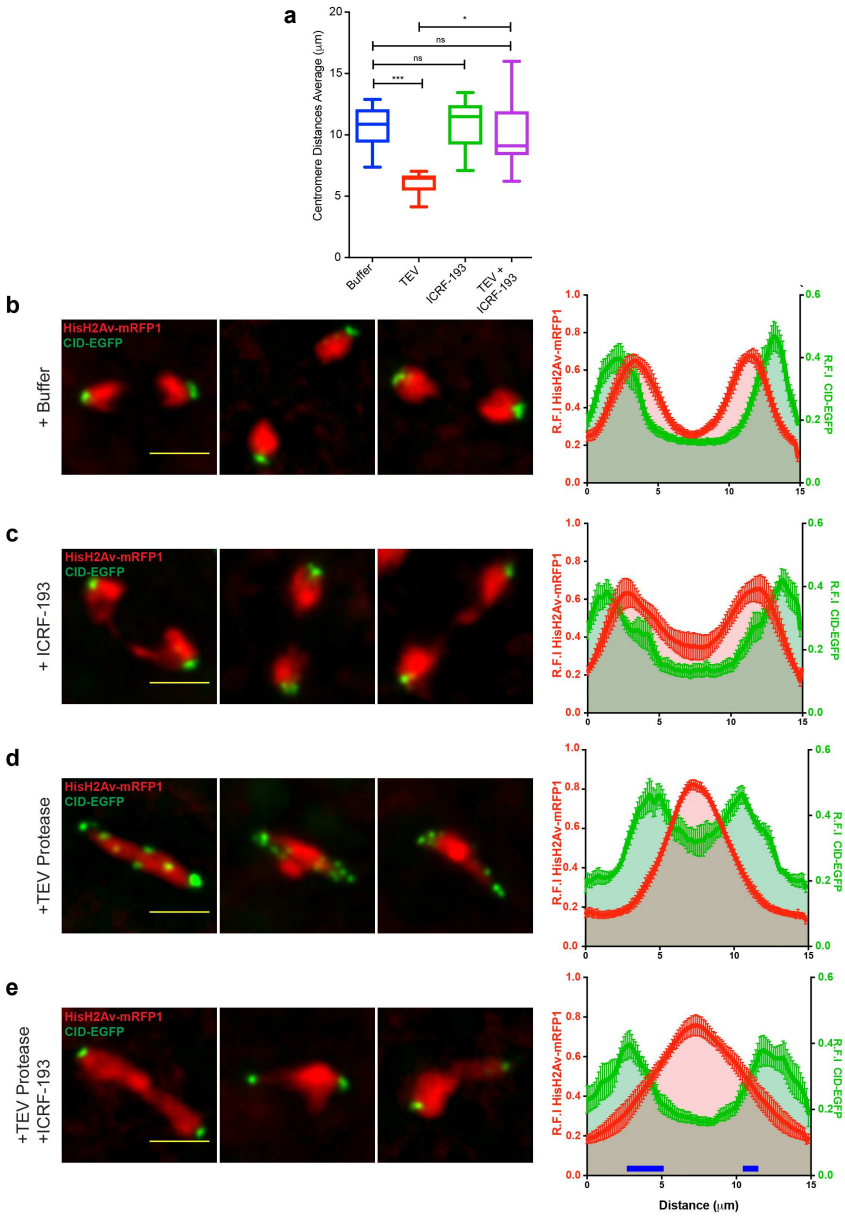


**Fig. 3.12. Condensin I inactivation results in topoisomerase II-dependent sister chromatids intertwines and segregation failure. (a)** Quantification of centromere distances during UbcH10$^{wt}$-induced anaphase as in (b-e). Graphs plot the distances between segregating centromeres measured 6 minutes after anaphase onset (n≥10 embryos for each experimental condition; for each embryo, at least 8 anaphases were analyzed). Statistical analysis was performed using mixed linear models. ns - not siginificant, *** p<0.001. *Legend continued on the next page*

**Fig. 3.12.** **(b-e)** Embryos were arrested with 12 mg/ml UbcH10$^{C114S}$ and injected with buffer **(b)**, 280 μM ICRF-193 **(c)**, 13 mg/ml TEV protease **(d)** or TEV + ICRF-193 **(e)**, while in metaphase; Embryos were subsequently injected with 14 mg/ml of a wild-type version of UbcH10 to release them from the arrest. Images depict representative images of the anaphase; Graphs plot the relative distribution of HisH2Av-mRFP1 and CID-EGFP across the 15 μm segregation plane, measured 4-6 minutes after anaphase onset. Graphs plot the average ±SEM of individual embryos (n≥10 embryos for each experimental condition). For each embryo, at least 8 anaphases were analyzed. The blue horizontal marks on the graph (e) illustrate ranges of distance where difference between TEV and TEV+ICRF-193 is statistically significant ($p<0.05$); details of this analysis are described in Materials and Methods section.

details for this interaction, however, remain elusive. Both condensins and topoisomerase II inactivation impair sister chromatid resolution (Uemura et al. 1987; Clarke et al. 1993; Bhat et al. 1996; Steffensen et al. 2001; Hagstrom et al. 2002; Hudson et al. 2003; Oliveira, Coelho, et al. 2005; Gerlich et al. 2006; Ribeiro et al. 2009), suggesting these two molecules have cooperative roles on chromosome resolution. In contrast, cytological analyses suggest that condensin and topoisomerase II have opposite roles in shaping mitotic chromatin (Samejima et al. 2012), raising further doubts on their functional interaction. It has long been hypothesized that condensin may impose directionality on topoisomerase II reactions (Coelho et al. 2003; Baxter, Sen, et al. 2011; Charbin et al. 2014; Leonard et al. 2015), as this enzyme is able to both decatenate and catenate DNA strands. But this model has been very difficult to formally prove. Studies in yeast using artificial circular mini-chromosomes, in which the levels of catenation can be directly measured, support that full decatenation by topoisomerase II requires condensin activity (Baxter, Sen, et al. 2011; Baxter and Aragón 2012; Charbin et al. 2014). Whether the same is true in large and linear native chromosomes remained to be addressed, particularly as circular chromosomes are under different topological constrains when compared to linear ones. The experimental approach used in our study allowed the manipulation of native chromosomes, in their natural environment, providing evidence that upon removal of condensin I, previously separated sister chromatids re-intertwine in a topoisomerase II - dependent manner. These findings are in agreement with a recent study that revealed that the resolution of sister chromatids from circular minichromosomes

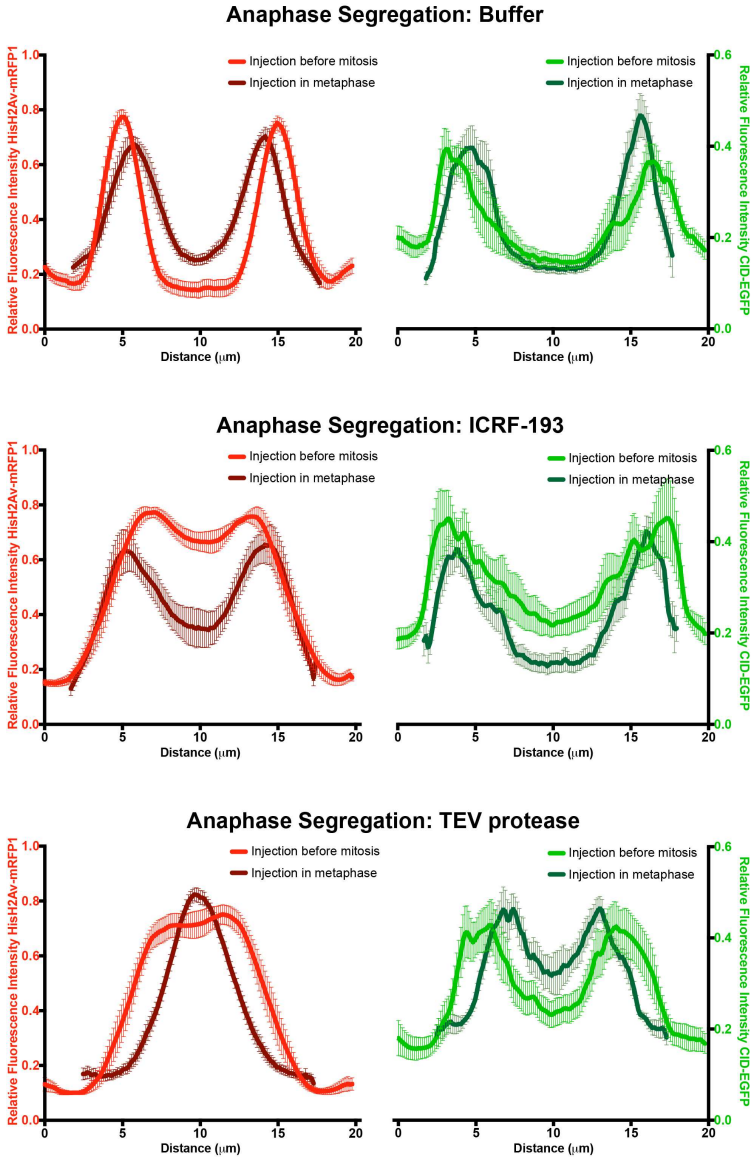


**Fig. 3.13. Comparative analysis of segregation efficiency for condensin and topoisomerase II inhibition before mitosis (light color) and during metaphase arrest/release (dark color).** Graphs plot the relative distribution of HisH2Av-mRFP1 (red) and CID-EGFP (green) across a 20 μm segregation plane, measured 4-6 minutes after anaphase onset.*Legend continued on the next page*

**Fig. 3.13.** Graphs plot the average ±SEM of individual embryos (n≥8 embryos for each experimental condition). For each embryo, at least 8 anaphases were analyzed.

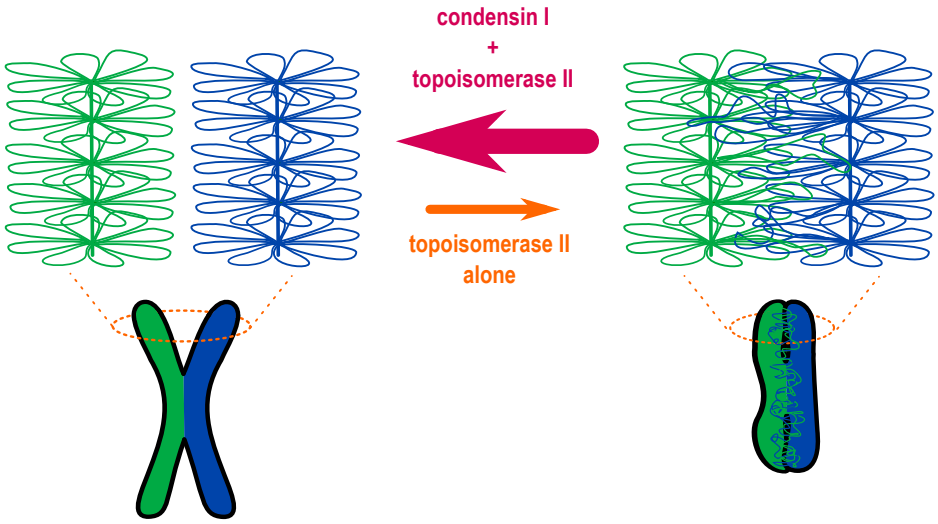


**Fig. 3.14. Proposed model of condensin I and toposimerase II influence on metaphase chromosomes.**

can be reverted by increased expression of topoisomerase II (Sen et al. 2016). All together, these results support that condensin I is not directly necessary for topoisomerase II catalytic activity, but rather to impose directionality on topoisomerase II reactions, favouring resolution of the sister DNA molecules rather than intertwining them. Upon condensin I removal, creation of new links between previously separated DNA strands leads to their increased proximity, which may underlie the observed increase in chromosome compaction. Importantly, our studies reveal that topoisomerase II is able to promote erroneous re-entanglements of sister chromatids throughout mitosis, an activity that needs to be constantly opposed by condensin I (Figure 3.14).

How condensin I is able to confer such directionality remains to be addressed. Condensins are enriched at the chromosome axis where they have been proposed to promote interactions within the same chromatid (Steffensen et al. 2001; Ono et al. 2003). Condensin I was shown to display significant turn-over on mitotic chromosomes (Gerlich et al. 2006; Oliveira, Heidmann, et al. 2007) highlighting

that its mode of action relies in dynamic reactions rather than statically holding chromatin loops. Bringing strands of DNA from the same chromatid in close proximity could alone favour sister chromatid decatenation by limiting the probability contacts between sister DNA molecules. Models that predict that DNA loops can extrude away from condensin have been hypothesized (Nasmyth 2001; Alipour and Marko 2012; Goloborodko et al. 2016) and are better at explaining the directionally issue, as they provide a mechanism that inherently explains how condensins distinguish intra- versus inter-chromosomal looping. Random intrachromatid linkages are also possible (Cheng et al. 2015; Cuylen et al. 2011), although in this case additional mechanisms may ensure that connections in *cis* are favoured over linkages between sister- (and nearby) chromatids. Condensin I- mediated supercoiling of the DNA molecule has also been proposed to change DNA structure to favour DNA decatenation activity (Baxter, Sen, et al. 2011; Baxter and Aragón 2012; Sen et al. 2016), although it is yet to be determined whether the supercoiling activity of this complex can account for all the phenotypes associated with condensin loss.

Our analysis further reveals that maintenance of chromosome architecture, particularly sister chromatid resolution, is not a unidirectional process but instead a much more dynamic reaction than previously anticipated. It is conceivable that the highly compacted chromatin state present in metaphase chromosomes could, on its own, shift topoisomerase II reaction towards sister chromatid re-entanglement given the increased proximity between DNA strands. Condensin I would thus counteract an inherent tendency of chromosomes to re-intertwine, a reaction necessary throughout metaphase. Additionally, it is possible that a dynamic balance of chromosome entanglements allows remodelling of chromosome architecture, providing chromosomes with plasticity to counteract the cytoplasmic drag faced during dynamic movements. Energy released during these reactions could potentially be used to further facilitate chromosome movement. Mitotic chromosomes should thus be visualized as highly dynamic structures during mitosis, whose re-shaping may be fundamental for the fidelity of their own segregation.

## 3.4 Materials and Methods

### 3.4.1 Constructing and testing UbcH10$^{S114C\ (wt)}$ plasmid

pET28 plasmid carrying human UbcH10$^{C114S}$ (catalytically dead) was kindly provided by M. Rape (Rape et al. 2006). To reverse the C114S mutation, following primers have been used: forward 5' GGTAACATATGCCTGGACATC 3', reverse 5' GATGTCCAGGCATATGTTACC 3'. Changing AGC to TGC produced UbcH10$^{S114C}$, as in the wild-type protein sequence, hence it is referred to as UbcH10$^{wt}$. The pET28 UbcH10$^{wt}$ plasmid was expressed and the protein was tested for catalytic activity by injecting the purified protein into UbcH10$^{C114S}$-arrested *Drosophila* embryo, in which case UbcH10$^{wt}$ was able to restore anaphase progression.

### 3.4.2 Fly strains

To destroy condensin by TEV protease-mediated cleavage, strains carrying solely TEV-sensitive Barren versions were produced (see Chapter 2). To destroy cohesin by TEV-protease we used strains carrying Rad21$^{TEV}$, previously described (Pauli et al. 2008; Oliveira et al. 2010). Fly strains also expressed His2AvD–mRFP1 or polyubiquitin His2B–RFP, to monitor DNA and EGFP–CID to monitor centromeres (Schuh et al. 2007). A list with detailed genotypes can be found in Materials and Methods of Chapter 2.

### 3.4.3 Microscopy

Aligned embryos on coverslips were covered with Series 700 halocarbon oil (Halocarbon Products Corporation). Time-lapse microscopy was performed with an inverted wide-field DeltaVision microscope (Applied Precision Inc.) at 18–20 °C in a temperature-controlled room. One stack of ~20 frames (0.8 μm apart) was acquired every 1 or 2 minutes using a 100×1.4 oil immersion objective (Olympus) and an EMCCD camera (Roper Cascade 1024). Widefield images were restored by deconvolution with the Huygens v15.10/16.10 deconvolution software using a calculated point-spread function (Scientific Volume Imaging). Movies were assembled using FIJI software (Schindelin et al. 2012) and selected

stills were processed with Photoshop CS6 (Adobe).

### 3.4.4 Microinjections

Microinjection experiments were performed as previously described (Oliveira, Hamilton, et al. 2010). 1–1.5 h old embryos (or 0–30 min for mRNA injections) were collected and processed according to standard protocols and embryos were injected at the posterior pole (up to three sequential injections) using a Burleigh Thorlabs Micromanipulator, a Femtojet microinjection system (Eppendorf), and pre-pulled Femtotip I needles (Eppendorf). Embryos were injected with buffer, drugs or proteins purified from *E. coli* at the following concentrations: buffer (20 mM Tris-HCl at pH 8.0, 1 mM EDTA, 50 mM NaCl and 2 mM DTT), 13 mg/ml TEV protease in TEV buffer, 12 mg/ml UbcH10$^{C114S}$, 14 mg/ml UbcH10$^{wt}$ and/or 280 μM ICRF-193 (Sigma), 280 μM ICRF-193 (Sigma-Aldrich) with 13 mg/ml TEV protease; 1mg/ml aclarubicin (Alfa Aesar); 2mM suramin (Merck Millipore); 5mM novobiocin (Sigma-Aldrich); 300μM merbarone (Sigma-Aldrich). Stock drugs were diluted in PBS to indicated concentrations, with no more than 2% DMSO in the final solution.

### 3.4.5 Protein purification

Purified TEV protease was described previously (Haering et al. 2008). Purification of UbcH10$^{wt}$ and UbcH10$^{C114S}$ was performed from BL21 cells as previously described (Oliveira, Hamilton, et al. 2010), with minor modifications, as follows. Bacterial cells were grown for 16 hours at 37°C, 225rpm. This preculture was used to inoculate fresh LB media and cells were allowed to grow until 0.8/1 ODs. Cultures were then induced with 1mM IPTG and after 4h of induction at 37°C, 225rpm, cells were harvested. Pellets were resuspended in Lysis Buffer (20mM Tris-HCL pH7.5, 0.5M NaCl, 5mM Imidazole with protease inhibitors) and sonicated 5× on ice in 30s cycles (power 5, Sonicator XL2020, Misonix). The soluble fraction of the extracts was then incubated in TALON Metal Affinity Resin (Takara), according to manufacturer's instructions. After several washes with Lysis Buffer, the resin coated with the protein was packed into a Poly-Prep Chromatography Column (Biorad). Proteins were eluted in the same buffer with 300mM imidazole. For buffer exchange, purified UbcH10wt

and UbcH10C114S proteins were dialyzed overnight, at 40C, in a Slide-a-Lyzer 7KDa Dialysis cassettes (Thermo Scientific). Final storage buffer was 20mM Tris-HCL pH 7.5, 0.3M NaCl). The purified proteins were concentrated in a Vivaspin 6 Centrifugal Concentrator MWCO 10.000KDa (GE Healthcare).

### 3.4.6 Electron microscopy of *Drosophila* embryos

*Drosophila* embryos from 1 hour collection were decorionated as described above. The embryos were subsequently aligned directly on a coverslip. To immobilize the embryos for injection, the anterior halves on embryos were covered with 2% low melting point agarose diluted in Schneider's medium. After drying for around 13 minutes, embryos were covered with halocarboin oil 700 (Sigma) and selected injected with UbcH10$^{C114S}$ protein to arrest in metaphase, than with 13mg/ml TEV protease or buffer. After around 15 minutes the embryos were cryo-protected with a solution of 20% (w/v) dextran (Alfa Aesar) and 0.5% (v/v) Tergitol type NP-40 (Sigma), frozen using a high-pressure freezer (M. Wohlwend GmbH) and stored in liquid nitrogen until processing. Before processing the embryos were pierced using a needle (0.4×19mM) at -160°C. The processing was done using an Automatic Freeze-Substitution System – EM AFS2 (Leica). Samples were immersed in a mixture of 0.3% (g/v) uranyl acetate in methanol (EMS), 0.3% (v/v) glutaraldehyde (EMS) and 3% (v/v) distilled water in acetone (Polysciences) at -90°C for 48 hours. Temperature was raised to -45°C using slope of 2°C per hour and further processed for 16 hours at this temperature. Three acetone washes were done at -45°C for 10 minutes each. Samples were infiltrated at -45°C using the methacrylate resin Lowicryl HM20 (Polysciences) in a graduated series of 10%, 25%, 50, 75% and 100% (v/v) in acetone. The polymerization was perform at -45°C for 48 hours under UV light and the temperature was raised from -45°C to 20°C using a slope of 5° C per hour. 70nnm sections of the embryos were cut on an EM-UC7 (Leica). The sections were post-stained with 2% (g/v) uranyl acetate in methanol (VWR) and Reynold's Lead Citrate (Alfa aesar) for five minutes each. Sections were imaged on a Hitachi H-7650 Transmission Electron Microscope (Tokyo, Japan) at 100 kV.

### 3.4.7 Quantitative analysis of compaction of chromosomes

For the quantification of chromosome compaction presented, deconvolved images were analyzed using Imaris v6.1 software (Bitplane). The same metaphase was tracked over time and average values for mean voxel intensity, volume and surface area were normalized to the first frame after injection. For the fluorescence profiles a wide 15 μm-long line was placed manually along the segregation plane and measured using the 'Plot Profile' function on FIJI software (Schindelin et al. 2012). For each data set, values were normalized to the maximum. Measurements of single chromatids width and length were performed on projected images (maximum intensity projection), using FIJI software and single chromatids mean voxel intensity measurements were performed using Imaris software.

### 3.4.8 Quantitative analysis of chromatids movements in time

Quantification of chromosome movement (Figue 3.9) was performed as previously described (Mirkovic et al. 2015). Briefly, HisH2B-RFP was imaged at 1 minute intervals. Images were segmented to select the chromosomal regions, based on an automatic threshold (set in the first frame after TEV injection), to create binary images. For each movie, a walking average of 3 frames was produced (using kymograph plug-in, written by J. Rietdorf and A. Seitz, EMBL, Heidelberg, Germany) creating a merged image in which the intensity is proportional to the overlap between consecutive frames. Intensity profiles were used to estimate the percentage of non-overlapping, 2- frame overlap and 3-frame overlap pixels. Graphic representation was performed using Prism 7 software (GraphPad).

### 3.4.9 Statistical analyses

The statistical analyses were performed using R.3.3.2 and Python 3.4. In general, our data consisted of quantifying parameters in multiple nuclei in several embryos in several experimental conditions. The variance between nuclei in common cytoplasm within an embryo is usually much smaller than variance between different embryos, also the measurements or various entities within the same embryos are not independent to each other, as the nuclei in syncytial embryo share

the same cytoplasm. The larger variation between embryos is in turn due to experimental variation of injection, such as amount of injected liquid, size of the embryo, precision of injection, etc., that can vary quite significantly from embryo to embryo because of technical limitations. The simplest model that takes into account such structure of data are linear mixed-effects models. In the analysis Python modules pandas and *rpy2* were used respectively to manipulate data frames and access R's packages *lme4* and *lmerTest*. To analyze data structure in such way, a script was written to first create a *lmer* model using *lme4* package. The formula taken into account single measurements (i.e. distances between centromeres) were nested into respective embryos, from which measurements were taken, and those embryos were nested into respective conditions (i.e. injected with buffer, TEV, ICRF or TEV-ICRF mix). Next, the model was analyzed by using anova method from *lmerTest* to perform ANOVA (Analysis of Variance Table of type III with Satterthwaite approximation for degrees of freedom). The significance values are 0 '***' 0.001 '**' 0.01 '*' 0.5 '.' 0.1 ' ' 1. The script is attached in Appendix 4. To analyze histone profiles we have applied the same strategy. Mixed linear models, as described above, were separately built data points of each discrete distance and tested for statistical significance between embryos injected with TEV and TEV+ICRF-193. The script for analysis of data per distance is attached in Appendix 5.

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# Chapter 4

# Exploring the external forces acting on mitotic chromosomes

Abstract Chapter 4

Mitotic chromosomes are subjected to variety of external forces during mitosis. Imbalance of those factors, through kinetochore disruption or spindle perturbations, can influence segregation efficiency. Chromosomes undergo a process of chromosome congression, which allows positioning of chromosomes in the segregation plane, located in the middle of the spindle. This process is proposed to be driven mostly by kinetochores and the mitotic spindle, but also other factors that potentially apply inwards pressure towards the nucleus could facilitate this process. In the light of the rapid overcompaction we have observed upon depletion of condensin I from metaphase chromosomes (see Chapter 3), we have questioned whether external forces may be driving or influencing the degree of *de novo* catenations. In order to explore this subject we have started by exploiting the congression forces acting in mitosis. We have shown that even after depolymerizing mitotic spindle by colchicine, sister chromatids have a strong tendency to rapidly clamp together. This leads us to propose that in *Drosophila* syncytial embryo there are other chromosome congression forces besides mitotic spindle, which may be an important factor to explain the overcompaction when biophysical properties of chromatin are suddenly changed. Those results open a new perspective path to explore the immediate chromosome's environment.

## Author contribution:

*All the experiments presented in this chapter were performed and analyzed by Ewa Piskadlo. The experiments were designed by Ewa Piskadlo and Raquel A. Oliveira. Alexandra Tavares prepared and purified proteins used in the experiments.*

## 4.1 Introduction

The fascinating result of Chapter 3 showed that *Drosophila* chromosomes lacking functional condensin I are increasing their compaction state very rapidly, within few minutes of injecting the TEV protease inactivating condensin I. At the same time we proposed that condensin I-depleted nuclei are accumulating a massive amounts of *de novo* catenation. Those catenations are most likely influencing compaction state by increased interlinking intra- and inter-sisters chromatids, as well as possibly connecting various chromosomes together, which causes chromosomes to clamp together and increase chromatids density. Although this could be solely responsible for overcompaction, the fast nature of this process is suggesting involvement of other factors. What are the forces driving the overcompaction is not clear, therefore our new direction of the research turned to exploring possible candidates.

In the nucleus there are various components that would be able to impose an inward force possibly leading to chromosomes congressing as a part of a normal mitosis. Chromosome congression is a process by which chromosomes are aligned in a spindle equator in prophase, a necessary step for achieving chromosome biorientation and perform a faithful division. Chromosome congression is believed to be driven by kinetochores and microtubules, in addition to motor proteins such as dynein, are responsible for the actual movement of kinetochores (and chromosomes on which they are assembled on) towards the middle of the spindle plane (as reviewed in (Auckland and McAinsh 2015 and Maiato et al. 2017). Nonetheless, it is perceivable that there are other factors facilitating chromosome congression in the middle of the spindle plane. In metaphase chromosomes are mostly stably bioriented and aligned at the spindle equator, and microtubules are applying pulling forces to the centromeres. As the pulling forces would likely contribute to stretching and decompaction the chromatin, we do not suspect that spindle forces are responsible for overcompaction. Yet, there may be other factors that cause the chromosome congression independent of the kinetochore-spindle pulling. In particular, proposal that microtubules are not necessary for chromosome congression was raised by a study in starfish meiosis (Lenart et al. 2005). It has shown that oocytes are still able to congress their chro-

mosomes in meiosis I even without visible attachments of astral microtubules to kinetochores of some chromosomes or after depolymerizing the spindle with nocodazol treatment. In this particular case, chromosome movement was driven by the actin-network (Lenart et al. 2005). Another possible candidate may be the spindle matrix, which is a dynamic, elastic hydrogel that encircles the spindle and contributes to its function, forming a unique environment for dividing chromosomes (as reviewed in Johansen et al. 2011 and Schweizer et al. 2014).

Such forces might be favoring chromosome congression by applying inwards force onto the chromatin mass. If so, such forces could drive mitotic chromosomes overcompaction in condensin I depletion conditions.

Driven by this logic we have started our investigation of alternative chromosome congression factors by observing whether there are any other forces, besides mitotic spindle, that act on mitotic chromosomes during metaphase.

## 4.2 Results

### 4.2.1 Isolated chromatids rapidly congress upon disruption of mitotic spindle.

To evaluate the forces acting on metaphase chromosomes we developed an experimental setup that allows dispersal of isolated chromatids throughout the spindle, followed by acute disruption of the spindle. *Drosophila melanogaster* embryos expressing cohesin's subunit Rad21 cleavable by TEV protease, HisH2Av-mRFP1 (histone marker), and CID-EGFP (centromere marker) were used in order to adopt a system in which congression forces would be clearly visible. In short, single sister chromatids are generated by injecting TEV protease into UbcH10$^{C114S}$-arrested embryos. Separated sister chromatids are subjected to dynamic shuffling by mitotic spindle, spreading chromatids over a large area. Perturbations of various cellular components can then be applied and following imaging allows capture changes for location or morphology of chromatids to look for factors potentially influencing chromosomes. This system scoring the chromosome congression forces is much easier than observing the unseparated chromosomes aligned as a metaphase plate. We have used colchicine to study effect of spindle depolymerization on chromatids behavior. The precise scheme

of the experimental set up is presented on Figure 4.1.

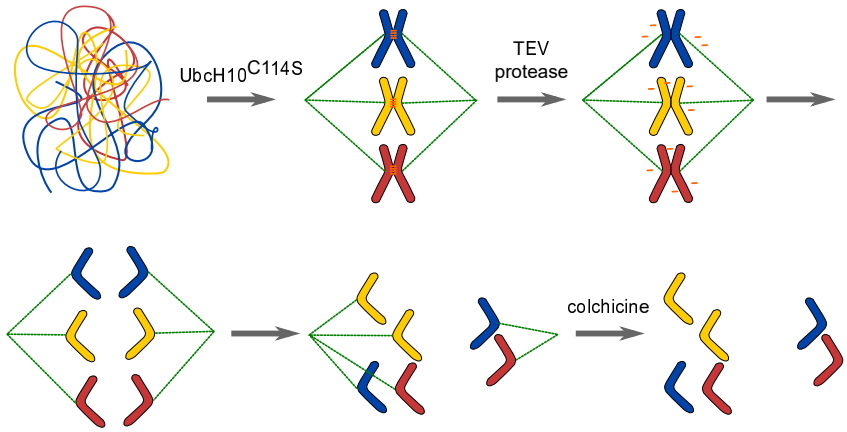


**Fig. 4.1. Scheme of experimental setup.** Interphase *Drosophila* embryos expressing TEV-cleavable Rad21 subunit of cohesin were injected with UbcH10$^{C114S}$ to induce metaphase arrest with an intact mitotic spindle. After metaphase was established, TEV protease was supplied to cause cohesin cleavage. This leads to a rapid cohesion loss, resulting in sister chromatids separation followed by their random shuffling. Around 8 minutes after TEV injection, 2mM colchicine was injected to depolymerize microtubules.

In short, embryos arrested in metaphase and subsequently injected with TEV protease to trigger sister chromatids dispersal. After several minutes microtubule depolymerization was triggered. If all congression forces were dependent solely on the spindle, than chromatids should halt their movements or freely diffuse in the cytoplasm. Instead, almost immediately after administering the spindle poison the chromosomes started to congress towards the center of their initial division plane (Figure 4.2, and Movie 13), confirmed by tracing centromere movements presented as kymograph on Figure 4.3. The experiment was repeated three times, yielding the same results in each case. Interestingly, chromatids always congress together with chromatids originating from the same nucleus. Chromatids do not seem to have tendency to congregate together with chromatids from other nuclei, even if two nuclei moved close to each other during the arrest in the common cytoplasm. This result strongly suggests that there are factors other than spindle that are able to impose forces towards the middle of the nuclei.

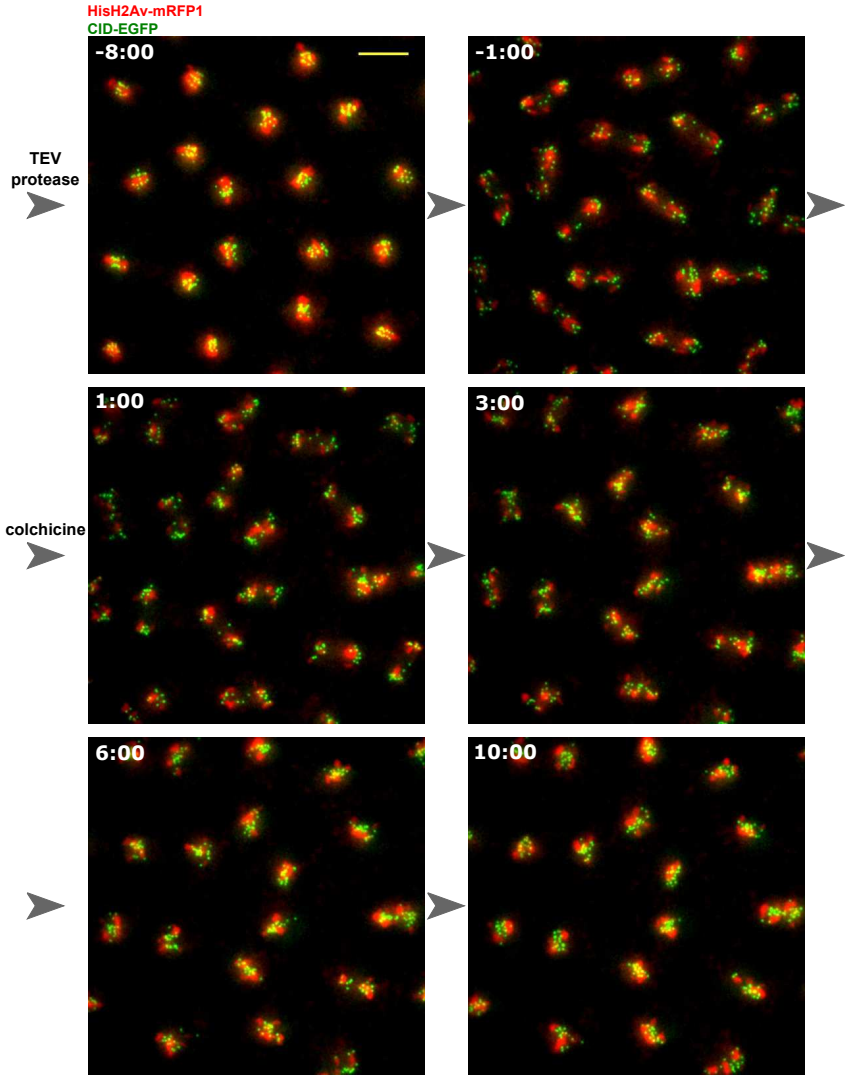


**Fig. 4.2. Isolated chromatids rapidly congress upon disruption of mitotic spindle.** Rad21$^{TEV}$ embryos were arrested in metaphase by 12 mg/ml UbcH10$^{C114S}$ and Rad21$^{TEV}$ subunit was cleaved by 13 mg/ml TEV protease injection. After several minutes, once chromatids separated completely, 2mM colchicine diluted in PBS was injected to depolymerize the spindle. The progress was followed by imaging nuclei in 3D every 1 minute. Time indicated on stills corresponds to time relative to colchicine injection (minutes:seconds). Scale bar is 10 μm.

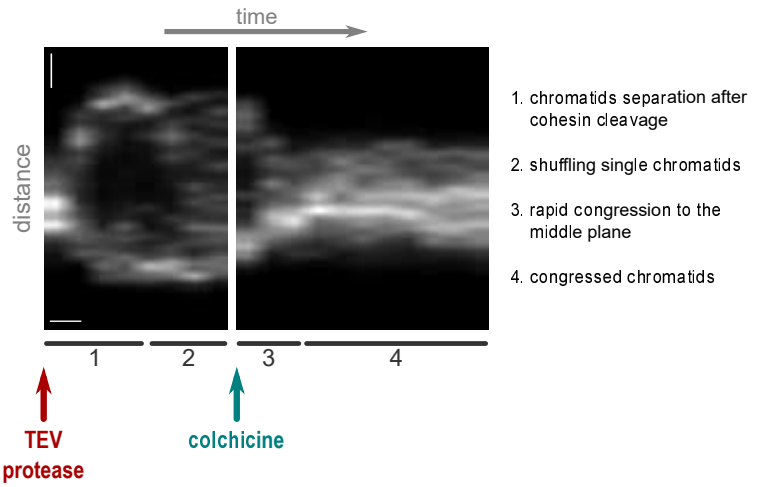


**Fig. 4.3. Quantification of centromere movement after disrupting mitotic spindle.** A representative kymograph showing centromere (CID-EGFP) movements after sister chromatids separation and after colchicine injection. Kymograph presents quantification of movement of a single metaphase plate from Figure 4.2. Vertical scale bar is 2 μm, horizontal scale bar corresponds to 1 min.

## 4.3 Discussion

Condensin I – depleted chromosomes were shown to lose their mechanical resistance to external forces, such as originating from a mitotic spindle. Chromosomes in such conditions were shown to be less rigid, resulting in their centromeres pulled away easily by mitotic spindle, as chromatin was apparently not able to resist the pulling forces (Oliveira, Coelho, et al. 2005; Gerlich et al. 2006; Ribeiro et al. 2009; Samoshkin et al. 2009). Such a change in biophysical properties of chromatin due to disrupting their internal organization may also explain why chromosomes rapidly overcompact in our system after condensin I inactivation. Softening the chromatin is likely due to removing loops that condensin I is stabilizing. This is most harsh to centromere region, where condensin I is probably contributing to form rigid centromere loops anchored to kinetochores (Lawrimore et al. 2015), hence we observe a rapid separation of centromeres once condensin I is removed (Figure 3.2). Notably this expanded distance between centromeres lasts even once the chromatin mass overcompacts, which means that overcompaction is not able to reverse this process. More

slowly than the abnormal centromere separation, chromosomes overcompact (Figure 3.3). As new links are being created in the absence of condensin I it is tempting to assign this to the extensive entanglements bringing DNA molecules progressively closer to each other and compressing DNA within the same chromosomes.

If chromosome entanglements were extensive enough to create such large overcompaction, it would also likely lead to secondary increase of chromatin rigidity after the initial loss of rigidity due to introducing multiple random links. It would be very interesting to explore in more details biophysical properties of mitotic chromosomes in a temporal manner to describe how exactly condensin I inactivation and the inferred introduction of de novo catenation affects the physical properties of chromosomes, leading to creating a better model of condensin's role in chromosome architecture. Chromosome self entanglements may not de driven solely by intrinsic chromosome behaviour, but additionally exarcebated by external factos. Those factors, such as microtubules, actin network, nuclear matrix components, and cytoplasm are interacting with the chromosomes. It is conceivable that in the normal circumstances chromosomes are organized in a way to withstand pressure from the outside and keep their normal shape and architecture. In condensin I-removal systems chromosomes are losing their rigidity. Although centromeres seem to be primarily affected, most likely the whole chromosome is compromised. As chromosomes lose their stiffness they do not have ability to balance the external forces pushing on them, which may cause the chromosomes to collapse inwards. This idea is supported by the fast kinetics of the overcompaction observed in our experiments, which would be expected in a process relying mostly on physical properties rather than enzymatic (topoisomerase II-dependent intertwines). On the other hand the same loss of stiffness and forcing DNA molecules to push on each other increases the probability contacts, making it easier to introduce catenations by topoisomerase II, especially in condensin I depletion background, where topoisomerase II loses its bias towards decatenation and is more likely to introduce entanglements. Therefore external forces increasing pressure on chromosomes may play an important part in accumulation of entanglements and overcompaction.

To better understand observed overcompaction, we have started to explore

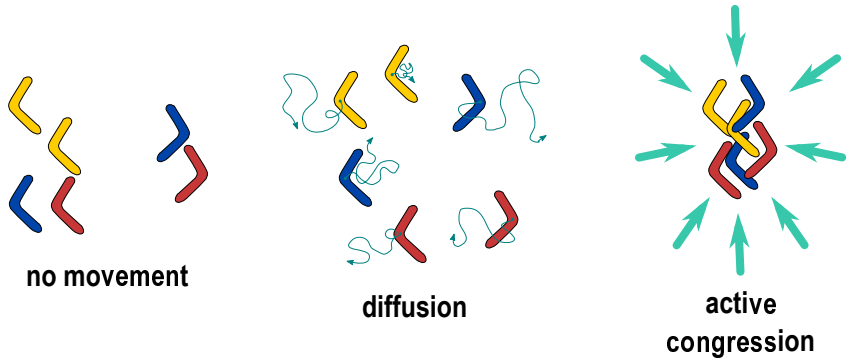


**Fig. 4.4. Predicted scenarios of chromatids' behavior following acute spindle disruption.**

in more depth the external forces acting on mitotic chromosomes in order to pinpoint the factor that might be responsible for aiding chromosome overcompaction in condensin I-inactivated nuclei. One of the most obvious source of forces affecting the chromatin organization are microtubules, mostly forming the mitotic spindle. We assumed that, according to current theories, they would be responsible for the majority of chromosome congression mechanism. We have tested how dispersed single chromatids would react to loss of mitotic spindle. We predicted several possible outcomes, presented on Figure 4.4. If congression forces are not effecting metaphase chromatids strongly, we would expect that dispersed sister chromatids would halt their movements. If the immediate environment of chromatids is very viscous and there are no strong external forces, chromatids may completely freeze their movement. Alternatively, chromatids may start feely diffusing once they lose attachments to microtubules, performing uncoordinated, slow movements. The last scenario is congression towards the middle of the division plane, which can be occur if there are some factors imposing inwards pressure onto isolated chromatids. Our experimental results clearly show that abolishing microtubules in the embryo by colchicine did not lead to disrupting chromosome movement. Quite the opposite, once microtubules were removed, the previously separated chromatids rapidly clamped together. This result strongly suggests that they are strong forces other than microtubules driving localization of chromosomes to the middle of the division plane in mitosis, at least in the absence of microtubules.

In addition chromatids always congress within their nucleus of origin, not being mixed with chromatids originating from other nuclei. The fact that chromatids keep their origin 'memory' confirms that there are still barriers between nuclei that prevents mixing of chromatids to some extent. One possibility is that nuclear envelope, that does not disintegrates completely in early stages of development of *Drosophila* during mitosis (Stafstrom and Staehelin 1984; Harel et al. 1989), constitutes an obstacle sufficient to prevent chromatids mixing. Also gel-like spindle matrix composed of multiple nuclear-derived protein is formed around each spindle. This was shown to contribute to compartmentalization of cytoplasm during mitosis, to separate internal spindle environment from the external cytoplasm and encapsulating each single nucleus as a single entity (Schweizer et al. 2014). Another possibility explaining rapid chromatids congression only within each nuclei may be that sister chromatids are not fully resolved at the metaphase stage, at the time that they were artificially separated by cohesin cleavage. It was previously shown that chromosomes can retain low level of catenations, especially in centromere region, that are only removed after the anaphase onset. Those catenations are not visible by standard DNA visualization methods and require staining for specific helicases, such as PICH or BLM (Broderick and Niedzwiedz 2015). It is therefore likely that in our experimental setup we would not be able to detect such fine bridges between sister chromatids. An argument therefore can be made that the rapid congression triggered by spindle depolymerization is caused by sister chromatids clamping together by elastic forces imposed by linking DNA catenations. Against this idea stands the fact of random distribution of chromatids before colchicine addition. As sister chromatids are no longer strongly cohesed and shuffled independently to both poles, there is quite a large chance of both sisters being located at the same side of the division equator. If thin elastic links were truly responsible for the observed congression, chromatids located at the same side would not congress to the middle after spindle depolymerization, instead collapse to each other on one side and stay away from the metaphase plane. As we do not observe such behavior, such DNA links are not likely to be a major driver of congression.

Congression forces are responsible for aligning chromosomes on the mitotic equator and they are believed to be based om spindle, kinetochores and mo-

tor proteins associated with them (Auckland and McAinsh 2015; Maiato et al. 2017). Nonetheless in some systems, such as starfish meiosis, microtubules are not required for chromosome congression (Lenart et al. 2005).This suggested that other factors are largely responsible for chromosome congression in this system. The same starfish meiosis study reported that a contractile actin network formed around the nucleus is indispensable for congressing the chromosomes in meiosis I, as both stabilizing or destabilizing actin filaments by drugs perturbed normal congression. It is therefore conceivable that also in mitotic *Drosophila* embryo actin may be able to impose contracting forces onto chromosomes. This could easily be exacerbated by removing spindle, thus changing the environment and breaking the force balance and causing actin network to collapse onto chromosomes, congressing chromosomes to the middle. Whether this hypothesis is valid and if so, whether actin network would be able to drive overcompaction of chromatin remains to be determined.

Another simple explanation of the observed rapid congression might be a simple manner of a rapid cytoplasmic flow. If a mitotic spindle is suddenly depolymerized, this would enforce reorganization of the region previously occupied by the spindle. It is possible that cytoplasm, maybe together with other small organelles inside it, would be able to cause an influx liquid and such a wave could drag chromatids inside and aggregating them to the middle. It is nonetheless doubtful whether cytoplasm could cause such a wave, since, as mentioned above, mitosis in early *Drosophila* embryo is semi-closed, therefore membranes of the nuclear envelope would likely limit such a sudden influx into the nuclear region.

Some less obvious targets potential candidates that may be responsible for chromosome overcompaction in condensin I-deprived chromosomes, but would likely not cause a massive chromosome congression, are proteins that coat the surface of the chromosomes. One of such proteins is Ki67 which in human cells is localized to the surface of mitotic chromosomes, forming a surfactant-like barrier around all the chromosomes in a nuclei (Cuylen et al. 2016). The pressure imposed by similar proteins coating the DNA onto the chromatin mass might explain overcompaction in case of sudden softening of underlying chromatin.

In the Chapter 3 we have discovered that acute inactivation of condensin I

leads to overcompaction of metaphase chromatin. At the same time we observe accumulation of de novo catenations between chromatids (and probably chromosomes). We propose that such links are driving overcompaction by forcing excessive and progressive entanglements between DNA molecules that cannot be efficiently resolved. Nonetheless chromosomes are embedded in a complex cellular environment and cannot be viewed as isolated entities, but are constantly subjected to interactions with other components of a cell. We hypothesize that the observed overcompaction may be partially caused by external factors that can exacerbate compaction by indirectly aiding in creating *de novo* catenation. Future work will be necessary to identify these mysterious forces. Once we understand the mechanical forces acting on metaphase chromosomes we will be able to dissect whether or not these forces contribute to the overcompaction observed upon condensin I inactivation.

## 4.4 Materials and Methods

### 4.4.1 Fly strains

To destroy cohesin by TEV-protease *Drosophila* strains were carrying Rad21$^{TEV}$, previously described (Pauli et al. 2008; Oliveira, Hamilton, et al. 2010). Fly strains also expressed His2AvD–mRFP1 to monitor DNA and EGFP–CID to monitor centromeres (Schuh et al. 2007).

### 4.4.2 Microscopy

Aligned embryos on coverslips were covered with Series 700 halocarbon oil (Halocarbon Products Corporation). Time-lapse microscopy was performed with an inverted wide-field DeltaVision microscope (Applied Precision Inc.) at 18–20 ∘C in a temperature-controlled room. One stack of ∼ 20 frames (0.8 µm apart) was acquired every 1 or 2 minutes using a 100×1.4 oil immersion objective (Olympus) and an EMCCD camera (Roper Cascade 1024). Widefield images were restored by deconvolution with the Huygens v15.10/16.10 deconvolution software using a calculated point-spread function (Scientific Volume Imaging).

Movies were assembled and analyzed using FIJI software (Schindelin et al. 2012) and selected stills were processed with Photoshop CS6 (Adobe).

### 4.4.3 Microinjections

Microinjection experiments were performed as previously described (Oliveira, Hamilton, et al. 2010). 1 — 1.5 h old embryos were collected and processed according to standard protocols and embryos were injected at the posterior pole (three sequential injections) using a Burleigh Thorlabs Micromanipulator, a Femtojet microinjection system (Eppendorf), and pre-pulled Femtotip I needles (Eppendorf). Embryos were injected with drugs or proteins purified from *E. coli* at the following concentrations: 12 mg/ml UbcH10$^{C114S}$, 13 mg/ml TEV protease in TEV buffer (20 mM Tris-HCl at pH 8.0, 1 mM EDTA, 50 mM NaCl and 2 mM DTT), 2 mM colchicine (Sigma-Aldrich).

### 4.4.4 Protein purification

TEV protease and UbcH10$^{C114S}$ were purified as described in Materials and Methods of Chapters 2 and 3.

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# CHAPTER 5

# General Discussion

Correctly assembled mitotic chromosomes are an absolute prerequisite for faithful segregation of the genome between daughter cells, a process that is the basis of all known life. To achieve such fidelity, in each cell cycle, every replicated and entangled interphase DNA molecule undergoes a strictly controlled series of changes leading to its compression into discrete rod-like entities. Chromosome condensation is a very complex process, which results in physical compaction of chromatin and ensures adequate biophysical properties necessary for withstanding forces to which a chromosome is subjected to during its segregation. Another crucial aspect of condensation is disentanglement from other DNA molecules and individualization of specific chromosomes and resolution of sister chromatids, preparing for convenient separation and minimalizing the risk of damaging the genetic material as a result of erroneous entanglements. If the condensation process is compromised, genomic stability of the cell's progeny can be in jeopardy.

Our knowledge of the exact internal architecture of mitotic chromosomes is still limited. Even though multiple various hypothesis exist for how chromosomes are organized, no model is able to fully explain chromatin folding on every scale of compaction. Because of the enormous importance of the mitotic process, understanding the mechanisms by which chromosomes are assembled and then maintained constitutes one of the greatest challenges in modern molecular biology.

Among many factors have been identified to drive or influence chromosome condensation, condensins have been proclaimed to be major organizers of chromatin both in interphase and in mitosis (Kschonsak and Haering 2015; Rana and Bosco 2017). In thesis we have presented the development of an innovative tool to study condensin I's function in *Drosophila melanogaster* system. This allowed us to explore function of condensin's role in metaphase chromosome structure maintenance and provided novel insights into the interplay of condensin I and topoisomerase II. We propose these two molecules dynamically regulate the higher order of architecture of metaphase chromosomes by modulating the amount of chromosome entanglements.

Even though condensins have a very suggestive name bringing to mind direct role in condensation, whether they actually involved in condensin chromosomes

was long debatable, as we described in more details in Chapter 1. In short, several decondensation phenotypes were noted in condensin removal situations (Hirano and Mitchison 1994; Hirano, Kobayashi, et al. 1997; Freeman et al. 2000; Lavoie et al. 2000; Petrova et al. 2013; Vagnarelli et al. 2006; Kruitwagen et al. 2015; Shintomi et al. 2015). Others noted only mild mitotic compaction defects in the absence of condensin, suggesting that chromosomes can still compact to a high degree without condensin (Bhat et al. 1996; Steffensen et al. 2001; Hudson et al. 2003; Oliveira, Coelho, et al. 2005; Gerlich et al. 2006; Ribeiro et al. 2009). More directly, condensin was shown to be able to compact isolated DNA *in vitro* (Strick et al. 2004; Cui et al. 2008; Eeftens et al. 2017). How can such discrepancies be explained? The key to answering this conundrum is introducing more precise definitions of condensation process itself. Condensation of interphase chromatin into fully formed mitotic chromosomes that can be observed in metaphase or anaphase, is not simply a matter of packing chromatin more tightly. Compaction is only one of the aspects of what we call condensation. Besides spatial compression chromosomes need to be re-organized to obtain specific internal structure which will equip chromosomes with defined mechanical properties, such as rigidity and elasticity. Another features of a correct condensation is achieving separating individual chromosomes (individualization) from interphase chromatin and disengagement of sister chromatids (resolution). All of those processes have been linked to condensin in various organisms.

The results presented in this thesis are compliant with the previous observations and expand our knowledge of condensin I's action to maintaining chromosome structure in metaphase. We broaden the crucial role of condensin's regulation of topoisomerase II in order to sustain the optimal amount of catenations within chromosomes. It was proposed that level of catenations are an important structural feature stabilizing metaphase chromosomes, as topoisomerase II-imposed (de)catenation influence stiffness and shape of isolated chromosomes (Kawamura et al. 2010; Bauer et al. 2012). Therefore topoisomerase II's ability to link and unlink DNA is not only involved in the process of simple, one-directional decatenation of heavily entangles sister chromatids to resolve them and allow smooth segregation. Instead, catenations imposed by topoisomerase II have an important structural role, which also explains severe condensation de-

fects of topoisomerase II removal or inhibition (Uemura et al. 1987; Andreassen et al. 1997; Chang et al. 2003; Carpenter and Porter 2004; Sakaguchi and Kikuchi 2004; Vas et al. 2007; Petrova et al. 2013). As topoisomerase II can perform its enzymatic reaction in both directions, its regulation is likely to be one of the most important level of control of chromosome properties and resolution state. Condensin was proposed to direct topoisomerase II's reaction towards unlinking DNA by globally introducing positive supercoiling, a preferred substrate for decatenation (Baxter, Sen, et al. 2011). Alternatively condensin may be preventing excessive links between DNA molecules by keeping already separated parts of sister chromatids (and other DNA molecules that should not be catenated, such as two different chromosomes) by physically keeping them far apart by looping those two molecules, therefore making the re-catenation by topoisomerase II highly improbable. Whatever is the molecular mechanisms of this collaboration, our results shown also that condensin's biasing is needed not only early in the condensation when the bulk of catenations are removed. If condensin I is impaired the topoisomerase II loses its favoritism towards unlinking and starts to introduce excessive catenations, even in metaphase chromosomes, that were previously almost completely decatenated. We propose that this exaggerated amounts of links between sister chromatids leads to their re-catenation and overcompaction. The overcompaction in such conditions at chromosomal arms may be secondarily exacerbated by the loss of structural stiffness of condensin I-depleted chromosomes, coupled with additional forces towards the chromosome mass (see 4). Loss of chromosomal stiffness is most notably visible in centromeric region, both in our system and many others (Oliveira, Coelho, et al. 2005; Ribeiro et al. 2009; Samoshkin et al. 2009) but this may simply be the result of spindle pulling forces and not a particular organization of this chromosomal locus.

At non-centromeric regions, one can also consider this situation from the opposite point of view – collapsing of mechanically softened chromosomes and clamping together is causing decreased distances between chromosomes and chromatids, which in turn is biasing topoisomerase II towards intertwining those molecules (Sen et al. 2016), leading to greater amount of catenations. All of those data suggest that condensin is responsible for such organization of chro-

matin that would allow efficient working of topoisomerase II, especially given that removal of condensin and topoisomerase II leads to similar phenotype (loss of stiffness, compaction and impaired resolution). Therefore our data suggest that both presumable separate functions of condensin – chromosome compaction and DNA decatenation – are in fact closely related and may be a consequence of the same function of chromosome organization. For example, DNA loops created by condensin I not only compact and stiffen the chromosomes by physically constraining the DNA molecules, but those loops create a topological landscape for topoisomerase II activity, controlling level of catenation, which regulates resolution and mechanical properties dependent of intrachromatid self-entanglements together with compaction.

How can condensins organize chromosomes? It was observed that condensins localize to the longitudinal axis of chromosomes (Ono 2004; Samejima et al. 2012). It was initially proposed that condensins are a fundamental ingredient of stiff chromosome scaffold, statically pinning the chromatin loops to form a defined axis (Adolph et al. 1977; Earnshaw 1983). Subsequent studies uncovered a dynamic nature of condensin I association to chromosomes (Gerlich et al. 2006; Oliveira, Heidmann, et al. 2007) which at first seemed to contradict condensin I's function as a structural component. In the light of a recent model of loop extrusion both of those ideas can be reconciled. Condensin may play a role of an active loop extruders, as suggested by several studies (Alipour and Marko 2012; Goloborodko et al. 2016), leading to accumulation of loops in the center of chromosomal axis where multiple extruders meet while pushing though the same DNA molecule. According to the model, those loops need to be dynamic by nature with the extruding factor binding on and off, perfectly fitting condensin I's behavior. Besides compaction and organization aspect, condensin I acting as a major loop extrusion factor in mitosis also explains efficient resolution of DNA and lack of erroneous entrapping two separate DNAs. Loop extrusion factor would only bind to a single loci, progressively enlarging the loop stemming from this single site. This behavior would prevent accidental capture of two separate chromatids or chromosomes, which could impair segregation either by condensin I entrapment or topoisomerase II-dependent catenation. Removing active condensin I in our system leads to creation of *de novo* catenation between

sister chromatids and impairing chromosome stiffness. Both of those observations are in agreement with loop extrusion model. Abolishing the loop extrusion factor would lead to eradiation of already existing chromatin loops and prevent the formation of new ones. As chromosomes likely depend on constraining DNA by its looping to provide rigidity, chromosomes lacking condensins become too soft to resist spindle forces and cannot withstand external factors that can lead to collapsing of chromatin. Also, assuming that condensin I is indeed extruding the loop to organize chromosomes, removal of loops is changing the architecture of chromatin, increasing contacts of various DNA molecules, which likely enables topoisomerase II to introduce unwelcome inter-DNA catenations.

For long time chromosomes were considered a passive entity in mitosis, acting as a simple cargo moved around by other forces (such as mitotic spindle). Our research underlines the dynamic character of dynamic chromosomes and the importance of constant control of chromosome architecture in order to execute perfect genome division. Even when chromosomes are correctly pre-established and reach their 'mature' condensed stage in metaphase they need constant maintenance to support adequate properties of the chromatin. Not only obvious errors in mechanical characteristics, such as excessive elasticity or unsound compaction, may be detrimental to cell division. Removal of catenations, which was usually seen as a one-directional process in living cell, leading to global resolution of links, turns out to be easily reversible upon condensin I removal, as we showed in this thesis. This means that precise governing of the level and placement of catenations is necessary for preferential intrachromatid catenation to stabilize the structure and prevent interchromatid and interchromosome catenation to ensure precise segregation in anaphase. Such bias is governed by the interplay of factors such as condensin I and topoisomerase II, whose continuous action on chromatin lasts throughout the whole mitosis to deliver ever changing, perfectly balanced architecture of resolved chromosomes able to withstand external forces. As the properties of those dynamic chromosomes influence highly the outcome of the mitotic division, we argue that chromosomes are not passive entities, but rather active players profoundly participating in their own segregation. Detailed description of how mitotic machinery reacts to sudden loss of condensin I in our system is one of the most interesting next stages of deeper characterization

of our condensin I inactivation system. At the same time by acutely removing condensin I in our system we gain a valuable tool to trace how external forces affect compromised chromosomes, allowing to infer the effect of those forces in an unperturbed situation.

We are now getting closer to gain full understanding of chromosome structure. Condensation of interphase chromatin into mitotic rod-like structures is a complex process with multiple factors contributing to it at various levels. Therefore it is of particular importance to identify all the players involved in condensation, characterize their molecular action and interplay between them in order to create a comprehensive model of condensation. The advances in techniques such as advanced imaging, chromosome confirmation capture (especially recent Hi-C), and molecular modelling allow us a take a better glimpse of the internal architecture of mitotic chromosomes (as reviewed in Piskadlo and Oliveira 2016). Alas, many questions about condensation remain unanswered not only in the broader chromosome organization, but also in a more specific condensin-centered field. Detailed molecular mechanism for DNA binding and enzymatic activity for example are still not understood. In contrast to cohesin, condensins do not seem to have a specific loader and the exact mode of their entrapping DNA is not known. Also obtaining more detailed description of condensin's enzymatic functionality dependent on ATP hydrolysis may provide additional hints for how condensin could impose compaction and organization. Condensin I works closely with topoisomerase II to ensure proper course of mitosis, as implied by this thesis and other publications (Charbin et al. 2014; Baxter, Sen, et al. 2011; Baxter and Aragón 2012). This thesis and other sources (Uemura et al. 1987; Hirano and Mitchison 1994; Hirano, Kobayashi, et al. 1997; Andreassen et al. 1997; Freeman et al. 2000; Lavoie et al. 2000; Carpenter and Porter 2004; Petrova et al. 2013; Kruitwagen et al. 2015; Shintomi et al. 2015) describe severe disruption of chromosome organization when condensin and/or topoisomerase II are removed. Pinpointing the exact molecular mechanism by which these proteins change topology and arrangement of DNA will bring a greater understanding of assembling and supporting the chromosome architecture.

Condensins seem to lie at the heart of genome organization. Expanding our knowledge about those fascinating complexes is bound to bring us closer to an

ultimate model of inner structure of mitotic chromosomes, one of the greatest challenges in modern cell biology.

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# Appendix

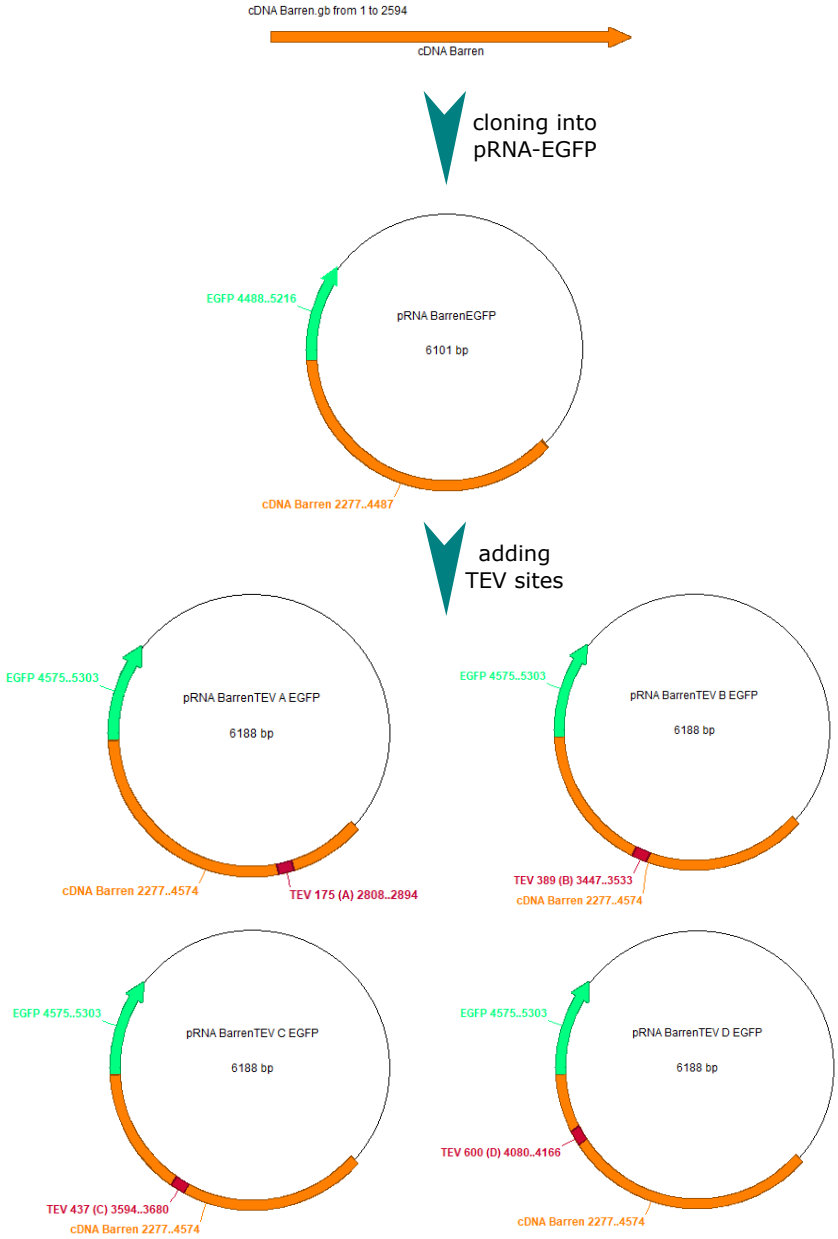
# .1 List of all Barren$^{TEV}$ fly lines.

All created Barren$^{TEV\ A\text{-}D}$ fly lines

| CHR # * | Genotype | TEV site | Chromosome |
|---|---|---|---|
| 1509 | Barr$^{(175\text{-}3TEV)}$myc10 ∇ II.1 / Cyo | A | II |
| 1510 | Barr$^{(175\text{-}3TEV)}$myc10 ∇ II.2 / Cyo | A | II |
| 1511 | Barr$^{(175\text{-}3TEV)}$myc10 ∇ III.3 / Tm3, Sb | A | III |
| 1512 | Barr$^{(175\text{-}3TEV)}$myc10 ∇ III.4 / Tm3, Sb | A | III |
| 1513 | Barr$^{(175\text{-}3TEV)}$myc10 ∇ III.5 / Tm3, Sb | A | III |
| 1514 | Barr$^{(437\text{-}3TEV)}$myc10 ∇ III.1 / Tm3, Sb | C | III |
| 1515 | Barr$^{(437\text{-}3TEV)}$myc10 ∇ III.2 / Tm3, Sb | C | III |
| 1516 | Barr$^{(437\text{-}3TEV)}$myc10 ∇ II.3 / Cyo | C | II |
| 1517 | Bar$^{(437\text{-}3TEV)}$myc10 ∇ X.4 / FM7i | C | X |
| 1518 | Barr$^{(600\text{-}3TEV)}$myc10 ∇ III.1 / Tm3, Sb | D | III |
| 1519 | Barr$^{(600\text{-}3TEV)}$myc10 ∇ II.2 / Cyo | D | II |
| 1520 | Barr$^{(600\text{-}3TEV)}$myc10 ∇ III.3 / TM3, Sb | D | III |
| 1521 | Barr$^{(389\text{-}3TEV)}$myc10 ∇ III.1 / TM3, Sb | B | III |
| 1522 | Barr$^{(389\text{-}3TEV)}$myc10 ∇ III.2/ TM3, Sb | B | III |
| 1523 | Barr$^{(389\text{-}3TEV)}$myc10 ∇ X.3/ FMi7 | B | X |
| 1524 | Barr$^{(389\text{-}3TEV)}$myc10 ∇ II.4/ CyO | B | II |

* Number of the laboratory's internal fly database.
List of all lines of *Drosophila* possessing insertions of Barren$^{TEV}$ variants mapped on various chromosomes.

## .2 Scheme of cloning of cDNA Barren$^{TEVA\text{-}D}$EGFP constructs



# .3 Scheme of cloning of genomic Barren$^{TEVA-D}$Myc constructs

## .4 Script for statistical analysis of centromere distance after induced anaphase using linear mixed models.

```
"""
Given a table in form of:
 Treatment | Embryo | Trait
    1          1      1
    1          1      2
    ...        ...    ...

builds a linear mixed model using R's lmer from lme4 package and checks for significance of Treatment
in Traits, while Traits are nested in Embryos.
"""

### import basic pandas/numpy
import pandas as pd
import numpy as np
### import rpy2 package to allow python to use R's functions

import os
from rpy2.robjects.packages import importr
import rpy2.robjects as robjects

### importing the utils to be able to install R's extra libraries, installing the extra libraries
utils = importr("utils")
#utils.install_packages('lme4')
#utils.install_packages('multcomp')

#utils.install_packages('lmerTest')
lme4 = importr('lme4')
multcomp = importr('multcomp')
lmerTest = importr('lmerTest')
r = robjects.r

### load the data frame, remove rows in which all the values are NA
df = pd.read_table('centromeredistanceforRALLtreatmentsEwa2.txt', header = 0, usecols = [0,1,3], decimal = ',')
df.dropna(how='all', inplace = True)
```

```
35
36 ### installing and activating the module for easy conversion of pandas'
       data frame into R's table
37 from rpy2.robjects import r, pandas2ri
38 pandas2ri.activate()
39 r_dataframe = pandas2ri.py2ri(df)
40
41 ### create a mixed linear model of Trait nested in Embryo
42 model1 =lmerTest.lmer('Trait ~ Treatment + (1 | Embryo)', data =
       r_dataframe)
43
44 #print(r.summary(model1))
45 ### perform the anova on the model
46 anv = lmerTest.anova(model1)
47 print(anv)
48 print('The p-value is: ' +str(anv[5]))
49
50 mcomp = multcomp.glht(model1, linfct=r.mcp(Treatment="Tukey"))
51 print(mcomp)
```

## .5 Script for statistical analysis of histone profiles after induced anaphase using linear mixed models.

```
"""
Given a table in form of:
 Distance | Treatment 1 | Treatment 1 | Treatment 1| ... | Treatment 2 |
     Treatment 2 | Treatment 2
 Embryo         1           1           2      ...       1           1
               2
    0.13        x           x           x      ...       x           x
                  x
    0.26        x           x           x      ...       x           x
                  x
    ...        ...         ...         ...     ...      ...         ...
               ...

builds a linear mixed model for each row of Distance using R's lmer from
    lme4 package and checks for significance
of Treatment in Traits, while Traits are nested in Embryos.

It adds a columns with the p-values (anova) to the table for each
    Distance value, together with stars significance
notation 0 '***' 0.001 '**' 0.01 '*' 0.5 '.' 0.1 ' ' 1 for a quick
    overview of the data.
"""

import pandas as pd
import numpy as np

import os

### import rpy2 package to allow python to use R's functions

from rpy2.robjects.packages import importr
import rpy2.robjects as robjects

### importing the utils to be able to install R's extra libraries,
    installing the extra libraries
utils = importr("utils")
#utils.install_packages('lme4')
```

```
#utils.install_packages('multcomp')
#utils.install_packages('lmerTest')
lme4 = importr('lme4')
multcomp = importr('multcomp')
lmerTest = importr('lmerTest')
r = robjects.r

### installing and activating the module for easy conversion of pandas' data frame into R's table
from rpy2.robjects import r, pandas2ri
pandas2ri.activate()

### load the data frame, remove rows in which all the values are NA
df = pd.read_excel('curves_excel.xlsx', header = None)
df.dropna(how='all', inplace = True)

### slice a given dataframe row by row; for each row take 'Treatment' row , 'Embryo' row and one of values for Distance
### (inx) the df_slice will be transposed to gain a format:
### Treatment | Embryo | Trait
###   1         1      1
###   1         1      2
###   ...       ...    ...
### becoming the input to build a mixed linear model of Trait in Treatment, where Trait is nested within Embryo, to
### which anova will be performed to test whether Treatment 1 vs Treatment 2 are different;
### each row of df (distance) will therefore become a small model with it's p values.
### The output of the function is a list of p values for each row (distance).
def lmer_anv(dframe):
    result = []
    for inx in range(2, len(df.ix[:,[1]])):
        df_slice = df.ix[[0, 1, inx], 1:].transpose()
        df_slice.columns = ['Treatment', 'Embryo', 'Trait']
        df_slice['Trait'] = pd.to_numeric(df_slice['Trait'])
        r_dataframe = pandas2ri.py2ri(df_slice)
        #print(r_dataframe)
        ### create a mixed linear model of Trait nested in Embryo
```

```
        model1 =lmerTest.lmer('Trait ~ Treatment + (1 | Embryo)', data =
            r_dataframe)
        #print(r.summary(model1))
        ### perform the anova on the model
        anv = lmerTest.anova(model1)
        result.append(anv[5][0])
    return result

### biuld a list of the p values for each row, also add two NA values for
    'index' rows (Treatment, Embryo), add the
### p-values list as a column to the df
P_val_list = lmer_anv(df)
P_val_list.insert(0, np.nan)
P_val_list.insert(0, np.nan)
df['P val'] = pd.Series(P_val_list)

### a function to give a quick overview of the significance levels in the
    data frame, creating a list of star - coded
### significance values: 0 '***' 0.001 '**' 0.01 '*' 0.5 '.' 0.1 ' ' 1 as
    given by the R's anova
def sign_stars(series):
    stars_list = []
    for n in series:
        if n < 0.001:
            stars_list.append('***')
        elif n >= 0.001 and n < 0.01:
            stars_list.append('**')
        elif n >= 0.01 and n < 0.05 :
            stars_list.append('*')
        elif n >= 0.05 and n < 0.1:
            stars_list.append('.')
        else:
            stars_list.append('')
    return stars_list
df['Stars'] = pd.Series(sign_stars(df['P val']))

### export the data frame to the excel file
df.to_excel('ceurves_nested_analysis_significance.xlsx')
```

## .6 Legends of the movies.

**Movie 1 –** Mitosis in *Drosophila* embryos. Embryos were injected with buffer in early interphase and monitored throughout the subsequent mitosis. Embryos express HisH2Av-mRFP1 (red) and CID-EGFP (green). Times are relative to injection time. Scale bar is 10 μm.

**Movie 2 –** Mitosis upon condensin I inactivation in *Drosophila* embryos. Embryos surviving solely on Barren$^{TEV}$ were injected with TEV protease in early interphase and monitored in the subsequent mitosis. Embryos express HisH2Av-mRFP1 (red) and CID-EGFP (green). Times are relative to injection time. Scale bar is 10 μm.

**Movie 3 –** Buffer injection in metaphase-arrested embryos. Embryos expressing solely Barren$^{TEV}$ were injected with 12 mg/ml of a dominant-negative form of the human E2 ubiquitin-conjugating enzyme (UbcH10$^{C114S}$), to induce a metaphase arrest, and subsequently injected with buffer. Embryos also express His2A–mRFP1 (red) and CID-EGFP (green); scale bars, 10 μm. Times (minutes:seconds) are relative to the time of buffer injection.

**Movie 4 –** Condensin I inactivation in metaphase-arrested embryos. Embryos expressing solely Barren$^{TEV}$ were injected with 12 mg/ml of a dominant-negative form of the human E2 ubiquitin-conjugating enzyme (UbcH10$^{C114S}$), to induce a metaphase arrest, and subsequently injected with 13 mg/ml TEV protease. Embryos also express His2A–mRFP1 (red) and CID-EGFP (green); scale bars, 10 μm. Times (minutes:seconds) are relative to the time of TEV injection.

**Movie 5 –** Topoisomerase II inhibition in metaphase-arrested embryos. Embryos expressing solely Barren$^{TEV}$ were injected with 12 mg/ml of a dominant-negative form of the human E2 ubiquitin-conjugating enzyme (UbcH10$^{C114S}$), to induce a metaphase arrest, and subsequently injected with 280 μM ICRF-193. Embryos also express His2A–mRFP1 (red) and CID-EGFP (green); scale bars, 10 μm. Times (minutes:seconds) are relative to the time of ICRF injection.

**Movie 6 –** Concomitant inactivation of topoisomerase II and condensin I in metaphase-arrested embryos. Embryos expressing solely Barren$^{TEV}$ were injected with 12 mg/ml of a dominant-negative form of the human E2 ubiquitin-conjugating enzyme (UbcH10C114S), to induce a metaphase arrest, and subsequently injected with a mix of 280 μM ICRF-193 and 13 mg/ml TEV protease.

Embryos also express His2A–mRFP1 (red) and CID-EGFP (green); scale bars, 10 μm. Times (minutes:seconds) are relative to the time of the second injection.

**Movie 7 –** Artificial induction of sister chromatid separation in metaphase-arrested embryos. Embryos expressing solely $Rad21^{TEV}$ and wild-type Barren were injected with 12 mg/ml of a dominant-negative form of the human E2 ubiquitin-conjugating enzyme ($UbcH10^{C114S}$), to induce a metaphase arrest, and subsequently injected with 13 mg/ml TEV protease. Embryos also express His2B–RFP; scale bars, 10 μm. Times (minutes:seconds) are relative to the time of the second injection.

**Movie 8 –** Effect of condensin I inactivation on isolated sister chromatids. Embryos expressing uniquely TEV-sensitive Rad21 and Barren were injected with 12 mg/ml of a dominant-negative form of the human E2 ubiquitin-conjugating enzyme ($UbcH10^{C114S}$), to induce a metaphase arrest, and subsequently injected with 13 mg/ml TEV protease. Embryos also express His2B–RFP; scale bars, 10 μm. Times (minutes:seconds) are relative to the time of the second injection.

**Movie 9 –** Induced anaphase in control embryos. Embryos expressing solely $Barren^{TEV}$ were injected with 12 mg/ml of a dominant-negative form of the human E2 ubiquitin-conjugating enzyme ($UbcH10^{C114S}$), to induce a metaphase arrest, and subsequently injected with TEV protease buffer (with no protease). After 14 minutes embryos were injected a wild-type version of UbcH10 to induce anaphase. Embryos also express His2A–mRFP1 (red) and CID-EGFP (green); scale bars, 10 μm.

**Movie 10 –** Induced anaphase after timely inhibition of topoisomerase II. Embryos expressing solely $Barren^{TEV}$ were injected with 12 mg/ml of a dominant-negative form of the human E2 ubiquitin-conjugating enzyme ($UbcH10^{C114S}$), to induce a metaphase arrest, and subsequently injected with 280 μM ICRF-193. After 14 minutes embryos were injected a wild-type version of UbcH10 to induce anaphase. Embryos also express His2A–mRFP1 (red) and CID-EGFP (green); scale bars, 10 μm.

**Movie 11 –** Induced anaphase after timely inhibition of condensin I. Embryos expressing solely $Barren^{TEV}$ were injected with 12 mg/ml of a dominant-negative form of the human E2 ubiquitin-conjugating enzyme ($UbcH10^{C114S}$), to induce a metaphase arrest, and subsequently injected with 13 mg/ml TEV pro-

tease. After 14 minutes embryos were injected a wild-type version of UbcH10 to induce anaphase. Embryos also express His2A–mRFP1 (red) and CID-EGFP (green); scale bars, 10 μm.

**Movie 12 –** Induced anaphase after timely inhibition of condensin I and topoisomerase II. Embryos expressing solely Barren$^{TEV}$ were injected with 12 mg/ml of a dominant-negative form of the human E2 ubiquitin-conjugating enzyme (UbcH10$^{C114S}$), to induce a metaphase arrest, and subsequently injected with a mix of 280 μM ICRF-193 and 13 mg/ml TEV protease. After 14 minutes embryos were injected a wild-type version of UbcH10 to induce anaphase. Embryos also express His2A–mRFP1 (red) and CID-EGFP (green); scale bars, 10 μm.

**Movie 13 –** Chromosome congression after spindle depolymerization. Embryos expressing solely Rad21$^{TEV}$ were injected with 12 mg/ml of a dominant-negative form of the human E2 ubiquitin-conjugating enzyme (UbcH10$^{C114S}$), to induce a metaphase arrest, and subsequently injected with 13 mg/ml TEV protease. After around 8-10 minutes embryos were injected 2 mM colchicine in PBS to depolymerize microtubules. Embryos also express His2A–mRFP1 (red) and CID-EGFP (green); scale bars, 10 μm. Times (minutes:seconds) are relative to the time of the first injection (UbcH10$^{C114S}$).

## .7 Source data files legends

**Source Data 1 –** Centromere displacement and chromosome compaction measurements upon condensin I and topoisomerase II inactivation. Individual measurements of centromeres displacement and relative Mean Voxel Intensity, relative volume and relative surface area. Each data set is presented on a separate sheet. Data were used to create following figures: Figure 3.3, Figure 3.7, and Figure 3.8.

**Source Data 2 –** Measurements of segregation efficiency and chromosome movement upon cohesin/condensin inactivation. Individual measurements of segregation efficiency and chromosome displacement Each data set is presented on a separate sheet. Data were used to create Figure 3.9.

**Source Data 3 –** Measurements of isolated chromatids upon cohesin/condensin inactivation. Individual measurements of chromosome thickness, length and mean voxel intensity upon TEV-mediated cleavage of Read21TEV and Rad21$^{TEV}$+Barren$^{TEV}$. Each data set is presented on a separate sheet. File includes descriptive statistics. Data were used to create Figure 3.10.

**Source Data 4 –** Measurements of segregation efficiency after metaphase-specific inactivation of condensin and/or topoisomerase II. Anaphase profiles for HisH2Av-mRFP1 and CID-EGFP measured 4-6 minutes after anaphase onset. Each measurement represents the average for independent embryos (resulting from at least 8 anaphases measured). Individual sheets include either the same measurement for the four experimental conditions or both CID-EGFP and HisH2Av-mRFP1 for the same experiment, as indicated.Data were used to create Figure 3.12.

**Source Data 5 –** Statistical analysis of segregation efficiency of centromeres after metaphase-specific inactivation of condensin and/or topoisomerase II. The distances between segregating centromeres measured 6 minutes after induced anaphase onset, for various treatments. Those input data were used to analyze data presented on Figure 3.12a using script from Appendix 4.

**Source Data 6 –** Statistical analysis of segregation efficiency of chromatin after metaphase-specific inactivation of condensin and/or topoisomerase II. Anaphase profiles for HisH2Av-mRFP1 measured 4-6 minutes after induced anaphase onset, for various treatments. Those input data were used to analyze data presented

on Figure 3.12e using script from Appendix 5.

**.8 Publication 1: Piskadlo, Ewa, and Raquel A Oliveira.** ***Novel Insights into Mitotic Chromosome Condensation.*** **F1000Research 5 (2016): F1000 Faculty Rev-1807.**

REVIEW

# Novel insights into mitotic chromosome condensation [version 1; referees: 2 approved]

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## Abstract

The fidelity of mitosis is essential for life, and successful completion of this process relies on drastic changes in chromosome organization at the onset of nuclear division. The mechanisms that govern chromosome compaction at every cell division cycle are still far from full comprehension, yet recent studies provide novel insights into this problem, challenging classical views on mitotic chromosome assembly. Here, we briefly introduce various models for chromosome assembly and known factors involved in the condensation process (e.g. condensin complexes and topoisomerase II). We will then focus on a few selected studies that have recently brought novel insights into the mysterious way chromosomes are condensed during nuclear division.

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## Introduction: why do chromosomes condense during mitosis?

Mitosis was first described in the 19th century and has captivated generations of scientists ever since. This fascinating process comprises the assembly of interphase chromatin into individual chromosomes and subsequently the equal separation of the genetic material between two daughter cells. Mitosis is undoubtedly an extremely complex operation that needs to be conducted and controlled precisely under the penalty of dismantling genome integrity. One of the key steps in mitosis is chromosome condensation – the compaction of the chromatin into well-defined rod-shaped structures (for other recent reviews, see 1–3). This process is cytologically very evident, yet both the internal structure of the mitotic chromosomes and the mechanisms by which this transformation is achieved remain quite elusive. To ensure that cell division is feasible within the cell space, vertebrate cells compact their DNA around 2–3 times more than in interphase, as estimated by chromatin volume measurements[4,5] and Förster resonance energy transfer (FRET)-based assays between histones[6]. Spatial compaction, however, is not the only important outcome of condensation. The structural reorganization during condensation leads to the separation of the identical sister chromatids from each other (known as sister chromatid resolution). Several topological constraints arise throughout interphase (most notably during DNA replication) that result in the entanglement of the two DNA molecules. The resolution of such intertwines (i.e. individualization) is crucial for efficient and faithful chromosome segregation during mitosis. Condensation of chromatin into sturdy chromosomes is also necessary to establish proper physical properties. Chromosomes must be stiff, resilient, and elastic enough to withstand forces coming from pulling microtubules and cytoplasmic drags during mitosis to prevent damage and breaks caused by external tensions.

Despite the utmost importance of chromosome condensation for the fidelity of mitosis, the molecular mechanisms that drive this process remain very unclear. Here we highlight recent findings regarding this process, discussed in the context of different models for mitotic chromosome condensation.

## Models for mitotic chromosome architecture

Over the past few decades, detailed characterization of metaphase chromosomes, using different cytological approaches, has led to the proposal of several models for mitotic chromosome assembly (Figure 1).

Classical views on chromosome organization postulate that mitotic chromosomes result from chromatin fiber folding. DuPraw suggested that fiber folding occurs randomly, transversely, and longitudinally, with no intermediate levels of compaction[7]. However, mitotic chromosomes fold into a reproducible structure in every mitosis, at least to some extent. Mitotic chromosomes acquire a

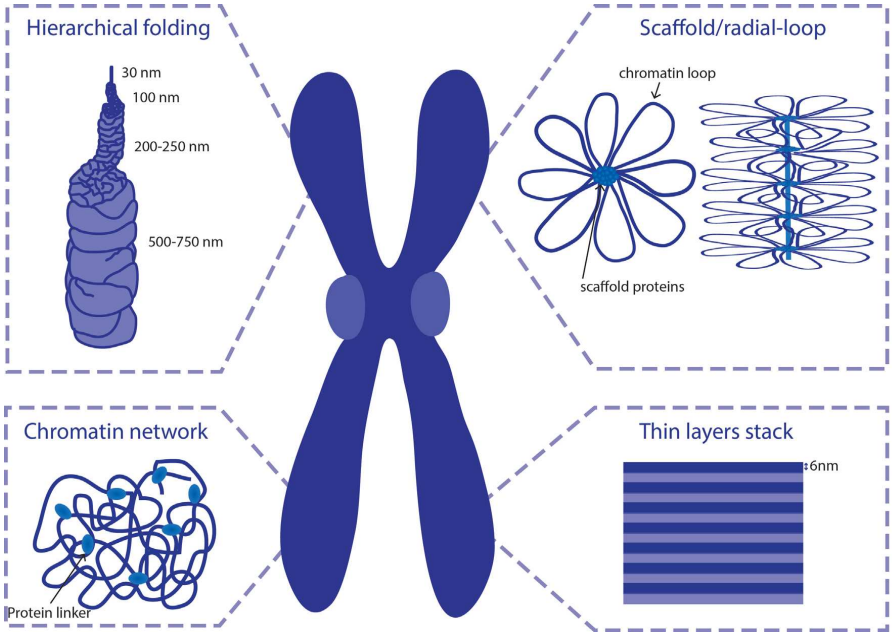


**Figure 1. Schematic representation of current models for mitotic chromosome condensation.** Adapted from Daban *et al.*[21]

reproducible length and display an invariable signature pattern of bands after staining with specific dyes, such as Giemsa. Moreover, specific DNA sequences occupy a reproducible position along the longitudinal and transverse axes of the chromosome[8]. Although some degree of randomness was observed within chromosomal domains[9,10], chromosome assembly cannot be explained as a purely random process.

Alternatively, it has been suggested that metaphase chromosomes result from helical coiling events (helical-coiling model). The nucleo-histone fiber is proposed to be coiled up into a helix, which is hierarchically wound up into larger helices to achieve the compactness of the mitotic chromosome (Figure 1)[11,12]. This model has been widely accepted, as lower levels of chromatin organization were long postulated to result from hierarchical folding: wrapping of DNA around nucleosomes forms a 11 nm bead-on-a-string structure that coils up into a 30 nm fiber. However, the existence of this 30 nm fiber *in vivo* is yet to be confirmed and has been recently highly debated[13–15].

Using electron microscopy (EM) studies, Paulson and Laemmli[16] provided a novel view on chromosome organization. Upon histone removal, chromosomes revealed a scaffold or core that has the shape of intact chromosomes, surrounded by loops of chromatin attached to this central core[17,18]. These and subsequent studies led to the consolidation of the scaffold/radial-loop model, which argues that radial DNA loops extend out from a protein element or scaffold positioned along the central axis of the chromatid.

In contrast to the scaffold model, analysis of the biophysical properties of mitotic chromosomes has challenged the idea that the continuity of mitotic chromosomes depends on its proteinaceous core. Taking advantage of the highly elastic behavior displayed by mitotic chromosomes, *in vitro* elasticity measurements revealed that the elastic response of mitotic chromosomes is lost after DNA digestion[19]. Mild protease treatment, in contrast, does not impair a reversible elastic response despite a progressively reduced force constant[19,20]. This led to the proposal of the chromatin-network model, in which chromatin itself is proposed to be the mechanical contiguous component of the mitotic chromosome.

More recent ideas for the internal folding of chromosomes suggest that mitotic chromosomes are arranged into stacks of 6 nm layers[21]. Those layers would be perpendicular to the chromosome axis and contain around 1 Mb of consequent DNA. Such arrangement of chromosomes has the advantage of explaining properties of G-bands and the geometry of chromosome translocations in a better way than other models.

Despite the differential contributions for chromatin/protein components within chromosome organization, these models might not be mutually exclusive and stacks, coils, and radial loops may co-exist within a less ordered structure.

## Known players of condensation

Despite the several unknowns on the precise molecular details of chromosome assembly, some key components are believed to be crucial for chromosome organization.

### Condensins

Condensins are a conserved group of multi-subunit proteins (Figure 2a) fulfilling many roles in chromatin organization throughout the cell cycle, but their most prominent function is to ensure efficient chromosome segregation (reviewed in 22–24). They were first isolated from *Xenopus* egg extract, and immunodepletion studies have suggested that this protein complex is required for proper chromosome condensation *in vitro*[25,26]. However, subsequent studies have challenged the view for condensin's requirement in chromosome condensation, as chromosomes do condense to a certain degree upon condensin's

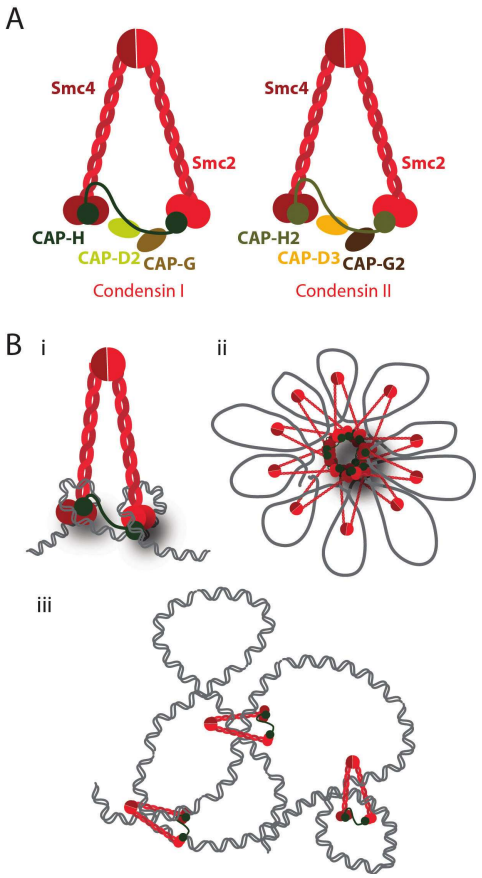


**Figure 2. Condensin complexes. A**) Schematic representation of the structure of condensin complexes. In metazoans, there are two types of condensins, condensin I and condensin II. The SMC2/SMC4 heterodimer is shared by both complexes, while the non-SMC subunits differ: CAP-D2, CAP-G, and CAP-H (Kleisin γ) for condensin I and CAP-D3, CAP-G2, and CAP-H2 (Kleisin β) for condensin II. **B**) Possible models for the condensins' role in DNA compaction include DNA supercoiling, loop-holder, and topological linker.

inactivation in several *in vivo* studies[27–32]. In addition to chromosome compaction, several studies revealed other roles for condensin in mitotic chromosome organization: maintenance of chromosomal structural integrity[28,30,33] and resolution of topological DNA entanglements[27,29–31,33]. Recent studies, using novel protein inactivation tools based on timely proteolytic cleavage of condensin complexes, revealed that condensin complexes (particularly condensin II) are indeed needed to support the structure of assembled meiotic chromosomes[34]. It should be noted that meiotic chromosomes are very different from their mitotic counterparts, as the mono-orientation of bivalents imposes pulling forces along the entire chromosome length (rather than simply at the pericentromeric regions). Thus, it remains to be addressed if condensins are required for chromosome condensation *per se* or simply to resist mechanical stress.

Importantly, it is yet to be determined how these different functions on chromosome organization are brought about, if they result from differential activities of condensins on mitotic chromatin, or, alternatively, if a single condensin-driven reaction may account for all the reported phenotypes. *In vitro* studies revealed that condensins are able to introduce positive supercoils on circular DNAs[35,36], which could account for chromosome compaction. Yet it is not clear if (and how) condensin supercoiling activity is required for *in vivo* chromosome condensation. Condensin subunits are also the major components of the chromosome scaffold[18,37], and it has thus been proposed to hold chromatin loops at the central axial core of chromosomes. However, condensin I (but not condensin II) displays a highly dynamic association with mitotic chromosomes[28,38], questioning the hypothesis that this complex is statically holding chromatin loops. Recent studies in budding yeast revealed that condensin complexes topologically embrace DNA molecules *in vivo*[39], providing strong evidence that condensins may work as an intra-chromosomal linker that brings together two distant segments of one sister chromatid and thereby promotes compaction. Further understanding on how condensin works on mitotic chromosomes is pivotal, not only to uncover the molecular mechanisms of these complexes but also to elucidate chromosome architecture itself.

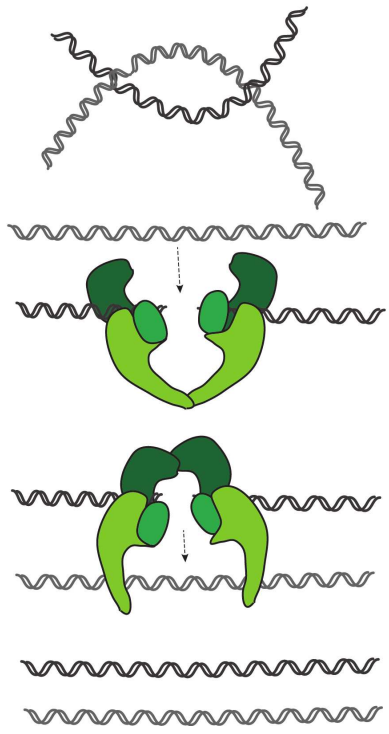

**Figure 3. Topoisomerase II: DNA decatenation reaction driven by topoisomerase II.** This enzyme cuts both strands of a DNA duplex and allows strand passage of a second duplex through the break. After strand passage, topoisomerase seals the break and releases both strands. It can thus promote the resolution of intertwines (catenations) between sister DNA molecules.

## Topoisomerase II

Topoisomerase II can introduce several changes in the topology of DNA molecules by driving both supercoiling and relaxing of the supercoils, and also the catenation and decatenation of DNA molecules[40]. Although some of these reactions can be brought about by topoisomerase I, only topoisomerase II can promote the resolution of catenated sister-DNA molecules. Topoisomerase II is able to decatenate intertwined DNAs by transiently cutting both strands of a DNA molecule, which are then resealed after passage through another DNA duplex (Figure 3). It is therefore essential for sister chromatid resolution and their efficient separation at the end of mitosis. Topoisomerase II is also a major component of the chromosome scaffold[41], and it has long been debatable whether or not this enzyme promotes chromosome compaction in addition (or in parallel) to sister chromatid resolution.

Topoisomerase II was reported to be dispensable for chromosome condensation in some model organisms (*Saccharomyces cerevisiae*[32], *Xenopus laevis*[42], and human cells[43]). Nevertheless, other studies provide evidence that topoisomerase II is necessary or at least contributes to establishing proper condensation and chromosome structure in *Schizosaccharomyces pombe*[44,45], *S. cerevisiae*[46], *X. laevis*[47,48], *Drosophila melanogaster*[49], chicken[50], hamster[51], or human[52,53] cells. How exactly topoisomerase II could facilitate condensation, however, remains unclear.

## Interplay between condensin I and topoisomerase II

Both condensin I and topoisomerase II localize to the central axis of mitotic chromosomes[54,55] and both complexes have the ability to alter DNA topology. Thus, it has been speculated that these proteins may cooperate (directly or indirectly) in establishing chromosome compaction and organization. Condensin I was initially proposed to directly interact with topoisomerase II[56], but later studies failed to provide evidence for a physical interaction between these proteins[26,47,57]. Nonetheless, depletion of condensins causes delocalization of topoisomerase II from the chromosome axis and decreases its decatenation activity[54]. Recent evidence further

supports the notion that during anaphase, topoisomerase is recruited to chromosome arms in a condensin-dependent manner[58].

Importantly, topoisomerase II was shown to be particularly efficient in decatenating (unlinking) supercoiled DNA molecules[59]. Given the condensins' ability to introduce positive supercoiling, it has been proposed that the topology generated by condensin I could be attracting topoisomerase II in order to drive global decatenation[59]. This notion is further supported by studies that measure the efficiency of decatenation of circular mini-chromosomes *in vivo*, revealing that condensin promotes DNA decatenation[57].

In contrast to the cooperation model, other studies support the idea that condensins and topoisomerase II may have antagonistic roles in chromosome assembly. Condensins were proposed to drive lateral compaction, while topoisomerase II was suggested to induce axial compaction[50,60]. The question of how condensins and topoisomerase II are able to cause directional compaction within separate sister chromatids without creating new links within individual sister chromatids and tangling them together remains.

### Kif4

Kif4 is a motor protein able to bind to mitotic chromosomes. Studies in vertebrate cells reveal that Kif4 contributes to the establishment of a correct morphology and structure of chromosomes[50,61]. It is proposed to cooperate or work alongside condensin in shortening the lateral axis of chromosomes, possibly by creating loops of chromatin[50], although little is known about the molecular mechanisms in this process.

### Histone modifications

During mitosis and concomitantly with chromosome condensation, the landscape of histone modifications is altered. Histone H1, the linker histone, is hyper-phosphorylated during mitosis[62,63], and it was initially thought to directly participate in condensation. However, subsequent studies suggest that histone H1 phosphorylation is not necessary for condensation[64,65] but nevertheless changes the overall chromatin structure[66,67]. Another key mitotic histone modification is the phosphorylation of serine 10 residue of histone 3 (H3 S10) by the mitotic kinase Aurora B[68]. The role for this modification in chromosome condensation has also been controversial[69–71], although recent evidence proposes that it drives the recruitment of deacetylase Hst2, which, in turn, induces deacetylation of lysine 16 of histone 4. This change in the properties of the histone 4 tail promotes interaction with histones H2A and H2B from other nucleosomes[72], thereby shortening the distance between neighboring nucleosomes. This would thus support the notion that histone modifications alone can promote the condensation of chromosomes. It should be noted that several histones and histone modifications were also described to be a chromosomal "receptor" for condensin binding[73–76]. Thus, some histone modifications may not be a direct contributor for chromosome compaction but rather a facilitator by promoting the binding of specialized proteins that model DNA topology.

## New insights from novel approaches

### Chromosome condensation revealed by high-resolution imaging and novel quantification methods

The chromosome condensation field has been largely dominated by cytological analysis. Yet, only recently, and with the advances in imaging and imaging analysis techniques, the field has started to adopt sophisticated quantification methods to estimate changes in chromosome structure during mitosis, revealing not only the compaction state but also the kinetics of the process.

Although chromosome condensation was often thought of as a linear and gradual process, a new study suggests that in the early mitosis stages, chromosomes undergo a series of subtle compaction and expansion steps[77]. The authors applied a series of sophisticated imaging and image analysis methods to describe changes in condensation throughout mitosis. Until mid-prophase chromosomes compact, but at late prophase stages their morphology changes and they expand at the same time sister chromatids are being individualized. This is followed by another compaction phase during prometaphase and metaphase. These observations were anticipated by a theoretical model of condensation that predicted this compaction-expansion cycle[78]. This hypothesis assumes that compaction is causing more "stress" to chromatin, as tethering segments together induces constraints and accumulates higher potential energy. The expansion stage, therefore, releases such stress and lowers the potential energy of chromosomes. The mentioned tethers causing physical constraints could be of various natures, such as protein linkers (cohesin, condensins) or DNA catenations. The authors propose that the stress cycle is ensuring the usage of the energy stored during the early compaction events for the energy-consuming drastic changes in chromosome structure, such as individualization of sister chromatids in late prophase. A recent study, however, revealed that the resolution of sister chromatids starts early during prophase, concomitantly with chromosome compaction[79]. The authors used sequential replication labeling with two distinct nucleotide derivatives to differentially label each DNA strand, which combined with quantitative advanced imaging allowed the assessment of the resolution process with unprecedented temporal resolution. Thus, the aforementioned compaction-expansion cycles may not necessarily correlate with differential processes throughout prophase.

In addition to the estimation of global compaction on entire chromosomes, recent quantitative microscopic assays were developed to assess local compaction[80]. Using a fluorescent reporter to target specific loci, this study reveals that the fluorescence intensity of the reporter varies depending on the compaction stage of chromosomes – the fluorescence is 2–2.5 times higher when chromatin is less compacted (interphase) than in mitotic, condensed chromosomes. This intensity variation was caused by quenching of bound fluorophore due to changes in the local environment created by packed chromosomes. The drop in fluorescence of reporters disappears if interactions between H2A and H4 histones are abolished, suggesting that the assay is primarily sensitive to

compaction at the level of neighboring nucleosomes. Therefore, it provides a convenient tool to study short-range condensation. Combining two different reporter genes along arms of a chromosome, it was possible to trace at the same time the axial (long-range) contraction of chromosomes along their longitudinal axis (distance between the reporter loci) and the short-range compaction of the marked regions. Remarkably, short-range and axial compaction have different kinetics during mitosis. In anaphase, short-range nucleosome-nucleosome compaction is happening before the axial decrease of chromosome length. Moreover, condensin depletion does not affect short-range compaction and, conversely, disturbing nucleosome-nucleosome interaction does not affect axial contraction. This led to the conclusion that short-range compaction and axial contraction are probably mostly independent and governed by different mechanisms. A common factor in both pathways is Hst2 deacetylase. By regulating H2A–H4 interaction, Hst2 promotes short-range nucleosome-nucleosome interactions and compaction. Additionally, Hst2 was shown to contribute to axial contraction by promoting condensin activity. This study proves that obtaining accurate quantification of microscopic data is very often challenging but can lead to novel discoveries.

### The minimal chromosome assembly system revealed by *in vitro* approaches

*In vitro* studies have brought major insights into many fields of biology. Separation of biological components into a controlled artificial environment with less complexity allows simpler and more precise interpretation of data. It is undeniably true that the *in vitro* results cannot be always directly translated back to the *in vivo* situation. Nevertheless, once the component or process (like chromosome condensation) is studied in the *in vitro* environment, it is easier to understand it in the *in vivo* context.

A breakthrough towards this idea was the identification of the minimal set of components that allows *in vitro* formation of a mitotic structure from uncondensed DNA in *Xenopus* egg extracts[48]. This reductionist approach demonstrated that out of thousands of possible proteins present in metaphase extract, only six factors, when combined, are sufficient to drive effective condensation. In addition to the "usual suspects" condensins and topoisomerase II, painstaking selection of other critical components further reveals the requirement of four other factors: nucleoplasmin, Nap1, and FACT (all of them are histone chaperone proteins) and embryonic core histones. In addition, the process was shown to be ATP dependent, which is necessary for enzymatic actions of condensins and topoisomerase II. This unique approach holds the promise of providing important insights into chromosome condensation by *in vitro* perturbations.

### Lessons from studies on isolated chromosomes

Isolated entire chromosomes can be micromanipulated and subjected to measurements of their mechanical properties. This approach, pioneered using large newt chromosomes[19,20,81,82], allows a direct measurement of the physical characteristics of chromosomes. Chromosomes can be assessed for their elastic properties in various conditions by stretching them and determining the force needed to double the chromosomal length. A major recent advance was the ability to perform similar studies on much smaller human chromosomes[83]. Importantly, most of the prior observations were confirmed in human chromosomes, further supporting the idea that a scaffold of protein crosslinkers is not necessary to keep chromosome structure together, which is instead sustained by a network of intertwined DNA. Yet the absence of these "modulating proteins" leads to significant changes in the properties and morphology of this chromosome network.

Another *in vitro* approach has also been recently used to understand the roles of DNA catenation in human mitotic chromosomes[84]. DNA catenations have long been speculated to be critical in mitotic chromosome structure, yet measuring DNA catenation *in vivo* has been a virtually impossible task. To test this, the authors used metaphase chromosomes isolated from human cells placed in a microfluidics lab-on-chip system, which allowed simultaneous imaging and environment control. When native metaphase chromosomes were treated with proteinase to remove all proteins, the resulting digested chromosomes were then challenged with various physical obstacles. The chromosomes preserved their canonical X-like shape and sister chromatids are kept together by thin DNA fibers in the centromeric region. Importantly, disrupting catenations, by chemical inhibition of topoisomerase II, caused drastic morphological changes along the entire length of the chromosome. Without functional topoisomerase II, the chromosomes become decondensed (elongated and rounded) and with less-defined axes along the arms. This led to the proposal that DNA catenation networks provided by topoisomerase II activity are crucial to maintain chromosome structure not only at the centromeres but also along the entire length of chromosome arms. It nevertheless remains to be determined if the same holds true *in vivo*, as it is possible that the *in vitro* manipulations may alone contribute to the observed phenotype.

### Internal chromosomal linkages revealed by Chromosome Conformation Capture methods

During interphase, chromosomes have their characteristic patterns of physical interactions of distinct regions within a single chromosome. It was recently shown in an elegant way that for the mitotic chromosome it does not really matter how it was previously folded during interphase[85]. When cells enter mitosis, each chromosome is somehow stripped of its interphase physical contact frequency pattern and acquires a homologous physical interaction pattern throughout its entire length (no compartmentalization of interaction within itself, meaning that only short-distance interactions occur). This absence of compartmentalization in mitotic chromosomes seems to be similar in all chromosomes, regardless of the chromosome identity or the cell type. The observed interaction map was confronted with models describing the folding, dynamics, and internal organization of mitotic chromosomes. Among others, it tackles the hierarchical model of packing DNA into chromosome structure and also a long-debated existence of internal scaffold in mitotic chromosomes. The authors argue that their experimental data do not fit with hierarchical folding models while models based on the existence of 80–120 kb long loops stay with the agreement with experimental work. Unfortunately, the authors were not able to anticipate whether or not chromosome structure contains a stiff scaffold around which chromatin is organized. A model in which the folding of interphase chromatin occurs in a two-step process, nevertheless, better explains their

findings. First, linear compaction occurs by creating loops of consecutive regions of the DNA of length 80–120 kb, possibly with the help of SMC complexes. The second step would be consequent axial compaction achieved by interactions of neighboring loops. It needs to be further supplemented with more detailed description of how this transition from interphase to mitotic chromosomes could be conducted inside living cells.

Biophysical modeling combined with interaction mapping has also been recently applied to study chromosome condensation in budding yeast[86]. A computational model was built to simulate the behavior of a large DNA piece (300 kb). The chromatin was modeled as a bead-spring polymer, in which beads (nucleosome) are connected by springs (the DNA linkers between nucleosomes). Such a defined nucleosome chain was subjected to basic physics laws (Hooke's law, Brownian movements, and others) without any additional *a priori* constraints. Simulations, further validated by *in vivo* measurements of loci proximity, indicate that yeast interphase chromatin behaves as an unconstrained nucleosome polymer. Addition of condensins (as stochastic intra-chromosomal linkers) promotes compaction of this array. Importantly, by modeling different modes for condensin binding, either connecting only two chromosomal regions or allowing interactions of two or three condensin-binding sites, the authors found that the binding of two (and no more) chromosomal regions reproduces the interaction maps found experimentally in mitotic cells. Moreover, these dynamic pair-wise interactions, in contrast to the attachment of more than two binding sites, were capable of promoting individualization of two separate DNA molecules by favoring intra-chromosomal interactions. Thus, this study further supports the notion that chromosomes may be assembled through a chromatin self-organization process, constrained by condensin interactions, rather than organized by higher order assemblies of condensin complexes within chromosomes.

## Conclusions and future perspectives

Mitotic chromosome condensation remains one of the greatest mysteries in cell biology. Recent advances in the field start to shed light onto this problem, although it is fair to assume that we are still far from understanding the rules that govern mitotic chromosome assembly. Nevertheless, recent advances to dissect metaphase chromosome compaction fail to provide solid evidence for classical models of hierarchical folding or rigid protein scaffolds at the core of chromosome assembly. A multidisciplinary perspective of the problem, combining advanced imaging with *in vivo* and *in vitro* controlled manipulations, along with biophysical studies and modeling may in the future provide an integrative view to understand how chromosomes fold at the onset of every cell division process.


## Competing interests

The authors declare that they have no competing interests.

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## .9 Publication 2: Piskadlo, Ewa, Alexandra Tavares, and Raquel A Oliveira. *Metaphase Chromosome Structure Is Dynamically Maintained by Condensin I-Directed DNA (De)catenation.* eLife 6 (2017): e26120.

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RESEARCH ARTICLE

# Metaphase chromosome structure is dynamically maintained by condensin I-directed DNA (de)catenation

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**Abstract** Mitotic chromosome assembly remains a big mystery in biology. Condensin complexes are pivotal for chromosome architecture yet how they shape mitotic chromatin remains unknown. Using acute inactivation approaches and live-cell imaging in Drosophila embryos, we dissect the role of condensin I in the maintenance of mitotic chromosome structure with unprecedented temporal resolution. Removal of condensin I from pre-established chromosomes results in rapid disassembly of centromeric regions while most chromatin mass undergoes hyper-compaction. This is accompanied by drastic changes in the degree of sister chromatid intertwines. While wild-type metaphase chromosomes display residual levels of catenations, upon timely removal of condensin I, chromosomes present high levels of de novo Topoisomerase II (TopoII)-dependent re-entanglements, and complete failure in chromosome segregation. TopoII is thus capable of re-intertwining previously separated DNA molecules and condensin I continuously required to counteract this erroneous activity. We propose that maintenance of chromosome resolution is a highly dynamic bidirectional process.
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## Introduction

Mitotic chromosome assembly, although poorly understood at the molecular level (Piskadlo and Oliveira, 2016), fulfils three major tasks essential for faithful chromosome segregation: First, it ensures chromosome compaction making cell division feasible within the cell space. Secondly, it provides chromosomes with the right mechanical properties (e.g. bendiness and rigidity) to facilitate their drastic movements during mitosis. Lastly, it ensures the resolution of the topological constrains that exist between the two sister DNA molecules, as well as between neighbouring chromosomes (chromosome individualization), a key requisite for efficient chromosome partitioning. At the heart of these structural changes are the condensin complexes. Condensin complexes, one of the most abundant non-histone complexes on mitotic chromosomes (Cuylen and Haering, 2011; Hirano et al., 1997; Ono et al., 2003), are composed of two structural maintenance of chromosome (SMC) proteins (SMC2 and SMC4) bridged by a kleisin subunit (Barren/Cap-H for condensin I and Barren2/Cap-H2 for condensin II)(Cuylen and Haering, 2011; Hirano et al., 1997; Ono et al., 2003). Despite extensive research over the last years, how condensins contribute to chromosome morphology is not completely understood. Biochemical and phenotypic analysis of condensin depletion suggest several possible activities for these complexes, including the resolution of DNA entanglements (Gerlich et al., 2006; Hagstrom et al., 2002; Hirano, 2006; Hudson et al., 2003; Oliveira et al., 2005; Ribeiro et al., 2009; Steffensen et al., 2001) and structural integrity by conferring chromosome rigidity (Gerlich et al., 2006; Houlard et al., 2015; Oliveira et al., 2005; Ribeiro et al., 2009). Whether or not these complexes also promote chromatin compaction remains controversial (Hagstrom et al., 2002; Hirano, 2006; Hirano et al., 1997; Hudson et al., 2003; Kimura and Hirano, 1997; Lavoie et al., 2002; Oliveira et al., 2005; Steffensen et al., 2001). The multiple

**eLife digest** Living cells can contain huge amounts of genetic information encoded in long strands of DNA. In total several metres of DNA are packed into a small space inside each human cell and these strands can easily become entangled and knotted. When a cell divides to produce new cells the DNA is duplicated and the two copies must be reliably separated, meaning all the knots must be undone. If the DNA strands are not properly separated it can cause extensive damage to genes when the cell tries to divide.

Enzymes called topoisomerases work to undo the tangles in DNA allowing it to be divided into two cells. A large protein complex called "condensin I" plays also an important part in organising DNA, and it has also been implicated in helping to resolve knots in the DNA. However, it was not known how condensin I contributes to the successful separation of DNA into new cells, or when in the course of a cell dividing the knots finally get untangled.

Cell division is similar in humans and the fruit fly Drosophila melanogaster, and so the fly is often used as a simple way to study this process in the laboratory. Now, Piskadlo et al. have examined the role of condensin I in dividing fruit fly cells by using recently developed techniques that rapidly shut down key molecular machineries while cells divide. The results show that condensin I and an enzyme called Topoisomerase II work together to separate entangled DNA. Topisomerase II can both entangle and disentangle DNA strands and it is condensin I that guides this process to ensure that ultimately all the knots are removed.

These findings show that successful cell division requires constant attention from condensin I to make sure Topoisomerase II aids cell division, rather than making the DNA more tangled. Overall this requires more active and constant work to disentangle DNA than expected, and further work is now needed to explain why. Understanding how cells avoid DNA damage during division clarifies why errors in this process cause diseases. For example, changes to condensin I are common in certain cancers and can also lead to disrupted brain development (e.g. microcephaly).

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phenotypes observed on mitotic chromosomes upon depletion of condensin complexes raise the possibility that these complexes may have distinct roles at different times of mitosis. Additionally, several lines of evidence support that these complexes also influence interphase chromosome structure (Cobbe et al., 2006; Hartl et al., 2008). Thus, it is difficult, if not impossible to interpret the results when condensins are depleted prior to mitotic entry using conventional depletion approaches. To circumvent this limitation, we adopt a 'reverse and acute' approach to dissect the role of condensin I in the maintenance of chromosome organization. We find that inactivation of one condensin I specifically during metaphase leads to over-compaction at the majority of chromosomal regions. We further demonstrate that upon condensin I cleavage previously separated sister DNA molecules undergo topoisomerase II-dependent re-intertwining and complete failure in chromosome segregation.

## Results

### A TEV-protease mediated system to inactivate condensin I in Drosophila melanogaster

To study the role of condensin complexes in the maintenance of chromosome structure, specifically during metaphase, we developed a system to enable analysis of chromosomal structural changes upon rapid and temporally controlled inactivation of condensin in Drosophila melanogaster. Our analysis focused on condensin I complex as prior studies reveal a minor role for condensin II in mitotic chromosome organization in Drosophila (Hartl et al., 2008; Herzog et al., 2013; Savvidou et al., 2005). We developed a fast inactivation system to disrupt condensin I in the living fly (Figure 1 and Figure 1—figure supplement 1), following a similar strategy previously used for the structurally related complex cohesin (Oliveira et al., 2010; Pauli et al., 2008; Uhlmann et al., 2000). This system is based on the use of an exogenous protease (Tobacco Etch Virus, TEV) to cleave an engineered protein of interest that contains TEV-cleavage sites and allows specific, rapid

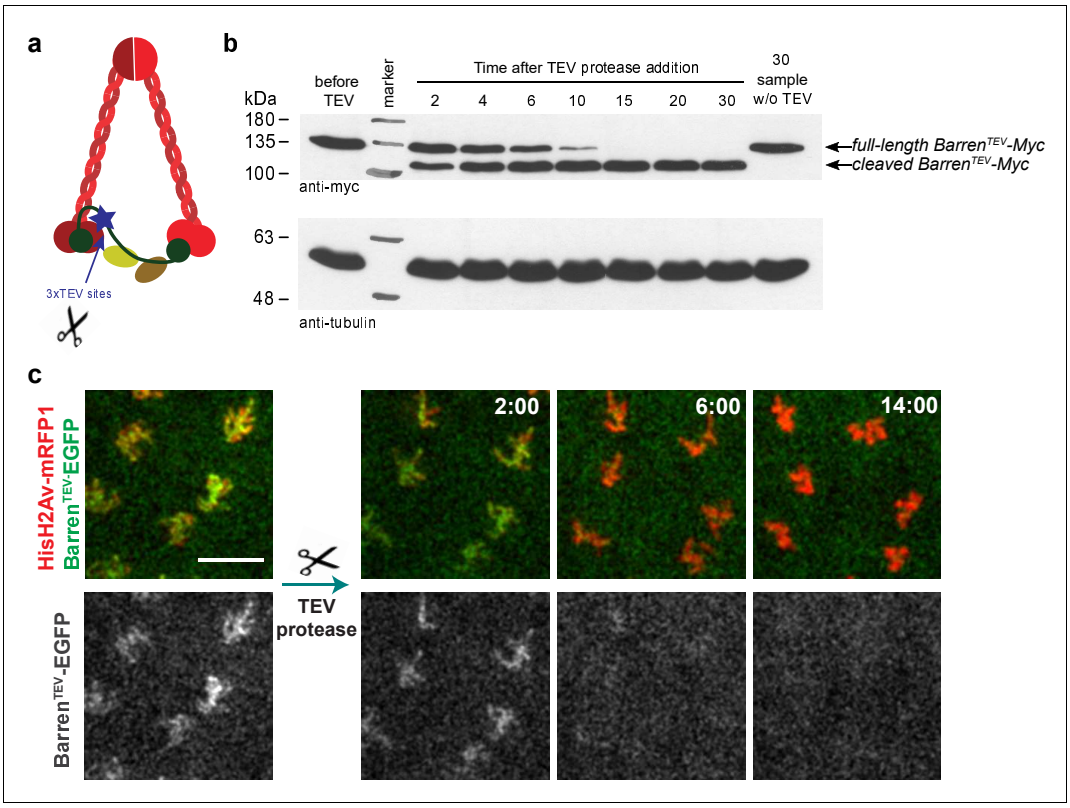


Figure 1. TEV-mediated cleavage of Barren disrupts condensin I function within a few minutes. (a) Schematic representation of condensin complex indicating the position of the 3xTEV cleavage sites in the kleisin subunit Barren (aa175). (b) In vitro cleavage of Barren$^{TEV}$-myc. Extracts were prepared from ovaries of flies expressing solely TEV-cleavable Barren and incubated with TEV protease for the indicated time points (periods of time). The presence of full-length and cleaved Barren was monitored by western blot using myc antibodies. Tubulin was used as loading control. (c) Early embryos (0–30 min old) expressing HisH2AvD-mRFP1 (red) were injected with mRNA coding for Barren$^{TEV}$-EGFP (green). Embryos were aged for 1 hr-1hr 30m to allow for protein expression. Embryos were injected with 12 mg/ml UbcH10$^{C114S}$ protein to arrest in metaphase and subsequently with TEV-protease; images depict the same region before and after TEV injection; times (minutes:seconds) are relative to the time of injection; scale bar is 10 mm.

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The following figure supplement is available for figure 1:

Figure supplement 1. A TEV-cleavable system to destroy condensin I.

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and efficient protein inactivation in a tissue- and/or time-dependent manner (Figure 1, Figure 1—figure supplement 1 and data not shown). To produce flies carrying solely TEV-sensitive condensin I complexes, we produced four versions of the kleisin subunit Barren that contain three consecutive TEV-cleavage sites at four different positions: aa175, aa389, aa437, aa600 (Figure 1—figure supplement 1). All versions are fully functional as they were able to complement the lethality associated with the Barren null allele Barr$^{L305}$ (Bhat et al., 1996) (Figure 1—figure supplement 1b and data not shown). In vitro cleavage experiments reveal that all modified proteins are efficiently cleaved by TEV protease (Figure 1B and Figure 1—figure supplement 1). The construct Barren$^{3xTEV175}$-myc

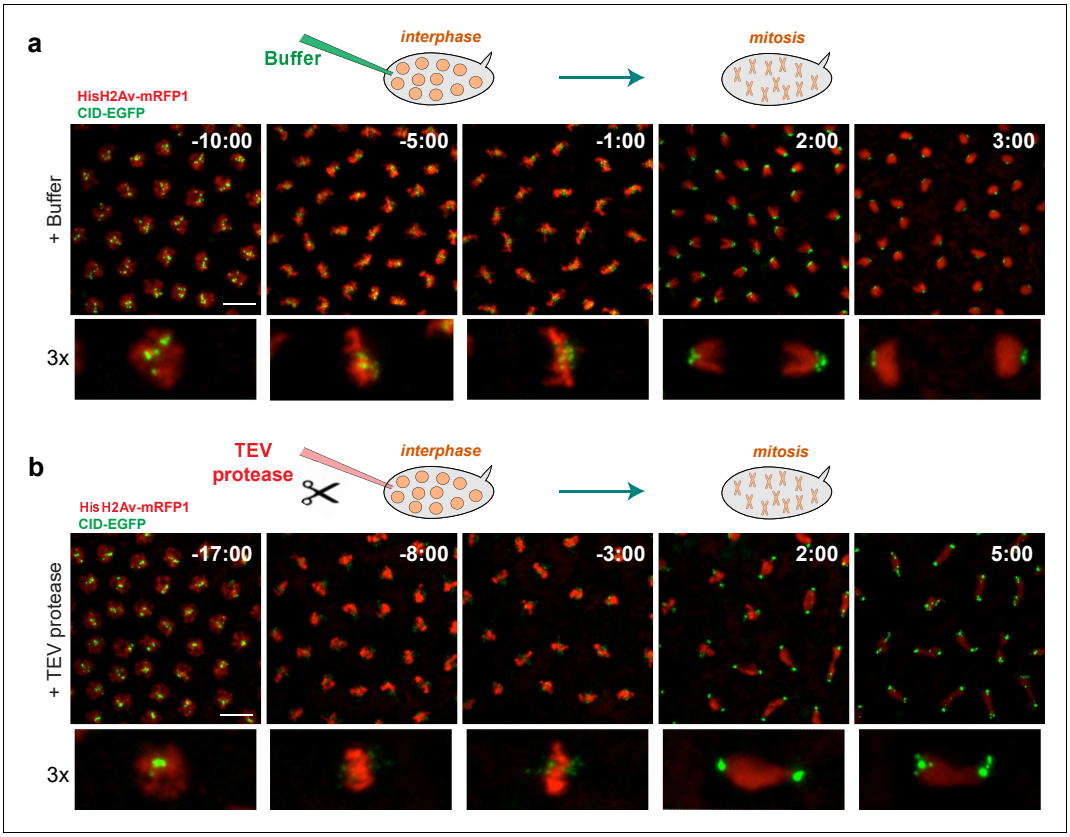


Figure 2. Condensin I inactivation prior to mitotic entry. Embryos surviving solely on Barren$^{TEV}$ were injected with buffer (a) or 13 mg/ml TEV protease (b) ~10–15 min before mitosis; Embryos also express His2A–mRFP1 (red) and Cid-EGFP (green); scale bars, 10 mm. Bottom rows show higher magnifications (~3x) of a single nuclear division. Times (minutes:seconds) are relative to the time of anaphase onset.
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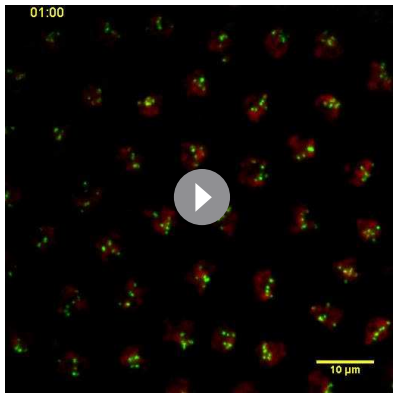
was chosen for future analysis based on the healthiness of the rescued strains (referred as Barren$^{TEV}$ hereafter).

TEV protease-mediated inactivation of condensin complexes has been previously successfully applied in yeast and mouse oocytes (Cuylen et al., 2011; Houlard et al., 2015). However, in both cases the inactivation of condensin complexes took place within over an hour after TEV protease induction. Direct injection of TEV-protease into syncytial embryos, in contrast, allowed cleavage and the removal of chromosome-associated Barren$^{TEV}$ within 8–15 min (Figure 1b,c), enabling the analysis of the immediate consequences upon disruption of this complex. To confirm that TEV-protease was able to inactivate condensin I efficiently within a few minutes, by cleavage of Barren$^{TEV}$, we injected TEV protease in embryos derived from females surviving solely on Barren$^{TEV}$ (ectopic expression of Barren$^{TEV}$ in a Barr$^{L305}$ null background). Injection of TEV-protease in early interphase embryos leads to complete failure of the subsequent mitosis (which takes place within ~15 min in these embryos). Although chromosomes were able to condense upon nuclear envelope breakdown, centromeres, monitored by the Cenp-A ortholog Cid-EGFP, display significant stretching upon




Video 1. Mitosis in Drosophila embryos. Embryos were injected with buffer in early interphase and monitored throughout the subsequent mitosis. Embryos express HisH2Av-mRFP1 (red) and Cid-EGFP (green). Times are relative to injection time. Scale bar is 10 um.
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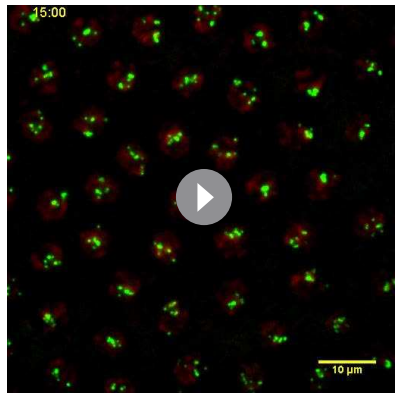


Video 2. Mitosis upon condensin I inactivation in Drosophila embryos. Embryos surviving solely on Barren$^{TEV}$ were injected with TEV protease in early interphase and monitored in the subsequent mitosis. Embryos express HisH2Av-mRFP1 (red) and Cid-EGFP (green). Times are relative to injection time. Scale bar is 10 um.
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microtubule attachment (Figure 2, Video 1 and Video 2). Moreover, resolution of sister chromatids is completely impaired, as chromatids appeared as a fused chromatin mass or display very thick bridges during the attempted anaphase (Figure 2, Video 1 and Video 2). These results are in accordance with previous findings for condensin I depletion (Gerlich et al., 2006; Hagstrom et al., 2002; Hirano, 2006; Hudson et al., 2003; Oliveira et al., 2005; Ribeiro et al., 2009; Steffensen et al., 2001), which ensures the developed system is efficient at promoting rapid condensin I inactivation.

## Condensin I inactivation in metaphase leads to increased chromosome compaction

To test the role of condensin I in the maintenance of chromosome architecture, we allowed mitotic chromosomes to assemble without any perturbation on their structure and subsequently disrupted condensin I during the metaphase-arrest. Embryos were arrested in metaphase, with a functional mitotic spindle, by the use of a catalytically dead dominant-negative form of the E2 ubiquitin ligase necessary for anaphase onset (UbcH10$^{C114S}$)(Oliveira et al., 2010; Rape et al., 2006). Arrested embryos were subsequently injected with TEV protease to destroy condensin I. Given the known role of condensin I in the rigidity of pericentromeric region of chromosomes (Gerlich et al., 2006; Oliveira et al., 2005; Ribeiro et al., 2009), we first tested the effect of TEV protease injection at those chromosomal sites. Whereas injection of buffer causes no change in the distance between centromeres, TEV protease injection in strains containing solely TEV-cleavable Barren results in rapid separation of centromeres, that appear to stretch towards the poles, leaving behind the majority of the chromatin mass (Figure 3, Video 3 and Video 4). These findings imply that condensin I is not only required for the establishment of a rigid structure at the pericentromeric domains prior to metaphase, but also for the maintenance of such organization.

Surprisingly, non-centromeric regions do not follow similar disorganization and in fact appeared to become more compacted. We defined chromosome compaction by degree of chromatin density, inferred from the signal of fluorescently labelled histone H2Av-mRFP1. To quantify the changes in chromosome compaction upon condensin inactivation, we used quantitative imaging analysis to monitor the mean voxel intensity, volume and surface area of each metaphase plate, over time, in

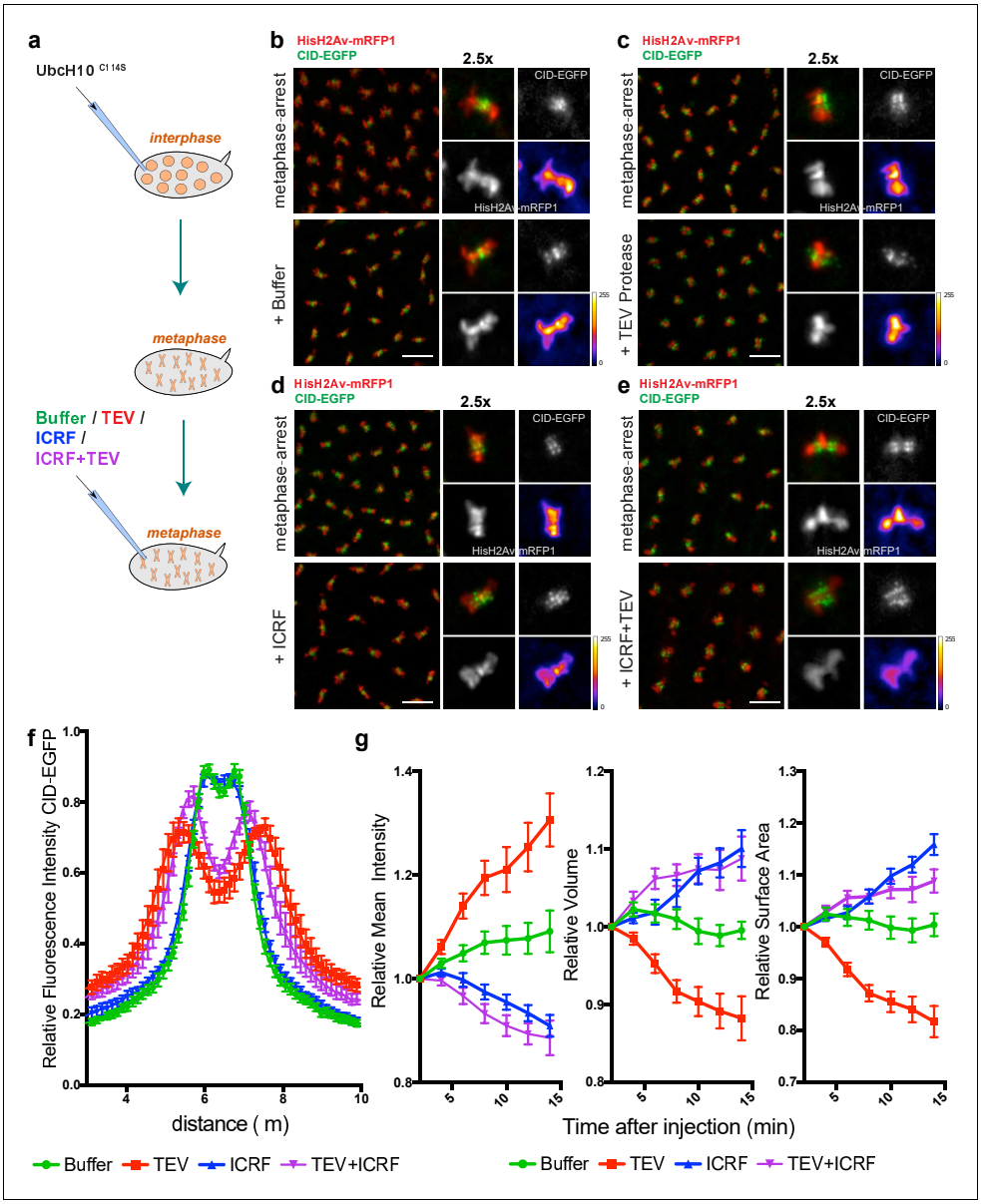


Figure 3. Condensin I inactivation in pre-assembled chromosomes leads to disruption of centromere structure and hyper-compaction of mitotic chromosomes. (a) Schematic representation of the experimental layout. Embryos expressing solely Barren$^{TEV}$ were injected with 12 mg/ml of a dominant-negative form of the human E2 ubiquitin-conjugating enzyme (UbcH10$^{C114S}$) to induce a metaphase arrest. Embryos were subsequently injected with buffer (b), 13 mg/ml TEV protease (c), 280 nM ICRF (d) or a mixture containing 13 mg/ml TEV protease and 280 nM ICRF (e); Images

Figure 3 continued on next page

Figure 3 continued

depict embryos before the second injection and 14 min after. Embryos also express His2A–mRFP1 (red) and Cid-EGFP (green); scale bars, 10 mm. Insets show higher magnifications (2.5x) of a single metaphase. Times (minutes:seconds) are relative to the time of the second injection. (f) Quantitative analysis of centromere positioning 10 min after the second injection, as above; graph shows average ± SEM of individual embryos (n ! 7 embryos for each experimental condition); for each embryo, a minimum of 8 metaphases was measured; (g) quantifications of mean voxel intensity, volume and surface area of the entire metaphase plate quantified in 3D, over time, and normalized to the time of the second injection. Graphs represent the average ± SEM of individual embryos (n ! 10 embryos for each experimental condition); for each embryo, a minimum of 8 metaphases was quantified.
DOI: 10.7554/eLife.26120.008

The following source data and figure supplement are available for figure 3:

Source data 1. Centromere displacement and chromosome compaction measurements upon condensin I and topoII inactivation.
DOI: 10.7554/eLife.26120.009

Figure supplement 1. - Chromosome condensation induced by TEV-protease depends on TEV cleavage sites present in Barren$^{TEV}$.
DOI: 10.7554/eLife.26120.010

3D (Figure 3d). We found that injection of TEV protease in strains surviving only on Barren$^{TEV}$ leads to an overall over-compaction of the entire chromatin mass, as evidenced by an increase in the mean voxel intensity and a decrease in both the surface area and the volume of the metaphase plate (Figure 3c,d). Injection of the protease in strains that do not contain TEV-cleavage sites does not result in any evident change in chromosome compaction relative to controls (Figure 3—figure supplement 1), implying that chromosome over-compaction is specific of condensin I inactivation.

In contrast, inactivation of Topoisomerase II (TopoII) using a small molecule inhibitor (ICRF-193), leads to rapid de-compaction of chromosomes (Figure 3d,g and Video 5). TopoII has been previously implicated in chromosome compaction although its role in the process remains controversial (Piskadlo and Oliveira, 2016). Although we cannot exclude that chromosome decompaction may be exacerbated by the fact that ICRF-193 traps TopoII onto chromatin, our results support that TopoII may contribute to chromosome compaction in metaphase, consistent with previous observations (Samejima et al., 2012), possibly by promoting self-entanglements within the same chromatid (Kawamura et al., 2010). Importantly, combined inactivation of both TopoII and condensin I results in chromosome decompaction similar to TopoII inhibition alone (Figure 3e,g and Video 6). This finding implies that chromatin hyper-compaction observed upon loss of condensin I depends on TopoII activity.

## Condensin I inactivation results in de novo sister chromatid intertwines

The unexpected finding that condensin I inactivation promotes further chromosome compaction, together with the observation that TopoII inhibition reverts this hyper-compaction phenotype, lead us to hypothesize that the observed increase in compaction stems from re-entanglements of DNA strands, which would consequently lead to an increase in chromatin density. Enzymatically, TopoII can promote both the decatenation and the concatenation of DNA strands. Efficient chromosome segregation requires that TopoII is strongly biased towards decatenation prior to anaphase onset but it is conceivable that TopoII can additionally drive the concatenation of native metaphase chromosomes, in vivo. To test


Video 3. Buffer injection in metaphase-arrested embryos. Embryos expressing solely Barren$^{TEV}$ were injected with 12 mg/ml of a dominant-negative form of the human E2 ubiquitin-conjugating enzyme (UbcH10$^{C114S}$), to induce a metaphase arrest, and subsequently injected with buffer. Embryos also express His2A–mRFP1 (red) and Cid-EGFP (green); scale bars, 10 mm. Times (minutes:seconds) are relative to the time of buffer injection.
DOI: 10.7554/eLife.26120.011


Video 4. Condensin I inactivation in metaphase-arrested embryos. Embryos expressing solely Barren$^{TEV}$ were injected with 12 mg/ml of a dominant-negative form of the human E2 ubiquitin-conjugating enzyme (UbcH10$^{C114S}$), to induce a metaphase arrest, and subsequently injected with 13 mg/ml TEV protease. Embryos also express His2A–mRFP1 (red) and Cid-EGFP (green); scale bars, 10 mm. Times (minutes: seconds) are relative to the time of TEV injection.
DOI: 10.7554/eLife.26120.012


Video 5. Topoisomerase II inhibition in metaphase-arrested embryos. Embryos expressing solely Barren$^{TEV}$ were injected with 12 mg/ml of a dominant-negative form of the human E2 ubiquitin-conjugating enzyme (UbcH10$^{C114S}$), to induce a metaphase arrest, and subsequently injected with 280 mM ICRF-193. Embryos also express His2A–mRFP1 (red) and Cid-EGFP (green); scale bars, 10 mm. Times (minutes:seconds) are relative to the time of ICRF injection.
DOI: 10.7554/eLife.26120.013

whether condensin I removal leads to re-catenation of previously separated sisters, we tested several predictions that arise from this hypothesis: First, the hyper-compaction observed in metaphase, if derived from sister-chromatid re-intertwining, should be dependent on the proximity between DNA molecules. The physical separation of sister chromatids will increase the distance between these two molecules, placing them too far apart, and consequently decreasing the likelihood of their re-entanglement, as recently proposed (Sen et al., 2016). Secondly, re-intertwining in late metaphase should lead to severe segregation failures. And lastly, DNA entanglements and the consequent segregation defects should depend on TopoII activity.

To evaluate the effect of sister chromatid proximity in chromosome condensation upon condensin inactivation we combined TEV-mediated cleavage of condensin I and cohesin by the use of strains carrying TEV-sensitive Rad21 (cohesin) and Barren (condensin) proteins. We took advantage of the fact that Rad21$^{TEV}$ cleavage is more efficient than Barren$^{TEV}$ (Figure 1—figure supplement 1), which allowed the analysis of changes in chromosome architecture upon condensin inactivation after artificial separation of sister chromatids. Upon TEV protease injection, pole-ward chromosome segregation is initiated within 2 to 5 min and with similar kinetics in both strains (Figure 4a).

After the initial pole-ward chromatid movement, isolated chromatids shuffle between the poles, consistent with previous observations (Oliveira et al., 2010). To quantify the degree of movement, we used a displacement-quantification method that infers chromosome movement by the overlap between chromosome positions on consecutive frames (Mirkovic et al., 2015). Cohesin cleavage alone leads to strong shuffling of isolated single chromatids, as previously described. However, concomitant inactivation of condensin and cohesin results in much slower chromatid movements, with chromatids accumulating in the middle of the segregation plane (Figure 4b,c). Condensin I is thus important for efficient movement of isolated chromatids. This may be due abnormal centromere/kinetochore structure and/or to a possible role for condensin in the error-correction process, as recently proposed (Peplowska et al., 2014; Verzijlbergen et al., 2014).


Video 6. Concomitant inactivation of Topoisomerase II and Condenin I in metaphase-arrested embryos. Embryos expressing solely Barren$^{TEV}$ were injected with 12 mg/ml of a dominant-negative form of the human E2 ubiquitin-conjugating enzyme (UbcH10$^{C114S}$), to induce a metaphase arrest, and subsequently injected with a mix of 280 mM ICRF-193 and 13 mg/ml TEV protease. Embryos also express His2A–mRFP1 (red) and Cid-EGFP (green); scale bars, 10 mm. Times (minutes:seconds) are relative to the time of the second injection.
DOI: 10.7554/eLife.26120.014

The reduced chromosome movement observed upon condensin I inactivation leads to considerable differences in chromatid positioning in both experimental set-ups. Thus, we restricted our chromosome morphology/compaction analysis to measurements of isolated single sisters, as quantifying the entire chromatin mass would include many confounding variables. Measurements of isolated single chromatids were performed at 20 min after injections and normalized to the values at 5 min after protease injection (once complete separation has occurred but no significant changes in chromosome structure was yet observed). Chromatids considerably change their shape, becoming thicker and shorter (Figure 5b,c, Video 7 and Video 8), as previously described (Green et al., 2012; Ono et al., 2003). To directly measure the degree of compaction of these isolated sisters, we measured their mean voxel intensity. This analysis revealed that despite the significant changes in chromatid organization, there is no overall change in the mean voxel intensity of single chromatids (Figure 5d), indicating that shape changes are not accompanied by an overall increase in chromatin compaction. We therefore conclude that condensin I inactivation does not promote further chromosome compaction if sister chromatids are physically apart, in contrast to the effect observed in metaphase-arrested chromosomes (Figure 3). These results support that over-compaction observed in metaphase chromosomes may arise from sister chromatid re-intertwining, consistent with previous observations using yeast circular mini-chromosomes (Sen et al., 2016). It is conceivable that condensin I inactivation also promotes abnormal re-entanglement in cis (between distal regions of the same chromatid). The shape changes observed upon condensin inactivation from isolated sisters (shorter and thicker chromatids) could indeed be explained by an excess of concatenation within the same DNA molecule, leading to the shortening of the longitudinal axis. However, our compaction measurements indicate that such changes, if occur, do not lead to detectable increase in chromatin density.

Next, we sought to evaluate chromosome segregation defects, which serve as an indirect readout for the amount of DNA catenations bridging DNA molecules. To monitor segregation defects when condensin I or TopoII are inactivated specifically in metaphase, we developed conditions in which unperturbed chromosomes would be transiently arrested in metaphase by the dominant negative UbcH10$^{C114S}$, for ~3–5 min, and subsequently injected with the respective perturbing factor in metaphase. Embryos were subsequently injected with a wild-type version of UbcH10 14 min later, which causes anaphase onset and mitotic exit in about 4–8 min (Figure 6a). We monitored the segregation efficiency during anaphase by quantitative analysis of the profile of Histone H2AvD-mRFP (to visualize chromatin separation) and Cid-EGFP (to infer centromere segregation) along the segregation plane (Figure 6). In this assay, injection of buffer causes virtually no defects in the segregation of sister chromatids (Figure 6b, Figure 6—figure supplement 1 and Video 9).

Inactivation of TopoII under these conditions leads mostly to the appearance of fine chromatid bridges (Figure 6c and Video 10). These residual bridges are insufficient to delay centromere separation (11,01 ± 2,03 mm upon ICRF-193 treatment compared to 10,72 ± 1,69 mm in buffer-injected embryos; Figure 6f). The extent of chromatin bridges observed upon metaphase-specific inactivation of TopoII is considerably lower when compared to experiments where this enzyme is inhibited

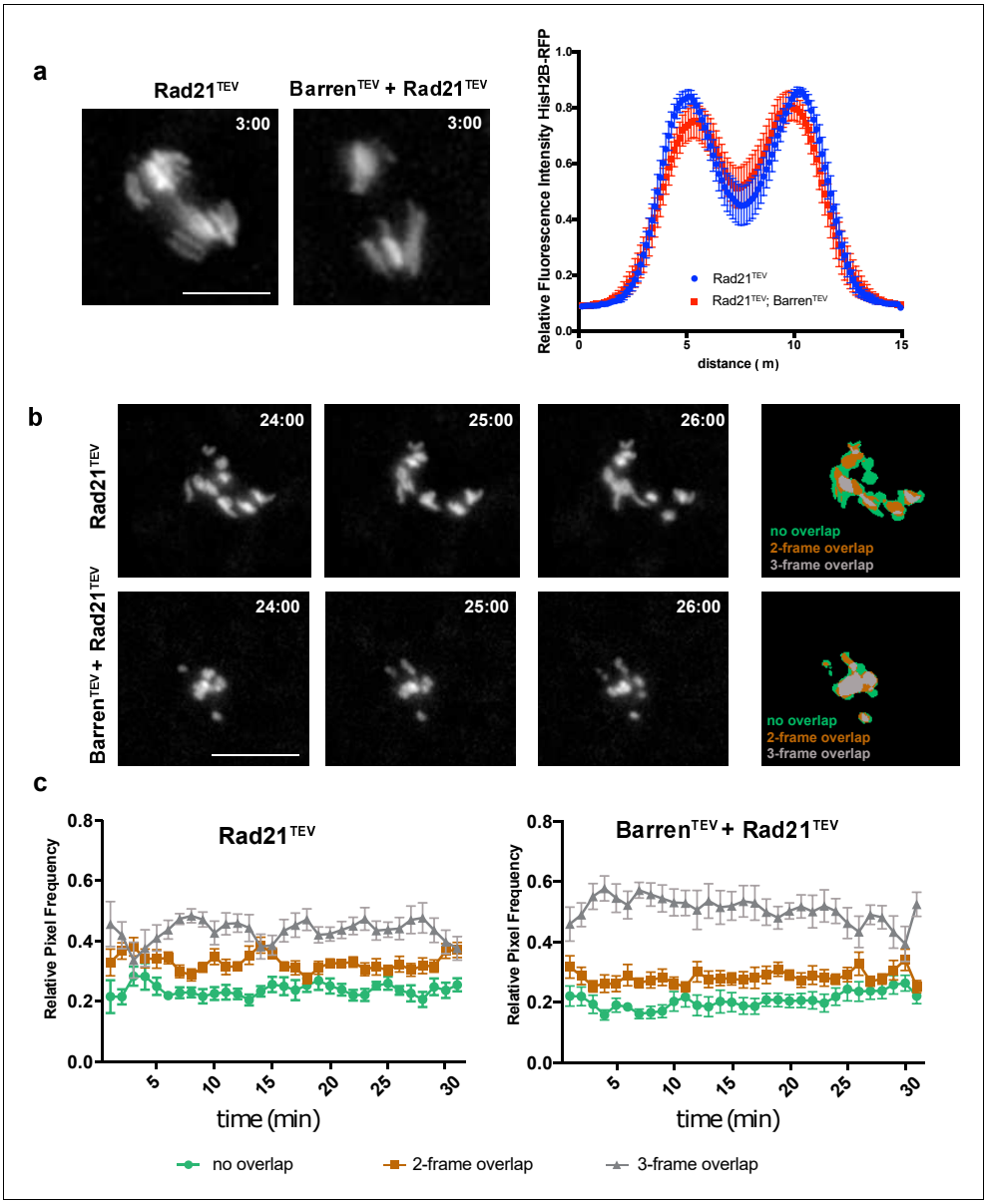


Figure 4. Condensin I inactivation in separated sister chromatids reduces their movement. (a) Representative images of the initial separation after TEV-mediated cleavage of Rad21$^{TEV}$ and Rad21$^{TEV}$ + Barren$^{TEV}$. Graph plots the relative distribution of HisH2B-RFP at the maximal state of sister chromatid separation triggered by TEV-mediated cleavage of Rad21$^{TEV}$, in strains that contain solely Rad21$^{TEV}$ or both Rad21TEV and Barren$^{TEV}$. A 15 mm line was used to measure plot profiles along the segregation plane, measured 3–5 min after TEV protease injection. Graphs plot the average ± SEM of

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Figure 4 continued

individual embryos (n ! 7 embryos for each experimental condition). For each embryo, between 8 and 12 anaphases were analysed. (b) Example of chromosome movement analysis; left panel represents average of the binary images of three consecutive frames, used to estimate chromosome displacements: blue, non-overlapping pixels; green, two- out of three-frame overlap; grey, three-frame overlap. Scale bar is 10 mm. (c) Frequency of overlapping pixels to estimate chromosome displacement (as in b), over time, after TEV protease injection.

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The following source data is available for figure 4:

Source data 1. Measurements of segregation efficiency and chromosome movement upon cohesin/condensin inactivation.

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prior to mitotic entry (Figure 6—figure supplement 1). These findings imply that in metaphase-arrested chromosomes the amount of unresolved catenations is residual. In contrast, inactivation of condensin I during metaphase leads to complete impairment of the segregation process, as revealed by the high frequency of 'fused' chromatin masses, with the chromosomes remaining in the centre of the segregation plane, and a significant decrease in the distance between segregating centromeres (6,08 ± 0,92 mm) (Figure 6d,f and Video 11). The degree of segregation defects observed in these metaphase-inactivation experiments, is even higher than the defects observed upon condensin inactivation prior to mitotic entry (Figure 6—figure supplement 1). The severity of segregation impairment upon metaphase-specific condensin I inactivation indicates that in the absence of this complex previously resolved sister DNA molecules undergo re-catenation.

To directly test this hypothesis, we accessed whether or not de novo chromatin intertwines take place upon condensin inactivation, as the formation of these new links should depend on TopoII activity. If TopoII-dependent re-catenation occurs upon condensin I inactivation, one would expect that the combination of TopoII and condensin I inactivation should reduce the amount of chromatin trapped in the middle of the segregation plane. On the contrary, if segregation defects result from pre-existing catenations, combined inhibition of condensin I and TopoII should increase, or at least maintain, the density of chromosome bridges and segregation impairment.

To address this issue, we used the same experimental layout as above but induced concomitant inactivation of condensin I and TopoII during the metaphase arrest. These experiments reveal a partial rescue of the retained chromatin mass, inferred by HisH2Av-mRFP1 profile (Figure 6e and Video 12). Chromosome positioning may not be linearly correlated with the amount of linkages bridging the two sister chromatids and thus the reduction on chromosome intertwines may be even more pronounced than inferred by histone profiles. In accordance with this notion, the efficiency of chromosome separation inferred by the position of centromeres returns to levels indistinguishable from wild-type (Figure 6e,f and Video 12). Thus, concomitant inactivation of condensin I and TopoII significantly reverts the defects associated with condensin I removal. We therefore conclude that the segregation defects observed upon metaphase-specific condensin I inactivation are caused by de novo TopoII-dependent re-intertwining of previously separated sister chromatids.

## Discussion

The role of condensin complexes in chromosome compaction has been extensively debated. Here we provide evidence that temporally controlled inactivation of condensin I, specifically during metaphase, causes an increase in the overall levels of chromosome compaction in non-centromeric regions. These findings strongly argue that condensin I is required to maintain chromosomal architecture but not to sustain their compacted state. Studies using similar inactivation techniques in mouse oocytes have proposed that condensins confirm longitudinal rigidity, as chromosomes disassemble upon condensin inactivation (particularly condensin II)(Houlard et al., 2015). At first sight, these findings may be perceived as in sharp contrast to our current observations. It should nevertheless be noted that meiotic chromosomes are under very different force-balance than their mitotic counterparts. In particular, spindle attachment on meiotic bivalents imposes stretching along the longitudinal axis of chromosomes, similarly to what we report here for the pericentromeric region in mitotic chromosomes. Our results now demonstrate that when chromosomes are not subjected to significant additional forces, condensin I inactivation results in an overall chromatin over-compaction rather than chromosome de-condensation. This force-dependent phenotype may explain several

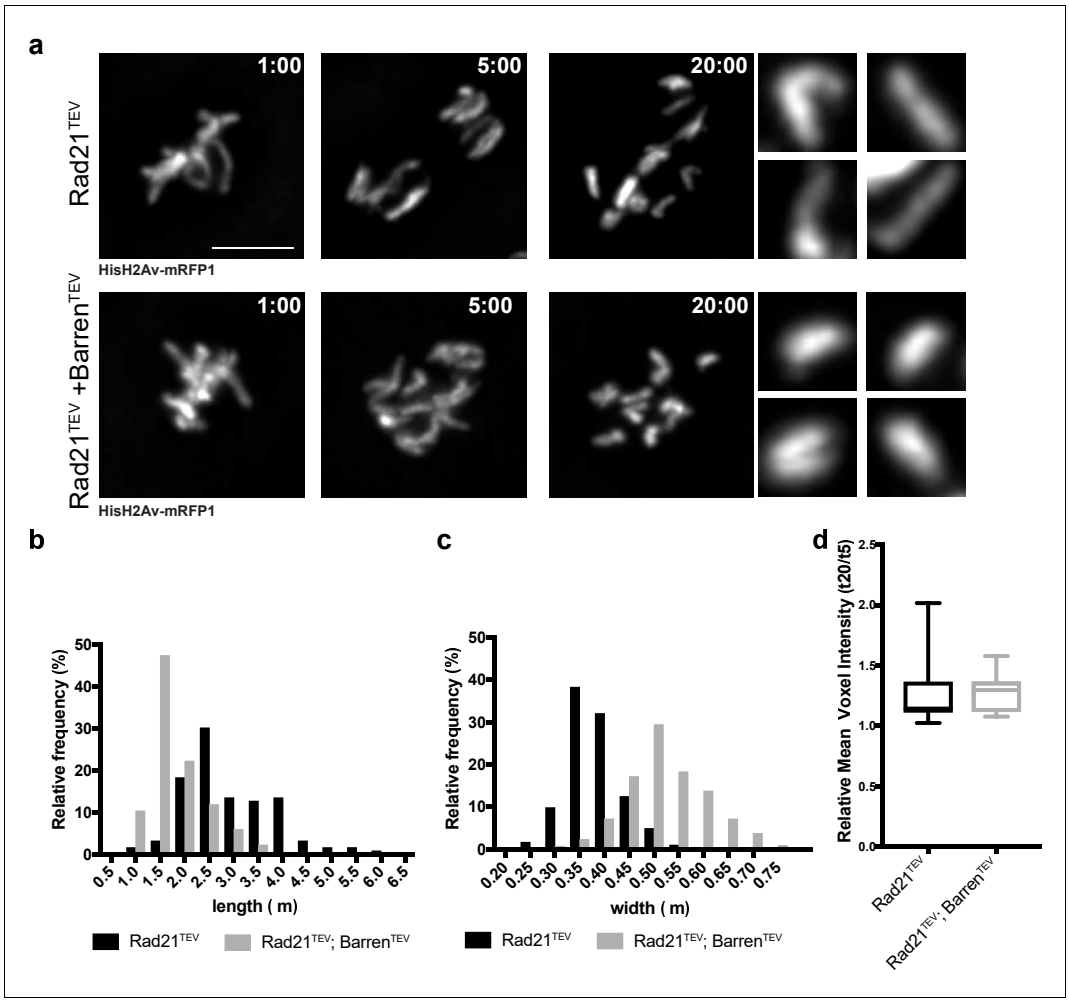


Figure 5. Chromosome over-compaction depends on sister-chromatid proximity. (a) Stills from metaphase-arrested embryos after injection of TEV protease in strains surviving solely on Rad21$^{TEV}$ (cohesin cleavage) or Rad21$^{TEV}$+Barren$^{TEV}$ (cohesin and condensin cleavage); embryos also express HisH2B-RFP; scale bars, 5 mm. Insets show higher magnifications (3x) of single chromatids 20 min after TEV injection. Times (minutes:seconds) are relative to the time of TEV injection. (b–c) Relative frequency of sister chromatid length (b) and width (c) at 20 min after TEV injections (n ! 120 single chromatids from seven independent embryos for each experimental condition). (d) Mean voxel intensity of isolated single chromatids 20 min after TEV injections, normalized to mean voxel intensity 5 min past injection. (n ! 10 embryos for each experimental condition).

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The following source data is available for figure 5:

Source data 1. Measurements of isolated chromatids upon cohesin/condensin inactivation.

DOI: 10.7554/eLife.26120.018

inconsistencies in prior analysis on condensins depletion that as sample preparation, chromosome state, presence/absence of microtubules, or even the cell division type (mitosis vs meiosis) may play a major role in chromosome morphology. In contrast to condensin I inactivation, TopoII inhibition leads to rapid chromosome decompaction. These finding are consistent with the idea that metaphase chromosome structure is organized as a chromatin network resultant from self-entanglements of DNA strands, as initially proposed by biophysical studies on isolated chromosomes (Kawamura et al., 2010). Restricting/favouring chromosome entanglements may thus dictate the state of chromosome compaction.

Condensin has been previously proposed to interplay with TopoII, both for chromosome compaction and sister chromatid resolution. The exact details for this interaction, however, remain elusive. Both condensins and TopoII inactivation impair sister chromatid resolution (Bhat et al., 1996; Clarke et al., 1993; Gerlich et al., 2006; Hagstrom et al., 2002; Hirano, 2006; Hudson et al., 2003; Oliveira et al., 2005; Ribeiro et al., 2009; Steffensen et al., 2001; Uemura et al., 1987), suggesting these two molecules have cooperative roles on chromosome resolution. In contrast, cytological analyses suggest that condensin and TopoII have opposite roles in shaping mitotic chromatin (Samejima et al., 2012), raising further doubts on their functional interaction. It has long been hypothesized that condensin may impose directionality on TopoII reactions (Baxter et al., 2011; Charbin et al., 2014; Coelho et al., 2003; Leonard et al., 2015), as this enzyme is able to both decatenate and catenate DNA strands. But this model has been very difficult to formally prove. Studies in yeast using artificial circular mini-chromosomes, in which the levels of catenation can be directly measured, support that full decatenation by TopoII requires condensin activity (Baxter and Aragón, 2012; Baxter et al., 2011; Charbin et al., 2014). Whether the same is true in large and linear native chromosomes remained to be addressed, particularly as circular chromosomes are under different topological constrains when compared to linear ones. The experimental approach used in our study allowed the manipulation of native chromosomes, in their natural environment, providing evidence


Video 7. Artificial induction of sister chromatid separation in metaphase-arrested embryos. Embryos expressing solely Rad21$^{TEV}$ and wild-type Barren were injected with 12 mg/ml of a dominant-negative form of the human E2 ubiquitin-conjugating enzyme (UbcH10$^{C114S}$), to induce a metaphase arrest, and subsequently injected with 13 mg/ml TEV protease. Embryos also express His2B–RFP; scale bars, 10 mm. Times (minutes:seconds) are relative to the time of the second injection.
DOI: 10.7554/eLife.26120.019


Video 8. Effect of condensin I inactivation on isolated sister chromatids. Embryos expressing uniquely TEV-sensitive Rad21 and Barren were injected with 12 mg/ml of a dominant-negative form of the human E2 ubiquitin-conjugating enzyme (UbcH10$^{C114S}$), to induce a metaphase arrest, and subsequently injected with 13 mg/ml TEV protease. Embryos also express His2B–RFP; scale bars, 10 mm. Times (minutes:seconds) are relative to the time of the second injection.
DOI: 10.7554/eLife.26120.020

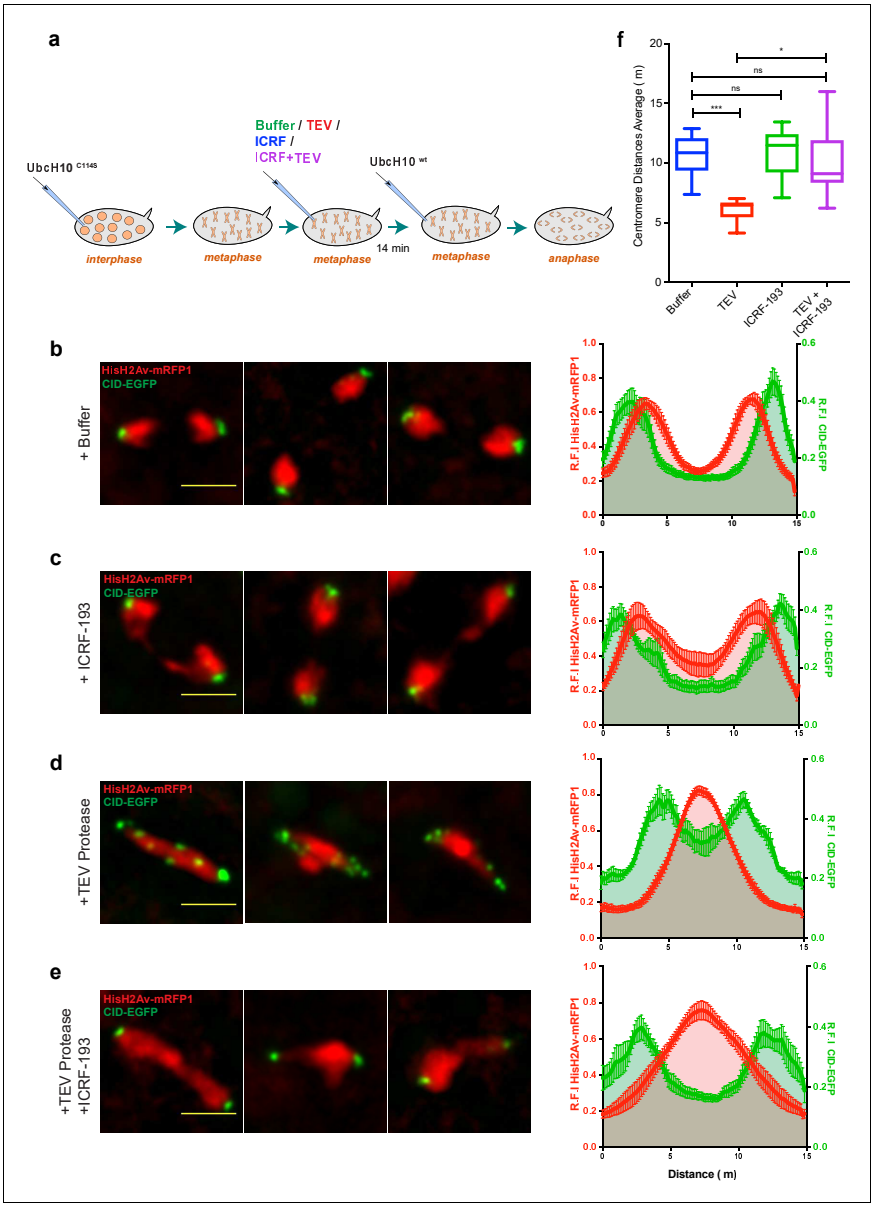


Figure 6. Condensin I inactivation results in TopoII-dependent sister chromatids intertwines and segregation failure. (a) Schematic representation of the experimental set-up. Embryos were arrested with 12 mg/ml UbcH10$^{C114S}$ and injected with buffer (b), 280 mM ICRF-193 (c), 13 mg/ml TEV protease (d) or TEV+ICRF-193, while in metaphase; Embryos were subsequently injected with 14 mg/ml of a wild-type version of UbcH10 to release them from the arrest. Images depict representative images of the anaphase; Graphs plot the relative distribution of HisH2Av-mRFP1 and Cid-EGFP across the 15 mm

Figure 6 continued on next page

Figure 6 continued

segregation plane, measured 4–6 min after anaphase onset. Graphs plot the average +_SEM of individual embryos (n ! 10 embryos for each experimental condition). For each embryo, at least eight anaphases were analysed. (f) Quantification of centromere distances during UbcH10$^{wt}$-induced anaphase as in (b–e). Graphs plot the distances between segregating centromeres measured 6 min after anaphase onset (n ! 10 embryos for each experimental condition; for each embryo, at least eight anaphases were analysed). Statistical analysis was performed using the non-parametric Kruskal-Wallis test; ns p >0.05, *p <0.05; ***p <0.001.

DOI: 10.7554/eLife.26120.021

The following source data and figure supplement are available for figure 6:

Source data 1. Measurements of segregation efficiency after metaphase-specific inactivation of condensin and/or Topoisomerase II.

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Figure supplement 1. – Comparative analysis of segregation efficiency for condensin and/TopoII inhibition before mitosis (light colour) and during metaphase arrest/release (dark colour); Graphs plot the relative distribution of HisH2Av-mRFP1 (red) and Cid-EGFP (green) across a 20 mm segregation plane, measured 4–6 min after anaphase onset.

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that upon removal of condensin I, previously separated sister chromatids re-intertwine in a TopoII-dependent manner. These findings are in agreement with a recent study that revealed that the resolution of sister chromatids from circular minichromosomes can be reverted by increased expression of TopoII (Sen et al., 2016). All together, these results support that condensin I is not directly necessary for TopoII catalytic activity, but rather to impose directionality on TopoII reactions, favouring resolution of the sister DNA molecules rather than their intertwine. Upon condensin I removal, creation of new links between previously separated DNA strands leads to their increased proximity, which may underlie the observed increase in chromosome compaction. Importantly, our studies reveal that


Video 9. Induced anaphase in control embryos. Embryos expressing solely Barren$^{TEV}$ were injected with 12 mg/ml of a dominant-negative form of the human E2 ubiquitin-conjugating enzyme (UbcH10$^{C114S}$), to induce a metaphase arrest, and subsequently injected with buffer. After 14 min embryos were injected a wild-type version of UbcH10 to induce anaphase. Embryos also express His2A–mRFP1 (red) and Cid-EGFP (green); scale bars, 10 mm.

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Video 10. Induced anaphase after timely inhibition of topoisomerase II. Embryos expressing solely Barren$^{TEV}$ were injected with 12 mg/ml of a dominant-negative form of the human E2 ubiquitin-conjugating enzyme (UbcH10$^{C114S}$), to induce a metaphase arrest, and subsequently injected with 280 mM ICRF-193. After 14 min embryos were injected a wild-type version of UbcH10 to induce anaphase. Embryos also express His2A–mRFP1 (red) and Cid-EGFP (green); scale bars, 10 mm.

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Video 11. Induced anaphase after timely inhibition of Condensin I. Embryos expressing solely Barren$^{TEV}$ were injected with 12 mg/ml of a dominant-negative form of the human E2 ubiquitin-conjugating enzyme (UbcH10$^{C114S}$), to induce a metaphase arrest, and subsequently injected with 13 mg/ml TEV protease. After 14 min embryos were injected a wild-type version of UbcH10 to induce anaphase. Embryos also express His2A–mRFP1 (red) and Cid-EGFP (green); scale bars, 10 mm.
DOI: 10.7554/eLife.26120.026

Video 12. Induced anaphase after timely inhibition of Condensin I and topoisomerase II. Embryos expressing solely Barren$^{TEV}$ were injected with 12 mg/ml of a dominant-negative form of the human E2 ubiquitin-conjugating enzyme (UbcH10$^{C114S}$), to induce a metaphase arrest, and subsequently injected with a mix of 280 mM ICRF-193 and 13 mg/ml TEV protease. After 14 min embryos were injected a wild-type version of UbcH10 to induce anaphase. Embryos also express His2A–mRFP1 (red) and Cid-EGFP (green); scale bars, 10 mm.
DOI: 10.7554/eLife.26120.027

Table 1. List of fly strains used in this study

| CHR#* | Genotype | Reference |
|---|---|---|
| 1418 | Barr$^{L305}$/CyO | Bhat et al. (1996) (RRID:BDSC_4402) |
| 1421 | Df(2L)Exel7077/CyO | Blommington #7850 (RRID:BDSC_7850) |
| 1513 | w;; Barr(175 - 3TEV)-myc10 III.5 | This study |
| 1509 | w; Barr(175 - 3TEV)-myc10 II.1; | This study |
| 1522 | w;; Barr(389 - 3TEV)-myc10 III.2 | This study |
| 1514 | w;; Barr(437 - 3TEV)-myc10 III.1 | This study |
| 1520 | w;; Barr(600 - 3TEV)-myc10 III.3 | This study |
| 1525 | w;; Barr(wt)-myc10 III.1 | This study |
| 1560 | w; Barr$^{L305}$/ Df(2L)Exel7077; Barr(175 - 3TEV)-myc10 III.5 | This study |
| 820 | w;; HisH2AvD-mRFP1 III.1, CGC (CID-EGFP) III.1 | Schuh et al. (2007) |
| 1564 | Df(2L)Exel7077 / CyO; HisH2AvD-mRFP1 III.1, CGC (CID-EGFP) III.1 | This study |
| | w; Barr$^{L305}$/ Df(2L)Exel7077; Barr(175 - 3TEV)-myc10 III.5/ HisH2AvD-mRFP1 III.1, CGC (CID-EGFP) III.1 | |
| 629 | w;; Rad21$^{ex15}$, polyubiq-H2B-RFP, tubpr-Rad21(550-3TEV) -myc10 | Oliveira et al. (2010) |
| 1646 | w; Barr$^{L305}$, Barr(175 - 3TEV)-myc10 II.1; +/+ | This study |
| 1648 | w; Barr$^{L305}$, Barr(175 - 3TEV)-myc10 II.1; Rad21$^{ex15}$, polyubiq-H2B-RFP, tubpr-Rad21(550-3TEV) -myc10 | This study |

*Reference number in our internal lab fly database
DOI: 10.7554/eLife.26120.028

TopoII is able to promote erroneous re-entanglements of sister chromatids throughout mitosis, an activity that needs to be constantly opposed by condensin I.

How condensin I is able to confer such directionality remains to be addressed. Condensins are enriched at the chromosome axis where they have been proposed to promote interactions within the same chromatid (Ono et al., 2003; Steffensen et al., 2001). Condensin I was shown to display significant turn-over on mitotic chromosomes (Gerlich et al., 2006; Oliveira et al., 2007) highlighting that its mode of action relies in dynamic reactions rather than statically holding chromatin loops. Bringing strands of DNA from the same chromatid in close proximity could alone favour sister chromatid decatenation by limiting the probability contacts between sister DNA molecules. Models that predict that DNA loops can extrude away from condensin have been hypothesized (Goloborodko et al., 2016; Nasmyth, 2001) and are better at explaining the directionally issue, as they provide a mechanism that inherently explains how condensins distinguish intra- versus inter-chromosomal looping. Random intrachromatid linkages are also possible (Cheng et al., 2015; Cuylen et al., 2011), although in this case additional mechanisms may ensure that connections in cis are favoured over linkages between sister- (and nearby) chromatids. Condensin I- mediated supercoiling of the DNA molecule has also been proposed to change DNA structure to favour DNA decatenation activity (Baxter and Aragón, 2012; Baxter et al., 2011; Sen et al., 2016), although it is yet to be determined whether the supercoiling activity of this complex can account for all the phenotypes associated with condensin loss.

Our analysis further reveals that maintenance of chromosome architecture, particularly sister chromatid resolution, is not a unidirectional process but instead a much more dynamic reaction than previously anticipated. It is conceivable that the highly compacted chromatin state present in metaphase chromosomes could, on its own, shift TopoII reaction towards sister chromatid re-entanglement given the increased proximity between DNA strands. Condensin I would thus counteract an inherent tendency of chromosomes to re-intertwine, a reaction necessary throughout metaphase. Additionally, it is possible that a dynamic balance of chromosome entanglements allows remodelling of chromosome architecture, providing chromosomes with plasticity to counteract the cytoplasmic drag faced during dynamic movements. Energy released during these reactions could potentially be used to further facilitate chromosome movement. Mitotic chromosomes should thus be visualized as highly dynamic structures during mitosis, whose re-shaping may be fundamental for the fidelity of their own segregation.

# Materials and methods

## Fly strains

To destroy condensin by TEV protease-mediated cleavage, strains carrying solely TEV-sensitive Barren versions were produced. A construct carrying a ~4.7 kb Barren genomic region was used as a starting point (kindly provided by Beat Suter, Institute of Cell Biology, University of Bern). This region contains the regulatory sequences and was previously shown to restore Barren function (Masrouha et al., 2003). This construct was engineered to add a 10xMyc sequence at the C-terminus of Barren. Three consecutive TEV recognition sites were placed at different positions (corresponding to a.a. 175, a.a. 389, a.a. 437 and a.a 600). Cloning details are available upon request. Each variant of genomic Barren with different TEV sites was cloned into pCaSpeR4 vector used for fly transformation. Transgenic flies were produced by P-element integration (BestGene Inc, Chino Hills, CA). Transgenes were placed in a $Barr^{L305}$ background, a Barren null allele (Bhat et al., 1996), over a deficiency for the corresponding genomic region (Df(2L)Exel7077, stock #7850 from Bloomington stock center). To destroy cohesin by TEV-protease we used strains carrying $Rad21^{TEV}$, previously described (Oliveira et al., 2010; Pauli et al., 2008). Fly strains also expressed His2AvD–mRFP1 or polyubiquitin His2B–RFP, to monitor DNA and EGFP–Cid to monitor centromeres (Schuh et al., 2007). A list with detailed genotypes can be found in Table 1.

## Microinjections

Microinjection experiments were performed as previously described (Oliveira et al., 2010). 1–1.5 hr old embryos (or 0–30 min for mRNA injections) were collected and processed according to standard protocols, and embryos were injected at the posterior pole (up to three sequential injections) using

a Burleigh Thorlabs Micromanipulator, a Femtojet microinjection system (Eppendorf, Germany), and pre-pulled Femtotip I needles (Eppendorf). Embryos were injected with buffer, drugs or proteins purified from E. coli at the following concentrations: Buffer (20 mM Tris-HCl at pH 8.0, 1 mM EDTA, 50 mM NaCl and 2 mM DTT), 13 mg/ml TEV protease in TEV buffer, 12 mg/ml UbcH10$^{C114S}$, 14 mg/ml UbcH10$^{wt}$ and/or 280 mM ICRF-193 (Sigma-Aldrich, St Louis, MO).

## Protein purification

Purified TEV protease was described previously (Haering et al., 2008). Purification of UbcH10$^{wt}$ and UbcH10$^{C114S}$ was performed from BL21 cells as previously described (Oliveira et al., 2010), with minor modifications, as follows. Bacterial cells were grown for 16 hr at 37˚C, 225 rpm. This pre-culture was used to inoculate fresh LB media and cells were allowed to grow until 0.8/1 ODs. Cultures were then induced with 1 mM IPTG and after 4 hr of induction at 37˚C, 225 rpm, cells were harvested. Pellets were ressuspended in Lysis Buffer (20 mM Tris-HCL pH7.5, 0.5M NaCl, 5 mM Imidazole with protease inhibitors) and sonicated 5x on ice in 30 s cycles (power 5- Sonicator XL2020, Misonix, Farmingdale, NY). The soluble fraction of the extracts was then incubated in TALON Metal Affinity Resin (Takara Bio Inc. , Japan), according to manufacturer's instructions. After several washes with Lysis Buffer, the resin coated with the protein was packed into a Poly-Prep Chromatography Column (Biorad, Hercules, CA). Proteins were eluted in the same buffer with 300 mM imidazole. For buffer exchange, purified UbcH10$^{wt}$ and UbcH10$^{C114S}$ proteins were dialyzed overnight, at 4˚C, in a Slide-a-Lyzer 7 KDa Dialysis cassettes (Thermo Scientific, Waltham, MA). Final storage buffer was 20 mM Tris-HCL pH7.5, 0.3M NaCl). The purified proteins were concentrated in a Vivaspin 6 Centrifugal Concentrator MWCO 10.000 KDa (GE Healthcare, Issaquah, WA).

## mRNA synthesis

Barren$^{175TEV}$-EGFP was cloned into a pRNA plasmid and mRNA was synthesized by in vitro transcription with the mMessage mMachine T3 kit (Ambion, Austin, TX), followed by purification with RNeasy kit (Qiagen, Germany), and elution in RNase-free water. To probe for the efficiency of Barren$^{TEV}$ removal (Figure 1C), 0–30 min old embryos surviving only on Barren$^{TEV}$-Myc were injected with Barren$^{TEV}$-EGFP mRNA in pure water at ~2.2 mg/ml. Embryos were left to develop at 22˚C for 1,5–2 hr, to allow for protein translation, before the subsequent injections.

## In vitro cleavage experiments

Ovaries were dissected from females and homogenized in PBS. Extracts were sonicated for 2 min in a water-bath (power 5- Sonicator XL2020, Misonix). After centrifugation for 10 min at 15.000 rpm at 4˚C, the supernatant was removed and adjusted to a final concentration of 2 mg/ml. For cleavage experiments, 80 ml of extract were incubated with 2 mg of TEV protease. At the indicated time points, 10 ml of the reaction were diluted with sample buffer, boiled and stored at À20˚C.

## Western-blot

Samples were loaded on a 10% SDS-gel for electrophoresis and transferred onto a membrane (Immun-Blot PVDF, Biorad). Western-blot analysis was performed according to standard protocols using the following antibodies: anti myc-tag (1:200, Santa Cruz Biotechnology, Dallas, TX, Cat# sc-47694 RRID:AB_627266), anti-a-tubulin (1:50.000, DM1A, Sigma-Aldrich Cat# T9026 RRID:AB_477593) and anti-Barren (1:3000, kindly provided by Hugo Bellen, (Bhat et al., 1996), RRID:AB_2567044).

## Microscopy

Aligned embryos on coverslips were covered with Series 700 halocarbon oil (Sigma-Aldrich). Imaging of embryos after mRNA injection (Figure 1c) was performed with a spinning disc Revolution XD microscope (Andor, UK) at 22˚C. Stacks of around 20 frames 1 mm were taken at indicated times using a 100 Â 1.4 oil immersion objective (Nikon, Japan) and iXon +512 EMCCD camera (Andor). Time-lapse microscopy was performed with an inverted wide-field DeltaVision microscope (Applied Precision Inc., Issaquah, WA) at 18–20˚C in a temperature-controlled room. One stack of ~20 frames (0.8 mm apart) was acquired every 1 or 2 min using a 100 Â 1.4 oil immersion objective (Olympus, Japan) and an EMCCD camera (Roper Cascade 1024, Roper Technologies,

Inc., Sarasota, FL). Widefield images were restored by deconvolution with the Huygens v15.10/16.10 deconvolution software using a calculated point-spread function (RRID:SCR_014237, Scientific Volume Imaging, The Netherlands). Movies were assembled using FIJI software (RRID:SCR_002285) (Schindelin et al., 2012) and selected stills were processed with Photoshop CS6 (Adobe Systems Incorporated, San Jose, CA).

### Quantitative imaging analysis

For the quantification of chromosome condensation presented in Figure 3g and Figure 3—figure supplement 1, deconvolved images were analyzed using Imaris v6.1 software (RRID:SCR_007370, Bitplane, Switzerland). The same metaphase was tracked over time and average values for mean voxel intensity, volume and surface area were normalized to the first frame after injection. For the fluorescence profiles presented in Figures 3f and 6b–e,a wide 15 mm-long line was placed manually along the segregation plane and measured using the 'Plot Profile' function on FIJI software. For each data set, values were normalized to the maximum. Measurements of single chromatids width and length were performed on projected images (maximum intensity projection), using FIJI software and single chromatids mean voxel intensity measurements were performed using Imaris software. Quantification of chromosome movement (Figure 4) was performed as previously described (Mirkovic et al., 2015). Briefly, HisH2B-RFP was imaged at 1 min intervals. Images were segmented to select the chromosomal regions, based on an automatic threshold (set in the first frame after TEV injection), to create binary images. For each movie, a walking average of 3 frames was produced (using kymograph plug-in, written by J. Rietdorf and A. Seitz, EMBL, Heidelberg, Germany) creating a merged image in which the intensity is proportional to the overlap between consecutive frames. Intensity profiles were used to estimate the percentage of non-overlapping, 2- frame overlap and 3-frame overlap pixels. Graphic representation was performed using Prism seven software (RRID:SCR_002798, GraphPad, La Jolla, CA).

### Statistical analysis

To compare the average of the centromere distances between each experimental condition (Figure 6f), at least 10 independent embryos were analyzed. Statistical analysis was performed using Prism seven software (RRID:SCR_002798). Given that some datasets did not pass the normality test (D'Agostino and Pearson normality test), multiple comparisons were performed using the non-parametric Krustal-Wallis test.

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### Author contributions

EP, Conceptualization, Resources, Formal analysis, Investigation, Methodology, Writing—review and editing; AT, Resources, Methodology, Writing—review and editing, Protein purification; RAO, Conceptualization, Formal analysis, Supervision, Funding acquisition, Writing—original draft


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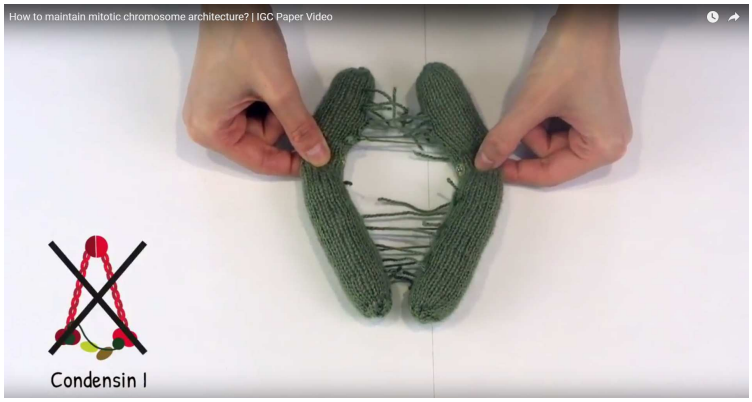
## .10 Outreach movie of the work

**Animated description of part of the work presented in the thesis, produced by the Instituto Gulbenkian de Ciência Outreach team.**


**English version:** https://youtu.be/HlbMfAc9WWE

**Portuguese version:** https://youtu.be/djW15tcVaSk

## .11 Knitting pattern of chromosomes presented on the cover of the thesis

**Abbreviations:**

k – knit

ssk – slip, slip, knit

k2tog – knit two together

m1L – make one left

m1R – make one right

()x – repeat x times

() at the end – stitch count

**Pattern:**

*First chromatid:*

1: cast on 32 stitches using the provisional cast on, join in the round

2 – 9: k (32)

10: k13, k2tog, k2, ssk, k13 (30)

11 – 18: k (30)

19: k12, k2tog, k2, ssk, k12 (28)

20 – 26: k (28)

27: k11, k2tog, k2, ssk, k11 (26)

28 – 33: k (26)

34: k10, k2tog, k2, ssk, k10 (24)

35 –39: k (24)

40: k9, k2tog, k2, ssk, k9 (22)

41 – 42: k (22)

43: k8, k2tog, k2, ssk, k8 (20)

44: k (20)

45: k7, k2tog, k2, ssk, k7 (18)

46-48: k (18)

49: k8, m1L, k2, m1R, k8 (20)

50: k (20)

51: k9, m1L, k2, m1R, k9 (22)

52 – 53: k (22)

54: k10, m1L, k2, m1R, k10 (24)

55 – 59: k (24)

At this point sew in the first elemnt of a snap to the flat part of the constriction, using a piece of tightly woven fabric as a backing to reinforce the snap in place.

60: k11, m1L, k2, m1R, k11 (26)

61 – 65: k (26)

66: k10, m1L, k2, m1R, k10 (28)

67 – 73: k (28)

74: k11, m1L, k2, m1R, k11 (30)

75–80: k (30)

81: k11, m1L, k2, m1R, k11 (32)

82 – 87: k (32)

88: (k3,k2tog)x8 (24)

89-91: k (24)

92: (k2, k2tog)x8 (16)

93-95: k (16)

93: (k, k2tog)x8 (8)

Break the yarn, pull it through the live stitches and pull to close the opening.

Unravel the provisional cast on and transfer the stitches to the needles.

Stuff the chromatid with a stuffing.

1 – 2: k (32)

3: (k3,k2tog)x8 (24)

4 – 7: k (24)

8: (k2, k2tog)x8 (16)

9 – 11: k (16)

12: (k, k2tog)x8 (8)

Stuff the chromatid to its full capacity. Break the yarn, pull it through the live stitches and pull to close the opening.

Then prepare the other chromatid, using the same instructions. Join two chromatids using the snaps.