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DOI
10.4233/uuid:560fb7a5-132b-4e74-85e8-1cead18ca28a

Publication date
2024

Document Version
Final published version

Citation (APA)
Hoencamp, C. (2024). *Chromosome metamorphoses: on the atypical roles of condensin II*. [Dissertation (TU Delft), Delft University of Technology]. https://doi.org/10.4233/uuid:560fb7a5-132b-4e74-85e8-1cead18ca28a

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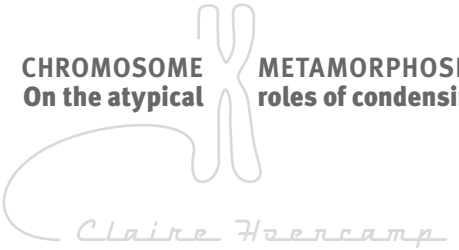

Claire Hoencamp

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ISBN 978-94-6483-646-2

Printing by thesis specialist Ridderprint, ridderprint.nl
Layout and design: Hans Schaapherder, persoonlijkproefschrift.nl

An electronic version of this dissertation is available at
https://www.publicatie-online.nl/publicaties/Claire-Hoencamp

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On the atypical roles of condensin II

Dissertation

for the purpose of obtaining the degree of doctor
at Delft University of Technology
by the authority of the Rector Magnificus, prof.dr.ir. T.H.J.J. van der Hagen,
chair of the Board for Doctorates
to be defended publicly on
Thursday 18 January 2024 at 15:00 o'clock

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Dr. J.A. Raaijmakers	Netherlands Cancer Institute

"My life amounts to no more than one drop in a limitless ocean.
Yet what is any ocean, but a multitude of drops?"

— David Mitchell, Cloud Atlas

CONTENTS

Chapter 1

General introduction: Shaping the genome in interphase and mitosis

The cell cycle

Every human once started out as one single cell. This single cell doubles to form two cells, which then continue to double until a full human body is formed, which exists of trillions of cells (Bianconi *et al.*, 2013). An important objective of a cell doubling is transmitting genetic information in the form of DNA molecules to both newly formed daughter cells. DNA is vital to most processes in the cell, and therefore its transmission is tightly controlled by a complex network of proteins. The series of events leading up to the actual cell division is called the cell cycle, and consists of multiple cell cycle phases (Fig. 1).

In G1-phase (Gap1-phase) of the cell cycle the cell grows, and proteins are manufactured to prepare for later stages of the cell cycle. At this stage internal and external cues determine whether the cell will proceed to the next stages of the cell cycle. If the cues are favorable, the cells progress into S-phase (Synthesis-phase). During S-phase, all DNA is duplicated to ensure there are two copies of all genetic information. The two copies are called sister chromatids, and these are held together by a ring-shaped protein complex called cohesin until their timely segregation at the end of the cell cycle. This is vital to ensure that both daughter cells will get one copy of the genetic information. After S-phase, cells enter into G2-phase (Gap2-phase), in which the cell prepares for the final step of the cell cycle: cell division. During G2-phase, the cell safeguards that all DNA is replicated and any existing DNA damage is repaired (Morgan, 2007). Once integrity of the genome is ensured, cells enter mitosis (Fig. 1).

Mitosis consists of multiple phases, each which contribute to chromosome segregation in their own way. First, in prophase, the DNA undergoes dramatic morphological changes to form rod-shaped mitotic chromosomes. Simultaneously, in the cytoplasm a collection of microtubules together forms the mitotic spindle. In prometaphase, the nuclear envelope breaks down, granting the mitotic spindle access to the chromosomes. The mitotic spindle is a network of microtubule fibers. In late prometaphase, these fibers start attaching to the chromosomes in a region called the centromere.

This centromeric region is of vital importance to proper chromosome segregation. Centromeres can be easily recognized in mitotic chromosomes as the constricted regions that bind the spindle fibers (Muller, Gil and Drinnenberg, 2019). The centromere is defined epigenetically by the specialized histone variant CENP-A. It functions as a docking station for the kinetochore: a large and intricate protein complex that binds microtubules to enable segregation (Cheeseman, 2014). At sequence level, the centromere is distinguished by repetitive DNA sequences such as alpha satellites, and they are surrounded by silenced DNA blocks termed pericentromeric heterochromatin (Hartley and O'neill, 2019). Whereas the copied sister chromatids separate from one another along the arms in early mitosis, centromeres are kept together by the cohesin complex (Haarhuis, Elbatsh and Rowland, 2014)(Fig. 3).

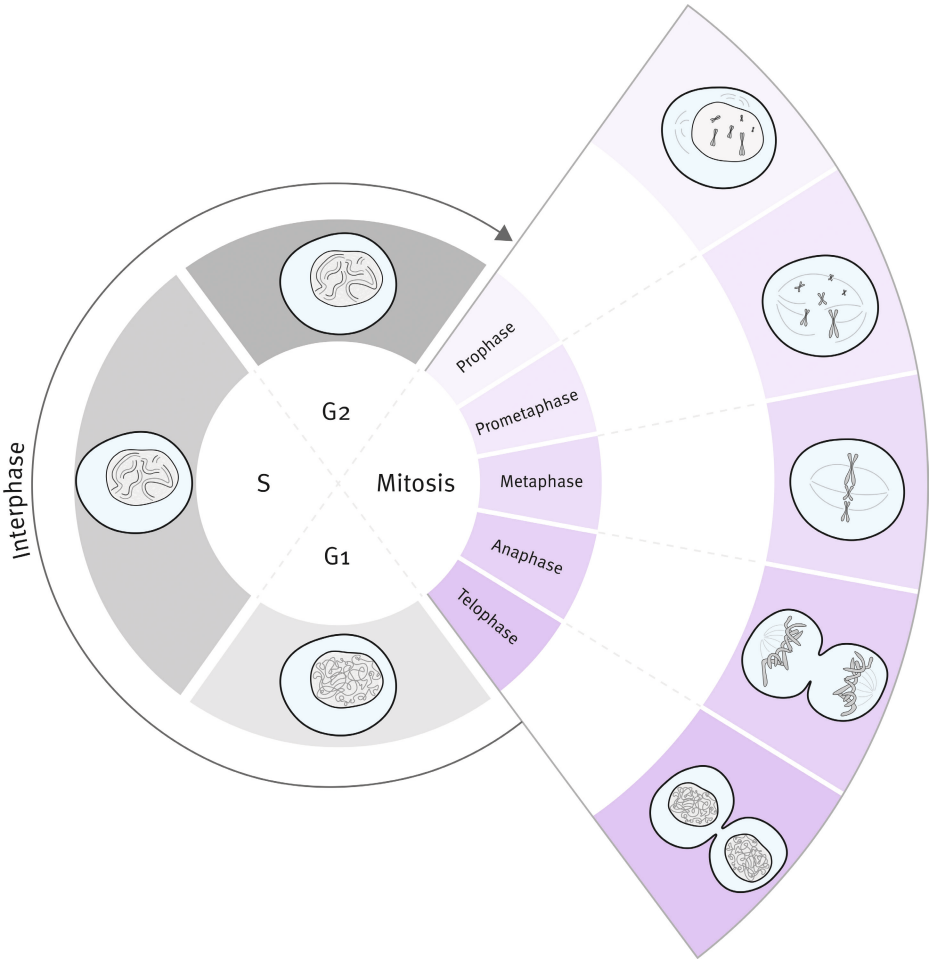

Figure 1 | The cell cycle. Schematic overview of the different stages of a typical cell cycle in eukaryotic cells. In G1 phase, cells grow and determine to proceed to the next phase of the cell cycle based on internal and external cues. In S phase, the DNA duplicates, forming two identical copies of each chromosome. In G2 phase, DNA damage is repaired and proteins are manufactured to prepare for mitosis. In mitosis, the two identical copies of the DNA are separated and divided among the two daughter cells. This process is subdivided in 5 main phases: prophase, prometaphase, metaphase, anaphase and telophase. In prophase, chromosomes condense to facilitate their segregation. In prometaphase, the nuclear envelope breaks down and the mitotic spindle forms and starts to bind chromosomes. In metaphase, all chromosomes align due to the pulling forces of the spindle. Upon entry into anaphase, connections between the sister chromatids are cleaved and they are pulled towards opposite sides of the cell. In telophase chromosomes decondense, new nuclear envelopes form and finally the two cells will be separated in a process called cytokinesis. Cells then re-enter G1 to continue another journey through the cell cycle.

Microtubules exert pulling forces, which cause chromosomes to align in the middle of the cell during metaphase. When each chromosome is attached to the spindle and aligned on the metaphase plate, the cohesin rings holding together the two chromatids are cleaved, transitioning the cell into anaphase. The chromatids are pulled towards opposite ends

of the cell, and in telophase two new nuclei are formed around each pack of chromatids. Chromosomes decondense within these two new nuclei, and the cytoplasm between the nuclei is split in a process called cytokinesis (Morgan, 2007). Two newly formed daughter cells are now ready for a new journey through the cell cycle, which will go on and on to form whole organisms (Fig. 1).

The cell cycle is vital for the development and growth of organisms. On top of that it promotes wound healing and facilitates reproduction. As such, problems in the cell cycle can lead to serious problems such as developmental defects, growth deficiencies or sterility. The cell cycle must however also be kept in check, as unrestrained cell division leads to cancer. Such uncontrolled growth can occur when the various regulatory mechanisms in the cell cycle fail. It is quite extraordinary that such a complex process as the cell cycle almost always transpires without any problems. This underlines how intricate cell cycle regulation must be, and how many fail-safes must be built into our cells to ensure proper cell division each and every time. Organisms rely on this complex network of cooperating proteins for every step in their life.

Genome folding

The whole cell cycle is designed to protect and propagate our genomic information, the DNA. DNA contains the essential information our cells need to survive, grow, divide and perform their specialized functions. It is thus not surprising that DNA needs to be stored in a manner that facilitates its protection and propagation, and simultaneously allows for DNA-based processes to take place. This is no easy feat, as a total of 2 meters of DNA is contained in each cell, while our cells only have a diameter of ~10 µM (Piovesan *et al.*, 2019). Therefore, the DNA needs to be tightly packaged and folded.

Throughout the cell cycle, the properties of the DNA must be adjusted to the cellular processes that occur. In interphase this means that the DNA has to allow for gene expression, DNA replication and DNA repair. As cells enter mitosis however, the chromosomes need to be structurally rigid and compact to enable cell division. Genome folding is thus starkly different between interphase and mitosis.

Interphase genome folding

To allow for the many DNA-based cellular processes in interphase, the genome is folded at different scales. At the smallest scale the DNA strand wraps around octamers of histones, forming a packaging unit called the nucleosome. Histones can be post-translationally modified with for example phosphorylation or acetylation. These modifications control the accessibility of the DNA and can recruit effector proteins, thereby regulating processes such as transcription and repair (Bannister and Kouzarides, 2011). DNA that is wrapped around nucleosomes is called chromatin.

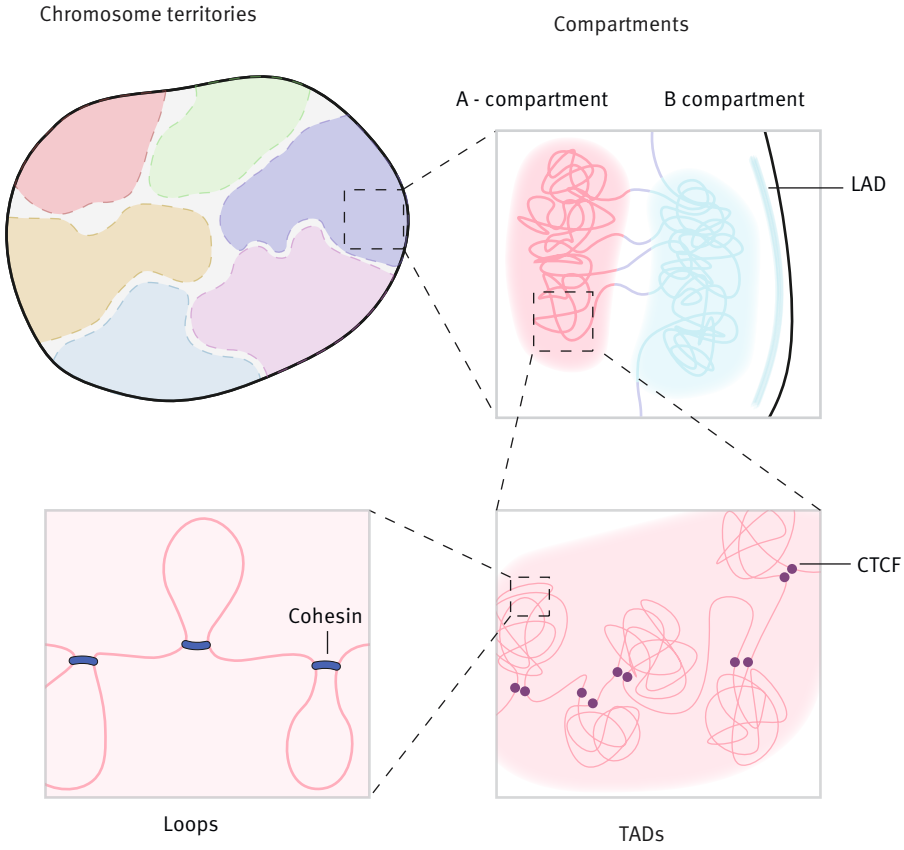

Figure 2 | Interphase genome organization. Schematic overview of the different levels of genome architecture in an interphase nucleus. Clockwise from the top left: in human cells, at the largest scale, chromosomes occupy distinct volumes within the nucleus termed chromosome territories. Because of these territories, there is relatively little intermingling between neighboring chromosomes. Zooming in in one such territory reveals that a chromosome divides into compartments based on their chromatin state. Transcriptionally active chromatin aggregates in so-called A compartments, whereas more silent and dense chromatin aggregates in B-compartments. These B-compartments often associate with the nuclear lamina in regions termed lamina associated domains (LADs). Within one compartment, the boundary elements CTCF demarcates regions which mostly interacts with themselves called topologically associated domains (TADs) or loop domains. One such TAD consists of a collection of loops that are formed by the cohesin complex.

Chromatin is shaped into chromatin loops by a protein complex called cohesin, a member of the SMC family of ATPases. In **Chapter 2** we discuss the wide range of processes that are controlled at the level of chromatin looping. These loops usually form within genomic regions that are flanked by boundary elements called CTCF, and a collection of loops between two such boundary elements is referred to as a loop domain or topologically associated domain (TAD)(Fig. 2). TADs represent functional units in which transcriptional regulation can take place (Dekker and Mirny, 2016; Rowley and Corces, 2018).

At a larger scale, the genome segregates into two compartments depending on the chromatin state (Lieberman-Aiden *et al.*, 2009). Transcriptionally active chromatin (euchromatin) clusters together in so-called A-compartments, whereas transcriptionally silent regions (heterochromatin) cluster into B-compartments (Fig. 2). These interactions between alike domains may help stabilize the respective chromatin states (Rowley and Corces, 2018). The heterochromatin compartments often associate with either the nuclear lamina or the nucleolar periphery, forming lamina associated domains (LADs) or nucleolus-associated domains (NADs) (van Steensel and Belmont, 2017)(Fig. 2).

At the largest scale, the human genome is divided into 46 chromosomes. These chromosomes each occupy their own, distinct, sub volume within the nucleus which is called a chromosome territory (Cremer and Cremer, 2010)(Fig. 2). Relatively little is known about how these chromosome territories are formed. In **Chapter 3** we reveal that the SMC complex condensin II promotes the formation of these chromosome territories in a manner that is likely evolutionarily conserved. In **Chapter 5** we investigate how condensin II performs its function in regulating genome architecture at the scale of whole chromosomes.

Mitotic chromosome formation

The word mitosis is derived from the Greek word μίτος (mitos), which means thread. This describes what Walther Flemming saw in the 1870s when he visualized mitotic chromosomes. From a seemingly unstructured chromatin mass in interphase, the chromatin changes into distinct thread-like chromosomes during mitosis.

Mitotic chromosomes have defined characteristics to promote the distinct processes that are needed to eventually form two new daughter cells. The rigid chromatin structures can resist the pulling forces of the mitotic spindle. Their individualization is essential to allow for proper chromosome segregation, increase chromatin motility and grant the microtubules access to the kinetochores. And finally, their compaction ensures that by the end of mitosis, the cleavage plane in cytokinesis is free of DNA. Any defects in the formation of mitosis chromosomes can lead to missegregations, which can lead to cell death or aneuploidy.

The chromosome compaction that occurs at the onset of mitosis is thought to be largely driven by changes in histone modifications that occur when cells enter into mitosis, such as histone deacetylation. On top of that the presence of divalent cations may play a role in this global compaction of the chromatin (Batty and Gerlich, 2019).

To ensure rigidness and individualization, the morphology of the chromatin completely changes in early mitosis. Any long-range interactions that were prevalent in interphase are lost from mitotic chromatin, including TADs and compartments. They are replaced by a rather uniform pattern of interactions (Gibcus *et al.*, 2018). These changes lead to the formation of rod-shaped and individualized mitotic chromosomes (Fig. 3). A handful of factors work together to form this mitotic chromatin. A set of proteins localize to a dynamic chromosomal

axis, which is surrounded by an (helical) array of chromatin loops. Central players are members of the SMC protein complex family– condensin I and condensin II, which build chromatin loops. Topoisomerase 2a ensures that DNA knots can be resolved, and thereby facilitates the continuation of loop formation by condensin complexes (Paulson *et al.*, 2021). In **Chapter 2** we further explore how these complexes work together to shape mitotic DNA.

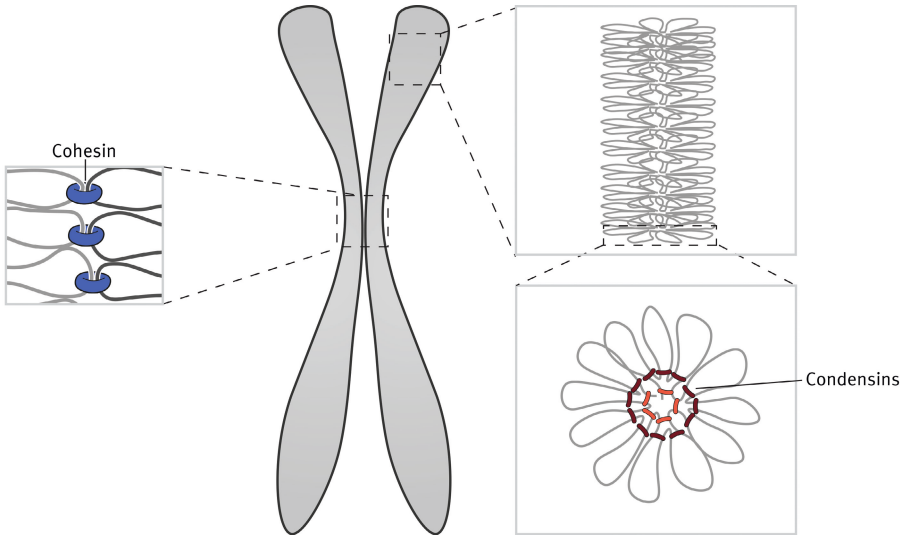

Figure 3 | Mitotic chromosome formation. Schematic overview of a mitotic chromosome. (Right) The mitotic chromosome consists of many chromatin loops, ensuring rigidness and individualization of the chromosome. Condensins (Red) and topoisomerase 2a are key players in shaping the mitotic chromosome. These proteins localize to the axis of the chromosome, and are surrounded by arrays of chromatin loops. (Left) The two sister chromatids must be kept together by cohesin from their formation in S-phase until anaphase onset.

The role of SMC complexes in shaping the 3D genome

Folding the genome throughout the cell cycle is a complex feat that must be tightly controlled. Key proteins in controlling genome folding are members of the SMC protein family. As described above, two of these SMC complexes have been implicated in genome organization in interphase and mitosis respectively. In **Chapter 2** we discuss the functions of the eukaryotic SMC complexes, cohesin, condensin and the SMC5/6 complex, throughout the different phases of the cell cycle. Apart from its role in mitotic genome organization, condensin II has been implicated in interphase genome organization. In **Chapter 3** and **Chapter 4** we investigate the role for condensin II in determining interphase 3D genome architecture. We not only do so by investigating the consequences of condensin II loss in human cells, but also by performing evolutionary 3D genomics across the tree of life. In **Chapter 5** we examine how condensin II exerts its function in shaping the 3D genome in interphase. In **Chapter 6** we discuss the implications of our findings and place the whole in a greater context.

SMC complexes are tightly controlled to ensure proper cell division, gene expression and DNA damage repair. This is illustrated by how mutations in these complexes lead to a range of human diseases. Mutations in the cohesin complex for example lead to cohesinopathies, an array of disorders which are often characterized by developmental and growth deficiencies (Piché *et al.*, 2019). Mutations in both condensin and cohesin are found in a wide array of cancers, probably due to their key roles in maintaining genome integrity (Yuen and Gerton, 2018). Condensin mutations are also widely found in microcephaly, a neurological disorder in which brain development is impaired (Martin *et al.*, 2016). This is interesting as MCPH1, the protein whose dysfunction was first identified to lead to primary microcephaly, is now known to be a negative regulator for condensin II in interphase. Apparently, both impaired condensin II activity and increased condensin II activity is detrimental for neurological development. On a cellular level, not a lot is understood about why MCPH1 must negatively regulate condensin II. In **Chapter 7** we therefore studied the consequences of MCPH1 loss and identified the processes which are defective in this setting.

Altogether, this thesis aims to further elucidate the function and regulation of the condensin II complex, with a focus on its non-canonical functions in interphase. By investigating the consequences of both condensin II loss and loss of its negative regulator MCPH1, we demonstrate that this complex plays an important role in processes ranging from interphase 3D genome organization to chromosome segregation.

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Chapter 2

Genome control by SMC complexes

Claire Hoencamp and Benjamin D. Rowland

**Adapted from Hoencamp, C. and Rowland, B.D. Genome Control by SMC complexes. Nature Reviews Molecular Cell Biology 24, 633-650 (2023)*

ABSTRACT

Many cellular processes require large-scale rearrangements of chromatin structure. Structural maintenance of chromosomes (SMC) protein complexes are molecular machines that can provide structure to chromatin. These complexes can connect DNA elements in *cis*, walk along DNA, build and processively enlarge DNA loops, and connect DNA molecules in *trans* to hold together the sister chromatids. These DNA-shaping abilities place SMC complexes at the heart of many DNA-based processes, including chromosome segregation in mitosis, transcription control, and DNA replication, repair and recombination. In this chapter, we discuss the latest insights into how SMC complexes such as cohesin, condensin and the SMC5–SMC6 complex shape DNA to direct these fundamental chromosomal processes.

INTRODUCTION

From the perspective of chromosome structure and function, few processes are as dramatic as mitosis. Four meters of DNA are converted into compact rigid structures that are then ripped in two, with each chromatid being pulled towards the opposite pole of the cell. This process ensures that the arising daughter cells receive equal karyotypes during cell division. Key to these major chromosomal processes is a family of protein complexes that drives a large portion of chromosome dynamics within the cell. These DNA organizing molecular machines are known as structural maintenance of chromosomes (SMC) protein complexes (Fig. 1A).

SMC proteins were discovered in the 1990s for their important role in chromosome segregation (Niki *et al.*, 1991; Strunnikov, Larionov and Koshland, 1993; Chuang, Albertson and Meyer, 1994; Hirano and Mitchison, 1994; Saitoh *et al.*, 1994; Saka *et al.*, 1994; Guacci, Koshland and Strunnikov, 1997; Hirano, Kobayashi and Hirano Michiko, 1997; Michaelis, Ciosk and Nasmyth, 1997; Losada, Hirano and Hirano, 1998). In the decades since it has become apparent that this family of protein complexes is not only essential for structuring chromosomes in mitosis, but also has key roles in many more cellular processes that require substantial changes in chromatin structure. Such processes include bringing together regulatory elements to facilitate long-distance gene regulation, and DNA replication, recombination and repair. These additional roles of SMC complexes have been studied intensely in recent years, yielding seminal insights into how and when SMC complexes control the genome.

In this chapter, we discuss the current understanding of how SMC complexes control genomic processes, with a focus on the eukaryotic SMC complexes known as cohesin, condensin and the SMC5–SMC6 complex. We also discuss the crucial roles that SMC complexes play in different fundamental aspects of chromosome biology.

Similarities and differences between SMC complexes

SMC complexes are conserved throughout the tree of life. The foundation of these complexes is a dimer of SMC proteins. Each SMC protein has a ~50 nm long coiled coil arm, with a dimerization interface on either end. The head domains of both SMC proteins of the dimer interact to form a composite ABC-like ATPase that can bind and hydrolyze two molecules of ATP. The head domains are bridged by a third protein, the kleisin subunit, to essentially form a ring-shaped complex (Fig. 1B).

The ring-shaped core complex is complemented by accessory subunits, which in case of cohesin and condensin includes HEAT repeat proteins associated with kleisin (HAWK) proteins (Wells *et al.*, 2017). In case of the SMC5–SMC6 complex and bacterial SMC complexes, the accessory subunits include kleisin-interacting tandem winged-helix element (KITE) proteins (Palecek and Gruber, 2015). The differences between these accessory subunits likely underlie the different mechanistic and temporal activities of the complexes. Although

cohesin and condensin are more similar to each other than to SMC5–SMC6, there are notable differences. In many species there are two variant condensin complexes, namely condensin I and condensin II. These variant complexes share the SMC2–SMC4 heterodimer, but each binds their own respective kleisin and two accessory subunits. Condensin I is associated with the kleisin CAP-H, and the HEAT proteins CAP-D2 and CAP-G, whereas condensin II binds CAP-H2, CAP-D3 and CAP-G2 (Fig. 1B).

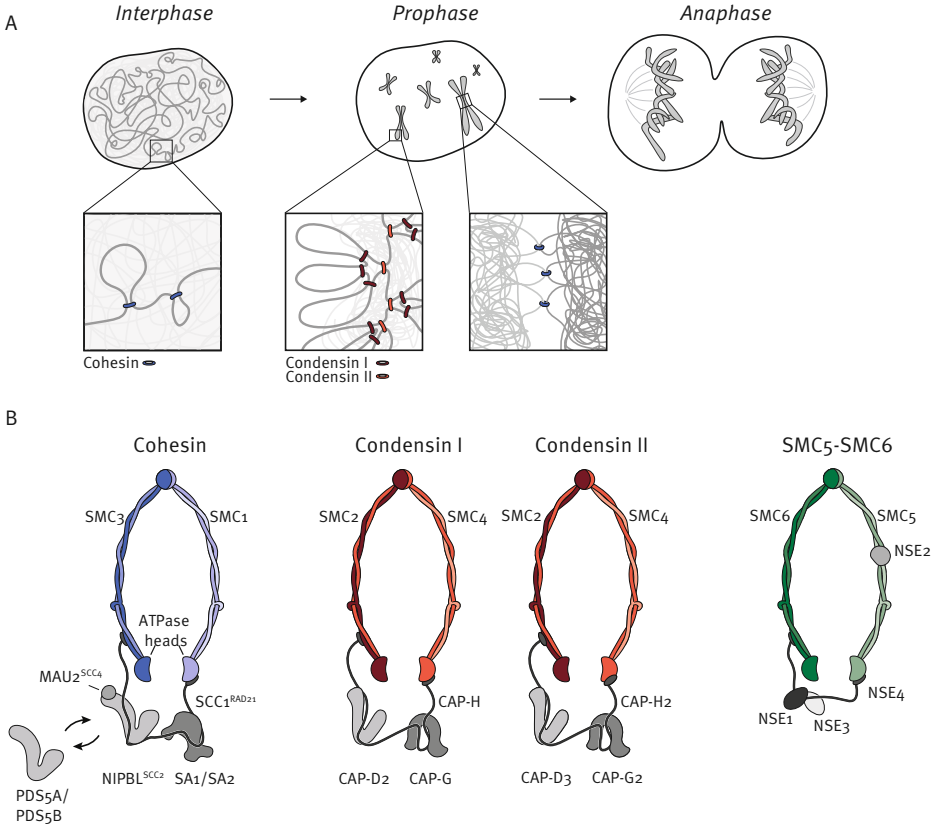


Figure 1 | The three eukaryotic structural maintenance of chromosomes complexes. (A) Structural maintenance of chromosomes (SMC) complexes act at different stages of the cell cycle and control a wide array of chromosomal processes. Cohesin shapes the genome in interphase by forming chromatin loops, and holds together the sister chromatids in mitosis. Condensin complexes shape mitotic chromosomes by forming nested loop structures. Ultimately, these SMC complexes ensure that each cell cycle can end with successful chromosome segregation. (B) Schematic overview of the cohesin, condensin I, condensin II and SMC5–SMC6 complexes. Each complex contains an SMC heterodimer, the two ATPase heads of which are bridged by a kleisin subunit. Cohesin also has HEAT repeat proteins associated with kleisin (HAWK) accessory subunits, SA1or SA2 as a stable subunit of the complex, and NIPBL^{SCC2}, or PDS5A or PDS5B. Binding to cohesin of NIPBL^{SCC2} and a PDS5 subunit is mutually exclusive. Condensin I and condensin II bind the HAWK accessory subunits CAP-D2 and CAP-G or CAP-D3 and CAP-G2, respectively. The SMC5–SMC6 complex binds the kleisin-interacting tandem winged-helix element (KITE) accessory subunits non-structural maintenance of chromosomes element 1 homolog (NSE1) and NSE3 and the SUMO E3 ligase NSE2.

It could be argued that condensin is simpler than cohesin due to its relatively stable subunit composition. The SMC1–SMC3–SCC1^{RAD21} trimer of cohesin is only stably associated with one HEAT protein, the SA subunit. At any given time, it also binds one of two variant HEAT subunits: NIPBL^{SCC2}, or PDS5 (Petela *et al.*, 2018). In its NIPBL^{SCC2} -bound state, cohesin appears to be enzymatically active. PDS5-bound cohesin is less well understood, and may reflect a paused or frozen state. The acetylation of cohesin's SMC3 subunit promotes this PDS5 bound state (Bastié *et al.*, 2022; van Ruiten *et al.*, 2022). The complexity of cohesin is further increased by the existence of two somatic variants of both the PDS5 subunit (PDS5A and PDS5B), and of the SA subunit (SA1 and SA2). Meiotic cells have even more cohesin variants in SMC1β, the kleisin variants REC8 and RAD21L, and SA3. These different variants presumably exist to enable context-specific control of the cohesin complex, which is likely not needed for condensin.

The SMC5–SMC6 complex associates with accessory subunits termed non-SMC elements (NSE). The KITE subunits are NSE3 and NSE1, and they associate with the complex through the kleisin NSE4. NSE3 and NSE1 have striking resemblances to accessory subunits of the prokaryotic SMC complexes ScpAB and MukBEF, suggesting that SMC5–SMC6 might be the evolutionarily oldest eukaryotic SMC complex (Palecek and Gruber, 2015). In contrast to cohesin and condensin, the SMC5–SMC6 complex has several enzymatic roles in addition to its ATPase activity. It has ubiquitin ligase activity through its NSE1 subunit (Kolesar *et al.*, 2022), and through its NSE2 accessory subunit it can also act as a small ubiquitin-related modifier (SUMO) ligase (Andrews *et al.*, 2005; Zhao and Blobel, 2005). In budding yeast, NSE5 and NSE6 interact with the SMC5–SMC6 complex to negatively regulate its ATPase (Hallett *et al.*, 2021; Taschner *et al.*, 2021). Their function may involve a role in loading SMC5–SMC6 onto chromatin (Taschner *et al.*, 2021), but overall is poorly understood. In mammals, SMC5–SMC6 complex localization factor protein 1 (SLF1) and SLF2 may perform a similar task, as they can recruit the SMC5–SMC6 complex to DNA lesions (Räschle *et al.*, 2015). The additional enzymatic subunits of the SMC5–SMC6 complex allow modulation of cellular processes in a manner that is somewhat different from the abilities of cohesin and condensin. It should be noted that whereas in recent decades a lot has been uncovered about the roles of cohesin and condensin, the key cellular roles of SMC5–SMC6 have remained more of a mystery.

As the core components of the SMC complexes are much alike, the mode in which SMC complexes can exert their role in shaping chromatin is likely largely the same. Over two decades ago, SMC complexes were proposed to shape chromatin by forming and processively enlarging DNA loops (Nasmyth, 2001). In recent years we have witnessed a great accumulation of both *in vivo* and *in vitro* evidence in favour of SMC complexes indeed performing such activity (Box 1). Through what is currently referred to as loop extrusion, these complexes can build loops by reeling in large segments of DNA (Ryu *et al.*, 2022), which at least in the case of cohesin and condensin can include histones and other potential extrusion obstacles (Pradhan, Barth, *et al.*, 2022).

Loop extrusion likely involves a cycle of concerted conformational changes that are driven by the ATPase machinery of the SMC subunits. Although recent studies have provided us with snapshots of specific stages of the looping reaction, we are far from fully understanding the mechanism by which the different conformational states together enable the formation and enlargement of chromatin loops. Our current mechanistic understanding has been covered in recent reviews (Davidson and Peters, 2021; Oldenkamp and Rowland, 2022). Although at a basic level all SMC complexes presumably build loops through a shared mechanism, the different complexes in fact control a range of very diverse chromosomal processes. Loop extrusion has a role in many of these processes, while other processes may involve other activities of SMC complexes. These differences are best illustrated by how SMC complexes act throughout different stages of the cell cycle.

CONTROL OF GENOMIC PROCESSES BY SMC COMPLEXES

Despite originally being identified as complexes important for mitosis, and early hints that these proteins may be important in DNA repair (Birkenbihl and Subramani, 1992), it has become apparent that SMC complexes have crucial functions in each cell cycle phase. These complexes shape the genome, bring together distant genomic regions, repair and prepare chromatin for mitosis, and then ultimately ensure proper chromosome segregation and the formation of daughter cells with identical karyotypes.

Looping together distant loci in interphase

The first SMC complex known to act when cells enter G1 phase is cohesin (Abramo *et al.*, 2019; H. Zhang *et al.*, 2019). Cohesin's action on DNA is dynamic and involves cycles of loading, the formation of small loops, their enlargement, and ultimately DNA release and concomitant loop loss. Cohesin loading depends on NIPBL^{SCC2}, which together with MAU2^{SCC4} forms the cohesin loader complex (Ciosk *et al.*, 2000). NIPBL^{SCC2} however is also essential for the loop extrusion process itself (Davidson *et al.*, 2019; Kim *et al.*, 2019). DNA release involves the cohesin release factor WAPL, which causes the opening of a DNA exit gate in cohesin rings (Gandhi, Gillespie and Hirano, 2006; Kueng *et al.*, 2006; Chan *et al.*, 2012a; Buheitel and Stemmann, 2013; Eichinger *et al.*, 2013; Murayama and Uhlmann, 2015; Beckouët *et al.*, 2016). The resulting loop loss enables cohesin to form new loops, which keeps the 3D configuration of the genome dynamic (Gassler *et al.*, 2017; Haarhuis *et al.*, 2017; Wutz *et al.*, 2017) (Fig. 2A).

Shaping chromatin by cohesin and CTCF

Cohesin complexes can form and processively enlarge loops of DNA. A collection of chromatin loops within a certain genomic region is generally referred to as a topologically associating domain (TAD) or loop domain (Box 1). TADs depend on cohesin, as removal of cohesin or NIPBL^{SCC2} leads to their loss (Gassler *et al.*, 2017; Haarhuis *et al.*, 2017; Rao *et al.*, 2017; Schwarzer *et al.*, 2017; Wutz *et al.*, 2017). When cohesin forms chromatin loops, it does so

in a bi-directional manner (Davidson *et al.*, 2019; Kim *et al.*, 2019; Golfier *et al.*, 2020). This means that cohesin enlarges a loop towards both sides of the DNA. Cohesin enlarges loops until it encounters CTCF, which can act as a barrier for cohesin (Davidson *et al.*, 2022; H. Zhang *et al.*, 2022). This presumably is why cohesin is found enriched at CTCF sites (Parelho *et al.*, 2008; Wendt *et al.*, 2008; Davidson *et al.*, 2016).

CTCF controls cohesin in multiple ways. CTCF binding sites are oriented in such a way that the N-terminus of the CTCF protein points towards the inside of loop domains (Nakahashi *et al.*, 2013; Rao *et al.*, 2014; Vietri Rudan *et al.*, 2015). This N-terminus turns out to bind directly to cohesin, and hereby directs the orientation in which cohesin can form or maintain loops (Li *et al.*, 2020; Nishana *et al.*, 2020; Nora *et al.*, 2020; Pugacheva *et al.*, 2020). CTCF binds a conserved interface of the cohesin complex that is formed by its SA HEAT subunit and the $SCC1^{RAD21}$ subunit (Fig. 2A). CTCF thus occupies a WAPL binding interface, which renders cohesin resistant to WAPL-mediated DNA release and therefore stabilizes cohesin at these sites (Li *et al.*, 2020). The maintenance of cohesin at CTCF sites requires the integrity of cohesin's ring structure, whereas cohesin binding in-between CTCF sites does not (Liu and Dekker, 2022). This difference suggests that cohesin at CTCF sites entraps DNA, and that cohesin at most other sites does not. Apart from the N-terminus, other regions of CTCF also control cohesin, possibly through RNA binding (Hansen *et al.*, 2019; Saldaña-Meyer *et al.*, 2019). The acetylation of the cohesin subunit SMC3 also affects cohesin at CTCF sites, by promoting the conversion of cohesin into its PDS5-bound state (Wutz *et al.*, 2020; Bastié *et al.*, 2022; van Ruiten *et al.*, 2022) (Fig. 2A). PDS5-bound cohesin presumably reflects a paused state that is impaired in its ATPase activity. PDS5 can also recruit SUMO proteases Ulp2 (in budding yeast) and its ortholog SENP6 (in human), which preserve cohesin stability (Wagner *et al.*, 2019; Psakhye and Branzei, 2021).

Quantification of CTCF and cohesin molecules on the chromatin reveals that cohesin and CTCF cannot occupy all CTCF sites in the genome at once (Hansen *et al.*, 2017; Cattoglio *et al.*, 2019; Holzmann *et al.*, 2019). Possibly because CTCF does not occupy all its genomic binding sites, cohesin-mediated interactions can frequently cross TAD boundaries and connect sequences that lie in neighbouring domains (Flyamer *et al.*, 2017; Stevens *et al.*, 2017; Bintu *et al.*, 2018; Finn *et al.*, 2019; Luppino *et al.*, 2020; Su *et al.*, 2020). There is however a preference for TAD boundaries to form at CTCF sites, which must be why loop domain structures are visible in bulk assays such as Hi-C (Bintu *et al.*, 2018). Live cell imaging shows that loops connecting CTCF sites are relatively rare and transient, and that cohesin is required to increase the frequency and duration of these contacts (Gabriele *et al.*, 2022; Mach *et al.*, 2022). An image emerges in which constant loop extrusion leads to interactions between different genomic regions, and in which CTCF acts as a boundary to insulate regions in a manner that is highly dynamic. Overall, it appears that chromatin looping by cohesin, and to a considerable degree its regulation by CTCF, lie at the heart of a major portion of chromatin interactions throughout interphase.

Directing long-range regulation of gene expression

Although until recently a general assumption in the field had been that loop extrusion by cohesin is needed for the expression of a large portion of genes, the reality turns out to be more nuanced. Loop domains and cohesin-mediated loop extrusion do not appear to control overall gene expression (Rao *et al.*, 2017; Schwarzer *et al.*, 2017). Instead, cohesin seems highly relevant for the expression of specific genes that require the bringing together of long-distance regulatory elements, such as genes involved in various developmental programmes. Loop domains enhance the possibility of a long-range promoter–enhancer pair to find one another (Symmons *et al.*, 2016), and CTCF forms a boundary between regulatory elements to prevent aberrant promoter–enhancer pairing (Lupiáñez *et al.*, 2015; Flavahan *et al.*, 2016; Hnisz *et al.*, 2016) (Fig. 2B). Disruption of CTCF sites through mutation or methylation can also lead to erroneous activation of genes, resulting in cancer or malformation syndromes (Lupiáñez *et al.*, 2015; Flavahan *et al.*, 2016; Hnisz *et al.*, 2016).

Cohesin appears to be recruited to the enhancers of a subset of genes, where presumably through loop extrusion it can facilitate promoter–enhancer pairing (Kagey *et al.*, 2010; Ing-Simmons *et al.*, 2015; Vian *et al.*, 2018; Barrington *et al.*, 2019; Thiecke *et al.*, 2020; Kane *et al.*, 2022; Rinzema *et al.*, 2022) (Fig. 2B). This pairing can lead to activation of the associated gene, possibly by modulating transcriptional bursting (Luppino *et al.*, 2020; Robles-Rebollo *et al.*, 2022). As discussed above, cohesin and its release from DNA by WAPL enables the dynamic cycle of forming, losing and reforming of loops. This turnover appears to be important for the expression of a subset of genes: preventing this cycle through WAPL depletion leads to a loss of cohesin localization near these genes and prevents their activation (Liu *et al.*, 2021).

The above-mentioned cohesin roles seem to be primarily important for establishing gene expression in certain developmental or differentiation programmes, in response to inducible transcription factors. Activation of such programmes by cohesin has been described in neurons, hematopoietic stem cells and progenitor cells and in response to activation of the glucocorticoid receptor (Cuartero *et al.*, 2018; Calderon *et al.*, 2022; Rinaldi *et al.*, 2022). The relevance of programme activation can be observed in mouse neurons, where cohesin and CTCF modulate the chromatin contacts between gene promoters and their regulatory elements following developmental cues, which is essential for learning and memory responses (Sams *et al.*, 2016; Yamada *et al.*, 2019). Intriguingly, cohesin also has an unexpected role in intragenic regions following stimulation with a hormone, possibly by forming a roadblock and causing pausing of transcript-elongating RNA polymerase II (Wang *et al.*, 2022).

Do other SMC complexes regulate gene expression? There is conflicting data regarding such a role for condensin. Whereas condensin I and II have been reported to regulate gene expression in different organisms (Chapter 4) (Lupo *et al.*, 2001; Li *et al.*, 2015; Zhang *et al.*, 2016; Yuen, Slaughter and Gerton, 2017; Hoencamp *et al.*, 2021; Lancaster *et al.*, 2021), others have reported only minor or no differences in gene expression in response to condensin

perturbation (Abdennur *et al.*, 2018; Macdonald *et al.*, 2022). It has been suggested that gene expression differences in these cells can be explained by chromosomal instability upon condensin loss (Woodward *et al.*, 2016; Hocquet *et al.*, 2018).

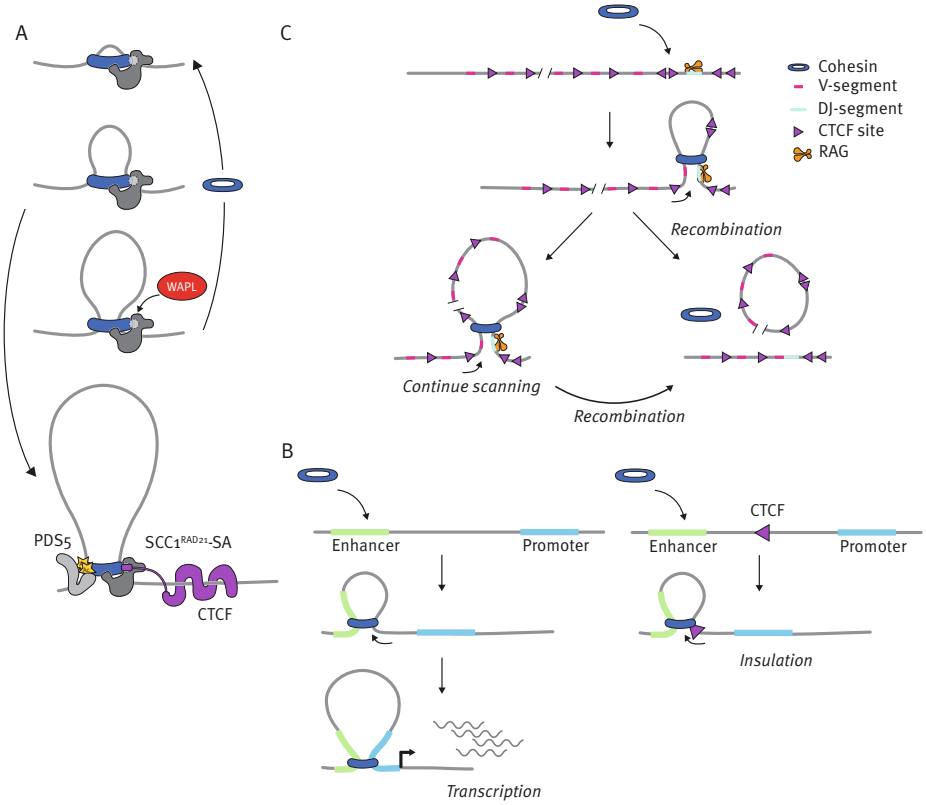


Figure 2 | Cohesin loops organize the genome and control gene expression and recombination. (A) Cohesin binds the DNA to form and enlarge chromatin loops. WAPL removes cohesin from the DNA by binding a composite interface (white circle) of the SCC1RAD21 subunit and the SA subunit. CTCF uses its N-terminus to bind this same cohesin interface, and therefore prevents WAPL-mediated cohesin release from the DNA. Cohesin can be further regulated at CTCF sites by acetylation (Ac) of its structural maintenance of chromosomes 3 (SMC3) subunit, which promotes PDS5 binding. (B) A role for chromatin looping by cohesin in promoter–enhancer pairing. (Left) Cohesin can load at enhancers, and through chromatin loop extrusion (small arrow) can scan the genome for promoters. (Right) CTCF boundaries can prevent cohesin from reaching promoters, thereby imposing transcriptional insulation between enhancers and promoters. (C) A role for loop extrusion in recombination. In B cells, cohesin loads at the Igh locus and scans the DNA to bring variable (V) DNA segments into close proximity of the diversity (D) and joining (J) segments. The endonuclease recombination activating gene (RAG) can then either recombine a DJ segment with a V segment (right), or continue scanning to recombine with it with a subsequent V segment (left).

However, some data show a specific function for condensin in response to external stimuli such as heat shock or estrogen addition (Li *et al.*, 2015; Lancaster *et al.*, 2021), in a manner quite analogous to cohesin's role in controlling gene expression in response to developmental

cues. As cohesin and condensin share their ability to form chromatin loops, it would not be surprising if condensin in certain contexts can also regulate transcription of a subset of genes. The condensin II complex in particular is a plausible candidate to perform such a function, as this complex is nuclear throughout the cell cycle. Despite all the advances over the past decade, a lot remains to be learned about how and when SMC complexes truly regulate gene expression.

Regulating adaptive immunity

Interestingly, cohesin-mediated loop extrusion turns out to be vital also for the immunological processes that ensure B-cell and T-cell receptor diversity, thereby facilitating our adaptive immune response to a wide array of pathogens (reviewed in (Y. Zhang *et al.*, 2022)). In B-cells, variable (V), diversity (D) and joining (J) segments need to be recombined into exons that encode the variable regions of antibodies. These V(D)J segments form large arrays of repeated segments, and the combination of different V, D and J segments leads to broad antibody diversity. Cohesin-mediated loop extrusion allows the recombination activating gene (RAG) endonuclease to travel along and scan across the DNA arrays to find V, D and J segments and enable their recombination (Guo *et al.*, 2011; Medvedovic *et al.*, 2013; Lin *et al.*, 2015; Jain *et al.*, 2018; Ba *et al.*, 2020; Hill *et al.*, 2020; Dai *et al.*, 2021). This recombination can only occur at segments near CTCF-bound sites, which presumably pause cohesin and RAG and allow recombination to take place (Fig. 2C). The IgH locus containing the V(D)J segments is exceptionally long. To ensure that all different V segments can be utilized, cohesin processivity is stimulated in pro-B cells by downregulation of WAPL (Hill *et al.*, 2020; Dai *et al.*, 2021). Increasing cohesin's residence time on DNA allows it to scan longer genomic segments.

V(D)J recombination ensures that there is a diverse range of antibody specificity. Once mature B cells have encountered a pathogen, they can initiate antibody class-switch recombination to express a different type of antibody. During this process, loop extrusion by cohesin also aligns different genomic elements and promotes their recombination in a process very analogous to V(D)J recombination (X. Zhang *et al.*, 2019). In thymocytes, cohesin controls several aspects of successful T-cell receptor rearrangements (Seitan *et al.*, 2011). Besides cohesin's role in immunological recombination, other SMC complexes also have unexpected roles in protecting cells against invading pathogens (Box 2).

Forming sister chromatid cohesion in S phase

DNA replication results in the formation of two identical sister chromatids that are held together by cohesin until mitosis, when the chromatids are divided equally between the newly formed daughter cells. Cohesin complexes in this setting entrap two DNA segments in *trans* (Haering *et al.*, 2008), in contrast to how they hold onto two DNA segments in *cis* during loop extrusion. Cohesin is the only SMC complex shown to form stable interactions in *trans*, which might explain why it has greater subunit complexity than condensin.

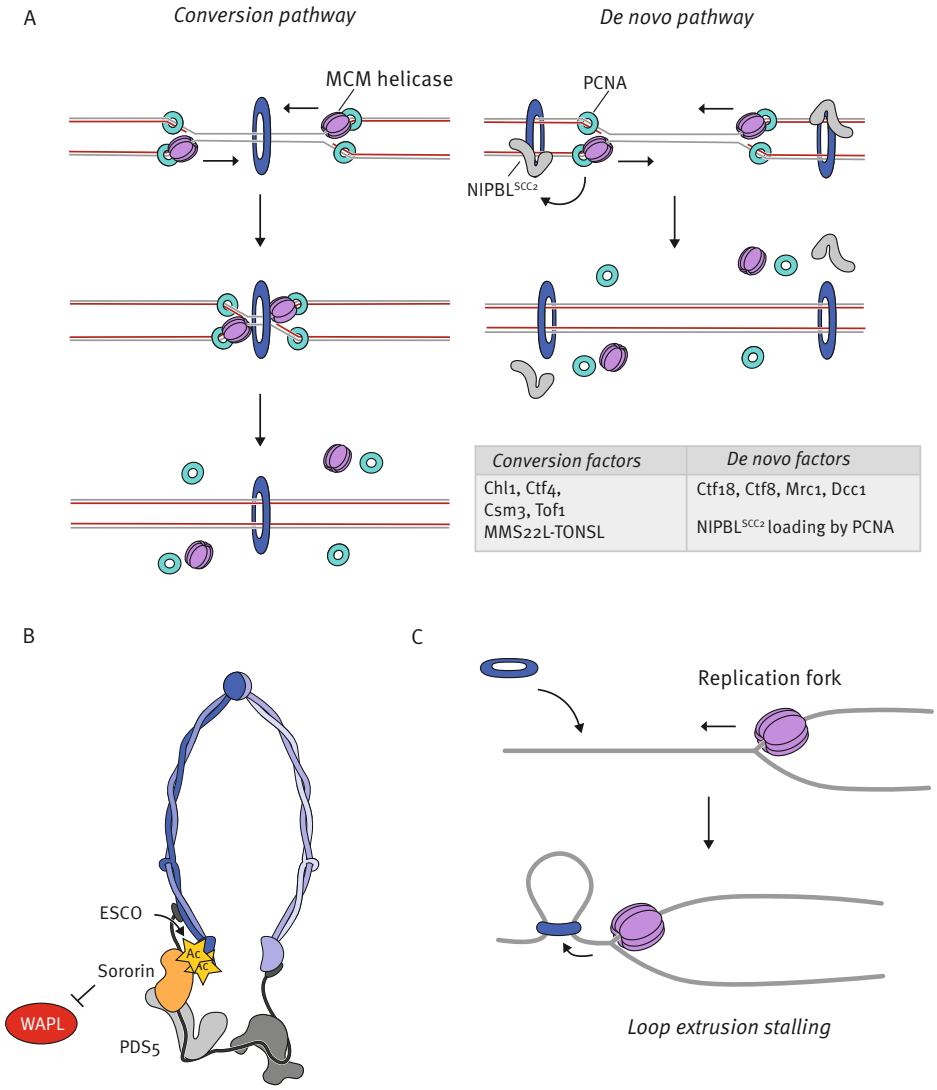


Figure 3 | Establishment and maintenance of sister chromatid cohesion. (A) Models for the two pathways of sister-chromatid cohesion establishment following DNA replication. Left: The conversion pathway, in which cohesin that is already bound to chromatin is pushed ahead of the replication fork. This cohesin is converted into cohesive cohesin at the site of replication termination. Right: The de novo pathway, in which PCNA recruits NIPBLSCC2 to establish cohesion on replicated DNA. The table lists yeast and human factors that are important for each of the two pathways. (B) The maintenance of sister chromatid cohesion involves the acetylation of the structural maintenance of chromosomes 3 (SMC3) subunit of cohesin, and the recruitment of sororin. Sororin prevents WAPL binding to the PDS5 subunit of cohesin, thereby stabilizing cohesin on DNA. (C) Replication fork components may act as barriers to loop extrusion by cohesin, possibly through interaction with MCM complexes.

How cohesin manages to connect specifically the sister chromatids of each chromosome remains poorly understood. Central to the establishment of sister chromatid cohesion are events that take place in the proximity of replication forks. *In vitro* experiments suggest that cohesin can first bind the double-stranded DNA (dsDNA) leading strand, then the single-stranded DNA (ssDNA) lagging strand, and that the conversion of the lagging strand from ssDNA to dsDNA stabilizes the 'cohesive' state of the chromatids (Murayama *et al.*, 2018). In budding yeast, two parallel pathways have been identified that aid cohesin in cohesion establishment (Xu, Boone and Brown, 2007). The first pathway, the conversion pathway, converts cohesin complexes that were already DNA-bound into cohesive complexes (Srinivasan *et al.*, 2020). A recent preprint elucidated that this conversion occurs at sites where converging replication forks terminate replication (Cameron *et al.*, 2022)(Fig. 3A). The conversion pathway depends on at least the proteins Chl1, Ctf4, Csm3 and Tof1. Another pathway, known as the *de novo* pathway, depends on the complex Ctf18–replication factor C (RFC) (Ctf18, Ctf8, Mrc1 and Dcc1). This pathway requires *de novo* loading of cohesin complexes by Scc2 (Xu, Boone and Brown, 2007; Rhodes *et al.*, 2017; Srinivasan *et al.*, 2019, 2020; van Schie *et al.*, 2023) (Fig. 3A). The Ctf18–RFC complex also seems to have a role in recruiting excess PCNA to replication forks, which allows the recruitment of the acetyltransferase Eco1, a factor required for the stabilization of cohesive cohesin complexes (see below)(Liu *et al.*, 2020). In vertebrates these two pathways have not been studied in great detail, but evidence is emerging that they are at least partially conserved (Faramarz *et al.*, 2020; Kawasumi *et al.*, 2021). In human cells, the conversion pathway also involves the MMS22L–TONSL complex (van Schie *et al.*, 2023). A recent preprint also indicates that PCNA recruits Scc2 to the replicated DNA, which is an essential and conserved step in the *de novo* pathway (Psakhye *et al.*, 2022).

Interestingly, sister chromatid cohesion is enriched at boundaries of loop domains, suggesting that CTCF also has a role in determining the sites of sister chromatid cohesion (Mitter *et al.*, 2020). This role could be related to the recent finding that cohesin at CTCF sites appears to topologically entrap DNA (Liu and Dekker, 2022). This particular binding mode could for example be required to convert cohesin complexes into cohesive complexes upon replication fork passage, which would not only explain why cohesion is enriched at these sites, but also why not all chromatin-looping cohesin seems to be converted into cohesive cohesin. Establishment of cohesion correspondingly seems to require the opening-up of the hinge domain of the cohesin complex (Collier and Nasmyth, 2022; Nagasaka *et al.*, 2022). Interestingly, a preprint reports that mutants in this hinge domain are less enriched at CTCF sites and are defective in sister chromatid cohesion (Nagasaka *et al.*, 2022), supporting a model in which cohesin at CTCF sites entraps DNA by opening-up its hinge interface, which might then be required for the establishment of sister chromatid cohesion.

Once cohesin has entrapped the two sister chromatids, they must be stably kept together until their separation in mitosis. Key to this process is the acetylation of SMC3 on two conserved lysine residues by acetyltransferases Eco1 (in yeast) or ESCO1 and ESCO2 (in

vertebrates) (Ivanov *et al.*, 2002; Ben-Shahar *et al.*, 2008; Unal *et al.*, 2008; Zhang *et al.*, 2008; Rowland *et al.*, 2009). In budding yeast cohesin acetylation is promoted by DNA nicks or flaps, which arise during DNA replication (Minamino *et al.*, 2023). Indeed, during S-phase a pool of cohesin that is highly stable on chromatin emerges (Gerlich, Koch, *et al.*, 2006). Acetylation of cohesin protects against WAPL-mediated DNA release, which ensures the maintenance of sister chromatid cohesion (Ben-Shahar *et al.*, 2008; Rowland *et al.*, 2009; Sutani *et al.*, 2009; Feytout *et al.*, 2011; Chan *et al.*, 2012b). Vertebrates, but not yeast, also require sororin for cohesion maintenance. Acetylation recruits sororin, which directly competes with WAPL for PDS5 binding and thus prevents DNA release (Rankin, Ayad and Kirschner, 2005; Schmitz *et al.*, 2007; Nishiyama *et al.*, 2010; Ladurner *et al.*, 2016) (Fig. 3B).

While a subset of the cohesin complexes becomes cohesive in S-phase, an even larger subset remains dynamically associated with the DNA to form loops. Recently, it has become clear that cohesin-mediated loop extrusion determines the location of origins of DNA replication (Emerson *et al.*, 2022). Replication fork components may in turn act as a barrier for loop extrusion by these complexes (Dequeker *et al.*, 2022; Jeppsson *et al.*, 2022), possibly through direct interaction with MCM helicase complexes, which are key factors in the replisome (Dequeker *et al.*, 2022) (Fig. 3C). DNA replication and cohesin apparently are interwoven in many ways. Future work will be needed to fully disentangle the many connections between replication, cohesin-mediated loop extrusion and sister chromatid cohesion.

Repairing DNA breaks

The genome is susceptible to the formation of DNA breaks, either as a consequence of endogenous processes such as DNA replication, or by exogenous sources such as ultraviolet radiation. It is vital that DNA breaks are repaired before cells enter mitosis, to avoid genome instability. G2 phase of the cell cycle is the optimal time to repair a DNA break, as an intact sister chromatid is available as a template for repair of double strand DNA breaks (DSBs) by the high-fidelity DNA repair pathway known as homologous recombination (HR). For HR to be successful, sister chromatids should be kept in proximity by cohesin. Depletion of cohesin subunits reduced the efficiency of DSB repair (Birkenbihl and Subramani, 1992; Sjögren and Nasmyth, 2001; Sonoda *et al.*, 2001; Bauerschmidt *et al.*, 2009), which could be related to cohesin's role in sister chromatid cohesion, but also to its role in chromatin looping.

When a DSB occurs, cohesin is recruited to the site in a manner dependent on the DNA damage response factors Mre11, γH2AX, Mec1 (known as ATR in animals and plants) and Scc2 (Kim *et al.*, 2002; Ström *et al.*, 2004, 2007; Ünal *et al.*, 2004; Unal, Heidinger-Pauli and Koshland, 2007). Sister chromatid cohesion can promote repair using the sister chromatid as a template, rather than using the homologous chromosome or other genomic regions in *trans* (Covo *et al.*, 2010; Piazza *et al.*, 2021). Although normally cohesion can only be established during S phase (Uhlmann and Nasmyth, 1998), in budding yeast DNA breaks initiate a new wave of cohesion establishment during G2, which occurs not only at the site of DNA damage, but throughout the genome, and in a manner that requires Eco1 (Ström *et al.*, 2007; Unal,

Heidinger-Pauli and Koshland, 2007). The relevance of this re-establishment of cohesion remains unknown, and has so far not been observed in human cells (Caron *et al.*, 2012).

Cohesin is important for DSB repair also in vertebrate cells (Sonoda *et al.*, 2001; Potts, Porteus and Yu, 2006; Schmitz *et al.*, 2007; Bauerschmidt *et al.*, 2009). NIPBL^{SCC2}–MAU2^{SCC4} is recruited to DSB sites by several independent pathways involving HP1γ and the kinases ATM and ATR (Bot *et al.*, 2017). This could suggest a role in DSB repair for cohesin recruitment, but potentially also for loop extrusion at these sites. Sister chromatid cohesion is important for promoting HR with specifically the sister chromatid (Gelot *et al.*, 2016). In addition to contributing to repair by connecting the sister chromatids, loop extrusion by cohesin may also contribute to other aspects of DNA repair, such as the controlled spreading of the DSB-associated histone phosphorylation mark γH2AX (Caron *et al.*, 2012; Arnould *et al.*, 2021). Interestingly, cohesin-SA2 but not cohesin-SA1 is recruited to DSBs and contributes to HR, suggesting that different cohesin variants have different roles in DSB repair (Kong *et al.*, 2014).

In yeast, SMC5–SMC6 complexes are also intricately involved at many stages of DNA repair (Lehmann *et al.*, 1995; Verkade *et al.*, 2001; Fujioka *et al.*, 2002; Andrews *et al.*, 2005). SMC5–SMC6 is loaded at DSB sites, which is facilitated by Mre11 (Betts Lindroos *et al.*, 2006). SMC5–SMC6 can recruit and SUMOylate cohesin at these DSBs, and hereby contributes to HR (De Piccoli *et al.*, 2006; Potts, Porteus and Yu, 2006; Wu *et al.*, 2012). SMC5–SMC6 complexes have additional roles in DSB repair. Through auto-SUMOylation, SMC5–SMC6 recruits and subsequently SUMOylates the Sgs1–Top3–Rmi1 complex (Bermúdez-López *et al.*, 2016; Bonner *et al.*, 2016; Agashe *et al.*, 2021). This complex in turn resolves HR intermediates known as Holliday junctions that occur during sister chromatid recombination (Bermúdez-López *et al.*, 2010, 2016; Chavez *et al.*, 2010; Menolfi *et al.*, 2015; Agashe *et al.*, 2021). SMC5–SMC6 can stably bind junctions of ssDNA and dsDNA, making it suited for binding Holliday junctions (Chang *et al.*, 2022; Tanasie *et al.*, 2022). This characteristic seems particularly important in stabilizing and rescuing stalled replication forks (Torres-Rosell *et al.*, 2005; Yong-Gonzales *et al.*, 2012).

SMC5–SMC6 seems to also regulate DNA repair in other, less understood manners. Recent papers show that the SMC5–SMC6 complex is required in S phase to prevent formation DNA gaps (Venegas *et al.*, 2020), and that NSE1's ubiquitylation function is required specifically during replication stress (Kolesar *et al.*, 2022). SMC5–SMC6 apparently also functions in the Fanconi anemia pathway to repair inter-strand crosslinks (Rossi *et al.*, 2020). Correspondingly, mutations in SMC5 or SLF2 lead to a neurodevelopmental disorder that is similar to disorders caused by mutations in the Fanconi anemia pathway (Grange *et al.*, 2022). A picture arises in which SMC5–SMC6 is a key player in supporting HR through the resolution of repair intermediates, thereby ensuring that DNA is repaired and replicated before cells enter mitosis.

Other lines of evidence suggest that condensin promotes DNA repair. Whereas condensin II might have a role in promoting HR (Wood *et al.*, 2007), condensin I has been reported to

cooperate with Poly [ADP-ribose] polymerase 1 in interphase to facilitate repair of single strand DNA breaks (Heale *et al.*, 2006; Kong *et al.*, 2011). Condensin I is also depleted from DNA at under-replicated sites in mitosis, possibly to allow for mitotic DNA synthesis and the completion of replication at these sites (Boteva *et al.*, 2020). Although recent years have clearly yielded new insights into how SMC complexes control DNA repair, we are only beginning to grasp how SMC complexes actually contribute to repair and how these different activities may then be regulated.

Shaping and dividing chromosomes during mitosis

When cells enter mitosis, chromosomes prepare for arguably the most dramatic act of chromosome gymnastics, namely the segregation of the sister chromatids to the opposite poles of the cell. SMC complexes have a crucial role in many of the processes leading up to chromosome segregation.

(Un)knotting DNA molecules

Catenanes are a natural consequence of replication. These linkages between sister chromatids need to be resolved before chromosome segregation through a process called sister chromatid resolution. The resolution of the sister chromatids begins from S phase onwards (Ono, Yamashita and Hirano, 2013; Nagasaka *et al.*, 2016) and is achieved by the enzyme topoisomerase 2A (TOP2A). A recent preprint suggests that cohesin-mediated loop extrusion initiates the resolution of the sister chromatids in G2 (Batty *et al.*, 2023). At the onset of mitosis, condensin can access the DNA and aid in this process by promoting TOP2A-dependent decatenation (D'Ambrosio *et al.*, 2008; Charbin, Bouchoux and Uhlmann, 2014; Dyson *et al.*, 2021) (Fig. 4A). Condensin might stimulate TOP2A by altering the chromatin environment (Baxter *et al.*, 2011) to provide directionality in DNA movement and to thereby promote the unknotting reaction over re-knotting of the close sister chromatids (Sen *et al.*, 2016; Piskadlo, Tavares and Oliveira, 2017). Condensin might also localize TOP2A to the chromatid axis (Coelho, Queiroz-Machado and Sunkel, 2003; Hudson *et al.*, 2003; Ono *et al.*, 2017). The SUMOylation activity of SMC5–SMC6 is also important for sister chromatid resolution, and this complex seems to physically interact with TOP2A (Verver *et al.*, 2016; Deiss *et al.*, 2019).

The activity of topoisomerases is not restricted to sister chromatid resolution. Following chromatid individualization, topoisomerases generate new entanglements within chromosome arms, which promotes the compaction of mitotic chromosomes (Shintomi and Hirano, 2022). Mitotic chromosome arms indeed exhibit many catenanes within their individual chromosome arms, as a recent preprint shows (Hildebrand *et al.*, 2022). Overall, the data would fit with a model in which condensin directs topoisomerases to individualize the chromatids by decatenation, and in which topoisomerases subsequently aid with chromosome compaction by catenating stacked DNA loops (Fig. 4A).

Condensation and segregation

Condensin complexes and topoisomerases are crucial for forming individualized and condensed mitotic chromosomes (Hudson *et al.*, 2003; Ono *et al.*, 2003, 2017; Shintomi and Hirano, 2011, 2022; Green *et al.*, 2012; Houlard *et al.*, 2015; Shintomi *et al.*, 2017; Samejima *et al.*, 2018)(Fig. 4A). Each of the two condensin complexes has its own role. At mitotic onset, condensin II becomes more stably bound to DNA, which allows it to form long chromatin loops (Gerlich, Hirota, *et al.*, 2006; Gibcus *et al.*, 2018). This shift in condensin II stability on DNA happens because the condensin II release factor microcephalin is inactivated, possibly through cyclin-dependent kinase 1 (CDK1)-mediated phosphorylation (Houlard *et al.*, 2021).

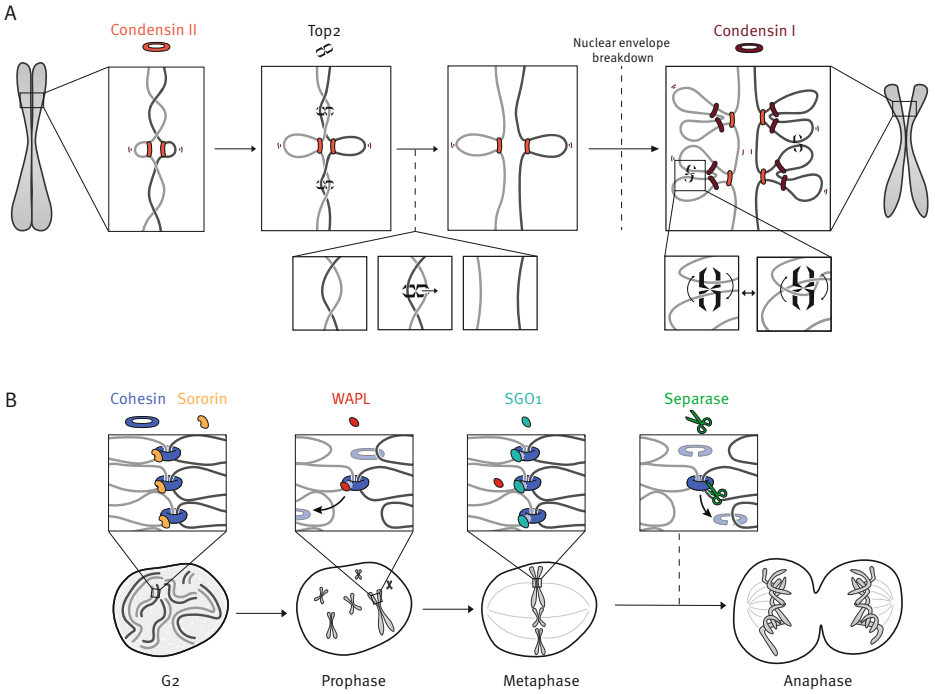


Figure 4 | Mitotic chromatin condensation, sister chromatid resolution and chromosome segregation. Schematic overview of key mechanisms involved in chromosome segregation. (A) At the onset of mitosis, condensin II starts forming chromatin loops, which promotes topoisomerase 2A (TOP2A)-mediated sister chromatid resolution (see inset) and formation of thread-like chromosomes in mitotic prophase. Following nuclear envelope breakdown, condensin I sub-divides the chromatin loops made by condensin II into smaller, nested loops. DNA regions that are close together (within the same sister chromatid) are knotted and unknotted by a cycle of TOP2A activity (see inset), which promotes further compaction of the chromatin. (B) Sister chromatids are held together by cohesin from S phase onwards. This cohesion is protected by sororin, and in mitosis centromeres are protected by shugoshin 1 (SGO1). When cells enter prophase, cohesin is phosphorylated (not shown) and WAPL can remove cohesin along chromosome arms through a process called the prophase pathway, which also allows the mechanisms described in part A to fully resolve the sister chromatids. At the metaphase-to-anaphase transition, separase is activated and cleaves the cohesin complexes, which remained at the centromeres. Cohesin cleavage triggers the segregation of the sister chromatids to the two newly formed daughter cells.

Most condensin I complexes are cytoplasmic and can only access DNA following the breakdown of the nuclear envelope, at the end of prophase (Hirota *et al.*, 2004; Ono *et al.*, 2004; Walther *et al.*, 2018). Condensin I then divides the long condensin II-mediated loops into smaller, nested chromatin loops (Gibcus *et al.*, 2018) (Fig. 4A). In contrast to condensin II, condensin I has a short residence time on chromosomes throughout mitosis (Gerlich, Hirota, *et al.*, 2006). Together these complexes form rod-shaped and rigid mitotic chromosomes, with an axis of condensin II and TOP2A surrounded by condensin I complexes (Ono *et al.*, 2003; Gibcus *et al.*, 2018; Walther *et al.*, 2018). In absence of functional condensin I and condensin II, no rod-shaped mitotic chromosomes can be formed, and instead the chromatin in mitosis becomes a single compact mass (Ono *et al.*, 2003; Samejima *et al.*, 2018). Depletion of either condensin I or condensin II gives quite distinct phenotypes. Whereas condensin I depletion results in fuzzy, undefined chromosomes, chromosomes lacking condensin II are elongated and lack a defined axis, with a zigzag-like appearance (Ono *et al.*, 2003; Shintomi and Hirano, 2011; Houlard *et al.*, 2015). The current model thus is that condensin II forms a chromosome axis and is required for axial shortening, whereas condensin I compacts the width of the chromosomes.

The rigidity of mitotic chromosomes seems essential for chromosome segregation, as depletion of condensin subunits leads to missegregations (Hudson *et al.*, 2003; Oliveira, Coelho and Sunkel, 2005; Green *et al.*, 2012; Piskadlo, Tavares and Oliveira, 2017). It seems that this rigidity is particularly important in the region surrounding the centromeres, as depletion of condensin results in severe stretching of the centromeric region and in a large increase in inter-kinetochore distance (Ono *et al.*, 2004; Oliveira, Coelho and Sunkel, 2005; Gerlich, Hirota, *et al.*, 2006; Houlard *et al.*, 2015; Sacristan *et al.*, 2022). If the centromere is not rigid enough, it cannot withstand the pulling forces from the mitotic spindle, resulting in improperly segregated chromosomes (Oliveira, Coelho and Sunkel, 2005; Samejima *et al.*, 2018).

Whereas condensin condenses and drives individualization of chromatids, the bulk of cohesin is removed from chromosome arms through a pathway known as the prophase pathway (Losada, Hirano and Hirano, 1998; Waizenegger *et al.*, 2000; Gandhi, Gillespie and Hirano, 2006; Kueng *et al.*, 2006). Phosphorylation of sororin and SA2 during early mitosis allows WAPL to release cohesin from chromosome arms (Losada, Hirano and Hirano, 2002; Sumara *et al.*, 2002; Giménez-Abián *et al.*, 2004; Hauf *et al.*, 2005; Nishiyama *et al.*, 2010, 2013; Dreier, Bekier and Taylor, 2011; Zhang *et al.*, 2011; Liu, Rankin and Yu, 2012) (Fig. 4B). The prophase pathway provides the opportunity for TOP2A to remove remaining catenanes between the sister chromatids at the chromosome arms, which are now no longer kept together by cohesin. Removal of cohesin from the chromosome arms also promotes the focusing of the kinase Aurora B to centromeres to promote correction of spindle-attachment errors (Haarhuis *et al.*, 2013; Tedeschi *et al.*, 2013; Chu *et al.*, 2022). The prophase pathway also appears to be important for evacuation of elongating RNA polymerase II, thereby shutting down transcription in a manner that is poorly understood (Perea-Resa *et al.*,

2020). The cohesin at centromeres however remains protected by shugoshin 1 (SGO1) (Salic, Waters and Mitchison, 2004; Tang *et al.*, 2004; McGuinness *et al.*, 2005), and these protected complexes are essential to keep the sister chromatids connected until the onset of anaphase (Fig. 4B). SGO1 has a dual protective function: it recruits protein phosphatase 2A to centromeres, to keep cohesin in a hypo-phosphorylated state at these sites (Kitajima *et al.*, 2006; Riedel *et al.*, 2006); and it also directly antagonizes Wapl by occupying cohesin's SA–Scc1 binding interface (Hara *et al.*, 2014). We note that the YxF motif that SGO1 uses to bind this SA-Scc1 interface is shared by CTCF and other cohesin regulatory factors (Li *et al.*, 2020; Dequeker *et al.*, 2022; García-Nieto *et al.*, 2023). Binding to the conserved SA–SCC1 interface could thus reflect a universal mechanism by which cohesin is locally regulated to control genome topology.

Once the chromosomes are attached to the mitotic spindle in a bi-oriented manner, the spindle assembly checkpoint is satisfied, and the anaphase promoting complex (also known as the cyclosome) will degrade its target proteins (Peters, 2006). The coordinated destruction of its two main targets, securin and cyclin B, is key to a successful mitosis. Destruction of securin unleashes the protease separase (also known as separin) so it can cleave the cohesin subunit $SCC1^{RAD21}$ (Fig. 4B). The destruction of cyclin B1 inactivates CDK1, which allows segregation to take place with stable microtubule–kinetochore attachments (Oliveira *et al.*, 2010; Vázquez-Novelle *et al.*, 2014). Recent work reveals that the CDK1–cyclin B–CKS1 complex is also a direct inhibitor of separase (Yu *et al.*, 2021). A third negative regulator of separase in mammals is the SGO2–MAD2 complex (Hellmuth *et al.*, 2020). Once the inhibitors of separase are all inactivated, it is free to proteolytically cleave $SCC1^{RAD21}$, which destroys the remaining sister chromatid cohesion and triggers anaphase and timely cell division by allowing the segregation of the sister chromatids to the opposite poles of the cell (Uhlmann, Lottspelch and Nasmyth, 1999; Uhlmann *et al.*, 2000; Oliveira *et al.*, 2010) (Fig. 4B). This process marks the beginning of what at face value appears to be a new, fresh journey through another cell cycle from G1 to mitosis: a process that can go on and on to shape whole organisms.

CONCLUSION AND FUTURE PERSPECTIVE

Over the past decades it has become obvious that the family of SMC complexes is indispensable for many major chromosomal processes. We have learned how these complexes not only act at the time of chromosome segregation, but prepare the chromosomes for this major event already during the preceding cell cycle phases. It is fascinating how the different SMC complexes can essentially function in a similar manner by forming DNA loops, but have evolved to control quite different processes, which begs the question: why have we evolved three unique complexes to perform all these functions? Why is there not just one SMC complex, which can be regulated to perform each of its functions through post-translational modifications?

Might the answer lie in the timing of when the complexes are most vital? Cohesin is removed from chromosomes just when condensin is working at its hardest to keep the chromosomes compacted. Or is the fact that the three complexes have distinct roles in DNA repair the reason for their divergence? Or might there be a yet unidentified essential interplay between the different complexes? It will be a wonderful adventure to learn more about these complexes and their many vital and distinct functions. New insights will without a doubt reveal how SMC complexes are regulated to adjust genome topology in different tissues and developmental stages.

Box 1: Evidence supporting loop extrusion by SMC complexes

The hypothesis that structural maintenance of chromosomes (SMC) complexes configure chromatin through a mechanism of processive loop enlargement was first put forward in 2001 (Nasmyth, 2001). This at the time hypothetical mechanism was later named loop extrusion (Alipour and Marko, 2012), and was shown by *in silico* modelling to explain in principle many aspects of chromosome biology (Sanborn *et al.*, 2015; Fudenberg *et al.*, 2016; Goloborodko, M. V. Imakaev, *et al.*, 2016). Over the past half-decade or so, a range of cellular and biophysical experiments have provided overwhelming experimental support that SMC complexes indeed shape chromatin by means of loop extrusion. Below we provide an overview of the key experimental insights in support of the loop extrusion model.

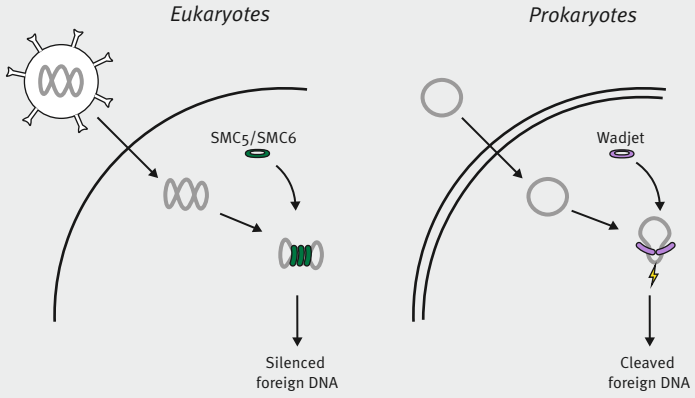
The chain of experiments that led to the demonstration that SMC complexes can shape chromatin through loop extrusion arguably started with the finding that CTCF binding sites are connected in a convergent orientation (Rao *et al.*, 2014; Vietri Rudan *et al.*, 2015). As such CTCF sites often lie far (up to megabases) apart, there had to be a linear DNA scanning mechanism that connects CTCF sites in convergent orientation. A key prediction of the loop extrusion hypothesis is that loops grow in size as a function of time. This prediction was tested by depletion of the cohesin release factor WAPL, which led to stabilization of cohesin on DNA and correspondingly to an increase in loop length genome-wide (Gassler *et al.*, 2017; Haarhuis *et al.*, 2017; Wutz *et al.*, 2017). Further key data revealed that in bacteria, movement of the Smc complex along chromosome arms corresponds with the tethering of DNAs, leading to the formation of a DNA loop (Wang *et al.*, 2017). The first direct evidence that SMC complexes can extrude loops was ultimately obtained with *in vitro* loop extrusion experiments using budding yeast condensin complexes (Ganji *et al.*, 2018). Since then, insightful *in vitro* experiments have shown that cohesin, condensin I, condensin II and, according to a recent preprint, also SMC5–SMC6 can extrude DNA loops (Davidson *et al.*, 2019; Kim *et al.*, 2019; Kong *et al.*, 2020; Pradhan, Kanno, *et al.*, 2022).

Many chromosomal features can be explained through loop extrusion by SMC complexes. Cohesin is required for the formation and maintenance of genomic loop domains known

as topologically associating domains (TADs)(Gassler *et al.*, 2017; Haarhuis *et al.*, 2017; Rao *et al.*, 2017; Schwarzer *et al.*, 2017; Wutz *et al.*, 2017). The existence of this genome-wide Hi-C feature can be explained if one considers that TADs could simply reflect collections of chromatin loops of different sizes between two convergent CTCF sites (Sanborn *et al.*, 2015; Fudenberg *et al.*, 2016). Additional chromosome conformation features appearing in Hi-C maps, such as architectural stripes and chromatin jets, can also be readily explained as one-sided cohesin-dependent loop extrusion from a CTCF site(Vian *et al.*, 2018; Guo *et al.*, 2022). Cohesin and its regulation by CTCF also has a vital role in processes such as V(D)J recombination and class switch recombination in adaptive immunity. This process requires a linear-DNA scanning mechanism, which again would be well explained by cohesin-mediated loop extrusion (Y. Zhang *et al.*, 2022).

Mitotic chromosomes are characterized by an array of consecutive chromatin loops (Naumova *et al.*, 2013), which are dependent on condensin complexes. Analogous to cohesin, increasing the stability of condensin II on the DNA likewise leads to the formation of longer-range DNA contacts (Houlard *et al.*, 2021). Computational modelling supports the notion that loop extrusion explains the compacted and individualized status of mitotic chromosomes (Alipour and Marko, 2012; Goloborodko, M. v. Imakaev, *et al.*, 2016). Other models cannot explain why condensin complexes would only form interactions within a sister chromatid, and not between sister chromatids. Loop extrusion best explains all the *in vivo* phenomena described above.

Box 2: Preserving and protecting the genome



In recent years, structural maintenance of chromosomes (SMC) complexes were found to be vital for the defense against pathogens. The best-known role for SMC complexes

in the immune system involves cohesin-mediated chromatin-loop extrusion in support of V(D)J recombination in B cells, as discussed in the main text (Fig. 2C). The SMC5–SMC6 complex also has an essential role in protecting our genome against a wide range of viruses. It does so not by structuring the human genome, but by binding to the invading viral genome (see the **Figure**). To date, SMC5–SMC6 has been identified as a host restriction factor against human immunodeficiency virus (Dupont *et al.*, 2021; Irwan, Bogerd and Cullen, 2022), hepatitis B virus (Decorsière *et al.*, 2016), human papillomavirus (Bentley *et al.*, 2018; Gibson and Androphy, 2020), herpes simplex virus (Xu *et al.*, 2018), Epstein Barr virus (Yiu *et al.*, 2022) and Kaposi's sarcoma-associated herpesvirus (Han *et al.*, 2022). In fact, various types of extrachromosomal DNA appear to be targeted by the SMC5–SMC6 complex (Decorsière *et al.*, 2016; Abdul *et al.*, 2022). To permit their propagation, many viruses hijack the host proteasomal degradation system in order to target SMC5–SMC6 for ubiquitylation and subsequent degradation (Decorsière *et al.*, 2016; Murphy *et al.*, 2016; Abdul *et al.*, 2018; Dupont *et al.*, 2021; Han *et al.*, 2022; Yiu *et al.*, 2022).

How does the SMC5–SMC6 complex defend against viral pathogens? SMC5–SMC6 transcriptionally silences the unintegrated and/or episomal viral DNA. It seems likely that the mechanism of silencing is common for all viruses, yet there are conflicting reports on whether this silencing requires SUMOylation by the SMC5–SMC6 accessory subunit NSE2 (Abdul *et al.*, 2022; Irwan, Bogerd and Cullen, 2022). Other functions of SMC5–SMC6, such as the compaction of the viral DNA, or changing the epigenetic landscape of the viral genome, also seem to be common in response to different viruses (Dupont *et al.*, 2021; Han *et al.*, 2022). Silencing is dependent on DNA binding and ATPase activity of the SMC5–SMC6 complex (Abdul *et al.*, 2022; Han *et al.*, 2022), possibly implicating SMC5–SMC6-mediated loop extrusion in this process. SMC5–SMC6 can also localize viral DNA to promyelocytic leukemia bodies, which might facilitate gene silencing (Abdul *et al.*, 2022).

SMC5–SMC6 interestingly appears to share its ability to protect against foreign DNA molecules with another SMC complex, the bacterial Wadjet SMC protein complex, which is an important part of the bacterial anti-plasmid defense (Doron *et al.*, 2018; Deep *et al.*, 2022). Wadjet contains a topoisomerase-like subunit, jetD, which can irreversibly cleave foreign DNA (Deep *et al.*, 2022) (see the **Figure**). This function is crucial for the anti-plasmid defense and was recently shown in a preprint to be dependent on the motor function of Wadjet, again implicating loop extrusion as a mechanism required for this protection (Liu *et al.*, 2022). Interestingly, Wadjet has a preference for closed circular DNA, with a maximum size of 100 kb (Deep *et al.*, 2022; Liu *et al.*, 2022). With cohesin promoting adaptive immunity through V(D)J recombination and class switch recombination, and SMC5–SMC6 and Wadjet protecting against foreign DNAs, it seems to be a common theme that SMC complexes are vital for life's defense mechanisms.

ACKNOWLEDGEMENTS

Due to space limitations, the authors apologize for not being able to cite all relevant articles or discuss all aspects of SMC biology. The authors acknowledge financial support from the European Research Council (772471-CohesinLooping) and from the Dutch Research Council (VI.C.202.098), and thank Anoek Friskes and members of the Rowland laboratory for critical reading of the manuscript.

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Chapter 3

3D genomics across the tree of life reveals condensin II as a determinant of architecture type

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Adapted from Hoencamp et al. 3D genomics across the tree of life reveals condensin II as a determinant of architecture type. Science* **372, *984-989 (2021).*

ABSTRACT

We investigated genome folding across the eukaryotic tree of life and find two types of three-dimensional (3D) genome architectures at the chromosome scale. Each type appears and disappears repeatedly during eukaryotic evolution. The type of genome architecture that an organism exhibits correlates with the absence of condensin II subunits. Moreover, condensin II depletion converts the architecture of the human genome to a state resembling that seen in organisms such as fungi or mosquitoes. The condensin II complex is best known for its role in mitotic chromosome formation. We here show that this mitotic role of condensin II is likely what prevents centromere clustering and thus determines architecture type of the subsequent interphase. This mechanism likely has been conserved since the last common ancestor of all eukaryotes.

INTRODUCTION

The proper organization of DNA in the 3D nucleus is crucial for controlling processes ranging from gene expression and DNA replication to chromosome segregation (Bonev and Cavalli, 2016; Dekker and Mirny, 2016; Piskadlo and Oliveira, 2016; Carré-Simon and Fabre, 2022). To facilitate these vital processes, DNA folding is regulated at different scales. At small-scale, chromatinized DNA is structured into DNA loops. Collections of loops between boundary elements are called loop domains or TADs. These loop domains are then segregated into active and inactive compartments, based on their transcriptional activity and histone modifications. At the largest scale, the genome is partitioned into chromosomes. While recent work has yielded important insight into the mechanisms that drive genome folding at the scale of chromatin loops (Haarhuis *et al.*, 2017; Rao *et al.*, 2017; Ganji *et al.*, 2018; Gibcus *et al.*, 2018; Davidson *et al.*, 2019; Golfier *et al.*, 2019; Kim *et al.*, 2019), and in the processes controlling compartmentalization (Larson *et al.*, 2017; Strom *et al.*, 2017; MacPherson, Beltran and Spakowitz, 2018; Plys *et al.*, 2019), the cellular mechanisms controlling "macro"-scale genome organization at the level of whole chromosomes remain poorly understood.

In human cells, each chromosome occupies a discrete sub-volume in the nucleus, excluding other chromosomes. Each of these sub-volumes is referred to as a chromosome territory (Cremer and Cremer, 2010; Dekker and Mirny, 2016; Rowley and Corces, 2018; Yatskevich, Rhodes and Nasmyth, 2019). In situ hybridization techniques such as FISH provided experimental evidence for the existence of such chromosome territories (Cremer and Cremer, 2010). It has been suggested that chromosome territories are a remnant of the individuality of chromosomes during mitosis (Boveri, 1909). The borders of chromosome territories do however partially overlap, providing opportunities to regulate gene expression in *trans*, but also for translocations to occur (Roix *et al.*, 2003; Branco and Pombo, 2006; Engreitz, Agarwala and Mirny, 2012; Zhang *et al.*, 2012; Roukos, Burman and Misteli, 2013; Bashkirova and Lomvardas, 2019; Rosin *et al.*, 2019).

The family of SMC complexes has proven to be vital in the organization of the 3D genome at different stages of the cell cycle. In interphase, the cohesin complex forms DNA loops and thereby create loop domains. In mitosis, condensin complexes also form loops to build rigid mitotic chromosomes, which is required for proper chromosome segregation (Uhlmann, 2016; van Ruiten and Rowland, 2018; Yatskevich, Rhodes and Nasmyth, 2019). Two condensin complexes work in a concerted manner to shape mitotic chromosomes: condensin I and condensin II. Both condensin complexes share the SMC2-SMC4 heterodimer, but differ in their accessory subunits. These complexes also differ in their cellular localization: condensin II is nuclear in interphase and can access the DNA throughout the cell cycle, whereas condensin I is cytoplasmic and can thus only reach the DNA upon nuclear envelope breakdown (Ono *et al.*, 2004). As condensin II is nuclear in interphase, and can theoretically shape the DNA by forming DNA loops also in this cell cycle stage, it has long been speculated condensin

II may control interphase genome organization (Fig. 1). Indeed, depletion of condensin II leads to changes in large-scale genome organization in a wide array of species, ranging from plants to mice (Bauer, Hartl and Bosco, 2012; Schubert, Lermontova and Schubert, 2013; Nishide and Hirano, 2014; Nguyen *et al.*, 2015; Iwasaki, Corcoran and Noma, 2016; Howard-Till and Loidl, 2018; Rosin *et al.*, 2018; Sakamoto *et al.*, 2019, 2022; Houlard *et al.*, 2021; Municio *et al.*, 2021). However, removal of condensin II from post-mitotic neurons does not affect the 3D genome (Abdennur *et al.*, 2018).

In this study, we reveal that condensin II is a key determinant of macro-scale genome architecture and that this is likely conserved throughout evolution. We use evolutionary 3D genomics to study genome architecture in species across the tree of life, and correlate this with condensin II status. We also directly examine the effect of condensin II loss on the 3D genome. This provides us with exciting new insights into the role of condensin II in interphase genome architecture.

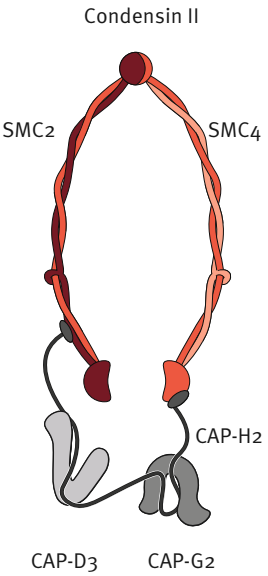

Figure 1 | The condensin II complex. A schematic overview of the different subunits of the condensin II complex.

RESULTS

Two major architecture types across the tree of life

To investigate the mechanisms controlling nuclear architecture at this scale of whole chromosomes, we performed *in situ* Hi-C on 24 species across the tree of life (Table S1). These species represent all subphyla of chordates, all 7 extant vertebrate classes, 7 out of 9 major animal phyla, as well as plants and fungi. Together, these species offer a comprehensive overview of nuclear organization since the last common ancestor of all eukaryotes.

The resulting maps reveal four features of nuclear architecture at the scale of whole chromosomes (Fig. 2 and Fig. S1). First, some species, such as the red piranha, exhibit enhanced contact frequency between loci on the same chromosome and depleted contact frequency between loci on different chromosomes. This is consistent with, though not necessarily identical to, classical chromosome territories. Second, species like the mosquitoes, exhibit prominent contacts between centromeres of different chromosomes. Third, species like the ground peanut, exhibit prominent contacts between telomeres. Finally, species like bread wheat exhibit an X-shape on the chromosomal map, indicating that the chromosome arms are folded back onto each other. We dub these last three features "Rabl-like", since they are reminiscent of the Rabl chromosome configuration (Rabl, 1885), in which centromeres cluster on one side of the nucleus and chromosome arms are arranged in parallel towards the other side of the nucleus.

Figure 2 | A comprehensive overview of genome organization across evolution. Aggregate chromosome analysis (ACA) on Hi-C maps of 24 species. ACA involves the scaling of chromosome arms to a uniform length and aggregating the signal of all intra- and inter-chromosomal contacts. The depicted species span three kingdoms: animals (yellow), fungi (blue) and plants (green); their evolutionary relationship is represented with a cladogram. Each corner shows an example ACA map and a schematic drawing of one of the four chromosome-scale features. The location of these example maps does not correspond to the architecture type of the closest species in the figure. Presence of the condensin II subunits in each species is indicated by solid black circles. Left to right: SMC2/SMC4/CAP-H2/CAP-G2/CAP-D3.

Chromosomes are widely diverse throughout evolution in terms of their size and the location of their centromere. This makes it difficult to identify or score the architectural features that these species exhibit. To aid in this process, we developed “Aggregate chromosome analysis” (ACA). This analysis rescales chromosome arms of all metacentric chromosomes to be the same length, and sums them up to form a representative aggregate map (Fig. S2). This map can then be used to score each of the four aforementioned features in an unbiased fashion, and thus identify which species exhibit what type of nuclear architecture (Fig. S3).

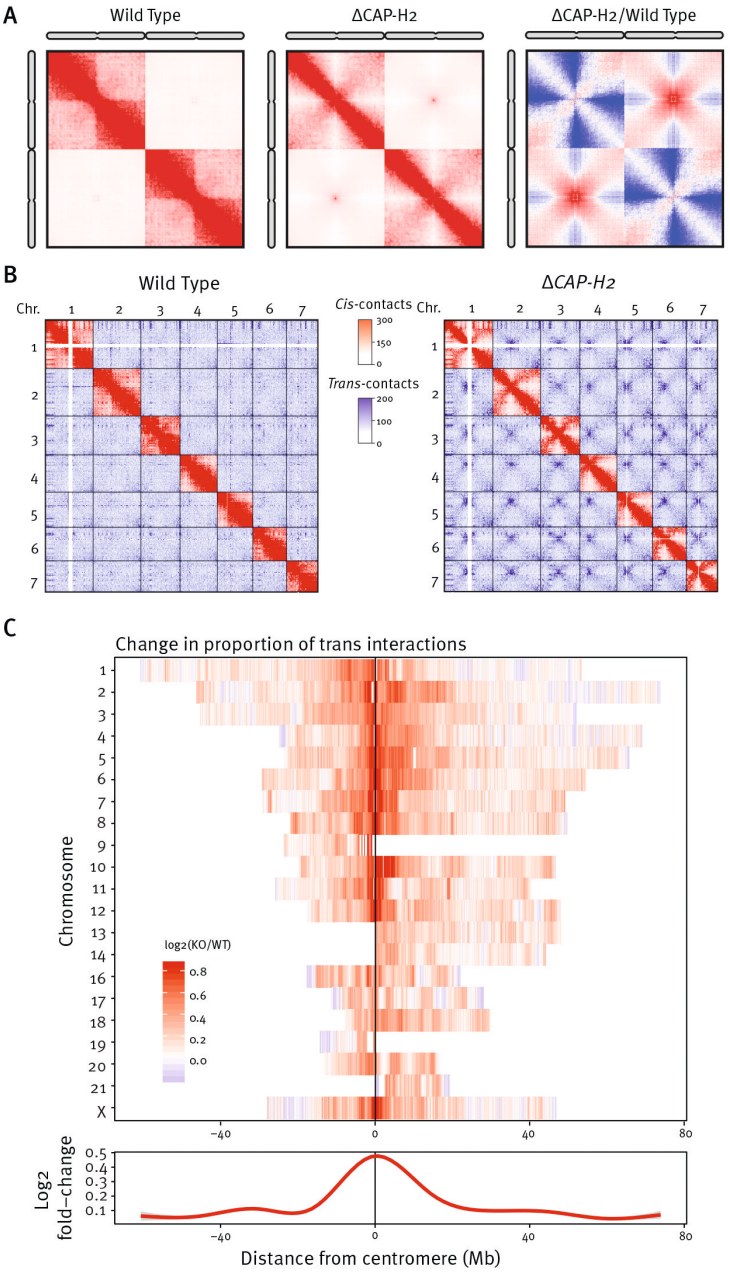

Figure 3 | Condensin II counteracts interactions between centromeres of different chromosomes. (A) ACA maps generated using the in situ Hi-C data from the experiment in Wild Type and ΔCAP-H2 Hap1 cell lines. Interactive version of this figure is available at https://tinyurl.com/y22r4m7g, and the 'native' Hi-C maps used to generate the ACA plots are available at https://tinyurl.com/y658rvkf. (B) Hi-C matrices of Wild Type and ΔCAP-H2 Hap1 cells. (C) Top panel: fold change (log2) of ΔCAP-H2 over Wild Type ratio of trans contacts to total contacts per chromosome at 100 kb bins. Lower panel: pile-up of the change in trans-contacts ratios across chromosomes binned at 100 kb. The median of changes is shown in red.

These results show that some features often co-occur, whereas other features rarely do. This allowed us to divide the features into two clusters, dubbed "Type I" and "Type II" architecture (Fig. S4). Type I architecture includes the three Rabl-like features: centromere clustering, telomere clustering, and a telomere-to-centromere axis. Type II architecture includes only chromosome territories. Species can thus also be subdivided depending on which features are more strongly exhibited (Table S2).

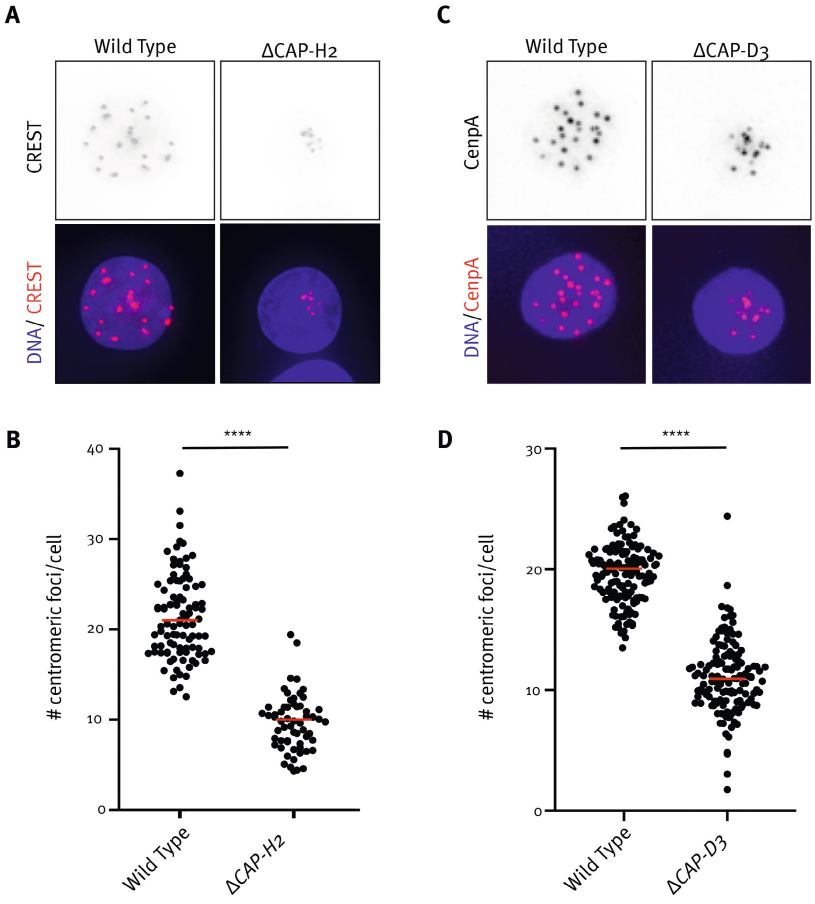

Figure 4 | Condensin II counteracts centromeric clustering. (A) Immunofluorescence of centromeres (CREST) and DNA (DAPI) in Wild Type and ΔCAP-H2 HAP1 cells as quantified in (B). (B). Quantification of the number of centromeric foci/cell in the depicted cell lines. Shown are 100 cells and the median. (C) Immunofluorescence of centromeres (CENPA) and DNA (DAPI) in Wild Type and ΔCAP-D3 HAP1 cells as quantified in (D). (D) Quantification of the number of centromeric foci/cell in the depicted cell lines. Shown are 100 cells and the median.

The data is consistent with a model in which individual chromosomal folding features appeared and disappeared over billions of years, as lineages switch between Rabl-like organization and territorial organization. From this it follows that it must be relatively

easy to switch from one type of architecture to the other, as it happened multiple times in evolution. This led us to investigate what this switch might be.

Condensin II loss and Rabl-like architecture have co-evolved throughout evolution

We noted that mosquitoes, which display Type I features (Fig. 2), also lack a subunit of the condensin II complex (King *et al.*, 2019). As condensin II can shape DNA in mitosis (Hirano, 2016), we wondered whether it can also regulate nuclear architecture type in interphase. We therefore searched for condensin II subunits in the genomes of all 24 species using a multi-step BLAST approach. Eight species lacked condensin II subunit(s) and all of these exhibited Rabl-like features (Fig. 2, Fig. S1 and Table S3). Since these organisms lie far apart on the evolutionary tree, Type I architectural features and the loss of condensin II subunits appear to have co-evolved repeatedly. Conversely, those species with more classical chromosome territories that do not display obvious *trans* contacts or foldback of the chromosome arms, mostly harbored all condensin II subunits. This striking correlation ($P<0.05$) could indicate that condensin II strengthens chromosome territories and/or counteracts Rabl-like features.

Humans exhibit Type II genome architecture, possessing strong chromosomal territories, and no Rabl-like features. Moreover, human genomes contain all condensin II subunits.

Would disruption of condensin II in human cells then interfere with chromosome territories and enhance the strength of Type I features? To test this, we performed *in situ* Hi-C on Hap1 cells lacking the condensin II subunit CAP-H2. Disruption of this core condensin II subunit prevents recruitment of the CAP-D3 and CAP-G2 subunits to the complex and renders the complex fully non-functional.

Δ*CAP-H2* cells exhibit striking changes in their large-scale chromosome organization (Fig. 3). First, there is a major increase in *trans* contacts, specifically between centromeres of different chromosomes (Fig. 3). As a consequence of the increase in *trans* contacts, chromosome territories are weakened. Moreover, the Δ*CAP-H2* cells have increased arm foldback, exemplified by the X-shape in the aggregate chromosome maps (Fig. 3A). The condensin II complex is thus required for counteracting *trans* contacts and promoting chromosome territories in human cells. The effects of this single disruption is particularly striking given that the most recent common ancestor of humans and any Type I organism lived 350 million years ago.

Condensin II controls large-scale genome architecture

Immunofluorescence microscopy further revealed that in Δ*CAP-H2* cells the centromeres are clustered together, confirming the results observed by Hi-C (Figs. 4A and 4B). Cells lacking the CAP-D3 subunit of condensin II exhibited centromere clustering to a similar extent (Figs. 4C and 4D, Fig. S5). It is thus disruption of the condensin II complex that transforms the folding of the human genome into a Type I-like configuration. This is a striking observation,

as it suggest that the relationship between condensin II loss and Type I organization is not merely a correlation, but might be causal throughout evolution.

Centromeric clustering requires the mitosis-to-anaphase transition

Condensin II is evidently essential for nuclear organization in interphase. This is a surprising finding, as previous research was not able to identify any functions for condensin II beyond mitosis (Abdennur *et al.*, 2018), and condensin II is negatively regulated in interphase by its removal factor MCPH1 (Houlard *et al.*, 2021). To assess whether condensin II determines nuclear architecture by acting in interphase, or whether its mitotic function is essential for shaping the interphase genome, we acutely depleted condensin II in HCT116 cells (Takagi *et al.*, 2018) at the G1/S transition, and either halted the cells prior to mitotic entry, or allowed the cells to progress through mitosis.

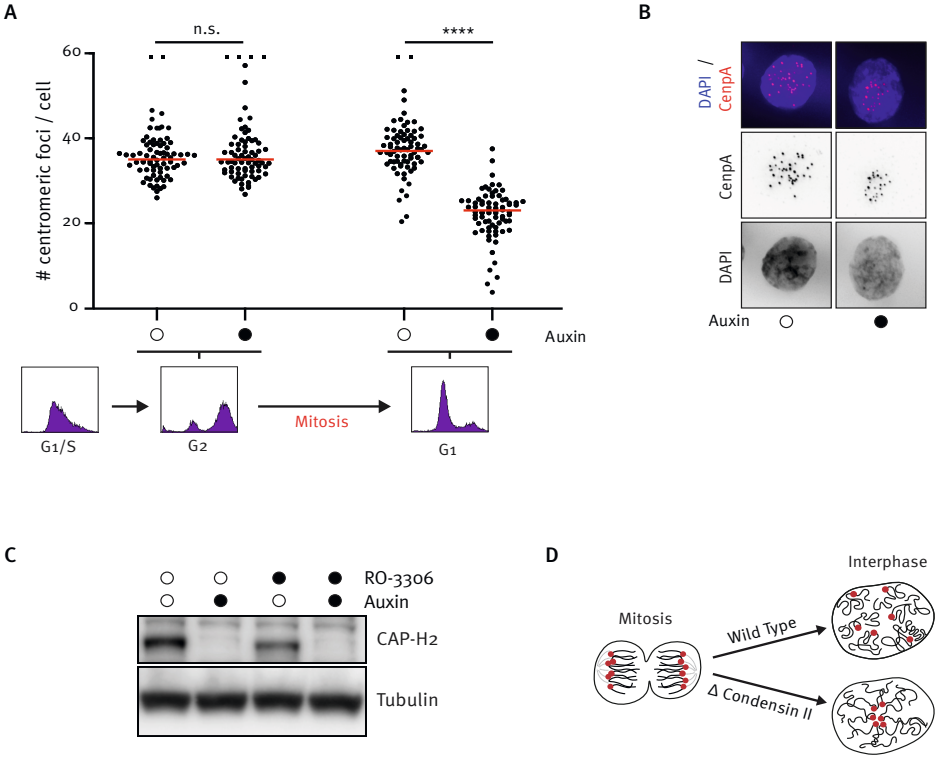

Figure 5 | Centromeric clustering requires mitosis-to-interphase transition. Circles depict drug treatment, black indicates addition of the indicated drug. (A) Quantification of centromeric foci before or after mitotic progression with/without condensin II. FACS plots depict cell cycle stages. Outliers (>60) were truncated and depicted as squares. n.s. = not significant. (B) Example images of G1 cells as quantified in (A). (C) Western blot analysis of the samples as quantified in (A). (D) Model depicting a role of condensin II in interphase genome organization.

When condensin II-depleted cells were halted prior to mitosis, centromeres did not cluster, consistent with condensin II depletion in post-mitotic cells not changing the 3D genome (Abdennur *et al.*, 2018). In contrast, progression through mitosis in absence of condensin II led to clear centromeric clustering in the subsequent G1 phase (Figs. 5A-C). This shows that condensin II must act in mitosis, or directly thereafter, to establish 3D genome organization for the next interphase (Fig. 5D).

DISCUSSION

Here, we provide key insights into the mechanisms by which large-scale genome architecture is regulated. Eukaryotes apparently can exhibit one of two architectural types: Rabl-like organization (Type I) or chromosome territories (Type II). These two architecture types have been gained and lost multiple times in evolution. We identify condensin II as a key player in determining the type of genome architecture a species exhibit. Removing condensin II in human cells, which naturally exhibit chromosome territories, leads to a switch in genome architecture to Rabl-like organization. We show that condensin II must act in or right after mitosis, as Rabl-like characteristics only appear when cells progress through mitosis without condensin II. Throughout evolution from yeast to mammals, condensin II absence correlates with Type I organization. We thus propose that condensin II is an evolutionarily conserved determinant of architecture type.

Preventing centromere clustering

We show that condensin II can prevent clustering of centromeres in human cells. However, the mechanism by which it does so remains unclear. Multiple hypotheses could explain this unclustering activity of condensin II. First, in various species, condensin can directly bind centromeres (Ono *et al.*, 2004; D'Ambrosio *et al.*, 2008; Nakazawa *et al.*, 2008). As SMC complexes are known to form DNA loops, condensin II might thus perform loop extrusion in the centromeric region. This could prevent clustering in two ways: (1) looping leads to constant movement of DNA sequences, and thereby might actively move apart parts of the centromere that otherwise would cluster together. (2) Activation of condensin II in interphase is thought to promote lengthwise compaction, which in turn could favor *cis* interactions over *trans* interactions (Bauer, Hartl and Bosco, 2012). Even though condensin II acts in mitosis rather than interphase, this hypothesis remains attractive. Lengthwise compaction of the chromatin might simply reduce the surface area of the centromeres, reducing the effective area that can cluster together.

Alternatively, condensin II might disrupt centromere clustering in a manner not related to chromatin looping. Possibly, condensin II's presence at the centromere may influence binding of other proteins at this location. These proteins may, when their binding is increased upon condensin II depletion, facilitate clustering directly or indirectly. As centromeric clustering requires mitotic progression, it is a possibility that such protagonists of centromeric clustering specifically localize to centromeres during mitosis.

An evolutionarily conserved mechanism for controlling genome architecture

Our finding that condensin II loss correlates with Type I architecture in species from yeast to mammals suggests that condensin II's function in keeping apart the centromeres is conserved throughout evolution. This is in line with previous key findings in a wide array of species. In the unicellular eukaryote *Tetrahymena thermophila* there are at least five different condensin complexes. Yet, depletion of the common SMC2 subunit leads to clustering together of the somatic homologs (Howard-Till and Loidl, 2018). While this is not the same as Rabl-like organization, it does underline the importance of condensin in disrupting *trans* interactions. *Schizosaccharomyces Pombe* (fission yeast) only has one condensin complex, which most resembles condensin I, and this species naturally exhibits Rabl-like organization. Yet further loss of condensin leads to more intermingling of chromosomes (Iwasaki, Corcoran and Noma, 2016). In plants, condensin II acts together with nuclear envelope proteins to facilitate centromere unclustering (Schubert, Lermontova and Schubert, 2013; Sakamoto *et al.*, 2019, 2022; Municio *et al.*, 2021). In mice condensin prevents the clustering together of heterochromatin chromocenters (Nishide and Hirano, 2014). And finally, in *Drosophila melanogaster* condensin II lacks the G2-subunit, and they naturally have Rabl-like architecture. Yet, condensin II still remains important for maintenance of chromosome territories and keeping apart centromeres (Bauer, Hartl and Bosco, 2012; Nguyen *et al.*, 2015; Rosin *et al.*, 2018). Excitingly, condensin II also is important for another type of 'unpairing activity' in *Drosophila Melanogaster* : it keeps apart the somatic homologs and thereby prevents transvection (Hartl, Smith and Bosco, 2008; Joyce *et al.*, 2012; Nguyen *et al.*, 2015; Vernizzi and Lehner, 2021). This might be due to its role in promoting territorialization and thereby individualization of chromosomes. Our preliminary data however suggests that in human cells, loss of condensin II does not lead to somatic homolog pairing of chromosomes 1 and 2 (Fig. S6). There might thus be additional factors that promote pairing in Drosophila, and that presumably are not active in humans.

All this data supports the notion that condensin II is a key player in large-scale genome organization. Yet, it cannot be the only determinant of architecture type. Whereas the species that lack condensin II all have Type I organization, there are also Type I species that do have condensin II, such as the ground peanut, stony coral or sea squirt. In these organisms, another change must allow for centromere clustering in presence of condensin II. Possibly other factors that normally counteract clustering have been lost in these species. Candidates for proteins with such centromere unpairing activity are nuclear lamina proteins, which are implicated in scattering of centromeres in both plants and yeast (Hou *et al.*, 2012; Hou, Kallgren and Jia, 2013; Sakamoto *et al.*, 2022). On top of that, recent comparative genomics in vertebrates has found a correlation between genome size and chromosome length with chromosome territory formation (Li *et al.*, 2022).

In this study, we have looked at a single type of material of each species for the *in situ* Hi-C, which differed between species. It must be noted that this is a limited view of an organism.

Since architecture type can be so easily switched back and forth, it is quite possible that different tissues may have different architecture types. An example for this can be seen in early spermatocytes of Drosophila, where condensin II promotes chromosome territory formation and centromere unclustering (Vernizzi and Lehner, 2021). A recent study described Rabl-like configuration for the Tamar Wallaby (Álvarez-González *et al.*, 2022), which is in contrast with our data in which we find chromosome territories in the Tamar Wallaby. This discrepancy might be explained by the different tissues, blood and a fibroblast cell line respectively, that were examined. This underlines the complexity of investigating 3D genome architecture in whole organisms. In different settings, different factors may contribute to architecture type. We may thus merely have scratched the surface in our understanding of this type of nuclear organization.

Interestingly, drastic changes in large-scale organization have been observed before in rod cells of nocturnal animals. These cells have inverted nuclei: a type of organization where euchromatin moves towards the nuclear exterior and heterochromatin towards the nuclear interior. This type of architecture is reminiscent of Rabl-like architecture (Falk *et al.*, 2019). This brings forth the exciting prospect that architecture type can be switched easily to facilitate specific processes in for example development or disease.

The role of condensin II in establishing the overall architecture of the genome appears to be among the most ancient capabilities defining genome folding in the eukaryotic lineage. Changes in condensin II have likely contributed to notable shifts from chromosome territories to Rabl-like features throughout the tree of life. As our exploration of the tree of life continues, one of the many fruits will be a deeper knowledge of our own cellular machinery.

ACKNOWLEDGEMENTS

This study is dedicated to the memory of our colleague, José Luis Gómez-Skarmeta. This is a SeaWorld technical manuscript contribution number 2020-12. We thank M. Takagi from the Cellular Dynamics Laboratory at RIKEN for sharing the CAP-H2-AID cell lines; M. Mertz from the NKI BioImaging Facility for advice on image analyses; the NKI Genomics core facility for sequencing; R. B. Gasser and P. K. Korhonen, Faculty of Veterinary and Agricultural Sciences, University of Melbourne, for their help with the oriental liver fluke sample; J. Alfoldi, J. Johnson, A. Berlin, S. Gnerre, D. Jaffe, I. MacCallum, S. Young,B. J. Walker, J. L. Chang, and E. S. Lander at the Broad Institute Genome Assembly & Analysis Group, Computational R&D Group, and Sequencing Platform for their contribution to the Aplysia californica draft genome assembly project; N. Watanabe at Kyoto University for providing the African clawed frog cell line to the Kornberg laboratory; V. Patel at Baylor Genetics for help with sequencing; and A. Fotos from adamfotos.com for help with figures.

MATERIALS & METHODS

Chromosome-length genome assemblies for 24 species across the tree of life

To learn about the general principles of nuclear architecture at chromosome-scale across the tree of life, we used *in situ* Hi-C (Rao *et al.*, 2014) to characterize the nuclear architecture of 24 species spanning three different kingdoms, 7 representative animal phyla including all 7 extant vertebrate classes (see Table S1).

In 10 out of 24 species, we generated the *in situ* Hi-C data and aligned it to previously published chromosome-length genome assemblies (for three of these, the yellow fever mosquito, the southern house mosquito and the ground peanut the assemblies are available from our prior work), see Table S1.

For the remaining 14 species (Burmese python, red piranha, bamboo shark, Arctic lamprey, European lancelet, purple sea urchin, water bear, California sea hare, oriental liver fluke, sea gooseberry, stony coral, common mushroom, tammar wallaby and moss animal) there was no existing chromosome-length reference genome assembly available.

Crucially, in addition to its use in examining nuclear architecture, *in situ* Hi-C data can also be used to reliably produce chromosome-length scaffolds from fragmentary genome assemblies (Burton *et al.*, 2013; Dudchenko *et al.*, 2017). We therefore combined the *in situ* Hi-C data with Juicer (Durand *et al.*, 2016), 3D-DNA (Dudchenko *et al.*, 2017) and Juicebox Assembly Tools (Dudchenko *et al.*, 2018), in order to produce highly accurate chromosome-length scaffolds for 12 species with available fragmentary genome assemblies. For the remaining two species the fragmented drafts were generated as part of this study. The software packages used were: w2rap-contigger (Clavijo *et al.*, 2017) for the tammar wallaby, contigging performed from short insert-size DNA-Seq Illumina library, and Flye (Kolmogorov *et al.*, 2019) for the moss animal, contigging performed from Pacific BioSciences long-read data. Small improvements were also made to the existing chromosome-length assembly of the sea squirt (Satou *et al.*, 2019).

iTOL Interactive Tree of Life tool was used to create phylogenetic trees in Figs. 2 and S1(Letunic and Bork, 2019). The topology is shown according to (Giribet, 2013) with additional data from (Baldauf and Palmer, 1993; Haussler *et al.*, 2009; Laumer *et al.*, 2015). (See (Yoshida *et al.*, 2017; Marlétaz *et al.*, 2019) for discussions on alternative resolution of Spiralia and Ecdysozoa.)

Aggregate chromosome analysis

To characterize chromosome-scale folding features we have developed a method dubbed Aggregate chromosome analysis (ACA) in which, for each metacentric chromosome in the genome, we rescale each chromosome arm to a uniform length, and then aggregate the

signal from all intra- and inter-chromosomal contacts (Fig. S2). For the purposes of this analysis a chromosome is considered metacentric if the centromere position C is such that $C > L/3$ && $C < 2L/3$ where L denotes the length of the chromosome.

The signal is processed in a fashion that removes asymmetries associated with any individual chromosome's order and orientation in the assembly. For example, consider a Hi-C contact between loci with genomic coordinates x on chromosome A (genomic length L_A, centromere position C_A, $x < C_A$) and y on chromosome B (genomic length L_B, centromere position C_B, $y < C_B$). Each contact $\{x, y\}$ will map onto 8 positions, $\{x^*, y^*\}$, $\{x^*, 1-y^*\}$, $\{1-x^*, y^*\}$, $\{1-x^*, 1-y^*\}$, $\{y^*, x^*\}$, $\{y^*, 1-x^*\}$, $\{1-y^*, x^*\}$, $\{1-y^*, 1-x^*\}$, where $x^* = \frac{1}{2} * x/C_A$ and $y^* = \frac{1}{2} * y/C_B$ (Fig. S2, https://tinyurl.com/y35qtu43). Similarly, each intrachromosomal contact point is mapped into 4 positions that symmetrize across chromosome arms (each scored x2 to control for coverage).

Note that when aggregation is performed across N chromosomes, $N > 2$, the intensity of the intra-chromosomal signal is proportional to $N/2$ while the intensity of the inter-chromosomal signal is proportional to $N*(N-1)/2$. As such, for the inter- and intra-chromosomal portions of the map to have relative intensities representative of those of any given pair of chromosomes, the intra-chromosomal signal has to be amplified by a factor of $N - 1$.

The result is an aggregate map that shows the interaction pattern representative of the genome, across two identical isochromosomes (Figs. 2 and S2). In addition to Fig. 2, ACA maps for all 24 species from this study are available at https://tinyurl.com/y5yrozfa.

The custom script to perform the aggregation is available on Zenodo as part of 3D-DNA suite release (Dudchenko, Singh Batra and Lieberman-Aiden, 2021) (supp/build-aca-hic.sh). The script uses the GNU Parallel tool for parallelization (Tange, 2011).

The ability to perform the aggregate analysis relies on knowing where the centromere is located on the chromosome. See next section for more details on how the position of the centromere was identified for the 24 species.

Centromere position identification

Our ability to judge chromosome-scale features such as centromere clustering and centromere-to-telomere axis requires that we know the position of the centromere in the genome assembly. This information is readily available for only a handful of genome assemblies among the 24 that have been used in this study (chicken, baker's yeast, fruit fly, and tammar wallaby, see Table S1).

To help with identifying the centromere position for the remaining genome assemblies, we have created a method that looks at the distribution of contacts of a given basepair sequence with loci across the genome. If the repeat sequence specifically associated with

the centromere is known as is often the case from CenH3 ChIP-Seq and FISH analyses, the distribution should peak near the centromere position in the genome assembly (https://tinyurl.com/y3b8kcex). Importantly, the method does not require the centromere per se to be assembled or the reads to be mapped to the centromere, but rather relies on the long-range information generated by the Hi-C experiment to find the anchored assembled sequences closest to the centromere.

In practice, the sequence in question is broken into overlapping k-mers, and the Hi-C read pairs are searched for exact k-mer matches (or their reverse complements). The associated custom script is available on Zenodo as part of the 3D-DNA suite release(Dudchenko, Singh Batra and Lieberman-Aiden, 2021) (supp/lookup-reads-matching-pattern.sh). The script uses the GNU Parallel tool for parallelization (Tange, 2011).

Centromere repeat sequence mapping shows that centromeres and loci associated with strong structural features such as focal inter-chromosomal interaction and foldback are one and the same (https://tinyurl.com/y3b8kcex). As such, in those cases where centromeric repeat sequences were not available but the Hi-C maps showed prominent 3D features suggestive of centromere clustering and/or telomere-to-centromere axis, the putative centromeres were identified based on these structural features (listed as "structural" for centromere coordinates source in Table S1). When neither centromeric repeat sequence was known nor there was a strong 3D signal suggestive of the centromere position (primarily Type-II genomes), the ACA plots were built based on two representative chromosomes, without rescaling (equivalent to centromere positions being assigned to the midpoints of each chromosome (cases listed as n/a for centromere coordinates source in Table S1). In the case of the holocentric roundworm, all chromosomes have been aggregated without arm rescaling.

It must be noted that in a few cases (southern house mosquito, red-bellied piranha and Arctic lamprey), even when the centromeric sequences have been suggested in the literature, the contact analysis track showed no meaningful enrichment anywhere along the chromosome. Many such cases came from the studies that identified centromeric repeats as the most abundant repeat in the sequencing data associated with the species (Melters *et al.*, 2013). Perhaps this hypothesis does not hold true in these species.

Scoring of architectural features

To help identify chromosome-scale folding features in an unbiased fashion, we created four scores to quantify the enhanced contact frequency at the relevant positions in the ACA plot, see Fig. S3.

The scores are defined as observed-to-expected ratios, where both observed and expected represent the total number of contacts across the relevant area in the ACA plot (Fig. S3). For

this calculation, the ACA contact map is binned with the bin size equal to 1/20 of the length of each of the ACA chromosomes, i.e., the ACA map is a 40x40 matrix.

The custom script to perform scoring is available on Zenodo as part of the 3D-DNA suite release (Dudchenko, Singh Batra and Lieberman-Aiden, 2021) (supp/score-aca.sh).

The resulting scores can be found in Table S3. The presence/absence of a features is defined when the score is higher than an empirically assigned threshold. The automatic annotations correlate well with the results of manual annotation (Table S3).

Heritability of architectural features

We sought to determine the extent to which chromosomal folding features were heritable. We found that adjacent species in our phylogeny were much more likely to share features than two species selected at random (CT: 0.8 vs 0.49; A: 0.8 vs 0.66, C: 0.6 vs 0.49, T: 0.8 vs 0.51, where CT, A, C and T stand for phylogenetic characters associated with chromosome territories, cetromere-to-telomere axis, centromere clustering and telomere clustering, respectively). At the same time, the most parsimonious account of the pattern of feature presence or absence across species involves each feature being gained or lost many times over the course of evolution (CT: ≥5, i.e., 1 gain or loss event per ‹360 million years; A: ≥4, 1 event per ‹450 million years; C: ≥5, 1 event per ‹360 million years; T: ≥7, 1 event per ‹257 million years).

For this calculation, adjacent species are defined as leaves on the phylogeny tree separated by exactly 1 node. There are 5 pairs of adjacent species as opposed to 276 random pairs that can be constructed from 24 species included in the phylogeny. The probabilities listed in the above paragraph are calculated as the ratios of the numbers of pairs in which the phylogenetic character is the same for both species in the pair divided by the total number of pairs, across neighboring pairs and across all pairs respectively.

The custom script to calculate the minimal number of gains and losses associated with each particular feature across the phylogenetic tree is shared on Zenodo (Dudchenko, 2021). The script uses the Phylogenetics package for Wolfram Mathematica to parse the Newick tree [https://github.com/IstvanZachar/Phylogenetics/releases/latest]. Presence/absence is assigned based on manual annotations from Table S3. The last eukaryotic common ancestor is assumed to have existed 1.8 billion years ago for the time estimates listed in the main text (Betts *et al.*, 2018).

Co-occurrence of architectural features and type assignment

We found that features fall into two groups, such that features in the same group were much more likely to co-occur, and features in different groups were less likely to co-occur. Group I includes the three Rabl-like features: centromere clustering, telomere clustering, and a telomere-to-centromere axis. We refer to this chromosome architecture as Type-I.

Group II (Type-II architecture) includes only the presence of chromosome territories. See Fig. S4 for conditional probabilities of the presence of one feature given the state of the other, calculated using the manual feature annotation set from Table S2.

The assignment to types I or II is made based on the presence of any one of the Rabl-like features in the manual annotation set (Table S2). Almost identical assignment can be made by empirically weighing the Rabl-like feature scores against the score associated with the strength of the chromosome territory-like feature, e.g., calculating $S(a) + S(c) + S(t) - S(ct)$, where $S(a),\ S(c),\ S(t)$ and $S(ct)$ are observed over expected scores associated with centromere-to-telomere axis, centromere colocalization, telomere clustering and chromosome territories, respectively. The higher weighted score is associated with Type-I arrangement while the lower score suggests that the species exhibit Type-II architecture (Table S2).

Conservation analysis

We analyzed conservation of condensin II complex subunits with a multistep BLAST approach. The searches were conducted from October 2019 to June 2020 as the assemblies became available, and were manually double-checked. All alignments were posted in (Hoencamp, 2021).

Our approach was based on a search strategy as used in earlier work (King *et al.*, 2019). We started by collecting publicly available protein sequences of the condensin I and II complex subunits of four diverse species from Uniprot: *Homo sapiens*, *Drosophila melanogaster*, *Caenorhabditis elegans* and *Arabidopsis thaliana*. As a positive control we searched for SMC2 and SMC4, and the condensin I subunits, which are thought to be essential in all species.

In the first alignment step, we used tblastn (Altschul *et al.*, 1990) to search with the translated protein sequences of the above species against the nucleotide collection (nr/nt) database of the target species. The Expect threshold was set at 0.05. We reported an alignment as a hit when it had an E-value of 1E-10 or less with multiple regions of alignment. If there was an alignment with less confidence, we did an extra validation step to confirm the alignment. This step entailed downloading the translated nucleotide sequence of the putative alignment and using tblastn to search against the genome of a closely related organism with an annotated genome. If this search yielded the putative protein we used as a bait, we considered the hit validated.

In the second alignment step we used the same approach, but we blasted against the wgs database of the target species. We again used 1E-10 as E-value cut-off. In the third step, only a few organisms still had missing subunits. To make an extra effort to find these subunits, we used the corresponding subunits of the nearest neighbor, which we identified in step 1 or 2, as bait. As the identified subunits were all nucleotide sequences, we used tblastx to translate these query sequences to protein sequences and blast against a translated nucleotide database. In this step we searched both the nr/nt database and the wgs database. As we were able to identify all SMC2/4 subunits, but still missed condensin II subunits we

are now fairly sure these organisms indeed miss these condensin II subunits. However, it is still possible these organisms do have all condensin II subunits, but with very low sequence conservation.

We were also able to identify the condensin I subunits in almost all species, with two notable exceptions (see Table S3). The Arctic lamprey lacked condensin I subunits CAPG and CAPD2. Because we were able to identify all condensin II subunits in this organism, we still included this species in our analysis. The other exception is the tardigrade. In this species we identified SMC2 and SMC4, but could not identify any of the accessory subunits of condensin I nor II. There are multiple possible explanations for this. On the one hand, it might have a biological explanation, for example in this organism condensin's accessory subunits have evolved beyond recognition with our methods, or this species indeed has lost both condensin I and II. On the other hand, the missing subunits may be explained by a technical issue, e.g. the quality of the databases. Therefore, we cannot with full certainty conclude that condensin II is indeed missing in the tardigrade, and this will need to be investigated further.

Cell culture

Hap1 cells were cultured in IMDM (Gibco), supplemented with 10% FBS (Sigma Aldrich), 1% Penicillin/Streptomycin (Gibco) and 1% L-glutamine (Gibco). HCT116 cells (gift of Naoko Imamoto lab (*24*)) were cultured in DMEM (Gibco), supplemented with 10% FBS (Sigma Aldrich), 1% Penicillin/Streptomycin (Gibco) and 1% L-glutamine (Gibco). All cells were cultured at 37°C with 5% CO_2.

For synchronization experiments, cells were synchronized at G1/S with 2.5 mM Thymidine (Sigma Aldrich) for 16 hours. The cells were then released from this arrest, in medium supplemented with or without 10 µm RO-3306 (Millipore) to arrest in G2 and with or without 0.5mM Auxin (Sigma Aldrich) to deplete CAP-H2. After 24 hours cells were harvested for immunofluorescence, flow cytometry and western blot analysis.

Generation of knock-out cell lines

CAP-H2 knockout cells were obtained as described in (Elbatsh *et al.*, 2019). To obtain CAP-D3 knock-out cells, Forward CACCGACTGTGTCAACCCATTCTAG and Reverse AAACCTAGAATGGGTTGACACAGTC sgRNAs were cloned into px330. The Hap1 cells were transfected with the sgRNAs together with a tia-blasticidin cassette, as previously described (Haarhuis *et al.*, 2017). Genomic DNA was isolated from picked clones and the desired mutations were scored for by Sanger Sequencing. All key results were validated in at least two independent *ΔCAP-H2* and *ΔCAP-D3* clones.

Hap1 in situ Hi-C experiment and data analysis

In situ Hi-C in Hap1 cells was performed as previously described (Rao *et al.*, 2014), with slight modifications as described in (Haarhuis *et al.*, 2017). Hi-C on the *ΔCAP-H2* cell line was performed in two biological replicates.

Raw sequence data were mapped and processed using HiC-Pro v2.9 (Servant *et al.*, 2015) with hg19 as reference. Two different libraries were prepared and combined after assessing their similarity. The valid pair files generated by HiC-Pro were used to create Juicebox-ready files using juicebox pre command from Juicer v1.9.8 (Durand *et al.*, 2016). Downstream analyses were performed using GENOVA (Brand *et al.*, 2021; Van Der Weide *et al.*, 2021) and custom R scripts (Sedeño Cacciatore, 2021), see below for details.

Statistics on the number of read pairs, valid read pairs and percentage of contacts in *cis* of the data generated are summarized in Table S7 of Hoencamp *et al*. (2021) (Hoencamp *et al.*, 2021).

Data analysis

ICE normalized contacts (Imakaev *et al.*, 2012) were normalized to 100 million contacts per sample for comparison across samples. For downstream analysis, reads mapped to chromosome Y and the mitochondrial genome were also excluded.

Hap1 cells are known to have translocations: a) reciprocal translocation between chromosome 9 and chromosome 22 (Andersson *et al.*, 1987) and b) an allele of a region of chromosome 15, that retains heterozygosity, inserted on chromosome 19 (Essletzbichler *et al.*, 2014). These rearrangements alter the primary sequence and, thus, the contact pattern in relation to other genomic elements. To avoid this affecting our genome-wide analysis, all contacts with or within the q-arms involving these translocations have also been excluded in our analysis.

- **ACA analysis of the ΔCAP-H2 cells**

In order to assess the change in the intensity of features associated with CAP-H2 knockout, we performed the ACA analysis of the Hi-C data generated in the Hap1 control and *ΔCAP-H2* cells (Fig. 3, https://tinyurl.com/y22r4m7g).

We processed the *in situ* Hi-C data with Juicer (Durand *et al.*, 2016), using hg19, with centromeric gaps removed to avoid the coverage anomalies those gaps would introduce, as a reference. In addition, chromosomes 1, 9, 16-20 were excluded from the analysis as their contact maps suggested a mismatch between the reference and the sample sequence (i.e., suggested a presence of either rearrangements in the sample or misassemblies in the reference). The centromere positions for the analysis were downloaded from the UCSC Table Browser ((Karolchik *et al.*, 2004); http://genome.ucsc.edu/).

The resulting maps are shown in Fig. 3. Interactive version of this figure is available at https://tinyurl.com/y22r4m7g, and the 'native' Hi-C maps used to generate the ACA plots are available at https://tinyurl.com/y658rvkf.

The same analysis was repeated using hg38 as a reference. The 'native' and ACA maps are available at https://tinyurl.com/y5nd49tt and https://tinyurl.com/yywg2tq6 respectively.

- **Ratio of trans interactions along chromosomes:**

Chromosome territories, defined as the increased probability of loci to interact with loci on the same chromosome, could be perturbed by an increase in *trans* interactions. Given the increased pericentromeric *trans* interactions in *ΔCAP-H2*, we wanted to test whether *trans* interactions are enriched in *ΔCAP-H2* cells genome-wide. *Trans* interactions were scored for contact data binned at 100 kb resolution by computing the ratio of *trans* contacts (involving a different chromosome) over total contacts per bin. The difference between *ΔCAP-H2* and wild type scores is shown as the logarithmic fold-change of these values per bin.

Microscopy

Cells were grown on 12 mm glass coverslip. Non-chromatin bound proteins were removed using PEM-T pre-extraction buffer (100mM PIPES pH 6.8, 1 mM MgCl2, 5 mM EGTA and 0.2% Triton) for 45 seconds and then samples were fixated with 4% PFA + 0.1% Triton for 10 minutes. Blocking was performed for 30 minutes in 3% BSA in PBS-Tween (0.1%). Incubation with primary antibody occurred overnight at 4°C. Slides were washed in PBS-Tween (0.1%) and incubated for 1 hour at room temperature with secondary antibody (mouse or rabbit Alexafluor 488, 568 or 647 from Invitrogen; 1:1000) and DAPI (Sigma-Aldrich; 1:1000). After washing the coverslips were mounted onto glass slides with Prolong Antifade Gold (Invitrogen). Deltavision Elite System (Applied precision) and Softworx software were used to acquire images.

Centromeric foci were quantified in ImageJ using a macro developed in-house(Van Den Broek, 2021) (Analyize_foci_in_nuclei_fixed.jim) All experiments were performed in three independent biological replicates, all showing the same results. In all quantifications, one experiment out of at least 3 independent experiments with a minimum of 50 cells is shown, and one other replicate in Fig. S24 of Hoencamp *et al*. 2021(Hoencamp *et al*., 2021), with the red lines indicating the median. For significance testing, **** indicates p<0.0001 in all cases. To calculate the statistical significance in Fig. 4B a Welch's t-test was performed. For Fig. 5A a one-way ANOVA, followed by Sidak's multiple comparisons test was performed. **** indicates a p–value of <0.0001 in the multiple comparison's test. For Fig. 4D, a t-test was performed.

Western blot

Cells were lysed using Laemmli buffer (120 mM Tris pH 6.8, 4% SDS, 20% glycerol) and boiling at 95°C. Lowry assay was used to quantify total protein concentration. Samples were loaded onto a NuPage 4-12% Bis-Tris gel and run in 1x MOPS buffer (Novex biologicals), transferred onto methanol activated PVDF blotting membrane (Immobilon) and visualized using Clarity ECL (BioRad) and the ChemiDoc (BioRad).

Fluorescence in-situ hybridization (FISH)

Cells were harvested with a mitotic shake-off after 1 hour incubation with nocodazole (1 µg/µl). The cell pellet was resuspended in 0.075M KCl with 200 uL fixative (MeOH : Acetic acid,

3:1) and incubated for 20 minutes at 37 °C. After this, the cells were washed with fixative twice, and resuspended in 50 µl fixative. Of this, 20 µl was dropped on a microscopic slide and dried on a 37 °C heat block. The chromosome spread was incubated with 2x Saline-sodium citrate buffer (20x SSC buffer, Sigma Aldrich) at 70 °C for 30 minutes. Cooled down microscope slides were subsequently incubated with 0.4x SSC-Tween (0.05%) buffer for 1 minute at room temperature, denatured with 0.07M NaOH for 1 minute at room temperature and then washed for 1 minute with 0.1x SSC buffer at 4 °C, and washed again for 1 minute with 2x SSC buffer at 4°C. After this, slides were incubated with increasing ethanol concentrations (70%, 95% and 100%) for 1 minute, air dried and then incubated with denatured FISH probes, XCyte 1 Human mBand probe and XCyte 2 Human mBAnd probe (Metasystems probe) respectively, for at least 24 hours at 37 °C. After this step, slides were washed for 2 minutes with 0.4x SSC buffer that was pre-warmed to 72°C, then with 2x SSC-Tween (0.05%) for 1 minute at room temperature. Cells were incubated for 10 minutes at room temperature with DAPI (Sigma-Aldrich;1:10000) in 2x SSC-Tween (0.05%). Slides were washed briefly with double distilled water and let air-dry. Slides were mounted with Prolong Gold Antifade (Invitrogen). Images were taken on a Leica Confocal system.

SUPPLEMENTAL FIGURES

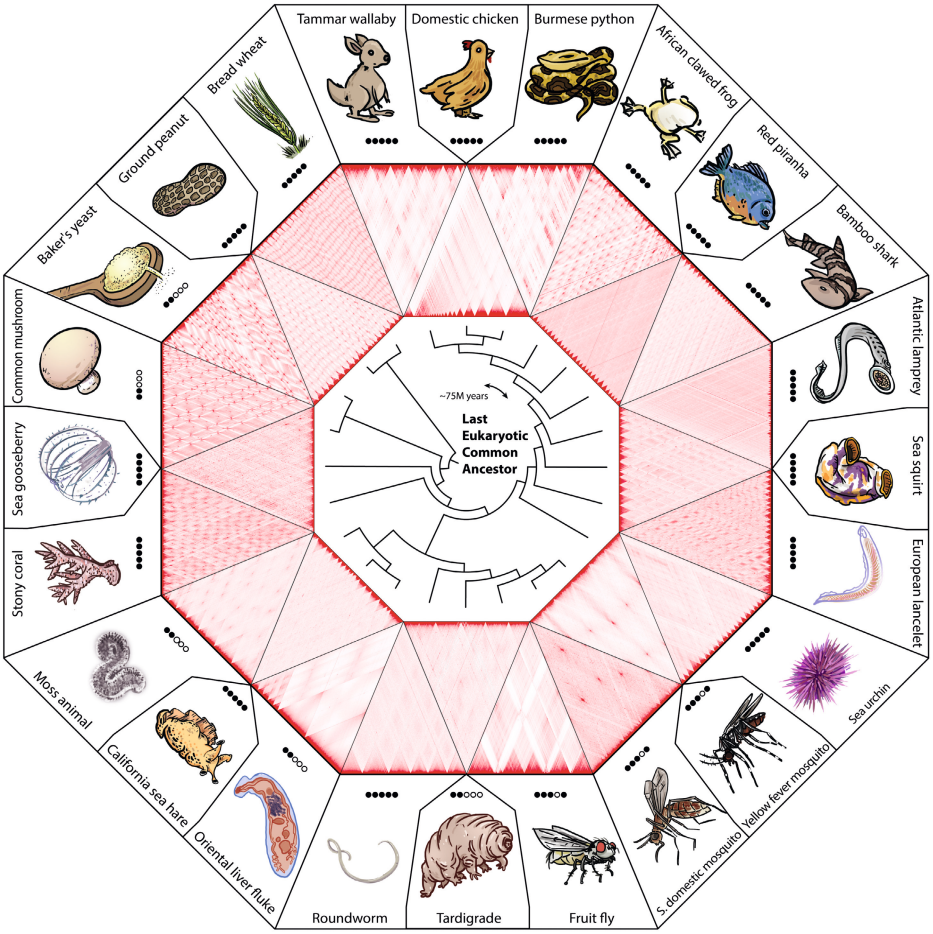

Figure S1 | Hi-C analysis of 24 species across the tree of life reveals nuclear architecture at chromosome-scale for 24 species. The phylogenetic relationship between the species is represented with a consensus cladogram at the center of the figure (2). Presence of the condensin II subunits in each species is indicated by solid black circles, indicating from left to right: SMC2, SMC4, CAP-H2, CAP-G2, CAP-D3. Interactive maps can be visualized using Juicebox.js and are available via https:// tinyurl.com/y5x44fjl and on www.dnazoo.org.

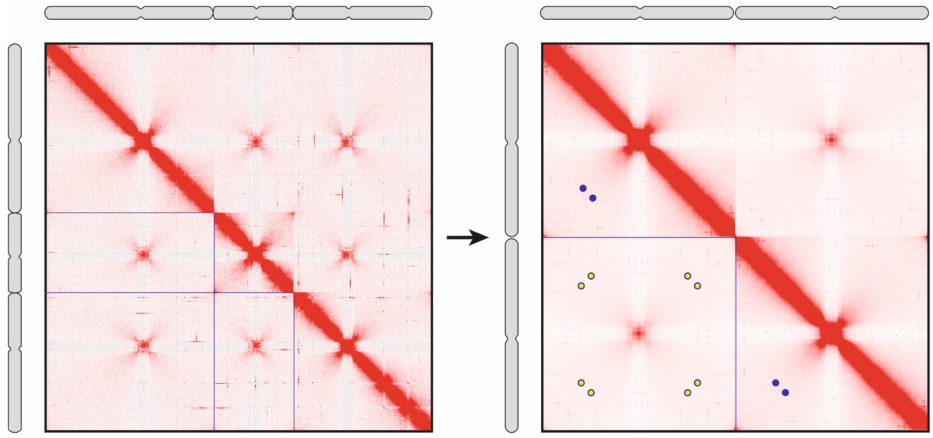

Figure S2 | Aggregate chromosome analysis helps highlight chromosome-scale folding features associated with a collection of chromosomes. On the left is an example 'native' contact map across the genome that comprises 3 metacentric chromosomes (chromosomes #3, #12 and #5 from the common mushroom genome assembly from this study), each one of different size and with p- and q-arms of different length. The ACA map symmetrizes across all possible orders and orientations of individual chromosomes to generate a representative isochromosome-by-isochromosome map, on the right. The points on the map highlight positions with exactly the same contact intensity. See also https://tinyurl.com/ y35qtu43. Interactive version of the ACA for every species is available at https://tinyurl.com/y5yrozfa.

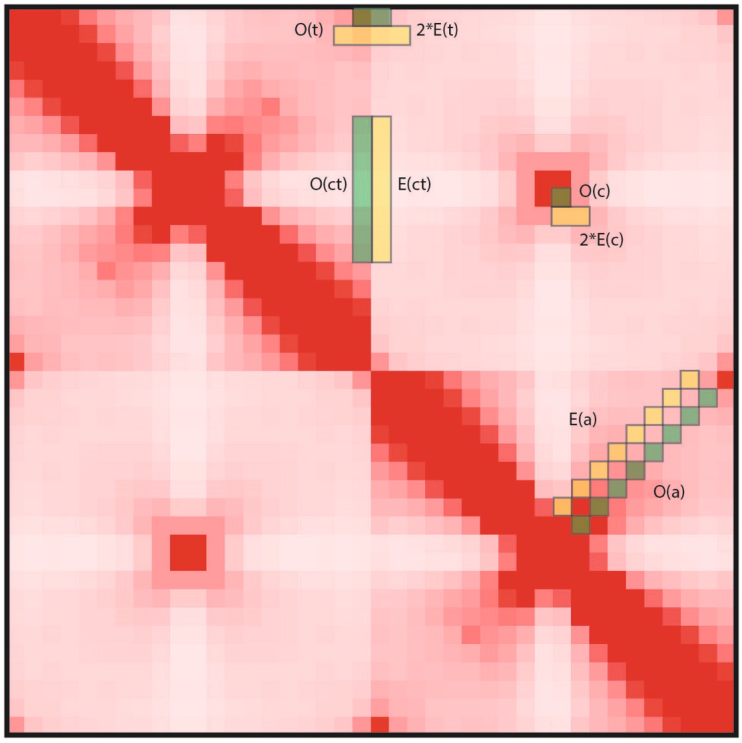

Figure S3 | Scoring of ACA maps for enrichment in contacts associated with the four chromosome-scale features. The presence or absence of enhanced contact frequency at the relevant positions in the ACA plots is quantified as an observed/expected ratio for each of the four architectural features: chromosome territories (ct), telomere-to-centromere axis (a), centromere (c) and telomere (t). The "observed" in each case is the sum of contacts across the area annotated in green. The expected score is calculated based on the total number of contacts across the area annotated in yellow. The contact map is binned with the bin size equal to 1/20 of the length of each of the ACA chromosomes, i.e., the ACA is a 40x40 matrix. The ACA map used in this figure is the common mushroom ACA map.

P(X|Y) -P(X)	ct	a	c	t
ct	0,38	-0,16	-0,13	-0,02
a	-0,49	0,71	0,40	0,44
c	-0,17	0,25	0,54	0,22
t	-0,03	0,31	0,24	0,58

Figure S4 | Type-I features, centromere-to-telomere axis (a), centromere clustering (c) and telomere clustering (t), are not likely to co-occur with chromosome territories (ct). The conditional probabilities table is calculated based on manual annotation set from Table S2.

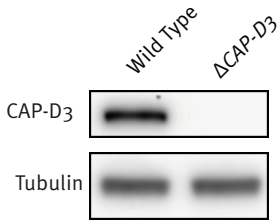

Figure S5 | Depletion of the condensin II subunit CAP-D3 leads to centromeric clustering. (A) Western blot analysis of CAP-D3 protein levels in wild type and ΔCAP-D3 cells.

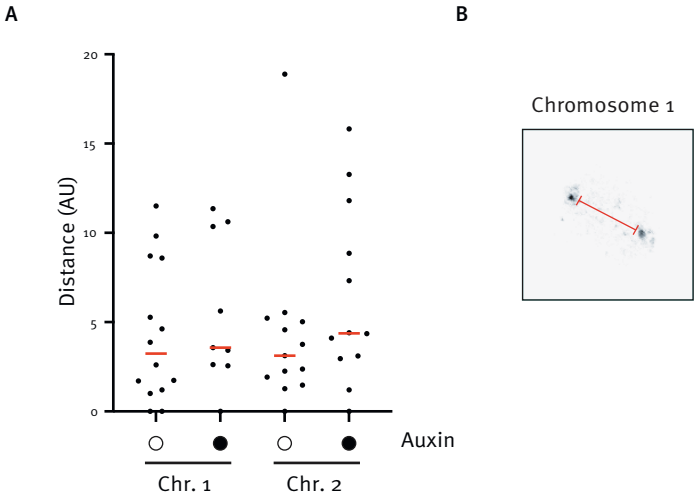

Figure S6 | Condensin depletion does not lead to somatic homolog pairing in human cells. (A) Quantification of Fluorescence in Situ Hybridization of chromosome 1 and chromosome 2 M-band staining. Distance between the two pairs of chromosome 1 and chromosome 2 in interphase was measured based on one channel of the FISH staining, as shown in the examplein (B). (B) Example of one colour of the Fluorescence in Situ Hybridization of chromosome 1.

SUPPLEMENTAL TABLES

Table S1 | Overview of the 24 species examined in Figure 1. Table contains the phylogenetic placement in kingdom and phyla/division, previously published genomics resources used, and GEO accession numbers associated with the resources generated as part of this study, including the chromosome-length fastas, 'native' Hi-C maps and the aggregate chromosome analysis (ACA) maps.

Kingdom	Phylum/Division	Species common	Species binomial	Material used for Hi-C
Animalia	Chordata	Tammar wallaby	*Macropus eugenii*	blood
		Domestic chicken	*Gallus gallus*	splenic-derived lympho-cytes
		Burmese python	*Python bivittatus*	blood
		African clawed frog	*Xenopus laevis*	fibroblast (XTC)
		Red piranha	*Pygocentrus nattereri*	muscle
		Bamboo shark	*Chiloscyllium punctatum*	blood
		Arctic lamprey	*Lethenteron camtschaticum*	muscle
		Sea squirt	*Ciona intestinalis/robusta*	tissue
		European lancelet	*Branchiostoma lanceolatum*	whole animal(s)
	Echinodermata	Sea urchin	*Strongylocentrotus purpuratus*	muscle
	Arthropoda	Yellow fever mosquito	*Aedes aegypti*	whole animal(s)
		Southern house mosquito	*Culex quinquefasciatus*	whole animal(s)
		Fruit fly	*Drosophila melanogaster*	whole animal(s)
	Tardigrada	Tardigrade	*Hypsibius dujardini*	whole animal(s)
	Nematoda	Roundworm	*Caenorhabditis elegans*	whole animal(s)
	Platyhelmynthes	Oriental liver fluke	*Clonorchis sinensis*	whole animal(s)
	Mollusca	California sea hare	*Aplysia californica*	mix of CNS cells
	Bryozoa	Moss animal	*Cristatella mucedo*	whole animal(s)
	Cnidaria	Stony coral	*Acropora millepora*	branch fragments
	Ctenophora	Sea gooseberry	*Pleurobrachia bachei*	tissue
Fungi	Basidiomycota	Common mushroom	*Agaricus bisporus*	fruiting body
	Ascomycota	Baker's yeast	*Saccharomyces cerevisiae*	cells
Plantae	Magnoliophyta	Ground peanut	*Arachis hypogaea*	leaves
		Bread wheat	*Triticum aestivum*	leaves

Draft genome source	Centromere coordinates source	GEO accession
n/a	*(92)*	GSM5182714
(93)	*(93)*	GSM5182715
(94)	*(95)*	GSM5182716
(96,97)	*(98)*	GSM5182717
https://www.ncbi.nlm.nih.gov/assembly/GCF_001682695.1/	n/a (HiC_scaffold_7 vs HiC_scaffold_15)	GSM5182718
(99)	structural	GSM5182719
(100)	n/a (HiC_scaffold_24 vs HiC_scaffold_36)	GSM5182720
(40)	structural	GSM5182721
(101)	n/a (HiC_scaffold_18 vs HiC_scaffold_19)	GSM5182723
https://www.ncbi.nlm.nih.gov/assembly/GCF_000002235.5 (contigs)	n/a (HiC_scaffold_17 vs HiC_scaffold_18)	GSM5182724
(102)	*(50)*	GSM5182725
(103)	structural	GSM5182727
(104)	*(104)*	GSM5182728
(47)	n/a (HiC_scaffold_1 vs HiC_scaffold_2)	GSM5182729
http://www.wormbase.org, release WS269, date 16-Nov-2018	n/a (all by all, holocentric)	GSM5182730
(105)	structural	GSM5182731
https://www.ncbi.nlm.nih.gov/assembly/GCF_000002075.1	*(50)*	GSM5182732
n/a	structural	GSM5182733
(106)	*(50)*	GSM5182734
(107)	structural	GSM5182735
(108)	structural	GSM5182736
(109)	*(109)*	GSM5182737
(110)	*(111)*	GSM5182738
(112)	*(113)*	GSM5182739

Table S2 | Enrichment scores calculated from the ACA maps help annotate chromosome-scale folding features. For each of the 24 species, the scores are calculated as described in the *Scoring of architectural features* section of the supplement. Binary assignment of features is listed based on the scores as well as manual annotation. The architecture type assignment is calculated based on the $S(a) + S(c) + S(t) - S(ct)$ for the automatic annotation and the presence of at least one manually annotated Type-I feature for the manual assignment of type.

Id	Species common	Species binomial	S(ct)	S(a)	S(c)	S(t)
1	Tammar wallaby	*Macropus eugenii*	6,179	1,026	1,103	1,482
2	Domestic chicken	*Gallus gallus*	10,33	0,982	0,97	1,558
3	Burmese python	*Python bivittatus*	7,485	1,024	1,236	1,93
4	African clawed frog	*Xenopus laevis*	2,085	1,161	1,626	1,551
5	Red piranha	*Pygocentrus nattereri*	6,258	0,986	1,005	1,327
6	Bamboo shark	*Chiloscyllium punctatum*	8,197	1,031	1,255	1,369
7	Arctic lamprey	*Lethenteron camtschaticum*	4,709	1,001	0,972	1,247
8	Sea squirt	*Ciona intestinalis/robusta*	3,57	1,009	1,982	1,506
9	European lancelet	*Branchiostoma lanceolatum*	3,12	1,082	1,092	1,052
10	Sea urchin	*Strongylocentrotus purpuratus*	3,789	0,988	0,881	1,296
11	Yellow fever mosquito	*Aedes aegypti*	1,109	1,317	2,226	2,391
12	Southern house mosquito	*Culex quinquefasciatus*	1,073	1,118	1,99	1,652
13	Fruit fly	*Drosophila melanogaster*	1,739	1,075	2,003	1,558
14	Tardigrade	*Hypsibius dujardini*	1,357	1,074	1,065	1,2
15	Roundworm	*Caenorhabditis elegans*	2,088	1,007	1,002	1,153
16	Oriental liver fluke	*Clonorchis sinensis*	1,693	1,054	1,262	1,647
17	California sea hare	*Aplysia californica*	5,687	0,952	1,004	1,225
18	Moss animal	*Cristatella mucedo*	2,236	1,02	1,687	1,408
19	Stony coral	*Acropora millepora*	1,993	1,112	1,379	1,541
20	Sea gooseberry	*Pleurobrachia bachei*	1,591	1,006	3,254	1,326
21	Common mushroom	*Agaricus bisporus*	1,572	1,622	2,244	2,291
22	Baker's yeast	*Saccharomyces cerevisiae*	3,181	1,241	2,514	1,617
23	Ground peanut	*Arachis hypogaea*	6,285	1,123	1,351	1,782
24	Bread wheat	*Triticum aestivum*	1,35	1,419	1,2	1,642

	auto_ CT	auto_ A	auto_ C	auto_ T	manual_ CT	manual_ A	manual_ C	manual_ T	auto_ type	manual_ type
	1	0	0	0	1	0	0	0	II	II
	1	0	0	1	1	0	0	0	II	II
	1	0	0	1	1	0	0	0	II	II
	0	1	1	1	0	1	1	0	I	I
	1	0	0	0	1	0	0	0	II	II
	1	0	0	0	1	0	0	0	II	II
	1	0	0	0	1	0	0	0	II	II
	1	0	1	1	1	0	0	1	I	I
	1	0	0	0	1	0	0	0	II	II
	1	0	0	0	1	0	0	0	II	II
	0	1	1	1	0	1	1	1	I	I
	0	1	1	1	0	1	1	1	I	I
	0	0	1	1	1	0	1	0	I	I
	0	0	0	0	0	0	0	1	I	I
	0	0	0	0	0	0	0	0	n/a	n/a
	0	0	0	1	1	1	0	1	I	I
	1	0	0	0	1	0	0	0	II	II
	1	0	1	0	1	0	1	0	I	I
	0	0	1	1	0	0	1	1	I	I
	0	0	1	0	0	0	1	0	I	I
	0	1	1	1	0	0	1	1	I	I
	1	1	1	1	0	0	1	0	I	I
	1	1	1	1	1	0	1	1	II	I
	0	1	0	1	0	1	0	1	I	I

Table S3 | Conservation status of condensin I and II in the 24 species examined. Red indicates the subunit is absent. If the subunit is present, the accession number of the hit is depicted. The last two columns indicate the architecture type as called in Table S3. According to the Fisher's exact test, condensin II status and architecture type are dependent variables ($p<0.05$).

Species common	Species binomial	NCBI TaxID	SMC2	SMC4
Tammar wallaby	*Macropus eugenii*	9315	ABQO020509468.1	ABQO020668049.1
Domestic chicken	*Gallus gallus*	9031	NM_205230.1	AJ532587.1
Burmese python	*Python bivittatus*	176946	XM_007424550.3	XM_007429945.3
African clawed frog	*Xenopus laevis*	8355	XM_018231781.1	BC170550.1
Red piranha	*Pygocentrus nattereri*	42514	XM_017709350.1	XM_017691835.1
Bamboo shark	*Chiloscyllium punctatum*	137246	BEZZ01000259.1	BEZZ01000226.1
Arctic lamprey	*Lethenteron camtschaticum*	980415	APJL01017391.1	APJL01127184.1
Sea squirt	*Ciona intestinalis/robusta*	7719	XM_002119922.5	XM_026835111.1
European lancelet	*Branchiostoma lanceolatum*	7740	FLLO01000002.1	FLLO01000037.1
Sea urchin	*Strongylocentrotus purpuratus*	7668	XM_030996301.1	XR_004063544.1
Yellow fever mosquito	*Aedes aegypti*	7159	XM_001656739.2	XM_001653698.2
Southern house mosquito	*Culex quinquefasciatus*	7176	XM_001851439.1	XM_001842568.1
Fruit fly	*Drosophila melanogaster*	7227	NM_137151.4	AF185287.1
Tardigrade	*Hypsibius dujardini*	232323	LMYF01000006.1	LRSR01014097.1
Roundworm	*Caenorhabditis elegans*	6239	U96387.1	NM_065534.6
Oriental liver fluke	*Clonorchis sinensis*	79923	NIRI01000953.1	BADR02001200.1
California sea hare	*Aplysia californica*	6500	XM_005105826.2	XM_013081361.1
Moss animal	*Cristatella mucedo*	67896	WPIO01000018.1	WPIO01000065.1
Stony coral	*Acropora millepora*	45264	XM_029333563.1	XM_029354457.1
Sea gooseberry	*Pleurobrachia bachei*	34499	AVPN01001033.1	AVPN01020953.1
Common mushroom	*Agaricus bisporus*	936046	XM_006456494.1	XM_006453854.1
Baker's yeast	*Saccharomyces cerevisiae*	4932	CP004955.2	NM_001181973.1
Ground peanut	*Arachis hypogaea*	3818	XM_025815404.1	XM_025768302.2
Bread wheat	*Triticum aestivum*	4565	AK449180.1	AK335489.1

Condensin I-specific			Condensin II-specific			manual_type
CAP-H	CAP-G	CAP-D2	CAP-H2	CAP-G2	CAP-D3	
ABQO020138847.1	ABQO020040571.1	ABQO020832739.1	ABQO020353992.1	ABQO020088274.1	ABQO021027835.1	II
XM_015297458.2	XM_025150356.1	XM_025149149.1	XM_423939.6	XM_003640678.4	XM_004948007.3	II
XM_025168051.1	XM_025165890.1	XM_025167542.1	XM_015891298.2	XM_025173103.1	XM_007441393.2	II
BC068643.1	BC128686.1	BC170462.1	NM_001094379.1	XM_018266532.1	NM_001089625.1	I
XM_017709710.1	XM_017713705.1	XM_017699182.1	XM_017706405.1	XM_017681648.1	XM_017724920.1	II
BEZZ01000022.1	BEZZ01000737.1	BEZZ01212930.1	BEZZ01001544.1	BEZZ01000148.1	BEZZ01000025.1	II
APJL01010387.1			APJL01056009.1	APJL01081510.1	APJL01028273.1	II
XM_018813644.2	XM_018817121.2	XM_026838496.1	XR_001975089.2	XM_009861925.3	XM_002122724.4	I
FLLO01000168.1	FLLO01000007.1	FLLO01000106.1	AVPN01001448.1	FLLO01000008.1	FLLO01000145.1	II
XM_030985417.1	XM_030985404.1	XM_030986864.1	XM_030989363.1	XM_030999910.1	XM_030993832.1	II
XM_001651684.2	XM_001661280.2	XM_021838324.1	XM_021857587.1		XM_021855365.1	I
XM_001862134.1	XM_001848849.1	XM_001843177.1	XM_001847339.1		XM_001844488.1	I
NM_057758.5	BT032841.1	AY122183.1	NM_141757.2		NM_001169416.2	I
						I
NM_069977.6	NM_059727.5	NM_066978.5	NM_066326.7	NM_073679.4	NM_059137.4	n/a
BADR02003099.1	BADR02002496.1	BADR02000574.1				I
XM_005091027.2	XM_013085890.1	XM_013090798.1	XM_013084910.1	XM_013084386.1	XM_013089334.1	II
WPIO01000025.1	WPIO01000091.1	WPIO01000042.1				I
XM_029340469.1	XM_029357074.1	XM_029357028.1	XM_029329290.1	XM_029337148.1	XM_029331840.1	I
AVPN01000441.1	AVPN01000320.1	AVPN01000106.1	AVPN01001448.1	AVPN01000177.1	AVPN01002829.1	I
AEOK01000112.1	XM_006463442.1	XM_006462292.1		I		I
CP004647.2	CP004691.1	NM_001182159.1				
XM_025787414.2	XM_025782767.2	XM_025804965.1	XM_025838837.2	XM_025842802.2	XM_025790832.2	I
AK453908.1	AK335327.1	CALO01063642.1	AK335978.1	CALO01331996.1	CALO01816520.1	I

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Chapter 4

Chromosome-scale architecture as a separate unit of genome organization

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Adapted from Hoencamp et al. 3D genomics across the tree of life reveals condensin II as a determinant of architecture type. Science* **372, *984-989 (2021).*

ABSTRACT

In human cells, each chromosome occupies a discrete subnuclear region known as a chromosome territory. The function of these chromosome territories, or how they are regulated, remains an unsolved mystery. In chapter 3, we identifed condensin II as a key factor in promoting chromosome territories. Indeed, condensin II loss results in weakening of chromosome territories, and the strengthening of another architecture type called Rabl-like organization. In this study, we exploit these findings and use condensin II knock-out cells to investigate the effect of loss of chromosome territories on smaller-scale genome architecture and gene expression. We find that the major changes in large-scale organization hardly affect genome folding at the scale of compartments, TADs or loops. Condensin II depletion does lead to redistribution of the DNA that is associated with the nuclear lamina. We find a general trend of (centromeric) regions moving away from the nuclear lamina, and instead forming *trans* contacts with other centromeric regions. The expression of a subset of genes seems to be particularly sensitive to these changes in the large-scale 3D genome. All in all, our work suggests that chromosome territories seem to be an separate unit of genome organization, whose functions remain an enigma.

INTRODUCTION

The spatial organization of the nucleus is vital for controlling processes such as gene expression. Major technological advances in the fields of sequencing and imaging have led to the uncovering of the different scales of organization that make up the 3D genome (Kempfer and Pombo, 2019; Xie and Liu, 2021). From small to large, this folding gets increasingly complex. At a small scale, chromatin can form cohesin-dependent loops. A collection of such loops between convergently oriented CTCF sites together form a loop domain or a Topologically Associated Domain (TAD). These TADs represent functional units which facilitate gene regulation by determining which regulatory elements can interact with each other (Merkenschlager and Nora, 2016). The genome further segregates into blocks of transcriptionally active (A) and inactive (B) chromatin, forming so-called compartments (Lieberman-Aiden *et al.*, 2009). At the largest scale, the scale of whole chromosomes, two architecture types can be distinguished throughout the tree of life (**Chapter 3**). In humans, chromosomes form few *trans* contacts and are confined to discrete regions within the nucleus. This type of organization is called chromosome territories (Cremer and Cremer, 2010). In yeast on the other hand, chromosomes readily form *trans* contacts, mainly at the centromeres and telomeres. This type of organization is called Rabl-like organization (Rabl, 1885). It remains mysterious how these architecture types benefit specific cellular processes.

Over the past decades, many discoveries have led us to better understand the regulation and function of chromatin organization at scales ranging from chromatin loops to compartments. However, research into chromosome territories was hampered by our lack of knowledge on how to disrupt them. Only recently a factor was identified that controls chromosome territories in an evolutionarily conserved manner (**Chapter 3**). Condensin II promotes the formation of chromosome territories, and hereby prevents Rabl-like organization. Removing condensin II from human cells indeed induces a switch from territorial organization to Rabl-like organization. This is most apparent from the clustering together of the centromeres. These findings finally provide us with a tool to further examine the functions of chromosome territories, and gives us an exciting opportunity to start answering the open questions in the field.

RESULTS

Very little is known about the interplay between chromosome territories and the the other scales of genome organization. To investigate whether chromosome territories regulate smaller scale genomic structures, we performed Hi-C on condensin II knock-out cells that have lost their territorial organization. In the genome, transcriptionally active and transcriptionally more silent regions are spatially partitioned into A and B compartments respectively. These compartments are visible in Hi-C maps as a plaid-like pattern. We analyzed the changes in these compartments and visualized them at their location on the chromosome arms (Figs. 1A and 1B). Condensin II deficiency only mildly affects compartmentalization, and it does so

for the largest part in the regions surrounding the centromeres. Since centromeres undergo large reorganization in absence of condensin II, it is not surprising that those contacts are most affected. Nonetheless, these changes are very minor and reveal that despite a dramatic reorganization of the genome, compartmentalization remains quite stable.

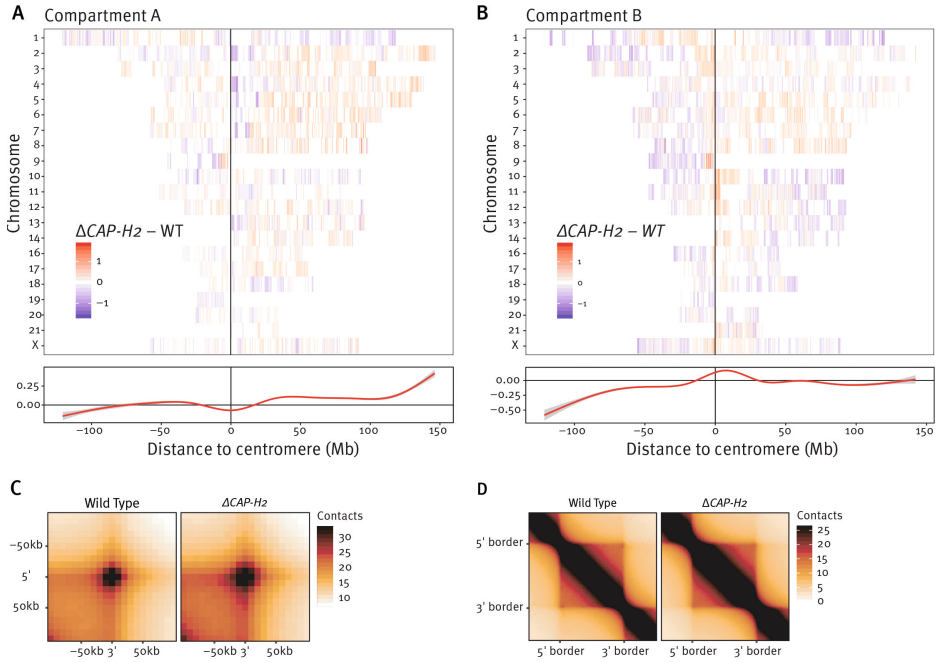

Figure 1 | Condensin II deficiency does not affect CTCF-anchored loops and loop domains, and mildly affects compartments. (A) Top panel: Differences in compartment score of compartment A regions at 100 kb bins between wild type and ΔCAP-H2 cells per chromosome. Lower panel: pile-up of the changes in compartment score across chromosomes binned at 100 kb. The median of changes is shown in red. (B) Top panel: Differences in compartment score of compartment B regions at 100 kb bins between wild type and ΔCAP-H2 cells per chromosome. Lower panel: pile-up of the changes in compartment score across chromosomes at 100 kb. The median of changes is shown in red. (C) Aggregate peak analysis (APA) depicting the average Hi-C signal for peaks, genomic locations with enriched interactions, as called from wild type by HICCUPs. (D) Aggregate TAD analysis (ATA) depicting the average Hi-C signal in wild type and ΔCAP-H2 cells across loop domains found in wild type.

At a smaller scale, the genome is folded into TADs - collections of chromatin loops that preferentially interact within a particular region that is demarcated by CTCF boundaries. These loops are formed by cohesin, another protein complex from the SMC family of complexes. As condensin and cohesin can function in a similar manner by forming chromatin loops, we asked if condensin II depletion also would lead to changes in these smaller scale structures. This was not the case, as CTCF-anchored loops and loop domains were unaffected in Δ*CAP-H2* cells (Figs. 1C and 1D). This is in strong contrast with loss of cohesin, which leads to a complete loss of such structures (Rao *et al.*, 2017). These observations reveal that loss of chromosome territories need not bring about major changes in smaller scale structures.

Nuclear organization is also dictated by interaction of the genome with other nuclear structures. One such structure is the nuclear lamina. Many regions of the genome have been identified to associate with the nuclear lamina, and as such are called Lamina Associated Domains (LADs). This nuclear periphery is associated with gene repression, and is known to control chromosome orientation within the nucleus (van Steensel and Belmont, 2017). We wondered whether LADs were impacted by the loss of chromosome territories.

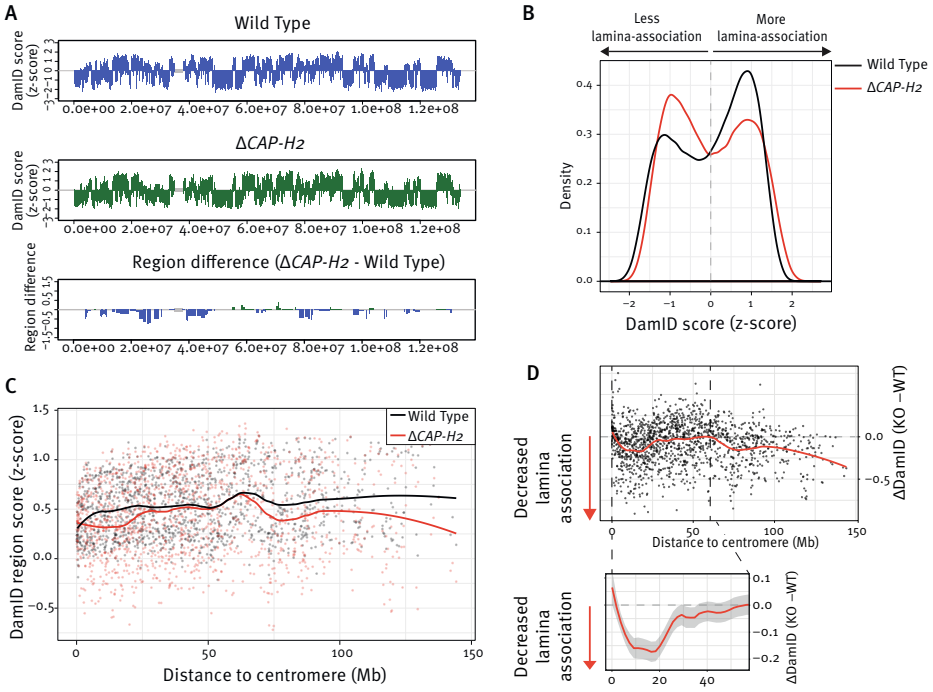

Figure 2 | Centromeres move away from the lamina in absence of condensin II. (A) Example genome browser screenshot showing scaled DamID scores of chromosome 12 for wild type and ΔCAP-H2 cells. The region difference track was created by averaging LAD scores and substracting ΔCAP-H2 from wild type cells. The grey box denotes the centromeres. (B) Density plot showing the mean distributions of the DamID data of wild type (n=4) and ΔCAP-H2 (n=2) cells, after scaling the data to mean=0, stdev=1. (C) Scatter plot showing DamID region scores as function of the distance to centromeres for wild type and ΔCAP-H2 cells. (D) Difference in DamID score relative to distance to centromere. Zoom-in includes 95% confidence interval of the mean in grey.

To identify DNA segments associated with the nuclear lamina (LADs), we performed DamID of LaminB1 (Guelen *et al.*, 2008). This technique allows for methylation specifically of regions that are close to the lamina, and these methylated regions can subsequently be identified by sequencing. It thus gives an image of which regions in the nucleus have come in close proximity to the nuclear lamina (LADs), and which regions are further away from the lamina (inter-LADs)(Fig. 2A). We find that, in general, condensin II loss leads to loss of lamina association (Figs. 2A and 2B).

We next wondered in which genomic regions the loss of lamina association mostly occurred. To investigate this, we plotted the chromosomal distribution of DamID scores, and found that genomic regions localizing up to 25 Mb from the centromeres appeared to move away from the lamina (Figs. 2C and 2D). This seems to overlap with the most striking changes in *trans* contacts surrounding the centromeres in absence of condensin II (**Chapter 3**). These regions surrounding the centromeres seemingly gain interactions with other chromosomes, and in turn lose contacts with the nuclear lamina, or *vice versa*. This shows that the switch from territorial organization to Rabl-like organization corresponds with more *trans* interactions and less lamina association. This is in line with the fact that Rabl-like organization is defined by clustering of different genomic regions in *trans*.

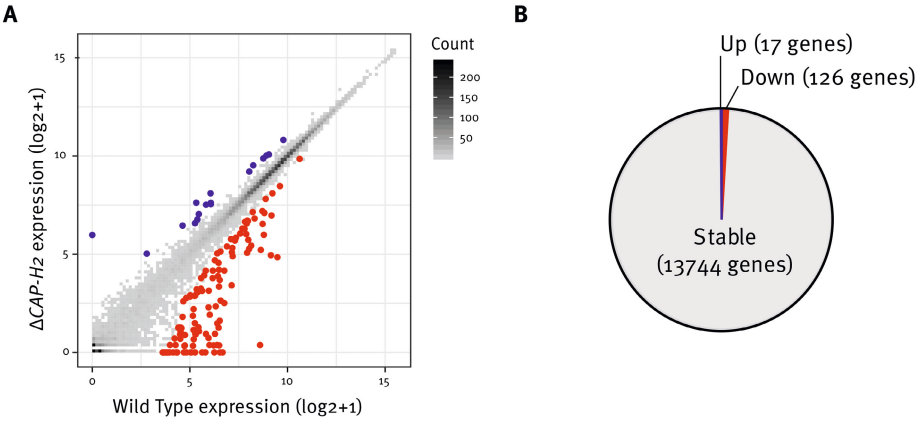

Figure 3 | Massive 3D genome changes hardly affect gene expression. (A) Gene expression of wild type relative to ΔCAP-H2. Unaffected genes depicted in grey, upregulated genes in blue and downregulated in red. (B) Number of genes in each category.

3D genome organization is thought to be important for gene regulation, although the extent to which this holds is true for chromosome territories remains a topic of debate. We thus examined whether condensin II disruption led to changes in gene expression. RNA-sequencing revealed that condensin II deficiency affected the expression of only a small fraction of genes (Fig. 3). This is quite surprising, considering the major changes in chromosome scale organization observed, and suggests that this large-scale organization does not play a major role in gene regulation.

However, there is a subset of genes that is affected by condensin II loss. Why are these genes particularly sensitive to condensin II depletion? We wondered whether these genes lie close to the centromeres, as we have so far observed the most changes in this region. This however did not seem to be the case (Fig. 4A). Further analysis interestingly revealed that affected genes are enriched within LADs and near LAD borders (Figs. 4B and 4C).

Do the changes in lamina association found upon condensin II loss correlate with the changes in gene expression? We tested this by correlating the RNA sequencing data with the DamID data, and indeed found that genes that were downregulated moved towards the nuclear lamina in Δ*CAP-H2* cells (Fig. 4D). As centromeres move away from the lamina upon condensin II loss, genes that are near or within these LADs can now potentially occupy the space that is vacated by these centromeres (Fig. 4E), which may in turn lead to their transcriptional repression. This model would explain why this subset of genes is particularly sensitive to large-scale reorganizations within the nucleus.

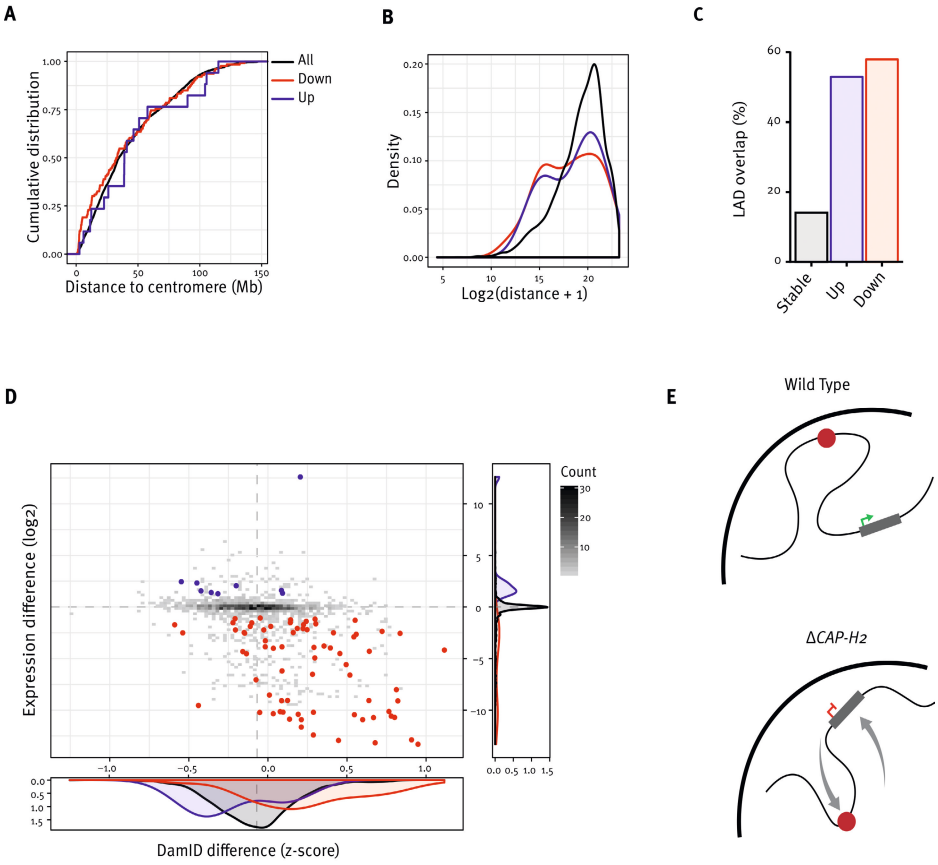

Figure 4 | Gene expression differences correlate with proximity to LAD borders. (A) Cumulative distribution of the distance of differential genes to the centromeres. (B) Density plot showing the distance of differentially expressed genes towards LAD border, filtered for active genes not overlapping LADs. (C) Percentage of active genes overlapping with LADs. (D) Intersection of differences in gene expression with differences in lamina association, depicting active genes within LADs. (E) Schematic model of centromeres (red) moving to the inner nucleus, and silenced genes that now localize to the lamina.

DISCUSSION

In this study, we aimed to better understand the role of chromosome territories in smaller scale genome architecture and gene expression. To answer these questions, we disrupt territorial organization by loss of condensin II. In **Chapter 3**, we showed that condensin II loss leads to a switch in genome architecture from territorial organization to Rabl-like organization. In this chapter, we reveal that despite these dramatic changes in large-scale organization upon condensin II loss, smaller scale genome organization is hardly affected. Instead, chromosomes apparently reposition, losing interaction with the lamina and gaining interactions with other chromosomes. The altered lamina associations seem to disrupt gene expression of a small but specific subset of genes.

These findings suggest that for most gene regulation, chromosome-scale genome organization is irrelevant, except for a small subset of genes. It must be noted that in this study we do not exclude the possibility that condensin II directly regulates these genes by binding them, rather than indirectly regulating them through overall genome architecture. Nascent RNA-sequencing after acute depletion of condensin II in interphase may elucidate whether this is the case. In this way, we can uncouple the loss of condensin II from the changes in large-scale organization, which only occur upon mitotic progression. However, since the expression changes of these genes correlate with their nuclear lamina changes after condensin II loss, it is likely that their nuclear repositioning is responsible for their misregulation. Alternatively, the gain in lamina interactions might be a consequence of the reduced expression of these genes (van Steensel and Belmont, 2017; Brueckner *et al.*, 2020). Why condensin II loss specifically regulates this subset of genes would then remain unknown.

All in all, this study reveals that chromosome territories operate largely independently from organization at the scale of compartments, TADs or loops. We also show that the major role of chromosome territories is likely not broad control of gene expression. What then is the function of chromosome territories remains a mystery, as we discuss below.

Condensin and cohesin: shaping the genome at different scales

Condensin II loss affects genome architecture at the scale of whole chromosomes, but not at the scale of TADs. Interestingly, another SMC complex, the cohesin complex, is essential for the formation of interphase loops and TADs (Gassler *et al.*, 2017; Haarhuis *et al.*, 2017; Rao *et al.*, 2017; Schwarzer *et al.*, 2017; Wutz *et al.*, 2017). A picture arises in which different SMC complexes are responsible for genome folding at different levels. This does raise the question of how two complexes that are so similar in structure and mechanism can control the genome a such different scales. One explanation for this could be the temporal regulation of these complexes. Cohesin complexes predominantly form loops in interphase, whereas condensin II is mostly active in mitosis. This suggests that the different scales of chromosome architecture are built during different stages of the cell cycle. Moreover, cohesin forms TADs in collaboration with the barrier protein CTCF, which binds at specific sites in the genome.

The lack of such binding factors for condensin II may additionally explain why condensin II cannot form structures such as TADs.

The function of chromosome territories

In this study, we have shown that dramatic changes in the 3D genome at the level of whole chromosomes only leads to minor changes in gene expression. Why then are chromosome territories promoted by condensin II, if not for widespread gene regulation? One possibility is that chromosome territories regulate a very defined set of genes required in a specific developmental pathway or cell type. Indeed, condensin II mutant mice have problems in T cell development, yet these problems seemingly arise due to genomic instability in these cells rather than gene regulation (Gosling *et al.*, 2007; Woodward *et al.*, 2016). Another possibility is that clustering of the centromeres is problematic when DNA damage occurs in these regions. As this DNA consists of many repetitive sequences, faulty homologous recombination may occur when many of these repeats are in close proximity of the break site. This would in turn lead to translocations, which can be detrimental to cells. Related to this, chromosome territories have been shown to protect against translocations, and loss of condensin II by itself indeed leads to more translocations (Rosin *et al.*, 2019).

As shown in **Chapter 3**, many species have evolved to naturally exhibit Rabl-like organization. Rabl-like organization must thus also have its benefits. In yeast, species that have lost their Rabl-like organization have defects in mitosis (Hou *et al.*, 2012). It is suggested that the clustering of centromeres facilitates rapid kinetochore capture, by limiting the region of the nucleus in which the microtubules have to find the centromeres (Hou, Kallgren and Jia, 2013). In that sense, Rabl organization may be specifically beneficial for species with rapid cell cycles such as yeast. Elaborate analysis of species with Rabl-like configuration in comparison with species with territorial architecture would be needed to begin to answer this important question. Future research will hopefully clarify many of these open questions, and continue to unravel the regulation and importance of genome architecture at the scale of whole chromosomes.

ACKNOWLEDGEMENTS

We thank the NKI Genomics core facility for sequencing.

MATERIALS & METHODS

Cell culture

Hap1 cells were cultured at 37°C with 5% in CO_2 in IMDM medium (Gibco), supplemented with 10% FBS (Sigma Aldrich), 1% Penicillin/Streptomycin (Gibco) and 1% L-glutamine (Gibco).

Generation of knock-out cell lines

Hap1 ΔCAP-H2 cells were generated as described in(Elbatsh *et al.*, 2019).

Hap1 in situ Hi-C experiment and data analysis

In situ Hi-C was performed and analyzed as described in Chapter 3.The same data was used for analyses in Chapter 3 and Chapter 4. For the analyses shown in this chapter:

- **Peak analysis:**

We employed loops called previously for Hap1 wild type in Haarhuis *et al.*(Haarhuis *et al.*, 2017). To visualize the genome-wide effect in loops we performed aggregate peak analysis(Rao *et al.*, 2014) as implemented in GENOVA. Briefly, for a set of loop coordinates a square submatrix is selected such that it is centered on the corresponding coordinates, with a 100kb flanking region upstream and downstream. These submatrices are then averaged to obtain a mean contact map for these locations.

- **TAD analysis:**

We employed TADs called previously for Hap1 wild type in Haarhuis *et al.*(Haarhuis *et al.*, 2017). Similar to the aggregated peak analysis, aggregate TAD analysis was done to visualize how TAD structures are affected by CAP-H2 loss. To compensate for the different sizes of TADs the selected regions are resized prior to averaging the contact maps. These regions are comprised of the TAD itself and a flanking region half its size.

- **Compartment score change:**

The compartment-score was calculated as previously described(Schwarzer, Abdennur, Goloborodko, Pekowska, Fudenberg, Loe-Mie, N. A. Fonseca, *et al.*, 2017). Briefly, we calculated the eigenvectors of the observed over expected (O/E) matrix minus 1 at a resolution of 100 kb. The first eigenvector was then multiplied by the square root of its eigenvalue to obtain the compartment score. We used the anti-correlation between LamB1 DamID data and the compartment score to correctly orient the compartment score.

The 100 kb bins used to compute the compartment score were segregated into A and B compartment based on their value in wild type cells. The difference between Δ*CAP-H2* and wild type compartment score for each bin was plotted with relation to their distance from the centromeres. A smoothed mean of these values was generated by fitting a generalized additive model to the data as implemented in the R package *mgcv 1.8-28*.

LMNB1 DamID-seq

DamID-seq and data processing were performed as reported previously, but mapped to hg19(Leemans *et al.*, 2019). The experiments were performed at least in three biological replicates. Briefly, Hap1 cells were transduced with lentivirus with Dam-only or Dam-LMNB1 constructs. Three days after transduction gDNA was isolated. To prevent adapter ligating to apoptotic fragments present in Hap1 cell culture, genomic DNA was first dephosphorylated with a phosphatase treatment. Up to 500 ng of gDNA was incubated in 10uL H_2O with 1x CutSmart buffer and 0.5U rSAP (NEB #M0371L) for 1 hour at 37°C, followed by heat inactivation for 10 minutes at 65°C. Next, we added 10U DpnI and continued with the protocol

as described before. Downstream analyses were performed in R with reads counted in 10kb bins. To account for translocations, the right arms of chromosomes 9, 15, 19 and 22 were removed from all analyses.

Analysis DamID data

We observed small differences in dynamic range between experiments. These differences were also present between biological replicates and thus most likely of technical origin. We accounted for this by scaling the data to a mean of zero and a standard deviation of one, without affecting the data distribution. For wild type and *ΔCAP-H2* cells, a consensus LAD model was determined with the requirement of being called in at least half of the replicates. The union of the two cell lines was used as our LAD definition. The LAD score was defined as the mean signal of z-transformed data tracks. In Fig. 4B, distance is defined as distance to the middle of the LAD.

RNA-sequencing

RNA sequencing was performed in three biological replicates. Total RNA from cultured Hap1 WT or *ΔCAP-H2* cells was extracted using RLT buffer supplemented with B-mercaptoethanol (Qiagen). Strand-specific libraries were generated using the TruSeq PolyA Stranded mRNA sample preparation kit (Illumina) with the following protocol. PolyA-RNA was purified using oligo-dT beads. RNA was purified, fragmented, and then reserve transcribed with random primers using SuperScript II Reverse Transcriptase (Invitrogen). The generated cDNA was 3' end-adenylated and ligated to Illumina Paired-end sequencing adapters and amplified by PCR using HiSeq SR Cluster Kit v4 cBot (Illumina). Libraries were checked using a 2100 Bioanalyzer (Agilent) and then sequenced on a HiSeq2000 (Illumina).

Data processing

Reads were processed with fastp 0.12.2 to remove potential adapter sequences and low-quality reads(Chen *et al.*, 2018). Filtered reads were aligned to hg19 and counted in gencode genes v19 with STAR 2.5.4a(Dobin *et al.*, 2013). Differential analysis was performed using DESeq2 1.24.0, where we defined significant differences as genes that have a Benjamini & Hochberg adjusted p-value lower than 0.05 when testing for a log2-fold change bigger than 0.5(Love, Huber and Anders, 2014). Log2 transformed normalized counts were used as a measure of gene expression. Active genes were defined as genes with a mean cell score of 2 or higher. To account for translocations, the right arms of chromosomes 9, 15, 19 and 22 were removed from all analyses.

To correlate changes in expression with changes in lamina association, we took the gene coordinates and determined the mean score of overlapping DamID bins. We extended the genes on both sides with 10kb to get more robust DamID estimates, especially for small genes.

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Chapter 5

Dissecting centromere clustering

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**Adapted from Hoencamp et al.*
3D genomics across the tree of life reveals condensin II as a determinant of architecture type.
Science **372**, *984-989 (2021).*

ABSTRACT

Condensin II shapes the interphase genome by counteracting centromere clustering during or directly after mitosis. We here aim to dissect what drives and prevents centromere clustering. We find that condensin II likely keeps apart the centromeres through controlling the shape of the chromosomes in mitosis. This function of condensin II can be in part be taken over by cohesin when it is stably associated with the DNA due to WAPL depletion. We propose a physical model in which the surface area of chromosomes during mitosis determines chromosome-scale genome architecture, with effects that are retained during the subsequent interphase.

INTRODUCTION

Each cell contains meters of DNA, which must be folded in an intricate manner to fit in the cell nucleus. Yet, the DNA still needs to be accessible to allow for vital DNA-based processes. SMC complexes control different aspects of genome folding and are vital for many such DNA-based processes. In interphase, the cohesin complex is a master regulator of genome architecture (Davidson and Peters, 2021; Oldenkamp and Rowland, 2022), which in this capacity regulates gene expression, DNA replication and DNA damage repair. In mitosis, condensin complexes build rigid mitotic chromosomes, that can withstand pulling forces and can successfully be segregated into two daughter cells.

The formation of rigid mitotic chromosome requires the distinct functions of the two condensin complexes: condensin I and condensin II. Condensin II is nuclear throughout the cell cycle (Hirota *et al.*, 2004; Ono *et al.*, 2004), but is inhibited in interphase by its negative regulator MCPH1 (Houlard *et al.*, 2021). In early mitosis, MCPH1 is phosphorylated, and can no longer inhibit condensin II (Houlard *et al.*, 2021). From this point onwards, condensin II can form long chromatin loops (Gibcus *et al.*, 2018; Walther *et al.*, 2018), promoting lengthwise shortening of the chromosome (Shintomi and Hirano, 2011; Green *et al.*, 2012; Gibcus *et al.*, 2018). Condensin I is mostly cytoplasmic throughout the cell cycle (Hirota *et al.*, 2004; Ono *et al.*, 2004), and accesses the DNA after nuclear envelope breakdown. It will then subdivide the long loops condensin II has formed into smaller, nested loops (Gibcus *et al.*, 2018; Walther *et al.*, 2018). Hereby it decreases the width of the chromosome (Shintomi and Hirano, 2011; Green *et al.*, 2012; Gibcus *et al.*, 2018).

We recently showed that condensin II also is a major determinant of interphase architecture type at the scale of whole chromosomes (see **Chapters 3 and 4**). In human cells, which normally exhibit territorial nuclear organization, removing condensin II leads to a switch towards Rabl-like genome architecture. We discovered that condensin II must act during, or directly after mitosis to counteract this centromeric clustering and promote territorial organization. As condensin II absence correlates with Rabl-like organization throughout evolution, both the forces that lead to a Rabl-like architecture, and condensin's ability to counteract this organization, presumably are evolutionarily conserved.

Many aspects of this switch in architecture type after condensin II removal remain poorly understood. In this study we aim to investigate and dissect centromere clustering in the absence of condensin II. We attempt to identify what drives centromere clustering in absence of condensin II. We provide evidence that shaping mitotic chromosomes by condensin II, or possibly other (SMC) complexes, is sufficient to prevent centromere clustering. These findings bring us one step closer to understanding the intricate regulation that decides how our genome is folded at the scale of whole chromosomes.

RESULTS

Seeking a driving force for centromere clustering

Centromere clustering is a characteristic of Rabl-like nuclear architecture, which occurs naturally in species ranging from mosquitoes to fungi. Often these species lack (subunits of) condensin II. Indeed, centromere clustering can be induced in human cells by depleting condensin II. Condensin II normally must act in mitosis or directly thereafter to keep centromeres apart. There apparently must be an attraction force that drives centromere clustering in absence of condensin II, which presumably (also) is present in mitosis. We set out to identify this centromeric attraction force.

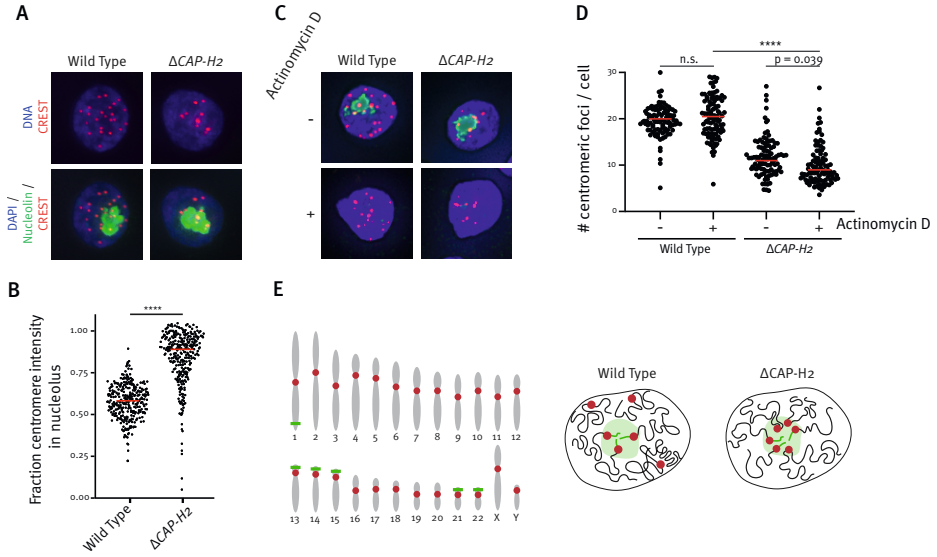

Figure 1 | Condensin II depletion leads to centromere clustering within the nucleolus. (A) Immunofluorescence of centromeres (CREST), nucleoli (Nucleolin) and DNA (DAPI). (B) Quantification of the fraction of centromere intensity within 0.4 µm of nucleoli as shown in (A). A Mann-Whitney non-parametric test was performed, ****=p<0.0001.(C) Centromeric clustering after disruption of the nucleolus by actinomycin D. Mitotic cells were replated and simultaneously treated with actinomycin D for 9 hours. Cells were stained for DNA with DAPI (blue), centromeres with CENPA (red) and the nucleolus by nucleolin (green). Representative immunofluorescence images of cells treated with or without actinomycin D. (D) Quantification of centromeric foci per cell of experiment shown in (C). Median is shown in red. ANOVA followed by a Tukey's multiple comparison test was perfomed, **** = p<0.0001, n.s.= not significant. (E) Left panel: schematic depiction of all 24 human chromosomes, indicating the locations of the centromere (red) and rDNA (green). Right panel: schematic depiction of a nucleus with chromosomes (black), centromeres (red), and rDNA (dark green). In wild type cells, only the centromeres close to rDNA localize in proximity of the nucleolus (light green). In ΔCAP-H2 cells, all centromeres cluster in the nucleolus.

In fruit flies, centromeres cluster naturally and localize to the nucleolus (Padeken *et al.*, 2013). Co-staining with a nucleolar marker revealed that in human Δ*CAP-H2* cells centromeres also cluster inside and nearby the nucleolus (Figs. 1A and 1B). The nucleolus is a membrane-less organelle inside the nucleus that is essential for ribosome biogenesis. Its assembly is

thought to be driven by liquid-liquid phase separation (Lafontaine *et al.*, 2020). In *Drosophila*, centromere clustering is dependent on the nucleolar protein NLP (nucleoplasmin-like protein) (Padeken *et al.*, 2013). This might mean that the nucleolus is also a driving force for centromere clustering in human cells.

To test whether nucleolar integrity is indeed required for centromere clustering, we treated the cells with Actinomycin D. This drug inhibits transcription elongation of RNA polymerase I, which causes the reorganization of nucleolar elements (Potapova and Gerton, 2019) and eventually the full disintegration of the nucleolus (Schoefl, 1964). Indeed, treatment with the drug disrupted the nucleolar structure in wild type and ΔCAP-H2 Hap1 cells (Fig. 1C). Despite the absence of a detectable nucleolus, the centromeres still clustered (Fig. 1C and 1D). Nucleolar integrity is thus not evidently required for centromeric clustering in human cells.

If the nucleolus is dispensable for centromere clustering, why do centromeres cluster in the nucleolus? We propose that the clustering of centromeres at the human nucleolus is because rDNA sequences, the genomic component of the nucleolus, often lie near centromeres in the human genome. Presumably, these centromeres would always be in proximity of the nucleolus. When centromeres cluster, they might therefore automatically localize near these centromeres at the nucleolus (Fig. 1E).

If not the nucleolus, what then causes the clustering? Regions surrounding centromeres are enriched for heterochromatin. This pericentromeric heterochromatin clusters upon condensin II depletion in both mice and fruit flies (Bauer, Hartl and Bosco, 2012; Nishide and Hirano, 2014). We thus wondered whether condensin II loss affected heterochromatin distribution in human cells. Indeed, condensin II loss in our Hap1 cells led to clustering of H3K9me3-containing heterochromatin, as observed by immunofluorescence staining (Figs. 2A and 2B). This indicates that condensin II plays a conserved role in the spatial organization of this repressive epigenetic mark.

Heterochromatin has the intrinsic property to cluster together. Could the centromeric attraction force thus be based on heterochromatin clustering? A key factor responsible for this clustering is heterochromatin protein 1 (HP1) (Canzio *et al.*, 2011; Larson *et al.*, 2017; Strom *et al.*, 2017; Biswas *et al.*, 2022). In mitosis HP1 is enriched at the centromeres (Minc *et al.*, 1999). HP1 is thus an interesting candidate for a driving force for centromeric clustering.

To test this hypothesis we depleted all three HP1 proteins, HP1α, β or γ, separately or together (Fig. 2D) and assessed the number of centromeric foci. Depletion of any of the HP1 proteins did not lead to centromere unclustering (Fig. 2C). HP1 thus presumably is not the driving force behind centromeric clustering. It should be noted that we were not able to assess knockdown efficiency for HP1α in all replicates, which leaves the option that we may not have sufficiently depleted HP1α, and that this factor in fact is important for centromere clustering. Alternatively, knock-down of any of the other HP1 proteins might

not have been sufficient to promote unclustering. On top of that, other heterochromatin factors may contribute to centromeric clustering.

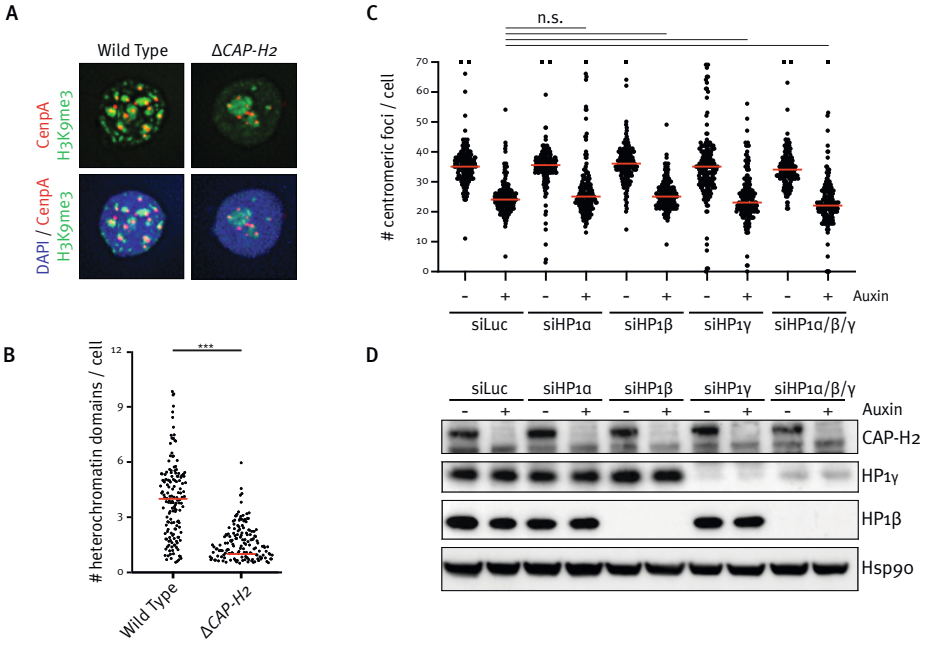

Figure 2 | Condensin depletion leads to heterochromatin clustering. (A) Immunofluorescence of centromeres (CenpA), heterochromatin (H3K9me3) and DNA (DAPI), as quantified in (B). (B) Quantification of the number of heterochromatin domains per cell. A Mann-Whitney nonparametric test was performed, ****=p<0.0001 (C) Quantification of the number of centromeric foci after the indicated siRNA treatment and CAP-H2 depletion by auxin overnight. Shown is the median and 164 cells. ANOVA followed by a Dunnett's multiple comparison test was performed, n.s. = not significant.(D) Western blot analysis of the samples quantified in (C).

We then sought other factors that may promote clustering of centromeres. A possible candidate was the protein Ki-67, which has a dual function in mitosis. In the beginning of mitosis, it functions as a biological surfactant that keeps mitotic chromosomes separated from one another (Cuylen *et al.*, 2016). However, due to a collapse of protein structure, during mitotic exit it can promote chromosome clustering (Cuylen-Haering *et al.*, 2020). We therefore hypothesized that this chromosome clustering might promote clustering together of the centromeres.

We depleted Ki-67 with siRNA treatment in absence of condensin II (Fig. 3B) to test whether Ki67-dependent chromosome clustering is indeed required for centromere clustering. In absence of Ki-67, centromeres still clustered (Fig. 3A). Ki67 is therefore not a main driver of centromere clustering, or at least not in such a manner that this clustering can be abolished by siRNA-mediated depletion.

Condensin II requires mitosis to uncluster centromeres

Next, we set out to investigate the mechanism by which condensin II can prevent centromere clustering. We know that condensin II depletion only causes centromere clustering when cells progress through mitosis (see **Chapter 3**). However, this result does not inform us whether condensin II can uncluster centromeres in interphase.

We addressed this question by using acute CAP-H2 depletion and re-expression experiments. We first acutely depleted condensin II with auxin for one day in HCT116 CAP-H2-AID cells. Centromeres indeed clustered after this auxin treatment (Fig. 4B, + Auxin day 1 condition). To next test whether re-establishment of condensin II would lead to unclustering, we washed out the auxin. This washout allowed restoration of CAP-H2 to normal levels (Fig. 4B, lower panel). Upon washout we either blocked the cells in RO-3306 to prevent mitotic progression, or let the cells progress through mitosis normally (Fig. 4A). Centromeres only unclustered when cells could progress through mitosis (Fig. 4B, washout condition without RO treatment), indicating that condensin II cannot uncluster centromeres in interphase.

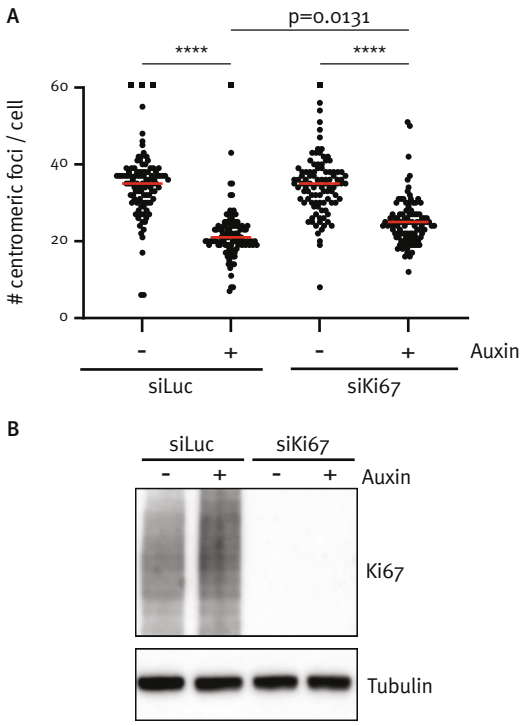

Figure 3 | Ki67 is not essential for centromere clustering. (A) Quantification of the number of centromeric foci after the indicated siRNA treatment and CAP-H2 depletion by auxin overnight. Shown is the median and 100 cells. Outliers are cut-off at 60 and depicted by squares. ANOVA followed by a Tukey's multiple comparison test was perfomed, **** = p<0.0001. (B) Western blot analysis of the samples quantified in (A).

There are multiple explanations for this mitosis-specific role of condensin II. First, centromere movement might be restricted during interphase due to space limitations in the crowded interphase nucleus. This could explain why they cannot be unclustered by condensin II in interphase when its levels are restored upon auxin washout. During mitosis chromosomes are more compact, the space restriction by the nuclear envelope is gone, and centromeres become more motile under the influence of the mitotic spindle. These factors could all contribute to condensin II's ability to uncluster centromeres specifically in mitosis. Whether or not this is the case will be quite hard to test. Alternatively, condensin II might only be able to uncluster centromeres in mitosis because it more stably associates with the DNA during this cell cycle phase (Gerlich *et al.*, 2006).

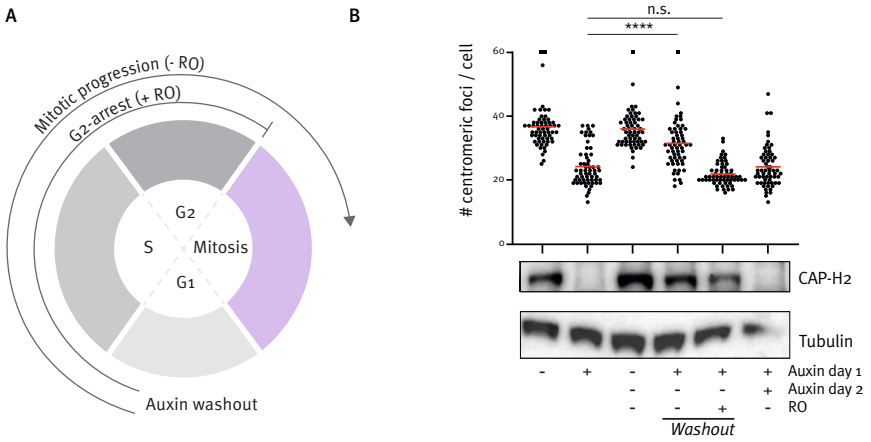

Figure 4 | Condensin II can only uncluster centromeres in mitosis. (A) Schematic overview of Auxin washout experiment performed in HCT116 CAP-H2-AID cells. Cells were or were not treated with auxin on day 1, and then were washed extensively on day 2. Control samples were taken at this moment to check for centromere clustering after auxin treatment. After these samples were washed, normal medium was supplemented (washout), or medium with auxin (positive control). The cells were allowed to progress through mitosis, or were stopped in G2 by RO treatment. (B) Top: Number of centromere foci in the samples indicated. The cells were stained by CENPA antibody and analyzed with immunofluorescence microscopy to score the number of centromere foci. ANOVA followed by a Sidak's multiple comparison test was perfomed, **** = p<0.0001, n.s. = not significant. Bottom: Western Blot analysis of CAP-H2 levels of the samples quantified in Top graph.

Condensin II, but not condensin I, counteracts centromere clustering

In mitosis, condensin II acts together with condensin I to shape mitotic chromosomes. To test whether the centromeric clustering phenotype is specific to condensin II, we depleted condensin I by auxin-mediated degradation of its kleisin subunit, CAP-H (Fig, 5B). Unlike condensin II depletion, acute depletion of condensin I did not lead to centromeric clustering (Figs. 5C and 5D), despite the fact that there was a condensation defect (Fig. 5A). Condensin II therefore has a specific role in counteracting centromeric clustering that it does not share with condensin I.

Lengthwise compaction can counteract centromere clustering

Condensin II, and not condensin I, promotes lengthwise compaction of mitotic chromatin. We thus asked whether this lengthwise compaction would in principle be able to keep apart centromeres. We used physical simulations to investigate whether this activity of condensin II can affect centromere clustering. In these simulations, chromosomes are polymers bisected by a centromere. These chromosomes are shaped by two forces: first, the ideal chromosome potential that models lengthwise compaction by condensin II (Di Pierro *et al.*, 2016; Zhang and Wolynes, 2016); and second, centromeric self-adhesion, which models heterochromatin's tendency to cluster (Larson *et al.*, 2017; Strom *et al.*, 2017; Falk *et al.*, 2019) and stabilizes inter-centromeric contacts in our setup. We simulated ten chromosomes with fixed centromere self-adhesion, and decreased lengthwise compaction to model condensin II depletion (Figs. 6A-E).

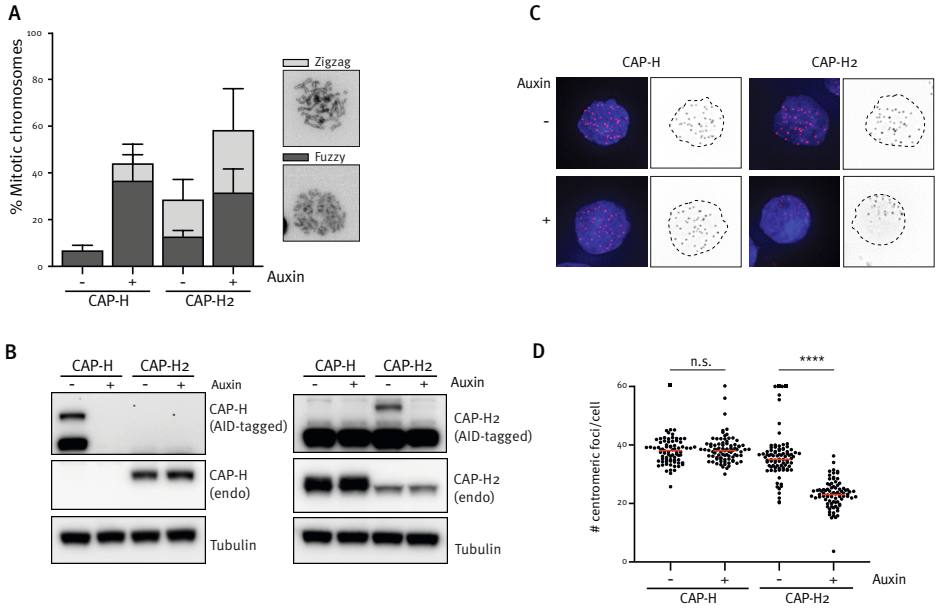

Figure 5 | Condensin II, but not condensin I, counteracts centromeric clustering. (A) Percentage of mitotic spreads with either fuzzy or zigzag-shaped chromosome morphology in HCT116 CAP-H- or CAP-H2-AID with or without 24 hours of auxin addition. Shown is mean + SD of three independent replicates. (B) Western blot analysis of the samples quantified in (D). (C) Representative images as quantified in (D). (D) Quantification of the number centromeric foci after CAP-H or CAP-H2 depletion by auxin for 24 hours, shown is median. Outliers are cut-off at 60 and depicted by squares. ANOVA followed by a Sidak's multiple comparison test was perfomed, **** = p<0.0001, n.s. = not significant.

We found that under high lengthwise compaction (which models condensin II presence) chromosomes form distinct and separate entities reminiscent of chromosome territories, and show little centromere clustering (Figs. 6A-B). On simulated Hi-C maps these setting resemble Wild Type cells (Fig. 6D), with a high ratio of *cis* over *trans* contacts (Fig. 6C), and little contacts between centromeres (Fig. 6E).

Correspondingly, lower lengthwise compaction (which models condensin II absence), leads to chromosome intermingling and centromere clustering (Fig. 6A-B), an architecture type more reminiscent of Rabl-like organization. Indeed, the Hi-C map of this setting is more like the ΔCAP-H2 cells, with less *cis* contacts and more *trans* contacts between centromeres (Figs. 6C-E, and **Chapter 3**: Figs. 3A and 3C). This physical model illustrates how the loss of lengthwise compaction might explain the observed clustering of centromeres in absence of condensin II.

A combination of condensin II disruption and simulation experiments now point to lengthwise compaction as one of the determinants of chromosome-scale genome architecture. Specifically, a decrease in lengthwise compaction that arises from condensin II loss appear to be associated with the shift from Type-II chromosome architecture to Type-I chromosome arrangement. We

wondered if a similar effect would be observed in systems that exhibit a dramatic change in chromosome length.

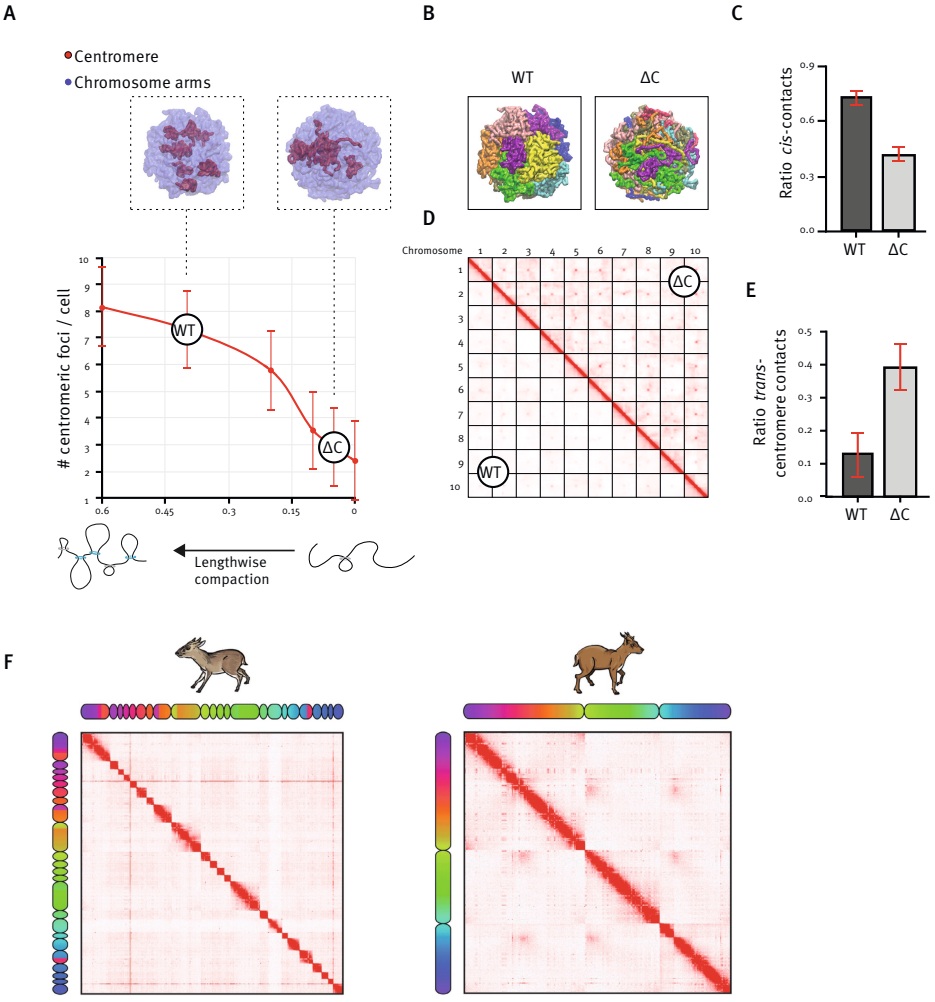

Figure 6 | Lengthwise compaction is required for centromere unclustering. (A) Number of centromere clusters upon varying lengthwise compaction. 'WT' and 'ΔC' correspond to higher and lower lengthwise compaction, best recapitulating the experimental data observed in wild type and ΔCAP-H2 cells. Top: representative models for both states. (B) Representative simulation snapshots depicting ten chromosomes in different colors. (C) Quantification of the ratio of cis-contacts. (D) Simulated Hi-C matrices depicting contacts between the respective chromosomes. (E) Quantification of the proportion of trans-centromeric contacts. (F) Hi-C contact maps for two fibroblast cell lines, the Chinese muntjac (left) and the Indian muntjac (right). The Chinese muntjac karyotype comprises 2n=46 acrocentric chromosomes. The Indian muntjac comprises one metacentric, one acrocentric and one submetacentric chromosome For interactive version of this figure see https://tinyurl.com/y6cwlh93.

Evolution has performed such an experiment in which chromosome length varies as a result of chromosome fusions rather than the loss of condensin II. The chromosomes of the Indian muntjac (*Muntiacus muntjak*) are unique among mammals due to their low diploid number (2*n*=6 in females, 2n=7 in males) and large size. It has been proposed that the karyotype of the Indian muntjac deer evolved from a related deer, the Chinese muntjac (*Muntiacus reevesi*), with a more "typical" diploid chromosome number of 2n= 46 (Brinkley *et al.*, 1984). Karyotype aside the Indian and Chinese muntjacs are morphologically similar and can even interbreed to produce viable (though sterile) offspring. As such, the comparison between the two deer offers a great opportunity to examine the effect of chromosome length on architecture.

By assembling the muntjac genomes, we found that the notable increase in chromosome length in the Indian muntjac coincides with the appearance of centromeric clustering (Fig 6F). This fits with the model that chromosome length during mitosis determines the type of genome architecture of the subsequent interphase, and has interesting implications for genome architecture in species with long chromosomes. Indeed, evolutionary genomics in mammals suggests that long chromosomes often exhibit centromere clustering (Álvarez-González *et al.*, 2022). It is worth noting that while the appearance of centromere clustering is consistent with the chromosome-length driven mechanism discussed in this study, other factors such as the increase of centromere size in the Indian muntjac (Brinkley *et al.*, 1984) may contribute to the effect.

Stable cohesin prevents centromere clustering

Condensin II normally is much more stably bound to chromatin than condensin I. This stability could potentially be required for lengthwise compaction and to counteract centromere clustering. To test this, we turned to another SMC protein complex, cohesin. This complex builds chromatin loops just like condensin II, but its residence time on DNA is limited by its release factor WAPL. Depletion of WAPL leads to cohesin binding more stably to the chromatin (Kueng *et al.*, 2006). This stable cohesin might in essence function as condensin II, and thus be able to rescue centromere clustering.

To test this hypothesis, we depleted WAPL with an siRNA in HCT116 CAP-H2-AID cells. Depletion of WAPL by itself led to longer mitotic chromosomes (Fig. 7C). Condensin II depletion in itself surprisingly did not evidently increase chromosome length in this experiment, but did lead to a 'zigzag' condensation defect (Figs. 7A and 7B). Co-depletion of WAPL and condensin II shortens the chromosomes to lengths similar to the control, and partially prevents the zigzag phenotype (Figs. 7B and 7C). Cohesin stabilization can thus partially make up for condensin II depletion, in that it can prevent the formation of zigzag-shaped chromosomes.

We next tested whether co-depletion of WAPL and CAP-H2 prevents centromere clustering. Excitingly, WAPL depletion in absence of condensin II prevents the clustering of the centromeres, albeit partially (Figs. 7D and 7E, Fig. S1B). We ensured that this is not because the cells progress

slower through the cell cycle (Fig. S1A). Centromere clustering can thus be counteracted by condensin II naturally, but in absence of condensin II it can also be artificially counteracted by increasing cohesin stability on the DNA.

Hyperactive condensin I cannot prevent centromere clustering

This data suggests that 'zigzag' condensation defects may promote centromere clustering, and that condensin II´s role in preventing these defects may counteract centromere clustering. The modeling experiments described above on the other hand suggests that lengthwise compaction by condensin II is sufficient to counteract centromere clustering. In an effort to solve this conundrum, we used the ATPase mutant $SMC4^{LV}$, in which these two phenotypes are uncoupled. In absence of condensin II, this mutant allows condensin I to shorten chromosomes, but it does not prevent zigzag condensation defects (Elbatsh *et al.*, 2019).

We then assessed whether this mutant could counteract centromere clustering in ΔCAP-H2 cells. The ΔCAP-H2/$SMC4^{LV}$ double mutant still exhibits centromere clustering (Figs. 7F and 7G). Apparently $SMC4^{LV}$ condensin I cannot take over the role of condensin II in counteracting centromeric clustering, despite the end-to-end length of chromosomes seemingly being shorter (Elbatsh *et al.*, 2019). This suggests that the zigzag phenotype could promote centromere clustering. Alternatively, condensin II's key function in keeping apart the centromeres might be performed before nuclear envelope breakdown, when condensin I cannot yet reach the DNA. It will be interesting to investigate whether localizing hyperactive condensin I to the nucleus during interphase may allow it to counteract centromere clustering.

DISCUSSION

Condensin II acts as a determinant for architecture type across evolution. Loss of condensin II results in a switch from chromosome territories to Rabl-like organization. One of the main characteristics of Rabl-like organization is centromere clustering. In this study we aimed to dissect centromere clustering in the absence of condensin II.

Centromeric attraction forces

For centromere clustering to occur, there must be a centromeric attraction force that promotes such clustering. In this study we attempted to identify this attraction force. Despite investigating several hypotheses, we were unsuccessful in identifying the driving force behind centromere clustering. This thus remains an open question.

We showed that HP1 likely is not responsible for centromere clustering. Despite this fact, other heterochromatin factors may still promote clustering, which might function in parallel with or independent of HP1. One viable candidate would be PRC1, which can phase separate *in vitro*, and thus could contribute to heterochromatin clustering (Plys *et al.*, 2019). PRC1 proteins are indeed enriched at pericentromeric heterochromatin in somatic cells (Abdouh *et al.*, 2016), supporting this hypothesis.

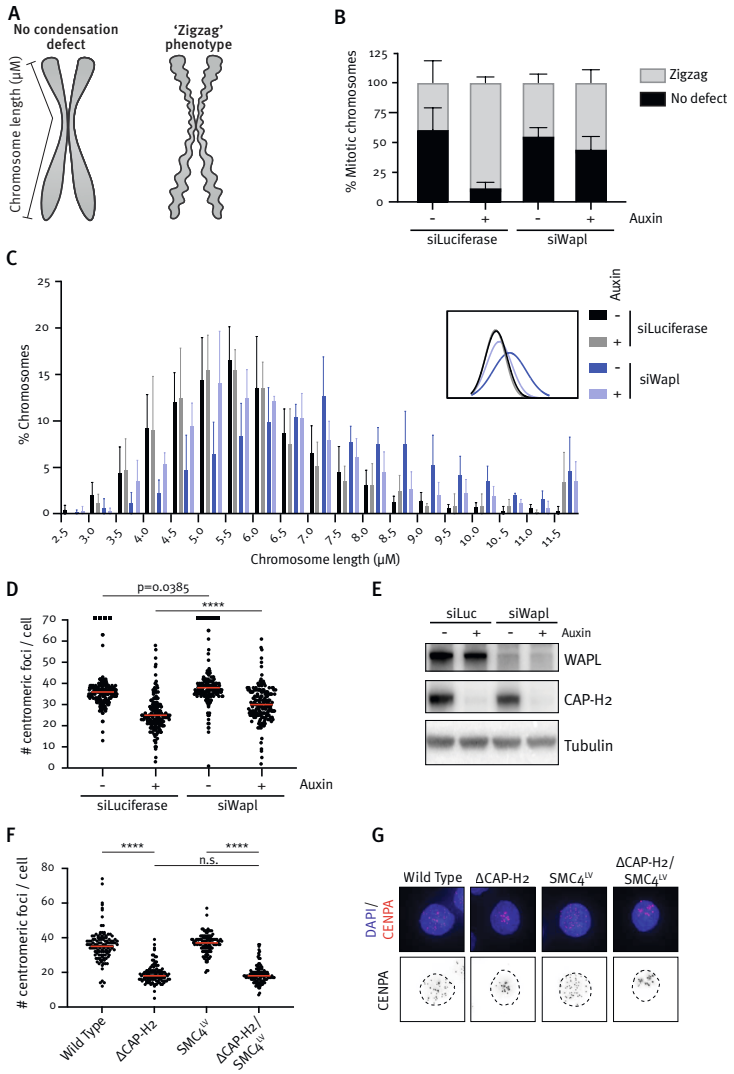

Figure 7 | Increasing cohesin stability can rescue centromere clustering. (A) Schematic overview of mitotic chromosomes with or without a 'zigzag' condensation defect, as quantified in (B). Chromosome length in (C) was measured according to the ruler drawn left of the chromosomes. (B) Percentage of mitotic spreads with either normal of zigzag-shaped chromosome condensation in HCT116 CAP-H2-AID cells with the depicted treatment. Shown is mean +- SD for four independent replicates, in which +- 50 chromosomes in each condition were counted. (C) Chromosome length measured as depicted in (A) for mitotic chromosome spreads in the depicted conditions. Shown is mean +- SD of four independent replicates. Measured were the six longest chromosomes of 30 spreads per condition in each replicate. (D) Quantification of the number of centromeric foci after treatment with siRNA targeting Luciferase or WAPL, and with or without auxin treatment. Shown is the median of 185 cells per condition. ANOVA followed by Sidak's multiple comparison test was performed, ****= p<0.0001. (E) Western Blot analysis of the samples used in (D). (F) Quantification of the number of centromere foci of the indicated genotypes. Shown is the median of 122 cells per condition. ANOVA followed by Tukey's multiple comparison test was performed, ****= p<0.0001, n.s.= not significant. (G) Representative snapshots of images analyzed in (F).

Apart from heterochromatin factors, there are many proteins that specifically localize to centromeres, such as kinetochore proteins. One of these proteins might thus promote clustering of this region in particular. In fact, centromeric protein CENP-B has recently been shown to be one factor that can promote centromere clustering (Chardon *et al.*, 2022).

With so many candidate genes that might drive centromeric clustering, the best way to identify such driving factors might be through an unbiased genome-wide screen. With recent advances in image analysis, it should be feasible to set up an image-based screen that identifies which factors lead to unclustering of centromeres. Such a screen would undoubtedly yield exciting insights into drivers of centromere clustering, and would advance our understanding into which factors control our genome at whole chromosome scale.

Counteracting centromere clustering

Condensin II counteracts centromere clustering in, or directly after, mitosis, but how exactly it keeps the centromeres apart remained a mystery. We here use a combination of cellular experiments and simulation modelling to dissect which function of condensin II is required for keeping apart the centromeres.

We find that condensin II, but no condensin I, is needed for keeping apart the centromeres. As one of the main roles of condensin II is promoting lengthwise compaction in mitosis, we wondered whether this compaction could prevent centromere clustering. Simulation modelling indeed supports the notion that lengthwise compaction in principle can counteract centromere clustering.

However, condensin II depletion by itself does not necessarily increase the end-to-end length of chromosomes (Fig. 6C). We moreover cannot counteract the centromere clustering by simply shortening the chromosomes end-to-end with a hyperactive condensin I (Fig. 7D). How do we reconcile these seemingly counterintuitive findings? Condensin II depletion also leads to a more irregular chromosome shape, the so-called 'zigzag' phenotype (Fig. 6A). Apparently, in absence of condensin II, other factors such as hyperactive condensin I can ensure end-to-end shortening of the chromosomes, but these factors cannot ensure the formation of wild type-like chromosome shapes. We hypothesize that loss of lengthwise compaction and zigzag condensation defects both lead to an increased surface area of chromosomes. This increased surface area in turn would allow for more *trans*-interactions to take place and could therefore promote clustering together of the centromeres, and Rabl-like organization of the interphase chromatin. Extremely long chromosomes, such as observed in the Indian muntjac, might also have a large chromosomal surface area, which could similarly promote *trans*-contacts. We elaborate further on this model in **Chapter 6**.

These findings, together with our earlier work (see **Chapter 3**), leads us to propose a model in which chromosome surface area in mitosis controls interphase architecture type (Fig. 8). Long and irregular chromosomes, caused by e.g. absence of condensin II, then allows

for centromeres to cluster together. The increased surface area of these chromosomes might also lead to more intermingling of the floppy chromosome arms. At mitotic exit these chromosome characteristics would be preserved, leading to Rabl-like genome architecture.

This phenotype is characterized by centromere clustering and more chromosome intermingling, as naturally can be observed in fruit flies or Indian muntjacs. Shortened and rigid chromosomes on the other hand, caused by e.g. presence of condensin II, counteracts centromere clustering in mitosis. The short chromosome arms have smaller chromosomal surface area, and thus will hardly intermingle. At mitotic exit these characteristics will be preserved and lead to chromosome territories, which can be naturally observed in humans and Chinese muntjacs.

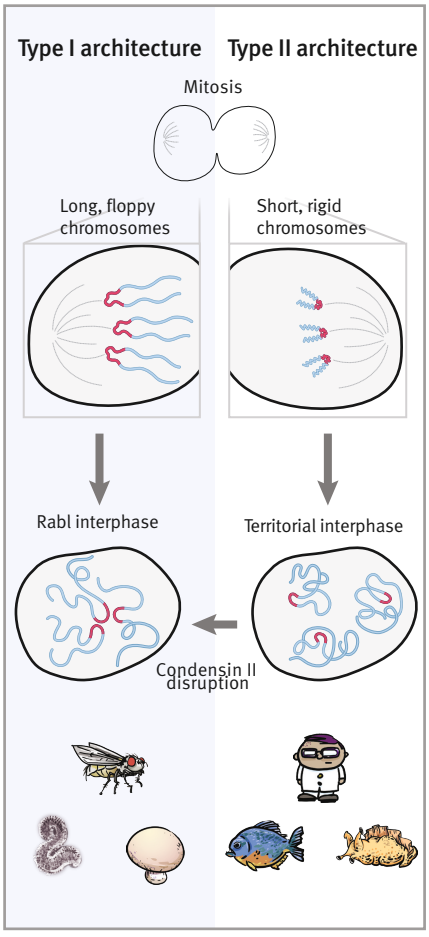

Figure 8 | Mitotic structure determines interphase architecture type. Model for the establishment of type-I and type-II genome architectures. Having shorter chromosomes during mitosis tends to interfere with adhesion between centromeres, leading to separate centromeres and territorial genome architecture in the subsequent interphase. Reducing lengthwise compaction, for example by condensin II disruption, leads to enhanced centromere clustering, loss of chromosome territories, and a Rabl-like genome architecture.

This model is in line with the fact that WAPL loss (partially) prevents centromere clustering. We show that more stable cohesin after WAPL depletion is able to prevent the formation of zigzag chromosomes. This is presumably because cohesin can now make large chromatin loops, thereby taking over this normal function of condensin II in shaping the mitotic chromosome. WAPL depletion however only partially prevents centromere clustering, suggesting that stable cohesin cannot rescue all of condensin II's functions. There are two feasible hypotheses to explain this. First, the cohesin-mediated loops in absence of condensin II might not fully recapitulate the same characteristics as a condensin-mediated chromatin loops in terms of chromosome loop length and density. This different structure might not be sufficient to fully prevent centromere clustering. It would be interesting to perform Hi-C on cells with co-depleted WAPL and condensin II, to investigate to which extent a cohesin-mediated and condensin II-mediated loops are alike. Second, cohesin is cleaved and thereby released from the DNA at anaphase onset, whereas condensin II remains chromatin-bound at this stage of mitosis. Maybe condensin II function from anaphase onwards is essential to fully prevent centromere clustering. To test this hypothesis, one could express an uncleavable variant of cohesin that would remain chromatin bound from anaphase onwards, and investigate whether this would fully rescue centromere clustering. It will be interesting to further investigate the fundamental similarities and differences between such closely related SMC complexes. Answering these questions may lead to a better understanding of why multiple SMC complexes have evolved to control different aspects of genome biology, rather than having one SMC complex that has been repurposed to control all genomic processes.

ACKNOWLEDGEMENTS

Chinese muntjac cells were kindly provided by W. R. Brinkley. Skin fibroblasts of Indian muntjac were obtained from JCRB (9100). We thank M. Takagi from the Cellular Dynamics Laboratory at RIKEN for sharing the CAP-H2-AID and the CAP-H-AID cell lines; S. Knemeyer and V. Yeung (SciStories, LLC) for help with Figure 8.

MATERIALS AND METHODS

Cell culture

All cells were cultured at 37°C with 5% CO_2. Hap1 cells are cultured in IMDM (Gibco), HCT116 cells (gift of Naoko Imamoto lab (Takagi *et al.*, 2018)) were cultured in DMEM (Gibco). Both IMDM and DMEM were supplemented with 10% FBS (Sigma Aldrich), 1% Penicillin/Streptomycin (Gibco) and 1% L-glutamine (Gibco). For the HCT116 CAP-H-AID and CAP-H2-AID cell lines, degradation of the AID-tagged protein was achieved by treatment with 0.5 mM Auxin (Sigma Aldrich).

For disruption of the nucleolus, actinomycin D (1 µg/ul) was added simultaneously with nocodazole (1 µg/µl) for 4 hours before mitotic cells were harvested and replated in a 6-well

plate treated with poly-L lysine. 7 hours after replating cells were fixed and stained using the immunofluorescence protocol.

For depletion of proteins by siRNA, we transfected the cells using Lipofectamine RNAiMAX reagent (Invitrogen) and Opti-MEM medium (Gibco) and cultured the cells for 4 days before harvesting for further experiments. When this siRNA treatment was combined with auxin-mediated condensin II depletion, auxin was added overnight the day before harvesting, with exception for Figs. 6A-C where auxin was added two days before harvesting.

The siRNAs used were: Ki67 HP1α, HP1β, HP1γ (all ON-TARGETplus Smartpool, Dharmacon), Luciferase (D-001100-01, Dharmacon), WAPL (J-026287-11, Dharmacon).

For the washout experiment, 10 µm RO-3306 (Millipore) was used to arrest the cells in G2.

Generating knock-out cell lines

Hap1 CAP-H2 knock-out cell lines were obtained as described in (Elbatsh *et al.*, 2019).

Immunofluorescence

Cells were grown on glass coverslips of 12 mm in diameter. Pre-extraction was achieved by either using PEM-T (100mM PIPES pH 6.8, 1 mM $MgCl_2$, 5 mM EGTA and 0.2% Triton) buffer for 45 seconds or 0.1% Triton in PBS for 1 minute. After this, samples were fixated with 4% PFA in PBS + 0.1% Triton. Cover slips were blocked in 3% BSA in PBS-Tween (0.1%) for 30 minutes. Primary antibody was incubated overnight at 4°C. Slides were washed in PBS-Tween (0.1%), before secondary antibody incubation for 1 hour at room temperature with antibody (mouse or rabbit Alexafluor 488, 568 or 647 from Invitrogen; 1:1000) and DAPI (Sigma-Aldrich; 1:1000). After this incubation, cells were washed with PBS-Tween (0.1%) and cover slips were mounted on glass slides with Prolong Antifade Gold (Invitrogen). To acquire images, Deltavision Elite System (Applied Precision), and Softworx software were used.

For quantification of centromere foci, an in-house macro was used with ImageJ(Van Den Broek, 2021). See Chapter 2 for further explanation of this macro. All imaging experiments were performed in at least three independent biological replicates, with the exception of the experiment in figure S1B which was performed twice. Replicates always showed the same result, and therefore only one representative replicate is shown for each experiment.

For the analysis of the amount of centromeres at the nucleolus, 3D Images were analyzed and quantified using Fiji (Schindelin *et al.*, 2012) in a fully automatic, unbiased manner, using a custom-made ImageJ macro (Van Den Broek, 2021) (Intensity_fraction_and_distribution_around_nucleoli.jim). First, each image was cropped 32 pixels from the edges to remove artifacts produced by the Deltavision deconvolution process. Next, cell nuclei were segmented in 2D using the DAPI channel, by consecutive application of a rolling ball background subtraction (radius 20 microns), 3D median filter (radii 200 nm (xy) and 400 nm (z)) and

maximum intensity projection, followed by thresholding (Otsu method) and a distance transform watershed operation to separate touching nuclei. At this point the macro allowed fixing segmentation mistakes by manual deletion/addition/combining of ROIs. Mitotic cells were removed from the analysis.

Automatic 3D segmentation of nucleoli was achieved by employing the built-in 3D Object Counter plugin. For every nucleus, a reliable threshold for 3D segmentation was established by setting it equal to the Otsu threshold level of the (background-subtracted) 2D maximum intensity projection of the nucleoli. For the centromere channel the same thresholding strategy was used, except that here a single threshold was determined using all valid nuclear ROIs in the image. Background fluorescence was removed by setting voxels with gray levels below half the found Otsu threshold to NaN.

For every remaining voxel in the centromere channel the intensity distribution around the nucleoli was calculated as follows. For every nucleus a set of 3D masks ('shells'), each on a certain distance from the nucleoli edge was created by utilizing the plugin '3D distance map' (EDT) in the 3D Suite plugins (Ollion *et al.*, 2013). These distance masks were then multiplied by the background-removed centromere stack of that nucleus to get the average intensity at different distances from the nucleoli edge, and were normalized by dividing by the total centromere intensity, to obtain the intensity distribution around the nucleoli. To decide which fraction of the centromeres where at the nucleolus we put a threshold at a distance of 0.4 µm from the nucleolus. In the figure, one experiment of three independent experiments is shown with median. We calculated statistical significance with a Mann-Whitney non-parametric test, and the p-value was ‹0.0001.

For Analysis of the H3K9me3 domains, 3D surface renderings were computed and analyzed using Imaris (Bitplane) in three independent experiments, at least 150 cells were quantified. In the graph, the median is depicted as a red line, outliers are depicted as dots and cut-off at the edge of the axis. All dotplots were made with ggplot2 and include random jitter to avoid overplotting. The calculate statistical significance, a Mann-Whitney nonparametric test was performed, and the p-value was ‹0.0001.

Antibodies

Immunofluorescence microscopy was performed using the following antibodies: Nucleolin (Abcam, ab70493), H3K9me3 (Abcam, ab8898) and CENPA (Abcam, ab13939 and ThermoFisher Scientific, MA1-20832).

Western Blots were performed using the following antibodies: KI67 (Abcam, ab15580), Tubulin (Sigma Aldrich, T5168), CAP-H2 (Bethyl, A302-275A), HP1γ (Millipore, 05-690), HP1β (Abcam, ab10478), Hsp90 (Santa Cruz, H-114), CAP-H (Novus Biologicals, NBP1-32573), WAPL (Santa Cruz, A-7).

Western Blot

Cells were lysed using one of two methods. Either they were lysed using Laemmli buffer

(120 mM Tris pH 6.8, 4% SDS, 20% glycerol) and boiling at 95°C. Or they were lysed using RIPA buffer (150 mM Sodium Chloride, 50 mM Tris-HCL (pH 8.0), 1% Nonidet P-40, 0.5% Sodium deoxycholate, 0.1 % SDS) and incubation on ice for 30 minutes. Protein concentration was quantified with the Lowry assay. Samples were loaded on a NuPage 4-12% Bis-Tris gel and run in 1x MOPS buffer (Novex biologicals), transferred onto a methanol-activated PVDF membrane (immobilon) or a nitrocellulose membrane (GE healthcare) and visualized using Clarity ECL (BioRad) and a Chemidoc Gel Imaging System (BioRad).

Chromosome spreads

Mitotic cells were harvested by mitotic shake-off after treatment with nocodazole (1 µg/µl) for 1.5 hours. Cells were treated with 0.075 M KCl and 6% fixative at 37ºC for 20 minutes and subsequently spun down at 1500 rpm for 5 minutes. Cells were fixed in fixative (MeOH: AA in 3:1) and centrifuged for 5 minutes at 1500 rpm. This procedure was repeated once before addition of fixative with DAPI (1:1000). Allowing the drops to fall from 30 cm height onto glass slides and tilting the slides created the chromosome spreads. Spreads were mounted using Prolong antifade Gold (Invitrogen) and analyzed using the Metafer software. The mean +SD of at least three independent experiments is shown.

Centromere clustering in the Indian muntjac

We used 3D-DNA (Dudchenko *et al.*, 2017) and Juicebox Assembly Tools (Dudchenko *et al.*, 2018) to upgrade the draft assemblies for the Indian and the Chinese muntjacs from (Chen *et al.*, 2019) to chromosome-length using Hi-C. (Recently an independent set of chromosome-length genome assemblies for the species has been generated in (Mudd *et al.*, 2020). In accordance with karyotype data, the Chinese muntjac assembly comprised 23 chromosome-length scaffolds and the Indian muntjac comprised 3.

Fig. 6F (https://tinyurl.com/y6cwlh93) shows the corresponding Hi-C contact data aligned to the new chromosome-length genome assemblies, with the map on the left representing the Chinese muntjac (23 acrocentric chromosomes), and the one on the right – the Indian muntjac (one metacentric chromosome, one acrocentric and one submetacentric). Remarkably, the map on the right has bright spots in positions that correspond to centromere-to-centromere interactions consistent with centromere clustering, a Type-I chromosome architecture feature. No such signal is visible in the Chinese muntjac contact map that displays a typical Type-II contact pattern with chromosome territories as a dominant architecture feature. Notably, the chromosome territories signal is attenuated in the Indian muntjac as compared to the Chinese, also consistent with the shift from Type-II to Type-I architecture type with the dramatic increase of chromosome length.

Simulation model

We simulate chromosomes as array of monomers, where individual monomers of diameter σ are chromatin "blobs" (typically containing tens of nucleosomes) that undergo stochastic Langevin dynamics (Di Pierro *et al.*, 2016). Our simulation setup consists of 10 chromosomes of 400 monomers each, where each chromosome possesses a 40 monomers-segment at the center as centromere.

The force fields characterizing the motion of each monomer may be divided into the three categories (Di Pierro *et al.*, 2016): first, the interactions that originate from polymeric constraints, such as FENE (finite-extensible nonlinear elastic) bond stretching interaction between nearest neighbors. These interactions are primarily responsible for a "random coil"-like behavior of the chromosome polymer.

Second, inter-monomer contact energy, which stabilizes contact between any pairs of centromeric monomers. We use an adhesive interaction (see the contact function used in (Di Pierro *et al.*, 2016)) with strength -0.25ε for any two pairs of centromeric monomers, whereas, there is a generic stickiness of -0.2ε for all other types of pairwise monomer-monomer contact (ε is the unit of energy in our simulations).

Finally, the third component is the lengthwise compaction or the ideal chromosome term (Marko, 2009; Di Pierro *et al.*, 2016; Zhang and Wolynes, 2016). This potential depends on the distance along the polymer chain between a pair of loci, and generates the forces that compact the chromosome polymer in a lengthwise manner, i.e., compaction along the chromosome contour. For lengthwise compaction, inspired by the ideal chromosome potential (Di Pierro *et al.*, 2016; Zhang and Wolynes, 2016), we implement a potential of the form: $-0.2*exp\left(-\frac{s}{10}\right) - B/s\,\hat{}\,0.5$, that decays monotonically along the contour length s (measured in σ units), leading to a contraction force that drives contour wise folding of individual chromosomes. Decreasing the parameter B leads to steady-state chromosome conformations corresponding to lower condensin II activity. Inter-centromeric self-adhesion, unlike lengthwise compaction, may act across chromosomes and lead to significant inter-chromosomal contacts; whereas, lengthwise compaction is only intra-chromosomal and drives predominance of cis-chromosomal contacts.

Chromosome territorialization signal

Territorialization of chromosomes is identified as the ratio of cis-contacts to total contacts per chromosome. We enumerate "contacts" based on a Voronoi-tessellation scheme where each chromosome monomer has about 8 surrounding neighbors that it makes "contacts" with. For a territorial chromosome, most of its monomers lie within the territory volume and all their neighbors are monomers of the same chromosome; only the monomers constituting the surface of the territory have about half their neighbors belonging to different chromosomes.

Centromere clustering analysis

We use a hierarchical clustering algorithm to spatially cluster the ten centromeres. Clusters are identified by requiring that when any two centromeres are merged, the distance between the centroids of the merged and the individual centromeres is larger than *n*-times the average radius of gyration of a centromere. We used n=3, however a 20% change in *n* does not change our qualitative results. A conformational state with higher centromeric clustering also corresponds to a higher ratio of *trans*-centromeric to total centromeric contacts, as calculated from Voronoi tessellation mentioned above.

Code and Data Availability

Our simulation package can be found at (Contessoto *et al.*, 2021; Hoencamp *et al.*, 2021). The codes relevant to this work and sample simulation trajectories are also available at (Contessoto *et al.*, 2021; Hoencamp *et al.*, 2021).

Simulated contact maps

The simulated contact maps, similar to Hi-C maps, show the probability of contact between genomic segments, as measured from the simulation snapshots. Using previously published model parameters (Di Pierro *et al.*, 2016), we map 3D distance in a snapshot to contact probability and generate a contact probability matrix. These probability matrices were then used to make .hic files and plotted using Juicebox.

Cell growth assay

To assay cell growth after siRNA and auxin double treatment, a Lionheart FX automated microscope was used. In a 96-well plate 1000 cells were plated and transfected 6 hours later with siRNA. One day later auxin and SPY-DNA 650 (Spirochrome, 1:4000) was added to the cells 1.5 hours before the start of the movie. Cells were imaged every four hours to generate growth curves. During filming the microscope maintained a constant temperature of 37 °C, and 5% of CO_2. Quantification of the cell number was performed in an automated manner by Gen5 software (BioTek).

SUPPLEMENTAL FIGURES

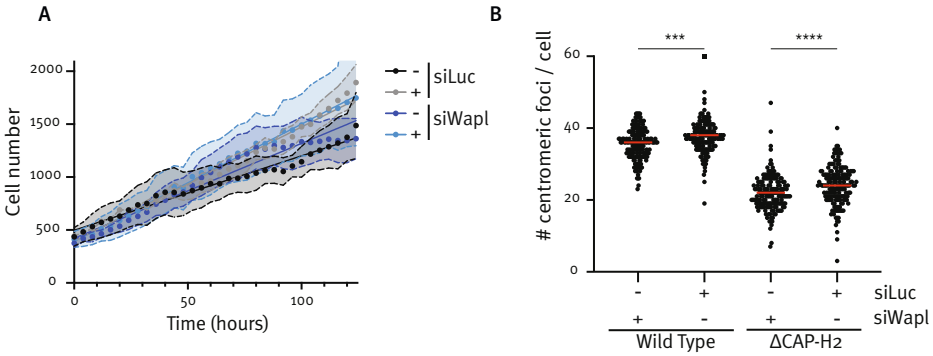

Figure S1 | Wapl depletion partially rescues centromere clustering in Hap1 cells. (A) Growth curves of HCT116 CAP-H2-AID with the indicated treatments as measured (B) Centromeric clustering after the indicated treatments with siRNA in Wild Type and ΔCAP-H2 Hap1 cells. Quantification of centromeric foci per cell of experiment, median is shown in red. Significance was calculated with an ANOVA followed by a Sidak's multiple comparison test, ***= p=0.0002 and ****=p<0.0001.

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Chapter 6

The opposing forces folding the genome

Claire Hoencamp and Benjamin D. Rowland

**Adapted from Hoencamp, C. and Rowland, B.D. Genome Control by SMC complexes. Nature Reviews Molecular Cell Biology 24, 633-650 (2023).*

ABSTRACT

SMC complexes are well known for their ability to build chromatin loops. By doing so, these complexes can regulate many DNA-based processes, ranging from DNA replication and repair, to building rigid mitotic chromosomes. In this chapter we consider how SMC complexes, by building chromatin loops, can also counteract the natural tendency of alike chromatin regions to cluster. SMC complexes thus control nuclear organization by participating in a molecular tug-of-war that determines the architecture of our genome.

INTRODUCTION

There are several unexpected connections between the way in which chromatin is structured in mitosis and the way in which chromosomes are organized in the subsequent interphase (Boveri, 1909). Recently it has become clear that also at the scale of whole chromosomes, the organization of the nucleus in interphase is instructed by the folding of chromosomes in mitosis (**Chapters 3 and 5**). A tug-of-war between two important nuclear forces turns out to regulate which type of genome organization will occur following cell division.

FIGHTING FOR TERRITORY

3D genome analyses across different branches of evolution have recently revealed that at the scale of whole chromosomes, genome folding can be categorized into either of two main types: Rabl-like organization or chromosome territories (**Chapter 3**) (Fig. 1). For a long time, it remained a mystery what controls genome organization at this scale. Research in a range of species suggests that condensin is one factor that controls chromosome-scale interphase genome architecture. One of the most beautiful examples of this exists in *Drosophila melanogaster* development, where maternal and paternal alleles are paired in a process called somatic homolog pairing (Joyce *et al.*, 2012). This pairing allows for transvection, a process in which gene expression is influenced by the other allele of the same gene. In mid oogenesis, this pairing is disrupted by the formation of chromosome territories. This process is dependent on condensin II, which antagonizes somatic homolog pairing and transvection, and instead promotes chromosome territorialization (Hartl, Smith and Bosco, 2008; Bauer, Hartl and Bosco, 2012; Joyce *et al.*, 2012; Buster *et al.*, 2013; Nguyen *et al.*, 2015; Vernizzi and Lehner, 2021). Territorialization leads to the loss of contacts between different chromosomes, including the loss of various types of *trans* contacts. An example of *trans* contacts that are lost are those between different centromeres and heterochromatin regions. Condensin promotes such loss of clustering of these regions not only in *D. melanogaster* cells (Nguyen *et al.*, 2015; Rosin *et al.*, 2018), but also in fission yeast (Iwasaki, Corcoran and Noma, 2016), *Tetrahymena thermophila* (Howard-Till and Loidl, 2018), plants (Schubert, Lermontova and Schubert, 2013; Sakamoto *et al.*, 2019, 2021; Municio *et al.*, 2021), mice (Nishide and Hirano, 2014) and humans (**Chapter 3**) (Houlard *et al.*, 2021).

The absence of condensin II throughout evolution correlates with the Rabl-like nuclear architecture (**Chapter 3**). It is therefore quite likely that the role for condensin II in controlling this architecture type is highly conserved. There are however some variations in how condensin can act to promote territories. In fission yeast, a species that lacks all condensin II subunits and exhibits Rabl-like organization, removal of its condensin complex (which more resembles condensin I than condensin II) leads to even more intermingling of chromosomes (Iwasaki *et al.*, 2010). *D. melanogaster* and mosquitos lack one subunit of the condensin II complex (**Chapter 3**) (King *et al.*, 2019), yet depletion of the other condensin subunits leads to more severe Rabl organization (Bauer, Hartl and Bosco, 2012). Finally, it must be noted that in

insects, loss of condensin II does not correlate with somatic homolog pairing (King *et al.*, 2019). Thus, although condensin II clearly is a key player in regulating chromosome-scale genome architecture, other, unknown processes must also contribute.

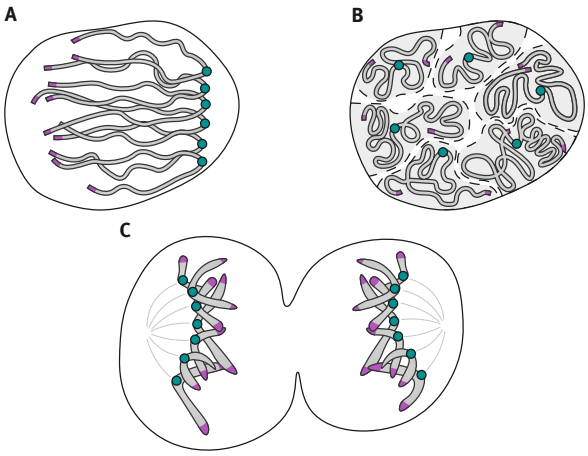

Figure 1 | Rabl-like organization versus chromosome territories. (A) Both Rabl-like organization and chromosome territories were hypothesized to exist over 100 years ago(Rabl, 1885; Boveri, 1909). Species with Rabl-like organization, such as yeasts, display centromere clustering at one side of the nucleus, with the chromosome arms stretching out in parallel to the other side of the nucleus, where the telomeres cluster. (B) Chromosome territories refer to a state, in which each chromosome occupies a distinct region within the nucleus, with few contacts occurring between chromosomes (Boveri, 1909; Longo and Roukos, 2021). (C) Both types of organization retain an aspect of the organization of chromosomes at the preceding mitosis (Boveri, 1909). Whereas Rabl-like chromosomes still exhibit the same orientation as mitotic chromosomes, chromosome territories organization to some degree reflects the individualization of chromosomes during mitosis.

What then allows condensin II to promote nuclear organisation into chromosome territories? Seminal work has linked chromosome axis compaction by condensin II to its unpairing activity (Bauer, Hartl and Bosco, 2012). This connection has an interesting parallel with sister chromatid resolution, where condensin II shortens the chromosome and thereby promotes *cis* interactions over *trans* interactions (Hirano, 2012). In *silico* modelling also shows that lengthwise compaction of chromosomes can be sufficient to counteract centromere clustering, which is one of the key features of Rabl-like organization (**Chapter 5**). All this supports a model in which condensin II's ability to shorten chromosomes explains its key role in nuclear organization. What remains unspecified is which force(s) promotes centromere clustering in the first place.

FORCES PROMOTING CLUSTERING

If condensin II prevents the formation of Rabl-like organization, what then promotes this type of organization? An attracting force between centromeres of different chromosomes is a strong candidate to drive this type of genome architecture (Fig. 2A).

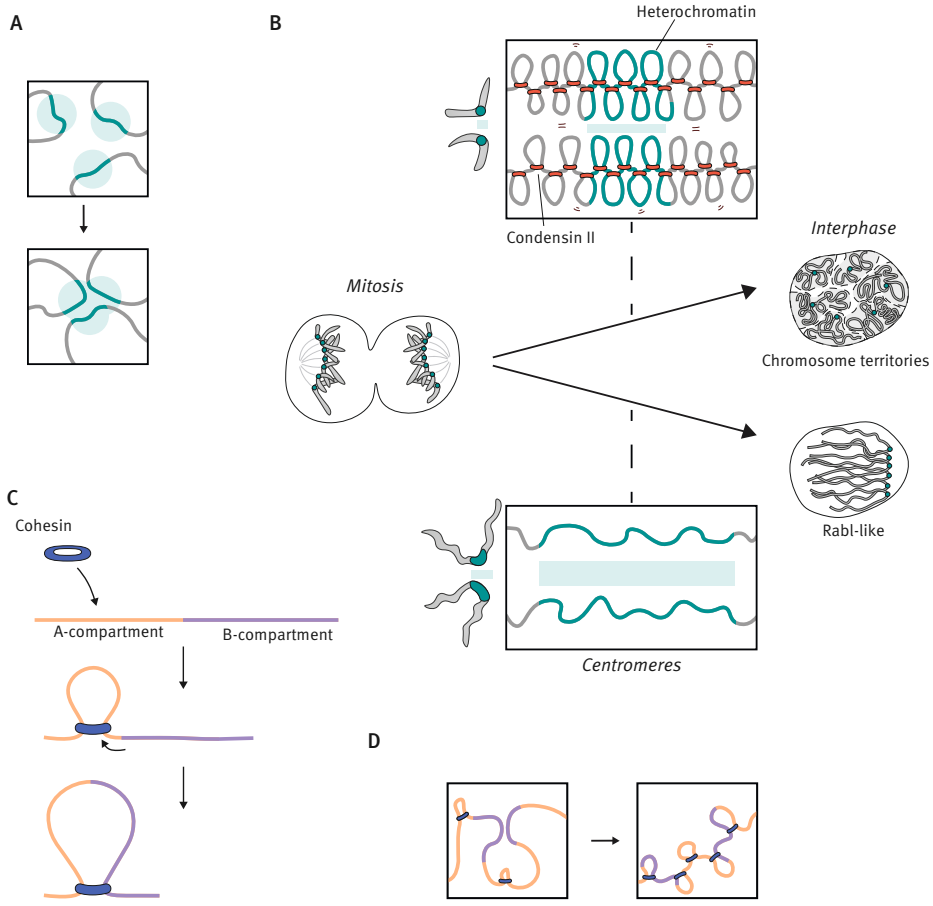

Figure 2 | Two opposing forces shaping the nucleus. (A) Model of heterochromatin attracting forces (turquoise) causing heterochromatin clustering. (B) Model of establishment of interphase genome architecture. In mitosis, different centromeres come into proximity due to the mitotic spindle forces. (Top) If condensin II is present during mitosis, loop extrusion occurs at centromeres, which may lead to smaller heterochromatin surface area. Alternatively, loop extrusion dynamics may limit the time centromeres can interact with each other. In either scenario, condensin II activity prevents centromere clustering, leading to formation of chromosome territories in the subsequent interphase. (Bottom) If condensin II is not present in the cell, heterochromatin attraction forces can cause the centromeres to cluster together. The result then is a Rabl-like nuclear architecture in the subsequent interphase. (C) By crossing the boundaries between euchromatin (A) and heterochromatin (B) compartments, cohesin can intermix these compartments and counteract compartmentalization. (D) Loop formation by cohesin could also prevent compartmentalization by increasing the rigidity of chromatin, thus counteracting long-range cis interactions.

Centromeres are flanked by regions known as pericentric heterochromatin, and heterochromatin regions that lie far apart from each other on the linear genome, tend to cluster together in the nucleus (Lieberman-Aiden *et al.*, 2009). Consequently, these heterochromatin-attracting forces are considered to be a major driving force of the spatial separation of euchromatin and heterochromatin in the nucleus: heterochromatin compartments occupy volumes in the nucleus that are distinct from those occupied by transcriptionally more active

regions (Lieberman-Aiden *et al.*, 2009; Falk *et al.*, 2019). Heterochromatin clustering also is responsible for the atypical nuclear organization observed in retinal rod cells, in which all heterochromatin resides in the nuclear interior and is surrounded by a layer of euchromatin (Falk *et al.*, 2019). Finally, in some species such as mice, heterochromatin aggregates in structures called chromocenters.

A key factor responsible for this type of clustering is heterochromatin protein 1 (HP1), which recognizes and binds nucleosomes with trimethylated histone H3 Lys9 (H3K9me3), an epigenetic mark involved in chromatin silencing. HP1 can contribute to clustering in multiple ways. First, it can multimerize into higher-order structures (Canzio *et al.*, 2011; Biswas *et al.*, 2022), and can thereby bridge different heterochromatin regions. *In vitro* HP1 can form liquid droplets through phase separation, which could also lead to heterochromatin clustering by merging of such droplets (Canzio *et al.*, 2011; Falk *et al.*, 2019; Biswas *et al.*, 2022). However, research *in vivo* failed to find evidence for such phase separation of HP1 within chromocenters (Erdel *et al.*, 2020; Gitler *et al.*, 2020), making it unclear whether HP1 can exert this function by undergoing phase separation. The binding of HP1 to nucleosomes also exposes certain residues on histones, which in turn promotes chromatin phase separation (Sanulli *et al.*, 2019). Each of these characteristics would be in line with the finding that HP1 is essential for the formation of Rabl-like architecture in early *D. melanogaster* development (Zenk *et al.*, 2021).

Polycomb repressive complex 1 (PRC1) can also undergo phase separation *in vitro* and thereby contribute to heterochromatin clustering (Plys *et al.*, 2019). Multiple additional factors have been discovered in *D. melanogaster* that promote chromocenter formation, presumably by bridging distal chromatin regions (Jagannathan, Cummings and Yamashita, 2019). All these mechanisms may work in concert to cluster together heterochromatin, thereby promoting interactions in *trans* and favouring Rabl-like organization over chromosome territories. Recently, it was discovered that the centromeric protein CENP-B can promote the clustering of centromeres (Chardon *et al.*, 2022). There might thus be other avenues unrelated to heterochromatin that promote Rabl-like organization.

Depletion of condensin II in interphase cells remarkably does not appear to affect nuclear architecture (**Chapter 3**) (Abdennur *et al.*, 2018). Pericentric heterochromatin in this setting apparently is not sufficient to lead to centromere clustering. Progression through mitosis in the absence of condensin II however leads to a shift from a territorial to a Rabl-like nuclear organization in the subsequent interphase (**Chapter 3**) (Fig. 2B). These data indicate that there are indeed other forces, specifically in mitosis, which enable centromere clustering. Possibly the type of chromosome folding, or the dramatic chromatin movement in mitosis, is required for heterochromatin domains to encounter each other. In species with no condensin II, the attraction forces could then be strong enough to keep centromeres clustered in the following interphase.

LOOP EXTRUSION VERSUS ATTRACTIVE FORCES

Condensin appears to be able to counteract long-range interactions between centromeres during mitosis. Condensin thereby allows for the establishment of chromosome territories in interphase. Excitingly, cohesin seems to be able to disrupt long-range interactions in an analogous manner. The transcriptionally active regions within the nucleus are referred to as A compartments, and the transcriptionally more silent regions are called B compartments. The different chromatin regions belonging to each compartment tend to cluster together. This process is known as compartmentalization (Lieberman-Aiden *et al.*, 2009), and is in part due to the heterochromatin clustering described above. However, phase separation promoted by chromatin factors such as bromodomain-containing protein 4 (BRD4), histone H1, histone acetyltransferases and the transcription machinery may all contribute to this segregation of the genome (Gibson *et al.*, 2019; Guo *et al.*, 2019). Modelling shows that loop extrusion could in principle counteract compartmentalization (Nuebler *et al.*, 2018), and indeed cellular work shows that cohesin counteracts the clustering together of compartment-alike genomic regions (Gassler *et al.*, 2017; Haarhuis *et al.*, 2017; Rao *et al.*, 2017; Schwarzer *et al.*, 2017; Wutz *et al.*, 2017). Analogous to condensin, cohesin-mediated loop extrusion can move apart chromatin regions, thereby hindering their clustering (Gassler *et al.*, 2017; Haarhuis *et al.*, 2017; Rao *et al.*, 2017; Schwarzer *et al.*, 2017; Wutz *et al.*, 2017). Furthermore, cohesin can extrude loops over boundaries between different compartments, thereby essentially intermixing these compartments and preventing their clustering (Fig. 2C). The stabilization of cohesin on DNA counteracts compartmentalization to the extreme, as B compartments and the corresponding heterochromatin domains can barely be formed in WAPL-deficient cells (Haarhuis *et al.*, 2022), possibly owing to the increasing stiffness of the chromosome, which no longer allows formation of the long-range contacts required for compartmentalization (Fig. 2D).

CONCLUDING REMARKS

SMC complexes are key factors that organize the 3D genome in both interphase and mitosis. Condensin and cohesin can both form chromatin loops, and hereby structure the genome at a relatively small scale. Condensin and cohesin can also indirectly regulate larger-scale genome organization. As a general theme, SMC complexes can antagonize attraction forces between alike sequences, such as those imposed by HP1 and BRD4 clustering. They can seemingly do so by performing loop extrusion. When forming chromatin loops, these complexes favour shorter-range *cis* interactions over longer-range interactions in *cis* or in *trans*. In the case of condensin, these can disrupt interactions between centromeres of different chromosomes. Cohesin functions in an analogous manner to disrupt long-range *cis* interactions, and on top of that can intermix compartments by extruding loops over compartment boundaries.

In the emerging model, the loop extruding complexes cohesin and condensin actively counteract attraction forces as part of a molecular tug of war that shapes the nucleus. It is an interesting notion that each of these complexes presumably acts in a similar manner, yet they control different aspects of larger-scale genome organization. It will be interesting to investigate whether this is for example due to their differential activity in the cell cycle. Whereas cohesin predominantly forms loops in interphase, condensin is most active in mitosis. Compartments and chromosome territories might then be established in different cell cycle phases, and therefore be controlled by either condensin or cohesin. Future research will surely provide us with exciting new insights into the dynamics of genome organization by SMC complexes.

ACKNOWLEDGEMENTS

Due to space limitations, the authors apologize for not being able to cite all relevant articles or discuss all aspects of SMC biology. The authors acknowledge financial support from the European Research Council (772471-CohesinLooping) and from the Dutch Research Council (VI.C.202.098), and thank Anoek Friskes and members of the Rowland laboratory for critical reading of the manuscript.

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Chapter 7

MCPH1 regulates topoisomerase and chromosome segregation through condensin II

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ABSTRACT

Condensin complexes are key for the formation of mitotic chromosomes and subsequent chromosome segregation. Yet, little is known about the factors that regulate condensins and ensure their timely activity. MCPH1 was found to be a negative regulator of condensin II. Loss of MCPH1 results in interphase condensation through increased condensin II stability. In this study, we investigate the consequences of this undue condensin activation. We find that loss of MCPH1 leads to micronuclei formation, possibly through mitotic defects. On top of that, MCPH1 loss also sensitizes to drugs targeting topoisomerase 2 in a condensin II-dependent manner. Our findings provide new insights on the link between topoisomerase and condensin II, and into why condensin II must be kept in check by MCPH1.

INTRODUCTION

In interphase, chromosomes form what through the microscope seems like an amorphous chromatin mass. When cells enter mitosis, chromosomes undergo a major transformation into individualized and rigid packages. Key factors responsible for this reorganization of mitotic chromatin are condensin complexes. Many organisms possess two distinct condensin complexes: condensin I and condensin II. These complexes share a SMC2-SMC4 heterodimer, but differ in their accessory subunits: condensin I binds CAP-H, CAP-D2 and CAP-G, whereas condensin II binds CAP-H2, CAP-D3 and CAP-G2 (Ono *et al.*, 2003).

An important difference between these two complexes is their cellular localization. Condensin I is mainly cytoplasmic, whereas condensin II is nuclear throughout the cell cycle. Despite being nuclear, condensin II cannot condense DNA in interphase. Condensation only occurs in mitosis, presumably because condensin II then binds DNA in a more stable manner (Gerlich *et al.*, 2006). In mitosis, condensin II builds long chromatin loops, which are subdivided into smaller chromatin loops by the more dynamic condensin I (Gibcus *et al.*, 2018; Walther *et al.*, 2018). For a long time, it remained a mystery what promotes condensin II's transition from dynamic interphase binding to stable mitotic binding. A recent study revealed that MCPH1 (also known as BRIT1 or microcephalin) is responsible for keeping condensin II dynamically associated with the DNA in interphase (Houlard *et al.*, 2021).

MCPH1 was originally discovered because it is mutated in autosomal recessive primary microcephaly (Jackson *et al.*, 2002), and in the related premature chromosome condensation (PCC) syndrome (Neitzel *et al.*, 2002; Trimborn *et al.*, 2004). These patients exhibit a marked reduction in brain size and mild mental retardation (Jackson *et al.*, 2002; Neitzel *et al.*, 2002). On top of that, MCPH1 is mutated in many cancers, and is generally associated with more aggressive tumors and worse patient outcome (Chaplet *et al.*, 2006; Rai *et al.*, 2006; Tervasmäki *et al.*, 2019; Denu and Burkard, 2020; Alsolami *et al.*, 2023).

MCPH1 has been implicated in many cellular processes. MCPH1 localizes to DNA damage foci to facilitate the recruitment of many repair factors such as BRCA2, 53BP1, Rad51 and RPA (Rai *et al.*, 2006; Wood *et al.*, 2007, 2008; Liang *et al.*, 2010). Moreover, MCPH1 promotes the stability of Rad51 filaments *in vitro* (Chang *et al.*, 2020). It is thought to exert this function at least in part through recruiting the SWI-SNF complex, which remodels nucleosomes around the break site (Peng *et al.*, 2009). MCPH1 also plays a role at telomeres, and was identified independently in a screen for transcriptional repressors of hTERT (human telomerase) (Lin and Elledge, 2003). We now know that it promotes homology directed repair at dysfunctional telomeres and facilitates telomere replication (Cicconi *et al.*, 2020). Apart from its various roles in DNA damage, MCPH1 also localizes to centrosomes to coordinate their duplication (Zhong, Pfeifer and Xu, 2006; Denu and Burkard, 2020; Kristofova, Ori and Wang, 2022).

Strikingly, patients with defective MCPH1 exhibit condensation in G1- and G2-phase of the cell cycle (Neitzel *et al.*, 2002; Trimborn *et al.*, 2004, 2005). This interphase condensation, a phenotype which has previously been called 'prophase like cells (PLC)' or 'premature chromosome condensation (PCC)', is dependent on condensin II (Trimborn *et al.*, 2006). This supports the notion that MCPH1 is a negative regulator of condensin II. Expression of MCPH1 in Xenopus egg extracts results in the loss of condensin II from the DNA, and reduced rigidity of mitotic chromosomes (Yamashita *et al.*, 2011). Loss of MCPH1 leads to stable DNA binding of condensin II in G1 and G2 (Houlard *et al.*, 2021). Apparently, in absence of MCPH1, condensin II somehow is no longer kept in check and can now readily condense chromatin during interphase. These findings establish MCPH1 as an important negative regulator of condensin II.

How does MPCH1 then regulate condensin II turnover? Turnover of cohesin, a protein complex closely related to condensin, is regulated by its release factor Wapl. This factor is thought to function by opening up the ring-shaped complex at a so-called 'exit gate'. Interestingly, fusion of the analogous interface in condensin II renders it resistant to MCPH1 (Houlard *et al.*, 2021). This suggests that MCPH1 controls condensin II in a manner similar to how Wapl controls cohesin. When cells enter mitosis, MPCH1 is thought to be phosphorylated, preventing its binding to condensin II (Houlard *et al.*, 2021). Condensin II is then no longer inhibited, and can stably associate with the DNA. This is likely the switch that is required to condense chromatin and form rigid and individualized mitotic chromosomes.

These intriguing findings lead to many new questions. Why is condensin II nuclear in interphase, but prevented from binding DNA in a stable manner? Which defects arise in absence of MCPH1, when condensin II is stably associated with the DNA throughout the cell cycle? In this study, we set out to answer these questions. We use single and double knock-out cell lines for MCPH1 and CAP-H2 to investigate which defects occur when condensin II is stabilized on the DNA. Using a combination of immunofluorescence imaging and an unbiased screening approach, we aim to better understand the biological relevance of MCPH1-mediated condensin II removal. This will likely not only further our fundamental understanding of condensin biology, but might also yield important insights into the processes that are affected in diseases such as primary microcephaly and cancer.

RESULTS

MCPH1 prevents condensin II-mediated interphase condensation

To investigate the relevance of condensin II regulation by MCPH1, we generated MCPH1 and CAP-H2 single knock-outs and MCPH1/CAP-H2 double knock-outs in Hap1 cells, using CRISPR-Cas9 (Fig. 1A). MCPH1 depletion causes hypercondensation in both mitosis and interphase in a condensin II-dependent manner (Trimborn *et al.*, 2004; Arroyo *et al.*, 2015). We wanted to confirm that these phenotypes also occurred in our cell lines. First, we measured mitotic chromosome length in ΔMCPH1 and control cells. Mitotic chromosomes are indeed

shorter in ΔMCPH1 cells (Fig. 1B). We next examined interphase condensation in absence of MCPH1, or in absence of both MCPH1 and CAP-H2. Wild type nuclei do not show any interphase condensation, but have a diffuse DNA stain which is indicative of uncondensed DNA. ΔMCPH1 cells on the other hand exhibit clear interphase condensation in ~%50 of nuclei. This interphase condensation is dependent on condensin II, as the ΔMCPH1/ΔCAP-H2 cells did not display this phenotype (Figs. 1D and 1E, Fig. S1A). MCPH1 deficiency thus also leads to untimely condensation in Hap1 cells.

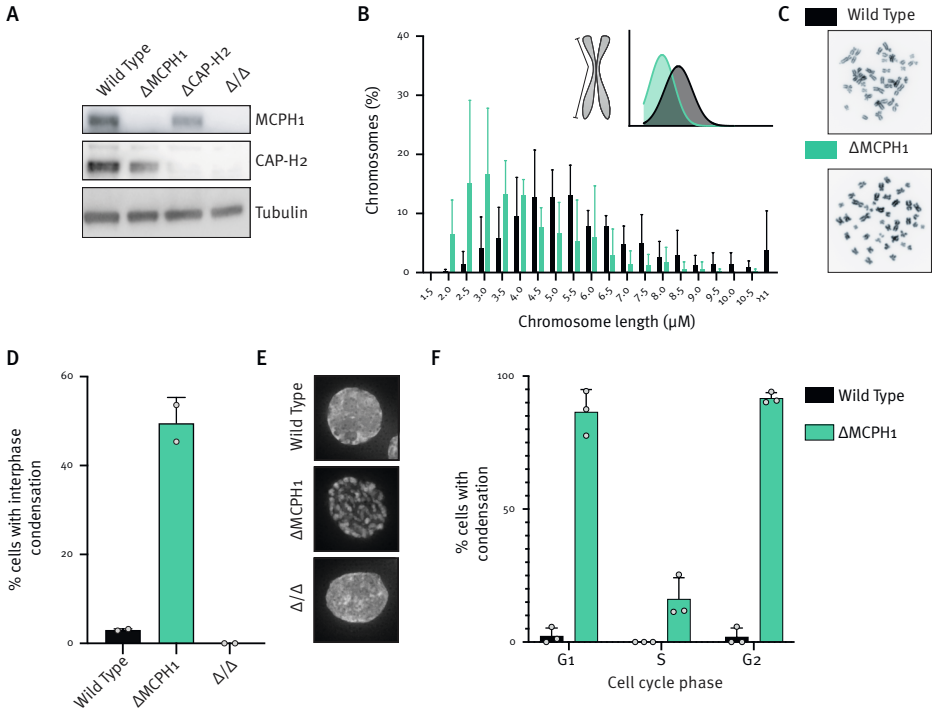

Figure 1 | MCPH1 loss leads to condensin-dependent hypercondensation. (A) Western Blot analysis of the indicated Hap1 ΔMCPH1, ΔCAP-H2, or ΔCAP-H2/ΔMCPH1 (Δ/Δ) cell lines. (B) Quantification of chromosome length in mitotic chromosome spreads (see panel C) of the indicated cell lines. Chromosomes 1, 2 and 3 were measured as indicated in the schematic overview of the chromosome. Shown is the Mean + SD of three independent experiments in which at least 150 chromosomes per condition were measured. Inset shows the histogram of the length distribution. (C) Example images of mitotic chromosome spreads as quantified in (B). (D) Quantification of the amount of cells with interphase condensation of the indicated cell lines. Interphase condensation is defined as irregular DNA staining, as can be observed in the example image of the ΔMCPH1 cell line in (E). Shown is the mean + SD of two independent experiments in which at least 50 cells were scored. Grey circles depict the mean of each independent experiment. (E) Immunofluorescence microscopy of the indicated cell lines. Cells were stained with DAPI to visualize DNA. (F) Quantification of the amount of cells with condensation in G1-, S- or G2-phase of the cell cycle, as visualized by immunofluorescence microscopy. Shown is the mean + SD of three independent experiments, a total of at least 110 cells were scored per cell line. Grey circles depict the mean of each independent experiment. Example images can be found in (Fig. S1).

We next investigated in which cell cycle phases this interphase condensation occurs. Using immunofluorescence microscopy of cell-cycle specific markers (CDT1, Geminin and EdU), we find that interphase condensation occurs throughout G1- and G2-phase, but rarely in S-phase (Fig. 1F, Figs. S1B-D). This is in line with data showing that MCPH1 defects lead to premature condensation and delayed decondensation (Neitzel *et al.*, 2002; Trimborn *et al.*, 2004, 2010). It is highly likely that these phenotypes arise because condensin II binds DNA much more stably in absence of MCPH1, and therefore can condense chromosomes in an untimely manner. Interestingly, this data also suggests that another regulator controls condensin II turnover during S-phase.

Mitotic defects in absence of MCPH1

Little is known about the biological consequences of hyper-condensation in interphase and mitosis. Since condensin II is best known for its role in mitotic chromosome condensation, which is essential for successful chromosome segregation, we wondered whether MCPH1 loss would lead to mitotic defects. When imaging ΔMCPH1 cells, we observed many small DNA fragments outside of the nucleus (Fig. 2A, Fig. S2A). These so-called 'micronuclei' can be a consequence of chromosome instability and mitotic defects (Krupina, Goginashvili and Cleveland, 2021). Interestingly, the increase in micronuclei in the ΔMCPH1 cells are condensin II-dependent, as they are absent in ΔMCPH1/ΔCAP-H2 cells.

To examine whether these micronuclei are indeed a consequence of segregation errors, we monitored mitotic progression by live cell imaging. ΔMCPH1 cells indeed have more mitotic defects than control cells, and these defects predominantly include lagging chromosomes and chromosome bridges (Figs. 2B and 2D, Fig. S2B). These defects could in principle cause micronuclei. From our data we cannot conclude whether the mitotic defects are dependent on condensin II, because of the high variation between the replicates of the ΔMCPH1/ΔCAP-H2 cells . It must be noted that depletion of condensin II alone already leads to an increase in segregation errors, as observed before (Elbatsh *et al.*, 2019). If the defects in ΔMCPH1 cells were dependent on condensin II, double depletion of MCPH1 and CAP-H2 likely exhibits a similar phenotype as CAP-H2 loss alone. On the other hand, if the defects of MCPH1 loss are not caused by condensin II, one would expect the defects in the individual knock-outs to have an additive effect in the ΔMCPH1/ΔCAP-H2 cells.

ΔMCPH1 loss can lead to problems in chromosome alignment in metaphase (Arroyo *et al.*, 2017). In our ΔMCPH1 cells we also observed cells which had difficulties to congress all chromosomes to the metaphase plate (Fig. 2D, lower panels). To quantify this, we measured the time from the start of mitosis to anaphase onset. ΔMCPH1 cells experience a delay in mitotic timing, which can be the result of difficulties in chromosome alignment (Fig 2C). Co-depletion of condensin II did not restore the duration to the time as observed in wild type cells, but instead to a time that is similar to condensin II loss by itself. As the defects of MCPH1 loss and CAP-H2 loss do not seem additive, MCPH1 loss likely leads to a defect in chromosome alignment in mitosis in a condensin II dependent manner.

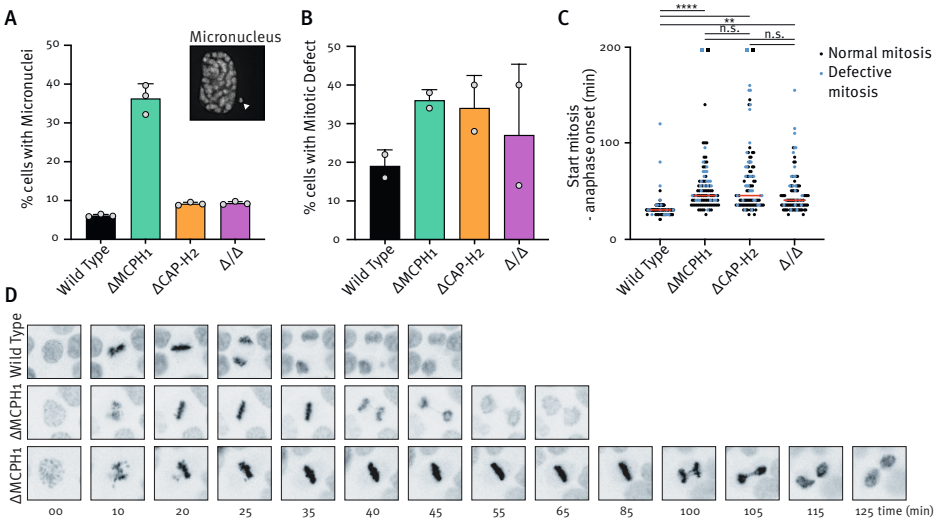

Figure 2 | Mitotic defects in absence of MCPH1. (A) Quantification of the percentage of cells with micronuclei, as visualized with a DAPI DNA-stain on immunofluorescence microscopy, of the indicated Hap1 ΔMCPH1, ΔCAP-H2, or ΔCAP-H2/ΔMCPH1 (Δ/Δ) cell lines. Inset shows an example of a cell with a micronucleus (white arrow). Shown is the mean + SD of three independent experiments in which at least 126 cells were scored. Grey circles depict the mean of each independent experiment. (B) Quantification of the percentage of mitotic cells that display a mitotic defect, as visualized by live-cell microscopy of the DNA-stain SiR-DNA. Shown is the mean + SD of two independent experiments in which at least 50 cells were scored. Grey circles depict the mean of each independent experiment. (C) Quantification of the time between the start of mitosis and anaphase onset in minutes as visualized by live-cell microscopy of the DNA-stain SiR-DNA. Colors indicate if the mitosis measured resulted in a mitotic defect or not. Shown is the median of the 100 cells scored in two independent replicates. ANOVA followed by a Sidak's multiple comparison test was perfomed, **** = p<0.0001, ** = p=0.0024, n.s. = not significant. (D) Examples of live-cell microscopy of a normal mitosis (top, wild type) and two defective mitoses (bottom, ΔMCPH1). DNA was stained by SiR-DNA.

A link between condensin II and topoisomerase II

MCPH1 loss leads to increased condensin II stability in G2. In G2, condensin II is thought to promote sister chromatid resolution (Ono, Yamashita and Hirano, 2013), though recent findings question this (Batty *et al.*, 2023). Either way, a strong link exists between condensin II and the protein responsible for decatenation of the sister chromatids: topoisomerase IIα (TOP2A). Condensin localizes TOP2A to chromatin axes in mitosis (Coelho, Queiroz-Machado and Sunkel, 2003; Hudson *et al.*, 2003; Ono *et al.*, 2017) and promotes TOP2A-mediated decatenation (D'Ambrosio *et al.*, 2008; Charbin, Bouchoux and Uhlmann, 2014; Dyson *et al.*, 2021). Increased condensin II stability might thus alter TOP2A behavior. Earlier findings upon MCPH1 loss support this notion. A largescale CRISPR-based screen found that MCPH1 loss sensitizes cells to drugs targeting TOP2 (Olivieri *et al.*, 2020). Cells without MCPH1 also exhibit different cell cycle progression in response to TOP2 inhibitors compared to wild type cells (Arroyo *et al.*, 2019, 2020).

We wondered if the sensitivity of ΔMCPH1 cells to TOP2 drugs was condensin II mediated. To test this, we used two drugs that target TOP2A in different manners. Etoposide is a

TOP2 poison which prevents re-ligation of the broken DNA, resulting in covalent TOP2-DNA complexes (Montecucco, Zanetta and Biamonti, 2015). ICRF-193 on the other hand is a catalytic inhibitor of topoisomerase, which prevents dissociation of topoisomerase 2 from the DNA (Fig. S3A) (Hajji *et al.*, 2003). We performed a growth assay in presence of increasing doses of these drugs and stained cells with crystal violet. MCPH1 loss sensitizes the cells to both etoposide and ICRF-193 treatment (Figs. 3A-D). The IC50 of these drugs decreases about 75% compared to control cells (Fig. 3E). Excitingly, this defect is dependent condensin II, as ΔMCPH1/ΔCAP-H2 displayed similar sensitivity to these drugs as wild type cells. This indicates that in ΔMCPH1 cells, condensin II displays an activity that is problematic for survival in the presence of drugs targeting TOP2.

MCPH1 and condensin II have previously been linked to homologous recombination (Wood *et al.*, 2008). Both etoposide and ICRF-193 treatment can induce DNA damage (Hajji *et al.*, 2003; Montecucco, Zanetta and Biamonti, 2015). We therefore tested whether the increased sensitivity to TOP2A drugs could be explained by defects in the DNA damage response.

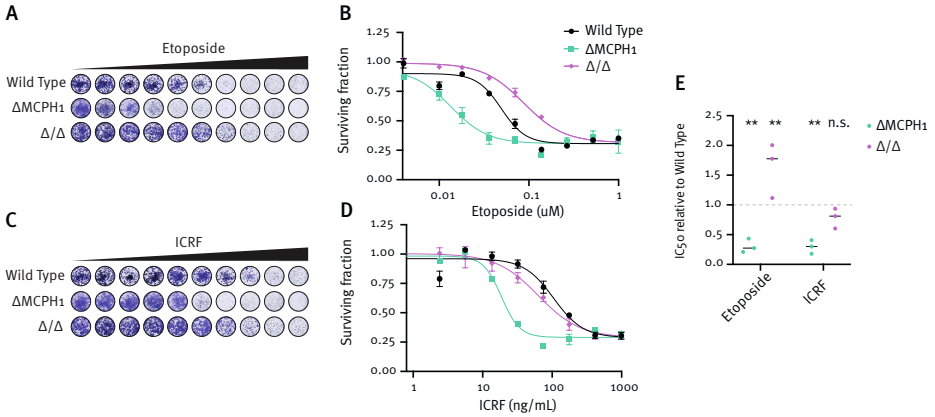

Figure 3 | Increased condensin-dependent toxicity of TOP2 drugs in ΔMCPH1 cells. (A) Growth assays of the indicated cell lines with increasing doses of Etoposide, a topoisomerase 2 poison, of the indicated Hap1 ΔMCPH1, ΔCAP-H2, or ΔCAP-H2/ΔMCPH1 (Δ/Δ) cell lines. Cells were stained with Crystal violet. (B) Quantification of the growth assays shown in (A). Shown is the mean +- SD of one representative experiment in which at least 3 technical replicates were scored. In total at least 3 biological replicates were performed with similar results. (C) Growth assays of the indicated cell lines with increasing doses of ICRF-193, a topoisomerase 2 catalytic inhibitor. Cells were stained with Crystal violet. (D) Quantification of the growth assays shown in (C). Shown is the mean +- SD of one representative experiment in which at least 3 technical replicates were scored, and the nonlinear. In total at least 3 biological replicates were performed with similar results. (E) IC50 values of the indicated cell lines relative to wild type IC50 value, as calculated from three independent biological replicates (of which one is shown in (B) and (D)). Shown is the overall mean, circles depict the mean of the technical replicates of three individual experiments. The IC50 values were based on the growth curves such as those shown in (A-D). ANOVA followed by Tukey's multiple comparison test was performed, indicated is the significance of the difference between wild type and the indicated genotype. **= p<0.01, n.s.= not significant.

We first performed growth assays with a panel of different drugs that either induce damage (Olaparib; a PARP inhibitor and Hydroxyurea), or inhibit the DNA damage response by inhibiting non-homologous end joining (M3814; DNA-pk inhibitor) or homologous recombination (BO2; Rad51 inhibitor)(Figs. 4A and 4B). None of these drugs showed an obvious condensin II-dependent sensitivity in ΔMCPH1 cells (Figs. 4A-C). This is in line with earlier work, in which MCPH1 loss sensitized to treatment with TOP2 targeting drugs, but not to any other genotoxic agents (Olivieri *et al.*, 2020).

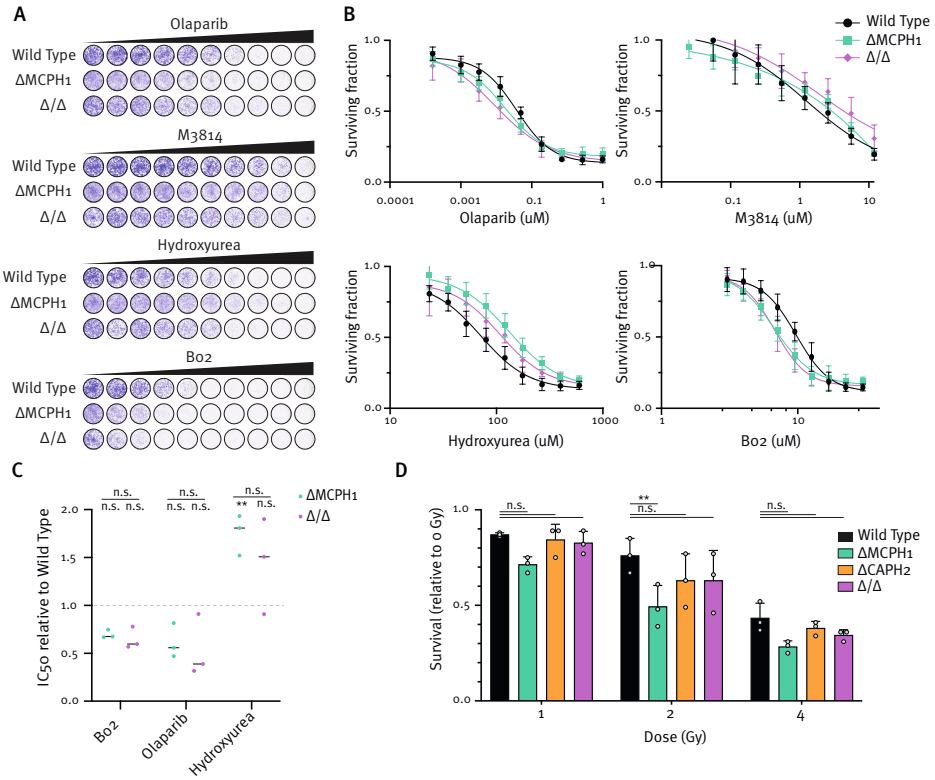

Figure 4 | Consequences of MCPH1 loss for DNA damage repair. (A) Growth assays of the indicated Hap1 ΔMCPH1, ΔCAP-H2, or ΔCAP-H2/ΔMCPH1 (Δ/Δ) cell lines with increasing doses of Olaparib, the DNA-pk inhibitor M3814, Hydroxyurea or the Rad51 inhibitor BO2. Cells were stained with Crystal violet. (B) Quantification of the growth assays shown in (A). Shown is the mean +- SD of at least six technical replicates which were spread over 2-3 biological replicates. (C) IC50 values of the indicated cell lines relative to wild type IC50 value. Shown is the overall mean, circles depict the mean of the technical replicates of three individual experiments. The IC50 values were based on the growth curves as shown in (A). ANOVA followed by Tukey's multiple comparison test was performed, indicated is the significance of the difference between wild type and the indicated genotype, or between ΔMCPH1 and dKO. **= p‹0.01, n.s.= not significant. IC50 could not be calculated for M3814. (D) Cell survival after increasing doses of irradiation, relative to survival after 0 Gy treatment. Shown is the mean + SD of three independent experiments. Grey circles depict the mean of each independent experiment. ANOVA followed by Tukey's multiple comparison test was performed. **= p‹0.01, n.s.= not significant.

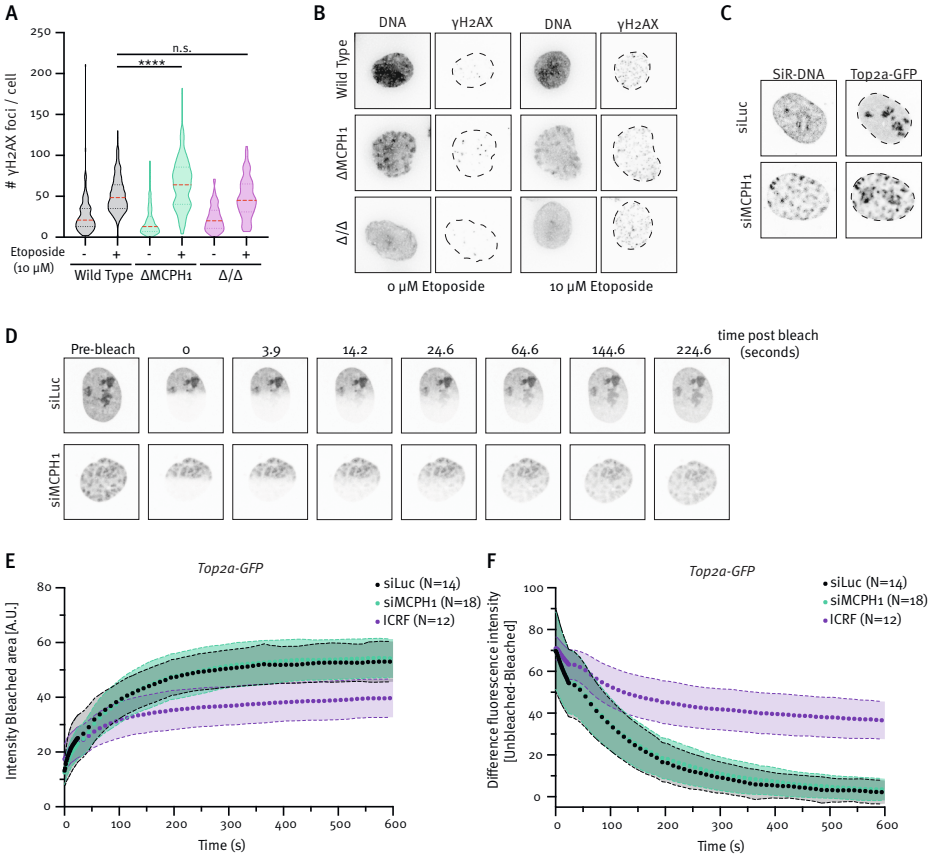

Figure 5 | MCPH1 loss affects TOP2 function through condensin II. (A) Quantification of the amount of DNA damage (γH2AX) foci per cell, with or without treatment with 10 μM Etoposide for 30 minutes. Shown is the mean +- the interquartile range of the combined data of four independent experiments in the indicated Hap1 ΔMCPH1, ΔCAP-H2, or ΔCAP-H2/ΔMCPH1 (Δ/Δ) cell lines. ANOVA followed by a Dunnett's multiple comparison test was performed, **** = p ‹ 0.0001, n.s. = not significant. Example images can be found in (B). (B) Immunofluorescence microscopy of the indicated cell lines with or without etoposide treatment. DNA was stained with DAPI, DNA damage was visualized by γH2AX staining. (C) Immunofluorescence microscopy of U2OS cells after treatment with siLuciferase or siMCPH1 for ~48 hours. DNA was visualized by SiR-DNA, TOP2A was GFP-tagged. (D) FRAP analysis on Top2a-GFP of U2OS cells treated with siLuciferase or siMCPH1for ~48 hours, or ICRF-193 for ›2 hours. Cells were synchronized in G2-phase by overnight treatment with RO-3306. Shown are example images pre- and post-bleach. FRAP was quantified in (E) and (F). (E) FRAP analysis of TOP2A-GFP as shown in (D). Quantification of the recovery of the fluorescence intensity in the bleached area after bleaching half of the nucleus. Shown is the mean +- SD of the indicated amount of cells measured in three independent experiments. (F) FRAP analysis of TOP2A-GFP as shown in (D). Quantification of the difference in fluorescence intensity between the bleached and unbleached areas after bleaching half of the nucleus. Shown is the mean +- SD of the indicated amount of cells measured in three independent experiments.

We also induced double strand breaks via increasing doses of irradiation, and measured survival. ΔMCPH1 cells are only slightly more sensitive to this induction of DNA damage, in a manner dependent on condensin II (Fig. 4D). This confirms earlier findings that MCPH1

and condensin II cooperate in DNA damage repair (Wood *et al.*, 2008). This increased sensitivity to irradiation is minor compared to the sensitivity to Etoposide or ICRF. Therefore we consider that sensitivity to TOP2 drugs might only partially be explained by defective DNA damage repair.

We instead propose that MCPH1 loss affects TOP2A biology in a more direct manner. Condensin II is known to promote TOP2A activity. Therefore, we hypothesize that more stable condensin II leads to more TOP2A activity. If there are more active TOP2A complexes, poisoning these complexes by etoposide would lead to more TOP2A-DNA covalent complexes and thus more DNA damage. To test if this hypothesis is true, we stained for DNA damage marker γH2AX after a short etoposide treatment be condensin II dependent, as it is restored to wild-type levels in the ΔMCPH1/ΔCAP-H2 cells (Fig. 5A). This indeed suggests that there are more active topoisomerase 2 complexes on the DNA when condensin II is stably bound. A direct measurement of TOP2A activity via an *in vitro* assay may provide a more robust test of this hypothesis.

Since condensin II binds DNA more stably in absence of MCPH1, and because condensin II is known to be responsible for TOP2A localization, we next investigated whether TOP2A turnover on the DNA was also changed. We visualized the complex with a GFP-tag in a U2OS cell line and depleted MCPH1 through siRNA treatment. Notably, TOP2A localizes in a much less diffuse manner upon MCPH1 treatment (Fig. 5C). TOP2A seemingly localizes to the most condensed regions of the genome, possibly under the influence of condensin II. To test whether TOP2A was now also binding chromatin more stably, we performed Fluorescence Recovery After Photobleaching (FRAP) experiments to asses turnover (Figs. 5D-F). We used ICRF-193 as a positive control, as this drug should prevent most turnover of TOP2A. ICRF-193 indeed stabilized TOP2A, but MCPH1 loss did not affect TOP2A turnover. Apparently, increased condensin II stability does not translate to increased TOP2A stability.

Since there might be more processes which are affected when condensin II is no longer kept in check by MCPH1, we aimed to identify these processes with a synthetic viability screen (Blomen *et al.*, 2015). In such a screen, gene-trap viruses randomly integrates in the genome of haploid cell lines, essentially generating a polyclonal collection of knock-out cell lines. If genes are important for cell viability, cells with disruptions in these genes will be depleted from the population over time (Fig. 6A). We performed this screen in both wild type and ΔMCPH1 Hap1 cells to specifically identify genes that become more or less important for viability in absence of MCPH1.

This screening approach yielded some fascinating hits, which essentially can be subdivided into three categories (Figs. 6B and 6C). Category 1 entail hits that are mitotic in nature. BUB1B, MAD1L1, CENPP and CENPO all become more important in absence of MCPH1, whereas BUB3 becomes less essential. These hits are particularly interesting in the context of our findings that MCPH1 display mitotic defects in a manner that is possibly dependent

on condensin II. Category 2 is comprised of hits that play a role in DNA damage repair or replication: DBF4, FANCL and FANCD2. FANCD2 and FANCL specifically are key factors in the Fanconi Anemia pathway, which is responsible for repair of inter-strand crosslinks (Lemonidis *et al.*, 2022). These genes all become more essential in absence of MCPH1, and are interesting in connection to a role of MCPH1 and condensin II in DNA damage repair. Category 3 is comprised of factors with 'other' or unknown roles, and includes two yet uncharacterized proteins (KIAA0930 and KIAA1586), and a subunit of a cyclin dependent kinase (CKS1B) that regulates mitotic exit and the G1-S transition (Harper, 2001). Validation of these hits in other ΔMCPH1 clones, and in ΔMCPH1/ΔCAP-H2 cells, is a necessary first step into better understanding which processes are affected by MCPH1 deficiency. This may lead to the discovery of novel processes in which condensin II can play a role, or to the uncovering of novel roles for any of the hits mentioned above.

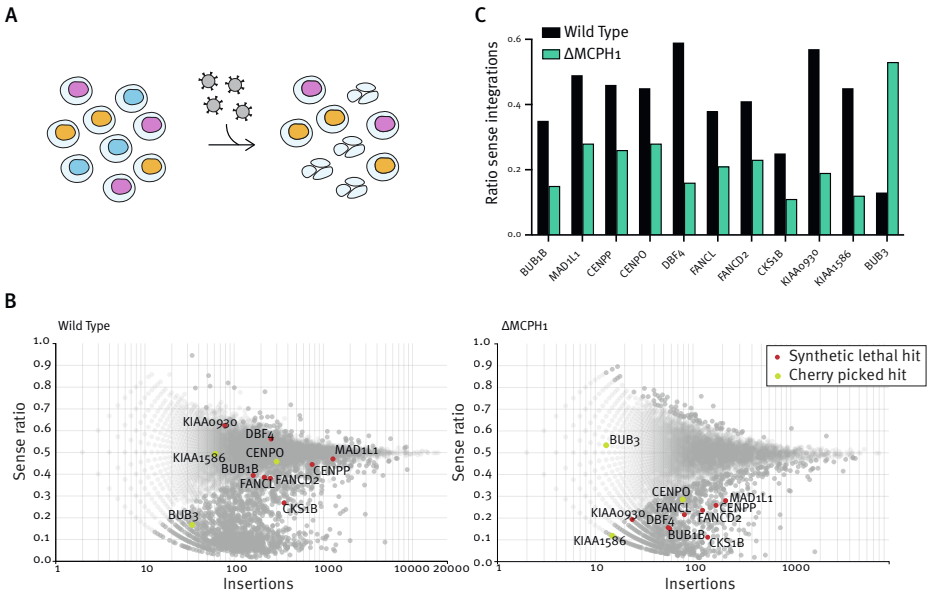

Figure 6 | Haploid genetic screen to identify vulnerabilities of ΔMCPH1 cells. (A) Schematic overview of the haploid genetic screening approach used. Cells are infected with genetrap viruses, that randomly integrate in the genome and disrupts the gene in which it lands. If a gene important for cell viability is disrupted, the cell dies. We performed this screening approach in both Hap1 wild type and ΔMCPH1 cells. (B) Fishtail plot of genetrap insertions in Hap1 wild type and ΔMCPH1 cells. Red dots depict genes that upon disruption lead to a cell survival disadvantage in ΔMCPH1 cells compared to wild type cells. Green dots depict genes that show differential effects on cell survival between wild type and ΔMCPH1 cells, but did not make the statistical cut-off to be called a hit. These genes were cherry-picked due to their biological function or because a member of the same complex was a hit. (C) Ratio of sense integrations in wild type and ΔMCPH1 cells of the genes highlighted in (B).

DISCUSSION

Recently, MCPH1 was identified to be the release factor of condensin II (Houlard *et al.*, 2021). Loss of MCPH1 leads to interphase condensation and to hypercondensation in mitosis. In this study we aimed to understand what the consequences are of these condensation defects. We find that increased condensin II stability leads to micronuclei formation and sensitizes to drugs targeting TOP2A. These findings shed a light on which processes require normal condensin turnover.

Cell cycle regulation of condensin II

We confirm that MCPH1 loss leads to interphase condensation specifically in G1- and G2-phase, but not in S-phase. This suggests that another regulatory factor probably keeps condensin II dynamic in S-phase. This factor has yet to be identified, but data from Drosophila has described Casein Kinase 1α as a negative regulator of condensin II. Upon loss of this protein, interphase condensation is observed (Nguyen *et al.*, 2015), though it is unclear whether this is specifically in S-phase. It will be exciting to investigate this factor and possible human homologs further, to see if the S-phase regulator of condensin II can be identified.

Our findings suggests that MCPH1 negatively regulates condensin II in G2, mitosis and G1. It is unsurprising that MCPH1 functions in G2, as it is present and active during this cell cycle stage. When cells enter mitosis, MCPH1 is presumably phosphorylated and as such can no longer bind condensin II (Houlard *et al.*, 2021). We thus propose that mitotic hypercondensation in ΔMCPH1 cells is a result of the earlier onset of condensation in G2, rather than of more active mitotic condensin II.

However, it is quite surprising that MCPH1 loss results in chromosome condensation in G1, as MCPH1 is a degradation target for the APC/Cdh1 complex in mitosis (Meyer *et al.*, 2019). This could suggest that decondensation normally is a passive process, which is hampered when chromosomes are hyper-condensed. Our data might on the other hand also be compatible with a model in which the low levels of MCPH1 that remain in G1 generally are sufficient to allow for chromosome decondensation. More extensive characterization of MCPH1 levels throughout the cell cycle coupled with acute depletion experiments of MCPH1 could clear up these apparent discrepancies.

Chromosome segregation requires tight regulation of condensin II

One of the striking defects upon MCPH1 loss is the formation of micronuclei. Why stable condensin II may lead to such micronuclei remains unclear. The observed segregation errors and mitotic delay cannot fully explain this phenotype, as condensin II loss by itself causes similar defects, but does not lead to micronuclei formation. One possible explanation could be that micronuclei arise from defects outside of mitosis, for example from DNA replication-related DNA damage (Krupina, Goginashvili and Cleveland, 2021). This would fit with the roles of MCPH1 and condensin II in DNA damage repair (Wood *et al.*, 2008). Another explanation

could be that micronuclei arise from particular mitotic defects that are not visible by low resolution live cell imaging, such as ultrafine bridges. Alternatively, the mitotic delay in ΔMCPH1 cells might have a different cause than the defects in ΔCAP-H2 cells. We observed clear defects in chromosome congression to the metaphase plate in ΔMCPH1 cells (Fig. 2D), which is less obvious in ΔCAP-H2 or ΔMCPH1/ΔCAP-H2 cells. Interestingly, cells that fail at chromosome alignment in absence of KIF18A form micronuclei (Fonseca *et al.*, 2019). This defect in chromosome congression might thus explain the formation of micronuclei specifically in ΔMCPH1 cells.

Several hits from the synthetic lethality screen also point towards a role for MCPH1-mediated condensin removal to promote successful segregation. BUB1B, MAD1L1 and BUB3 are involved in the mitotic checkpoint (Lara-Gonzalez, Westhorpe and Taylor, 2012). Interestingly, earlier work has shown synthetic lethal interactions between members of the spindle assembly checkpoint and proteins that have defects in chromosome alignment (Raaijmakers *et al.*, 2018). As MCPH1 has defects in chromosome alignment that are possibly condensin II-dependent, this is a link that is worth further investigating. CENPP and CENPO are both members of the CENP-O/P/Q/R/U subcomplex of the Constitutive Centromere Associated Network. The exact function of this subcomplex is unknown (Hara and Fukagawa, 2017), thus it might be interesting to investigate why these factors become more important for viability upon MCPH1 loss. Condensin II activity evidently must be tightly balanced to ensure successful chromosome segregation, as too little condensin (ΔCAP-H2) or too stable condensin (ΔMCPH1) both lead to mitotic defects.

A link between condensin II and TOP2

Intriguingly, increased condensin II stability appears to sensitize cells against drugs targeting TOP2. This increased sensitivity can have two explanations. First, these cells might rely more on TOP2 because they have a major increase in catenanes due to for example problems in DNA replication or DNA damage repair. When these catenanes are not resolved, they will lead to massive mitotic defects and subsequent cell death. Alternatively, the sensitivity can also be increased because TOP2 is more active. This could be simply caused by the fact that the catenanes that are present are under more tension due to loop extrusion activity, which in turn recruits TOP2 complexes to allow strand passage and thus decatenation (Fig. 7). Alternatively, condensin II might directly promote the enzymatic activity of TOP2 complexes in a manner that has yet to be identified. Active complexes are the complexes that are targeted by both etoposide and ICRF-193, and these complexes will get stuck on the DNA upon drug treatment. Both drugs will eventually, directly or indirectly, lead to massive DNA damage which can lead to cell death. Since condensin II is known to promote TOP2A decatenation activity, we hypothesize that the latter explanation might be true. MCPH1 loss would then lead to increased TOP2 activity in a condensin II-dependent manner. To test this hypothesis, *in vitro* decatenation assays with extracts from ΔMCPH1 cells could be helpful.

The fact that MCPH1 loss sensitizes to TOP2A drugs in a condensin II-dependent manner is quite an exciting finding, and one with possible clinical implications. MCPH1 is a tumor suppressor which is mutated in a wide array of cancers (Alsolami *et al.*, 2023). TOP2 poisons such as etoposide are successful anticancer drugs that are widely used in the clinic, yet they cause serious side effects like secondary malignancies or cardiotoxicity (Delgado *et al.*, 2018). Our findings may suggest that treatment of tumors that harbor MCPH1 loss of function mutations can be treated with lower dose of anti-TOP2 drugs, greatly reducing the harmful side effects. It will be interesting to test this concept in a panel of cancer cell lines with defective MCPH1. All in all, further investigating the biological relevance of condensin II removal by MCPH1 may lead to important insights into condensin biology and could provide clinically relevant insights for cancer treatment.

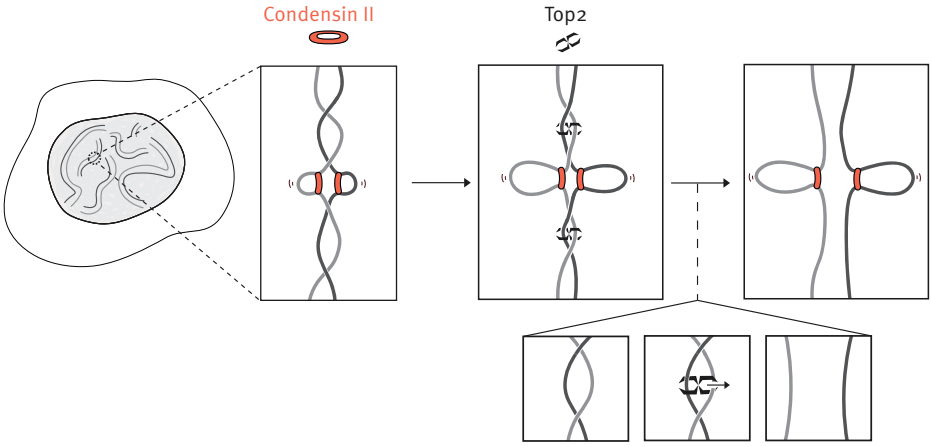

Figure 7 | The interplay between condensin II and topoisomerase 2. Schematic model of the possible interplay between condensin II and TOP2. Upon MCPH1 loss, condensin II can perform loop extrusion in G2-phase of the cell cycle. This leads to increased tension upon DNA catenanes, which in turn recruits TOP2 complexes to allow for strand passage.

ACKNOWLEDGEMENTS

We thank Louise Janssen for fruitful discussions about topoisomerase 2 biology, and the Rowland lab for technical and scientific input on the project.

MATERIALS AND METHODS

Cell culture and siRNA treatment

Hap1 cells were cultured in IMDM (Gibco) and TOP2A-GFP U2OS cells (gift of Louise Janssen and Jonne Raaijmakers) were cultured in DMEM (Gibco). Both media were supplemented with 10% FBS (Sigma Aldrich), 1% L-Glutamine (Gibco) and 1% Penicillin/Streptomycin (Gibco). All cell lines were cultured at 37°C with 5% CO_2.

For treatment with Etoposide (Figs. 5A and 5B) cells were incubated with 10 µM Etoposide for 30 min. For ICRF treatment (Figs. 5E and 5F) cells were incubated with 2 µg/mL ICRF-193 for at least 2 hours before the start of the FRAP experiment. All drugs (Etoposide, ICRF, B02, HU, M3814, Olaparib) were dissolved in DMSO and used at the indicated concentrations.

siRNA transfections were performed using RNAiMax (Invitrogen) according to the manufacturer's protocol. The following siRNAs were used: siLuc (D-001100-01, Dharmacon) and siMCPH1 (ON-TARGETplus SMARTpool, Dharmacon).

Generation of knock-out cell lines

ΔCAP-H2 cells were generated as previously described in (Elbatsh *et al.*, 2019). The MCPH1 knock-out and CAP-H2/MCPH1 double knock-out cells were obtained by CRISPR/Cas9 mediated genome editing. Guide-RNAs (Forward sequence: CACCGAAAGCTCGTTTCGGTGCTCT, Reverse sequence: AAACAGAGCACCGAAACGAGCTTTC) targeting MCPH1 were cloned into the px330 vector. Parental Hap1 cells, and CAP-H2 knock-out cells were both transfected with this px330 targeting MCPH1 and a puromycin cassette, as previously described (Haarhuis *et al.*, 2017). Genomic DNA was isolated from single clones and the desired knock-out was assessed by PCR and Sanger sequencing.

Western Blot

Cells were harvested for Western blot and lysed with RIPA buffer (150 mM Sodium Chloride, 50 mM Tris-HCL (pH 8.0), 1% Nonidet P-40, 0.5% Sodium deoxycholate, 0.1 % SDS) for 30 minutes on ice. Total protein content was measured by the Lowry assay. Samples were loaded on a 4-12% Bis-Tris gel and run in 1x NuPAGE SDS MOPS Running buffer (Invitrogen). Proteins were transferred on a PVDF membrane, and blocked with 5% milk in TBS with 0.1% Tween before incubating with the indicated antibodies.

Antibodies used for Western Blot were: MCPH1 (Cell signaling, 4120S, 1:1000), CAP-H2 (Bethyl, A302-275A, 1:1000), Tubulin (Sigma, T5168, 1:10000).

Chromosome spreads

Cells were treated with nocodazole (1 µg/µl) for 1.5 hours before performing mitotic shake-off to harvest mitotic cells. Chromosome spreads were prepared by lysing the cells with 0.075M KCl + 6% fixative (MeOH:Acetic Acid, 3:1) for 20 minutes at 37°C. After this, cells were spun down for 5 minutes 1500 rpm and resuspended in fixative. Cells were spun again, resuspended in fixative + DAPI (1:1000) and spread dropwise on a glass slide. Spreads were mounted with Prolong Gold Antifade (Thermo Fisher), imaged and analyzed using the Metafer software.

Immunofluorescence

Cells were grown on 12mm cover slips. When imaging only DNA, or H2AX foci, pre-extraction was performed using PBS + 0.1% Triton for 1 minute, followed by fixation with 4% PFA for 7

minutes. When imaging CDT1 or Geminin, cells were first fixed with 4% PFA for 10 minutes, followed by permeabilization by PBS-0.1% Triton. After this, in both protocols cells were washed twice with PBS + 0.1% Tween and blocked in 3% BSA. The indicated antibodies were incubated for 1 hour at room temperature, followed by three washes with PBS-Tween. Secondary antibody incubation was also performed at room temperature for 1 hour (mouse or rabbit Alexafluor 488, 568 or 647 from Invitrogen; 1:1000) and DAPI (Sigma-Aldrich; 1:1000). After another three washed with PBS-Tween, slides were mounted with Prolong Gold Antifade, and images were obtained with Deltavision Elite System (Applied Precision) using Softworx software or a THUNDER Imager (Leica Microsystems).

Antibodies used for IF were CDT1 (Abcam, ab202067, 1:1000), Geminin (Cell signaling, #52508, 1:1000), γH2AX (Millipore, 05-636, 1:500).

In the experiments to look at S-phase condensation, and H2AX foci, we stained S-phase cells using an EdU. In these instances, we incubated the cells with EdU (used at a concentration of 10 µm, Molecular Probes) for 30 minutes before fixing the cells. EdU was subsequently stained using a staining buffer (100 mM Tris-HCl (pH 8.5), 1mM $CuSO_4$, 100 mM ascorbic acid and AF-647 Azide (Invitrogen, 1:1000). Slides were incubated for 30 min at room temperature in this buffer and washed two times in 3% BSA before proceeding with primary antibody staining.

Live cell imaging

Cells were plated in an 8-well LabTekII-chambered cover glass (Thermo Scientific Nunc). At least two hours before imaging SiRDNA (used at a final concentration of 25 nM, Spirochrome) and Verapamil (used at a final concentration of 1nmol/µL, Spirochrome) were added to the cells. Cells were imaged using a THUNDER Imager (Leica Microsystems), during the filming cells were maintained at 37°C and 5% CO_2.

When cells were filmed after RO release, they were treated overnight with 10 µm RO-3306 (Millipore), which was washed out directly before start of the imaging.

Crystal Violet + drug sensitivity assays

The indicated cell lines were plated in 96-well plates, at a density of 1500 cells/well (Wild type and ΔMCPH1/ΔCAP-H2) or 1800 cells/well (ΔMCPH1 and ΔCAP-H2) in the morning. In the afternoon, drugs were added to the well at the indicated concentrations using a Tecan D300 Digital Dispenser. After 5 days, cells were fixed in 100% ice-cold methanol for 10 minutes, and stained with crystal violet at room temperature for a minimum of 5 hours. Plates were scanned using and the intensity of the staining was measured with Fiji imaging software.

IR sensitivity

The indicated cell lines were plated in a 6-well plate, at a density of 100.000 cells/well (Wild type and ΔMCPH1/ΔCAP-H2) or 125.000 cells/well (ΔMCPH1 and ΔCAP-H2). Cells

were γ-irradiated with the indicated dose using a Gammacell Exactor (Best Theratronics) with a ^{137}Cs source. After 4 days, cells were fixed and stained with crystal violet according to the protocol above. Plates were scanned and the intensity of the staining was measured using Fiji image software.

Fluorescence Recovery after Bleaching (FRAP)

Cells were plated in a 4-well LabTekII-chambered cover glass (Thermo Scientific Nunc) and transfected with the indicated siRNAs. After overnight synchronization in G2 using RO-3306, SiR-DNA (Spirochrome) was added at a concentration of 250 nM to the wells. Live cell imaging was performed on a Leica SP5 confocal microscope with a 63x oil objective. The experiment was performed in the LAS-AF FRAP Wizard. Five images were taken, before bleaching half of the interphase nuclear using 5 pulses of 100% emission of the 488-nm laser. After this, 20 images were taken at intervals of +- 1.2 sec, after this another 60 images were taken every 10 seconds. Fluorescence intensity was measured in the bleached and unbleached area using Fiji image software, and this was background corrected. Recovery can be measured by calculating the difference between the bleached and unbleached area over time.

Synthetic viability screen

The synthetic viability screen in Hap1 cells was performed as described previously (Blomen *et al.*, 2015).

In short, retroviral genetrap virus was produced using HEK293T cells by transfection of Gag-pol, pAdvantage, VSVg and the gene trap plasmid with an GFP cassette. Virus was harvested for two subsequent days and concentrated using Amicon concentrator tubes. Hap1 cells were transduced with this concentrated genetrap retrovirus. Cells were passaged for 11 days, when cells were harvested and sorted for haploid cells in G1 phase of the cell cycle based on DNA content. Genomic DNA was isolated from these cells using a DNA mini kit (Qiagen). To identify where the genetrap virus integrated, we amplified the insertions using a linear amplification PCR. Insertions were mapped to the human genome using bowtie. Gene-trap insertions were intersected with coordinates of Refseq gene coordinates to determine intragenic integrations. To identify which genes were differentially enriched in wild type versus ΔMCPH1 cells, we used a FDR-corrected P value cutoff of p=0.005. Some hits, as indicated, did not make the cut-off, hence the term ‘cherry-picked’.

SUPPLEMENTAL FIGURES

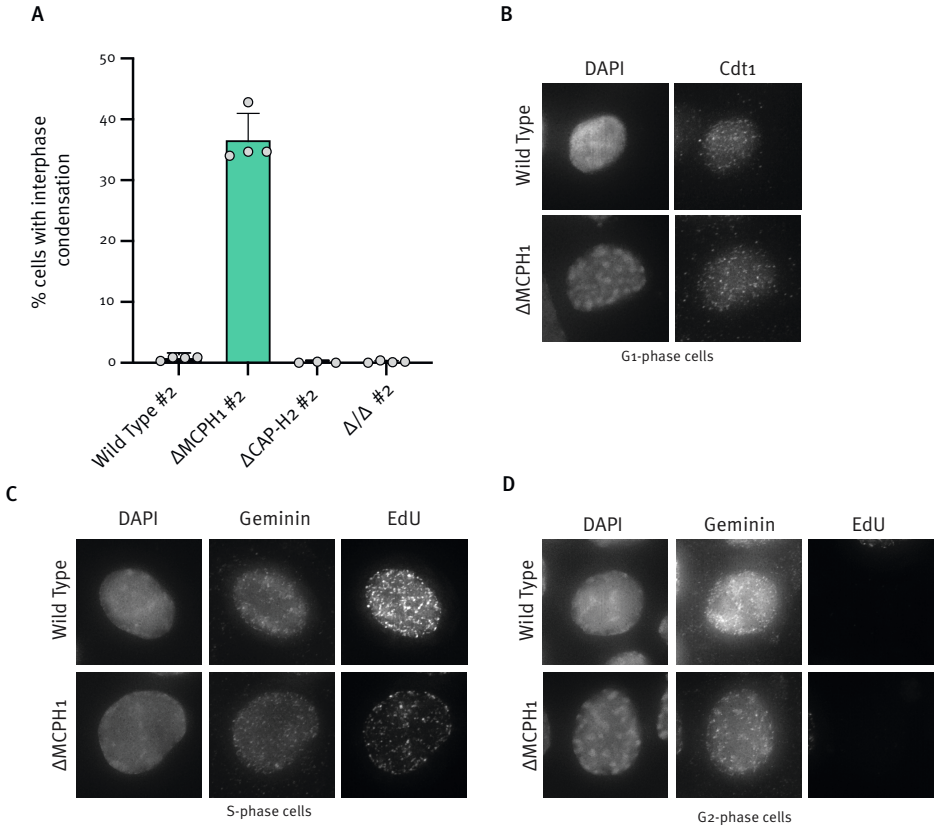

Figure S1 | MCPH1 loss leads to hypercondensation in G1- and G2-phase. (A) Quantification of the amount of cells with interphase condensation of the indicated Hap1 ΔMCPH1, ΔCAP-H2, or ΔCAP-H2/ΔMCPH1 (Δ/Δ) cell lines, a replicate of Fig. 1D. Shown is the mean + SD of a minimum of three independent experiments in which at least 397 cells were scored. Grey circles depict the mean of each independent experiment. (B-D) Example images of the experiment in Fig. 1F. (B) Immunofluorescence microscopy of G1-phase cells. G1-phase cells were recognized by positive Cdt1 signal, condensation was assessed based on the DNA-stain DAPI. (C) Immunofluorescence microscopy of S-phase cells. S-phase cells were recognized by positive Geminin and EdU signal, condensation was assessed based on the DNA-stain DAPI. (D) Immunofluorescence microscopy of G2-phase cells. G2-phase cells were recognized by positive Geminin and negative EdU signal, condensation was assessed based on the DNA-stain DAPI.

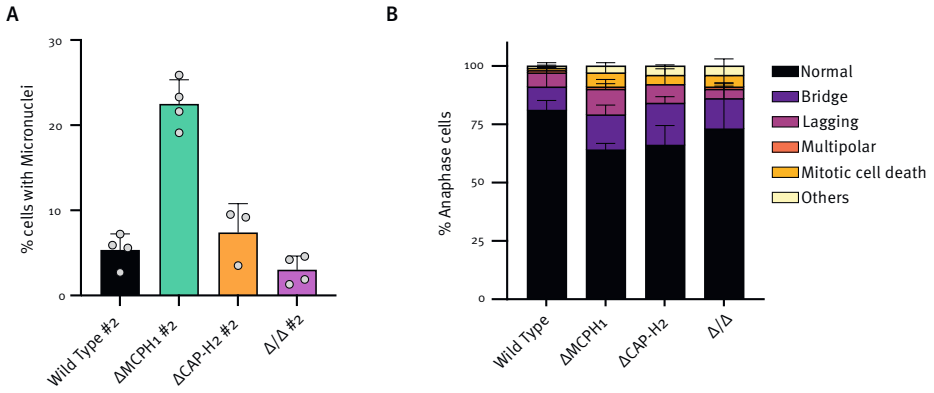

Figure S2 | Mitotic defects in absence of MCPH1. (A) Quantification of the percentage of cells with micronuclei of the indicated cell lines, as visualized with a DAPI DNA-stain on immunofluorescence microscopy, of the indicated Hap1 ΔMCPH1, ΔCAP-H2, or ΔCAP-H2/ΔMCPH1 (Δ/Δ) cell lines. Shown is the mean + SD of a minimum of three independent experiments in which at least 397 cells were scored. Grey circles depict the mean of each independent experiment. (B) Quantification of the percentage of mitotic cells that display a particular mitotic phenotype, as visualized by live-cell microscopy of the DNA-stain SiR-DNA. This figures shows the details of the data depicted in Fig. 2B. Shown is the mean + SD of two independent experiments in which at least 50 cells were scored. Grey circles depict the mean of each independent experiment.

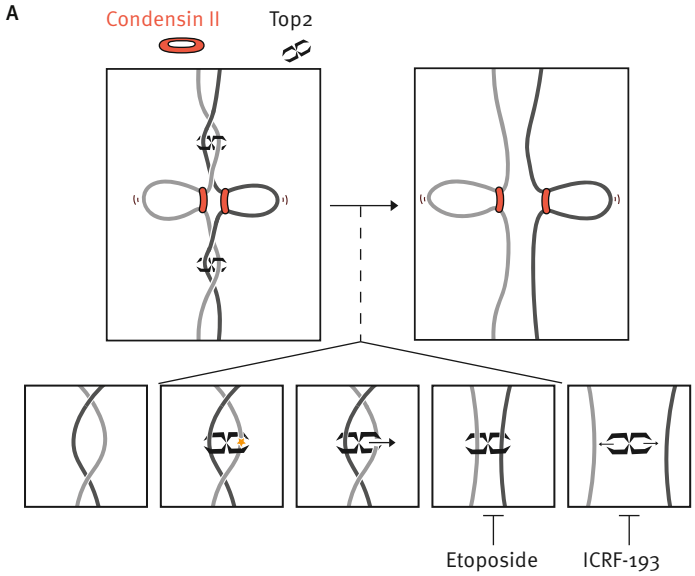

Figure S3 | MCPH1 loss sensitizes to drugs targeting topoisomerase II. (A) Schematic overview of how topoisomerase II functions. TOP2 binds catenated DNA and induces a double strand break in one of the catenated strands. The catenated strands can now pass one another to decatenate. Etoposide prevents the religation of the broken strand and thus directly leads to DNA damage, whereas ICRF-193 prevents the release from TOP2 from the DNA after the full decatenation reaction.

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Chapter 8

General Discussion

ABSTRACT

Condensin complexes are important for the dramatic transition of interphase chromatin into rigid mitotic chromosomes. In the past decades our insights into the mechanism by which these complexes compact mitotic chromatin have rapidly expanded. It remained a mystery however if condensin complexes also control other processes in addition to mitotic chromosome organization. Especially condensin II had been speculated to have additional functions, as it is nuclear and can thus reach DNA throughout the cell cycle. In this thesis, we set out to investigate these 'non-canonical' functions of condensin II. In **Chapters 3, 4** and **5** we revealed that condensin II-mediated mitotic chromosome organization determines large-scale interphase genome architecture in a manner that is evolutionarily conserved. In **Chapter 6** we discussed the implications of our finding in the context of genome architecture as a whole. While in the first chapters, we revealed the consequences of condensin II loss, in **Chapter 7** we investigated the other side of the coin, and find out what the biological consequences are losing the negative regulator of condensin II: MCPH1. In this thesis we thus aimed to illustrate the importance of balancing condensin II activity, as having either too little or too active condensin II is has substantial consequences for cells. In this chapter, I will discuss the significance of our findings, and put them in a broader perspective. I will also discuss the open questions in the field, the impact and importance of this research, and provide a future outlook.

SUMMARY

SMC complexes control a wide array of DNA-based processes. These complexes are very similar to one another in mechanistic terms, yet they somehow play roles in very different cellular processes. Whereas for example condensin controls chromosome architecture in mitosis, cohesin regulates interphase genome architecture. In **Chapter 2**, we introduced the three best studied SMC complexes in eukaryotes: condensin, cohesin and the SMC5/6 complex. We discussed the similarities and differences in the composition of these complexes, and described how they function by forming and enlarging chromatin loops. In a journey through the cell cycle we described the various functions of each complex, including gene regulation, DNA repair and protection against pathogens. It becomes clear that each of these complexes plays multiple vital roles in our cells, and that many additional functions are likely to still be discovered.

Condensin complexes were initially discovered as factors essential for shaping mitotic chromosomes. Without condensin complexes, chromosomes are not individualized when they enter into mitosis and instead form a single compact mass (Ono *et al.*, 2003; Samejima *et al.*, 2018). This unsurprisingly leads to missegregations, showing that condensin complexes are important for the fidelity of chromosome segregation (Hudson *et al.*, 2003; Oliveira, Coelho and Sunkel, 2005; Green *et al.*, 2012; Piskadlo, Tavares and Oliveira, 2017). As such, condensin complexes are vital in development. This is illustrated by the fact that full condensin loss leads to embryonic lethality (Houlard *et al.*, 2015), and partial condensin loss leads to cancer, developmental problems and infertility (Nishide and Hirano, 2014; Houlard *et al.*, 2015; Woodward *et al.*, 2016). Chromosomal instability is likely also a major reason why mutations in condensin complexes are enriched in tumors (Leiserson *et al.*, 2015).

Shaping mitotic chromosomes is clearly a vital function of condensin complexes. Yet, it has been widely speculated that especially condensin II might have functions outside of mitosis, as it is nuclear throughout interphase (Hirota *et al.*, 2004; Ono *et al.*, 2004). Indeed, studies in many different species indicate that condensin II controls the 3D organization of the interphase genome, especially that of the centromeres (Schubert, Lermontova and Schubert, 2013; Nishide and Hirano, 2014; Nguyen *et al.*, 2015; Iwasaki, Corcoran and Noma, 2016; Howard-Till and Loidl, 2018; Rosin *et al.*, 2018; Sakamoto *et al.*, 2019, 2022; Houlard *et al.*, 2021; Municio *et al.*, 2021). Condensin II seemingly promotes *cis*-contacts over *trans*-contacts, and hereby negatively regulates processes that require *trans*-interactions such as polytene chromosome assembly and transvection in *Drosophila melanogaster* (Hartl, Smith and Bosco, 2008). Condensin II is also required for the resolution of the sister chromatids, though whether this process already starts in interphase remains a topic of debate (Ono, Yamashita and Hirano, 2013; Batty *et al.*, 2023).

Despite being nuclear, condensin II is negatively regulated in interphase and thus might not be active. This is achieved by its release factor MCPH1, which keeps association of condensin

II with the DNA dynamic (Houlard *et al.*, 2021). However, the fact that it is negatively regulated in this manner, and not simply removed from the nucleus like condensin I, suggests that there might be instances in which condensin II acts and that MCPH1 may not inhibit condensin II. In this thesis, we investigated functions of condensin II outside of its canonical mitotic functions. We did so by studying the consequences of both the loss of condensin II, and of the loss of its negative regulator MCPH1.

In **Chapter 3** we revealed the existence of two recurring types of interphase genome architecture throughout evolution. In Type I architecture, "Rabl-like organization", centromeres and telomeres cluster together and chromosome arms fold back onto each other. In Type II architecture, "chromosome territories", chromosomes occupy a distinct volume in the nucleus and hardly display any *trans*-interactions. We found that condensin II loss correlates with Rabl-like architecture. Condensin II depletion in cells indeed induces a switch from chromosome territories to Rabl-like organization. Interestingly, this does not seem to be an interphase function of condensin II, as mitotic progression after condensin II loss is necessary to get centromere clustering.

Condensin II loss thus brings about major changes in 3D genome organization at the scale of whole chromosomes. In **Chapter 4** we investigated how these changes affect smaller scale genome architecture and gene expression. We found that chromosome territories function largely independent from the other levels of genome organization, as condensin II loss does not evidently affect loops and TADs. Gene expression was also hardly changed, as only a subset of genes seemed sensitive to the changes in condensin II, seemingly because of their differential association with the nuclear lamina.

How are others aspects of nuclear organization then affected? In **Chapter 5** we described that centromere clustering occurs in or in the proximity of the nucleolus. Heterochromatin also clusters in absence of condensin II, as observed before in mice (Nishide and Hirano, 2014). Yet, neither the nucleolus nor HP1 (an important heterochromatin protein) are not responsible for centromere clustering in the absence of condensin II. We then investigated how condensin II can drive centromere unclustering, and found that the lengthwise compaction of the chromatin axis likely is key to this process. Interestingly, we can partially bypass the need for condensin II in spatial organization of centromeres by stabilizing cohesin on the DNA. In **Chapter 6** we discussed our findings on condensin's role in interphase genome architecture in the context of the broader field, and propose a model to describe how lengthwise compaction prevents centromere clustering.

After discussing the consequences of condensin II loss, we shift gears. In **Chapter 7** we investigated what happens if we tip the balance to the opposite side, and stabilize condensin II by removing MCPH1. We found that this leads to genomic instability, and in changes in topoisomerase 2 function. Apparently condensin II must be tightly controlled to ensure successful genome organization, chromosome segregation and decatenation.

GENERAL DISCUSSION

A conserved function for condensin II in interphase genome architecture

The transition between interphase chromatin and mitotic chromosomes

The transition from a seemingly unstructured chromatin mass in interphase to separate and rigid mitotic chromosomes is one of the most dramatic processes that occurs during the cell cycle. Yet, there are more similarities between these two chromatin states than expected at first glance. In **Chapter 3**, we described two interphase architecture types that are recurrent throughout evolution. Interestingly, each of these architecture types retains characteristics of the preceding mitosis. Chromosome territories retain the individuality of the mitotic chromosomes, as in these interphase nuclei each chromosome still occupies a separate region of the nucleus with little overlap with other chromosomes. Rabl-like organization on the other hand retains the chromosome orientation upon segregation: centromeres cluster on one side of the nucleus, and chromosome arms align along their length towards the other side of the nucleus, where telomeres cluster (See **Chapter 6**, Fig. 1). This suggests that when chromosomes decondense at mitotic exit, they do so while maintaining general mitotic characteristics.

In **Chapter 3** we showed that the state of chromosomes during mitosis determines which of the two architecture types features more prominently in the subsequent interphase. Chromosomes that have been shortened and are more individualized because of condensin II activity, are more likely to retain these aspects in a subsequent interphase. This model fits with our finding that condensin II promotes chromosome territories. On the other hand, elongated chromosomes have more surface area to form *trans*-interactions, and these interactions are maintained in interphase leading to Rabl-like organization. This fits with our finding that condensin II loss leads to Rabl-like organization, but also with the finding that chromosome length correlates with this architecture type (Álvarez-González *et al.*, 2022).

Surface area and lengthwise compaction

The transition from interphase to mitosis is associated with two changes: a 2-3 fold compaction step and a transformation of the chromosomal structure. It has become clear that condensin is only is important for the latter step. SMC2 depletion does not lead to a change in chromatin volume, but does lead to a drastic change in surface area of the chromosome (Samejima *et al.*, 2018). Chromosome compaction is then largely facilitated by other processes in the cell, for example by histone deacetylation (Schneider *et al.*, 2022; Spicer and Gerlich, 2023).

We also found that overall chromosome length is not evidently affected by condensin II loss in our experiments, but we did observe a 'zigzag' condensation defect (**Chapter 5**). As a consequence of this zigzag phenotype, the surface area of the chromosome likely increases drastically. As described in **Chapter 6**, we propose that this increased surface

area promotes the formation of *trans* contacts during mitosis, which tips the balance to a Rabl-like organization.

In Muntjacs, long chromosome length does correlate with centromere clustering (**Chapter 5**), though the size of the centromere might be a confounding variable in this example. A recent comparative evolutionary study in mammals however also revealed also that centromere clustering correlated with chromosome length and low CTCF density (Álvarez-González *et al.*, 2022). This does suggest that simply having longer chromosomes also influences architecture type. These long chromosomes of course have a larger surface area. Condensin moreover exhibits species-specific spacing along chromosomes(Kakui *et al.*, 2022). An additional possibility is thus that condensin II loop density in species with these long chromosomes may furthermore be lower than in species with shorter chromosomes, which could contribute to a larger surface area. This has so far not been investigated. Future research must elucidate if this correlation between chromosome length and centromere clustering is indeed a causal relationship.

Apart from chromosome length and condensin status, there are likely to be other factors that contribute to the decisions underlying architecture type. Our model indicates that these factors are quite likely to act in mitosis, and possibly either function in regulation of chromosome axis length, or in modulating the attractive forces between different chromosomes. One such factor was recently identified as CENP-B: this factor controls compaction of the centromeric region and promotes clustering of centromeres (Chardon *et al.*, 2022).

Identification of new key regulators may contribute to our better understand of why evolution has 'chosen' one type of architecture over the other in some species. Architecture type seems to only mildly affect gene expression (**Chapter 4**). The question thus remains what the advantage is of either architecture type over the other. By expanding our knowledge on which factors contribute to this decision, we also expand the toolbox by which we can investigate the benefits of either architecture type.

Interphase regulation of condensin

Regulation of condensin complexes: novel insights and open questions

Even though condensin II is nuclear in interphase, it does not condense the chromatin during this time. This suggests that a switch must occur that activates condensin II when cells approach mitosis. Condensin I is phosphorylated extensively during mitosis, and this phosphorylation activates its DNA supercoiling activity (Kimura *et al.*, 1998). This phosphorylation is important, as it is the only post-translational modification that is required to reconstitute mitotic chromosomes in *Xenopus laevis* egg extracts (Shintomi, Takahashi and Hirano, 2015). It was therefore speculated that a multi-step phosphorylation cascade of condensin is the switch required to turn on condensation in mitosis (Bazile, St-Pierre and D'Amours, 2010). It would not be surprising if condensin II was regulated in a similar manner.

Indeed, Mps1-mediated phosphorylation of CAP-H2 promotes DNA binding of condensin II in a poorly understood manner (Kagami *et al.*, 2014). Moreover, CDK1 can phosphorylate CAP-D3 in early mitosis, which in turn promotes Plk1 recruitment and subsequent phosphorylation of all condensin II-specific subunits (Abe *et al.*, 2011). The initial CDK1-mediated phosphorylation of CAP-D3 seems required for condensation, whereas CAP-D3 phosphorylation by Plk1 is not. Possibly Plk1-mediated phosphorylation of the other complexes is more important. This suggests that for condensin II there also is a two-step regulatory phosphorylation cascade that promotes condensation specifically in mitosis.

More recently, MCPH1 has been identified as a negative regulator of condensin II. MCPH1 loss is sufficient to stabilize condensin II on DNA and to promote interphase condensation (Houlard *et al.*, 2021) even in the absence of mitotic kinases. How can we reconcile these seemingly contradictory findings? Possibly, phosphorylation does not directly affect the activity of condensin II, but instead counteracts MCPH1 function in mitosis. Indeed, phosphorylation of MPCH1 counteracts its binding to condensin II (Houlard *et al.*, 2021). It will be interesting to see if phosphorylation of the condensin complex also functions to attenuate its binding to MCPH1, or maybe even to other yet unidentified proteins. In cohesin, other post translational modifications such as acetylation have also been identified to be important in regulation of the complex. We know very little of whether such modifications also occur in condensin, but if they occur, this could also contribute to regulating condensin's activity through the cell cycle.

MCPH1 loss leads to interphase condensation in G1- and G2- phase respectively, but not in S-phase (**Chapter 7**). This suggests that another factor must regulate condensin II during S-phase. Such a S-phase specific negative regulator has yet to be identified, but earlier studies might provide hints towards interesting candidates. One of those is Casein kinase 2, which phosphorylates condensin I and hereby negative regulates it in interphase (Takemoto *et al.*, 2006). Interestingly, the related Casein Kinase I alpha is a negative regulator of condensin II in *Drosophila melanogaster*. Depletion of this kinase leads to a similar interphase condensation phenotype as MCPH1 loss (Nguyen *et al.*, 2015). It thus would be interesting to investigate Casein Kinases further as possible regulators of condensin II in human cells. Especially since an important binding interface of MCPH1 is in CAP-G2, and this protein is absent in *Drosophila,* which could indicate that MCPH1 does not regulate condensin II in that species. Maybe casein kinases were early regulators of condensins, and in vertebrates MCPH1 has evolved to function as an additional level of regulation of condensin II outside of S-phase.

Condensin regulation by MCPH1

Though many open questions remain on the topic of regulation of condensin II, we can now confidently state that MCPH1 is a negative regulator of condensin II (**Chapter 7** and work from others). MCPH1 maintains condensin II turnover on chromatin, and thereby normally prevents it from condensing the DNA in G2 (Houlard *et al.*, 2021). How MCPH1 loss leads to hypercondensation in mitosis and G1-phase remains unknown, as discussed in **Chapter 7**.

Mechanistically, MCPH1 is thought to function in a manner that is analogous to how Wapl releases cohesin from the DNA. MCPH1 would open up the interface between CAP-H2 and the neck of SMC2 to remove condensin II from the DNA, which fits with the data presented in a recent study (Houlard *et al.*, 2021). However, there are also differences between MCPH1 and Wapl that are not well understood, such as the surprising finding that MCPH1 cannot remove condensin complexes that have previously bound the DNA under particular circumstances. Future research must thus clarify the precise mode of action of MCPH1.

It also remains unclear which binding interfaces are important for condensin II inhibition by MCPH1. MCPH1 binds CAP-G2 through an interface in MCPH1's central domain (Wood *et al.*, 2008; Yamashita *et al.*, 2011; Houlard *et al.*, 2021), though there is conflicting data on whether this binding is essential for MCPH1's inhibitory effect on condensin II (Wood *et al.*, 2008; Houlard *et al.*, 2021). Another, more cryptic, binding interface exists between the N-terminal domain of MCPH1 and CAP-D3 (Yamashita *et al.*, 2011). The N-terminus clearly is important for the negative regulation of condensin (Wood *et al.*, 2008; Yamashita *et al.*, 2011), but as this binding appears difficult to identify in the context of the holocomplex, our understanding of this part of condensin II control remains limited (Yamashita *et al.*, 2011).

An open question Is why condensin II is kept in check during interphase by MCPH1. In **Chapter 7** we investigated the biological relevance of MCPH1-mediated condensin control. We found that this process is important to prevent micronuclei formation, and to regulate topoisomerase II function, both through yet unknown mechanisms. Apart from these processes, there might be others for which MCPH1-mediated condensin regulation is important. Interestingly, MCPH1 has been studied in the context of many cellular processes, including DNA damage repair, telomere maintenance and centrosome duplication. It is important for recruitment of many DNA damage repair factors to double strand breaks, and there are hints that this function is possibly exerted through condensin II (Wood *et al.*, 2008). This puts forward the idea that MCPH1 may also regulate condensin in specific processes, rather than only globally. This opens up the possibility that some of the other known MCPH1's functions are also exerted through condensin. MCPH1 would then locally regulate condensin II-mediated loop formation to allow other processes, such as telomere maintenance, to successfully take place. It will be interesting to find out which, if any, of the functions of MCPH1 outside of regulating mitotic chromosome condensation are performed by controlling condensin II.

SMC complexes: master regulators of chromosomal processes

Keeping the balance by regulating condensin

One of the take home messages of this thesis is that condensin II activity needs to be carefully balanced to control various genomic processes. Loss of condensin II leads to massive changes in interphase genome organization (**Chapter 3-5**), and also to missegregations due to problems in mitotic chromosome formation (Saka *et al.*, 1994; Hagstrom *et al.*, 2002; Hudson *et al.*, 2003). These missegregations can have detrimental effects for organisms,

as they can lead to for example lymphomas (Woodward *et al.*, 2016). Other consequences of condensin II loss are increased translocation rate (Rosin *et al.*, 2019) and mis-regulation of a subset of genes (**Chapter 4**). On the other side of the coin, losing the negative regulator of condensin II (MCPH1) also leads to a plethora of defects, as illustrated by the facts that MCPH1 loss leads to the disease primary microcephaly. This disease must be at least in part caused by condensin II-related defects, as condensin mutations can cause similar phenotypes (Perche *et al.*, 2013; Martin *et al.*, 2016; Kristofova, Ori and Wang, 2022). The strict balance of condensin II activity apparently is required to prevent disease and promote healthy development.

The interplay between condensin and cohesin

Keeping condensin in check during interphase might also have another function: to not interfere with proper cohesin function. Cohesin and condensin function in a similar manner by forming chromatin loops, but have widely different spatiotemporal regulation. When the prophase pathway releases the bulk of looping cohesin from the DNA in early mitosis, condensin II by contrast binds the DNA in a very stable manner. On the other hand, when cohesin controls the interphase 3D genome, condensin II only very transiently associates with the DNA. In **Chapter 5**, we investigated what happens when cohesin can also bind the DNA in a very stable manner during mitosis, much like condensin II. Upon WAPL depletion, chromosomes became a lot longer than in control cells, suggesting that highly stable cohesin somehow prevents chromosome shortening. Surprisingly, co-depletion of condensin II rescued this increase in chromosome length. Apparently, when both cohesin and condensin II stably associate with the chromosome, they intervene with chromosome shortening, while both complexes by themselves can promote chromosome shortening when they stably bind DNA. Modeling data has suggested before that too many loop extruding complexes on the DNA can indeed interfere with chromosome shortening. Too high abundance of complexes on the DNA could lead to many short chromatin loops, rather than to the long chromatin loops that contribute most to chromosome shortening (Goloborodko *et al.*, 2016).

Our findings imply that when these complexes encounter each other on the DNA, this may lead to collisions that prevent further chromosome shortening by these complexes. On the one hand this is quite surprising, as these complexes have been shown to be able to pass large obstacles on the DNA (Pradhan *et al.*, 2022). On the other hand, when two condensin complexes encounter each other they can traverse one another and form higher order DNA structures (Kim *et al.*, 2020). These so-called Z-loops possibly counteract compaction, as condensin complexes that are less efficient in forming these structures *in vitro* lead to much shorter chromosomes *in vivo* (Elbatsh *et al.*, 2019). It is thus possible that cohesin and condensin II can form such Z-loops when they are both forming chromatin loops, which may lead to less chromosome shortening. All in all, it would be very interesting to see how cohesin and condensin II act when they encounter each other *in vitro*.

When both condensin II and cohesin already stably bind the DNA in G2, as was shown in cells depleted of both WAPL and MCPH1, mitotic chromosome morphology drastically changes (Houlard *et al.*, 2021). The chromosomes form spring-like structures, where a cohesin axis forms a tightly wound coil. In this context, cohesin and condensin do not seem to counteract each other in a manner similar to what is observed upon single WAPL depletion, as mitotic chromosomes are seemingly more compact. This suggests that the interplay between condensin II and cohesin is regulated at multiple levels, and that this regulation might be different in different cell cycle phases. The interplay between SMC complexes has thus far not been studied in great detail, but future research will have to address fundamental questions about what happens when two different SMC complexes encounter each other on the DNA. This will provide important new insights into the forces that shape our genome.

FUTURE OUTLOOK AND SOCIETAL IMPACT

DNA is the template for all processes in our cells. Studying how its folding is regulated by condensin in both interphase and mitosis has been a fascinating endeavour. Many open questions remain as a consequence of our work and the work of many others, some of which have been outlined above. The coming decades surely will yield many advances in the knowledge of the biological processes described here, as well as more mechanistic insights into how the process of loop extrusion works. Linking the biological and mechanistic insights will be vital to fully understand how condensin II acts, and what it precisely controls.

Fundamental research such as this serves to broaden our understanding of how cells work. It is difficult to predict how this research will benefit society in the long run. It is likely that better understanding of DNA folding and chromosome segregation will yield valuable insights into disease such as cancer. In this thesis, at first glance the most clinically relevant finding is the increased sensitivity to known chemotherapeutical agents of cells which have lost the tumor suppressor gene MCPH1. Better understanding the mechanism behind this sensitivity can be valuable to provide targeted therapy or better understand mechanisms of treatment resistance. On the other hand, the findings in this thesis might also contribute to solutions for unanticipated problems. Maybe our findings on condensin in mosquitos will in fifty years contribute to pest control or malaria prevention. Or maybe it will not contribute directly to any treatments at all. Still the knowledge can be used to continue asking fundamental questions, and broadening the horizon of what we know transpires in our cells. I truly hope that with this thesis, I have contributed to this effort and helped broaden the horizon.

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Adderdum

NEDERLANDSE SAMENVATTING

Elk mens is ooit ontstaan uit één enkele bevruchtte eicel. Deze cel heeft zich eindeloos verdubbeld, en hiermee een mensenlichaam gevormd bestaande uit biljoenen cellen. Het proces waarin de cel zich verdubbeld en zo twee nieuwe dochtercellen vormt, heet de celcyclus.

De celcyclus bestaat uit interfase, waarin de cel zich voorbereid op het vormen van twee dochtercellen, en de uiteindelijke celdeling: mitose. Een van de voornaamste doelen van de celcyclus is ervoor zorgen dat ons genetisch materiaal, DNA, nauwkeurig wordt gedupliceerd en eerlijk wordt verdeeld over de twee nieuwe dochtercellen. Fouten in dit proces kunnen verregaande gevolgen hebben, zoals het ontstaan van kanker.

Een cel, met een diameter van zo'n 10 micrometer, bevat DNA dat bij elkaar zo'n twee meter lang is. Dit DNA is opgedeeld in 46 pakketjes, chromosomen genaamd. Deze chromosomen moeten strak opgevouwen zitten om in de cel te passen. Echter, tijdens interfase moet DNA ook toegankelijk zijn voor eiwitten zodat het gelezen kan worden en kan dienen als bouwinstructie voor nieuwe eiwitten. Maar in mitose moet het DNA nóg compacter opgevouwen worden om te zorgen dat het makkelijk verdeeld kan worden over de twee dochtercellen. In deze fase hebben de chromosomen de welbekende X-vorm. De metamorfose die het DNA ondergaat als de cel mitose in gaat wordt voor een belangrijk deel gedreven door een moleculaire machine genaamd condensin

Condensin is onderdeel van een familie van eiwitcomplexen, SMC-complexen genaamd. SMC complexen spelen belangrijke rollen in het vouwen van het DNA tijdens verschillende momenten in de celcyclus. In die hoedanigheid zijn het essentiële regulatoren van veel processen die zich op het DNA afspelen. In **Hoofdstuk 2** bespreken we uitvoerig hoe deze complexen werken en wanneer in de celcyclus ze een belangrijke rol vervullen. Condensin is voornamelijk bekend van zijn rol in het vormgeven van chromosomen in mitose. In mensen zijn er twee smaken condensin, condensin I en condensin II. Er is lang gespeculeerd of condensin II een rol speelt in processen in interfase, omdat het zich dan ook in de celkern bevindt en toegang heeft tot het DNA. In dit proefschrift hebben we onderzoek verricht naar de functies van condensin II buiten het vormgeven van mitotische chromosomen.

In **Hoofdstuk 3** hebben we gekeken naar hoe de chromosomen zich organiseren in de celkern tijdens interfase in 24 verschillende organismen, variërend van de pindaplant, bakkersgist tot aan Wallaby's. We vinden dat in sommige organismen, zoals in gist, chromosomen dubbelgevouwen in de cel liggen en relatief veel interacties hebben met elkaar. Dit soort organisatie noemen we Rabl-achtige organisatie. In andere organismen, zoals de Wallaby, ligt elk chromosoom als een soort van mie-nestje gevouwen in de celkern en overlapt weinig met andere chromosomen. Deze soort organisatie heet chromosoom territoria. Van de organismen die we onderzocht hebben die condensin II missen, hebben allen

Rabl-achtige organisatie. We vinden dat dit waarschijnlijk een causaal verband is, want het verwijderen van condensin II uit menselijke cellen zorgt ervoor dat hun organisatie verandert van chromosoom territoria naar Rabl-achtige organisatie. Een belangrijk kenmerk van deze Rabl-achtige organisatie is dat bepaalde delen van het chromosoom, centromeren genaamd, samen clusteren.

In **Hoofdstuk 4** bekijken we wat de functie is van de chromosoom territoria in menselijke cellen, door te onderzoeken wat het effect is van het verwijderen van condensin II. Hoewel chromosomen volledig anders georganiseerd zijn in de celkern, zien we dat als we inzoomen op de fijnmazigere organisatie van het DNA er weinig veranderd is. Hieruit concluderen we dat de verschillende niveaus van genoomorganisatie grotendeels onafhankelijk van elkaar opereren. We vinden ook dat het verwijderen van condensin II maar een klein deel van de expressie van genen beïnvloedt, wat suggereert dat chromosoom territoria niet of nauwelijks belangrijk zijn voor het reguleren van gen expressie.

Condensin II voorkomt normaliter het vormen van Rabl-achtige organisatie, en het samen clusteren van centromeren. In **Hoofdstuk 5** proberen we te begrijpen hoe condensin II dit normaliter tegen gaat. We vinden dat condensin II deze functie specifiek in of na mitose kan uitoefenen. Dit suggereert dat de organisatie van chromosomen in mitose bepaalt hoe de chromosomen zich in de volgende interfase organiseren. Onze data wijst er op dat specifiek de rol van condensin in het korter maken van de chromosoom-as belangrijk is voor het voorkomen van centromeer clusters. In **Hoofdstuk 6** bespreken we de bevindingen uit Hoofdstukken 3 tot 5 in de context van de wetenschappelijke literatuur, en beschrijven we in een model hoe condensin II mogelijk interfase organisatie controleert.

Na eerst gefocust te hebben op de effecten van het weghalen van condensin II, onderzoeken we in **Hoofdstuk 7** het tegenovergestelde. In interfase wordt condensin II geremd door de negatieve regulator MCPH1. Deze regulator voorkomt dat condensin II al in interfase het DNA gaat condenseren. Als we MCPH1 verwijderen, laten interfase cellen al duidelijke condensatie zien. In dit hoofdstuk onderzoeken we de biologische consequenties van deze interfase condensatie, met het doel te begrijpen wat er mis gaat als condensin II vroegtijdig actief is. We vinden dat cellen zonder MCPH1 hun DNA tijdens de celdeling niet meer netjes verdelen over de twee celkernen. Normaal gesproken werkt condensin II samen met een eiwit genaamd topoisomerase 2 om knopen in het DNA te ontwarren. We vinden dat wanneer MCPH1 verwijderd is uit de cel, dit ontwar-proces anders verloopt.

Al met al onderstreept dit proefschrift het belang van het goed balanceren van condensin II. We onderzoeken zowel het verlies van condensin II als de over-activatie van condensin II, en vinden dat beide verregaande effecten hebben op de cel. Ook identificeren we belangrijke functies van condensin II buiten het organiseren van chromosomen in mitose. Blijkbaar is condensin II ook vitaal voor de organisatie van chromosomen in interfase. Dit proefschrift draagt hiermee hopelijk bij aan nieuwe, fundamentele inzichten in condensin-biologie.

ENGLISH SUMMARY

Every human being has originated from a single fertilized egg cell. This cell has endlessly duplicated, forming a human body consisting of trillions of cells. The process in which the cell duplicates itself to form two new daughter cells is called the cell cycle.

The cell cycle consists of two major stages known as interphase and mitosis. During interphase, the cell prepares to form two daughter cells, and during mitosis the cell divides in two. One of the main goals of the cell cycle is to ensure that our genetic material, DNA, is accurately duplicated and evenly distributed among the two new daughter cells. Errors in this process can have far-reaching consequences, such as the development of cancer.

A cell with a diameter of approximately 10 micrometer contains DNA that is in total approximately two meters long. This DNA is divided into 46 packages, called chromosomes. These chromosomes must be tightly folded to fit inside the cell. However, during interphase, DNA must also be accessible to proteins so that it can be read and serve as building instructions for new proteins. But during mitosis, DNA must be even more compactly folded to ensure proper distribution to the two daughter cells. In this cell cycle phase, the chromosomes take on the well-known X-shape. This transformation of the DNA when the cell enters mitosis is largely driven by a molecular machine called condensin.

Condensin is part of a family of proteins compelexes called SMC complexes. SMC complexes play important roles in folding DNA at various points in the cell cycle. In this capacity, they are essential regulators of many processes that occur on DNA. In **Chapter 2**, we discuss extensively how these complexes work and at which moments in the cell cycle they play a significant role. Condensin is primarily known for its role in shaping mitotic chromosomes. In humans, there are two flavors of condensin; condensin I and condensin II. For a long time there has been speculation on whether condensin II plays a role in interphase processes, because it is present in the cell nucleus and has access to DNA during this time. In this thesis, we conducted research into the functions of condensin II outside the structuring of mitotic chromosomes.

In **Chapter 3**, we looked at how chromosomes organize themselves in the cell nucleus during interphase in 24 different organisms, ranging from the ground peanut and baker's yeast to wallabies. We found that in some organisms, such as yeast, chromosomes are folded onto themselves and have relatively many interactions with each other. This type of organization is called Rabl-like organization. In other organisms, such as the wallaby, each chromosome is folded in a kind of ball in the cell nucleus and has little overlap with other chromosomes. This type of organization is called chromosome territories. Organisms lacking condensin II appear to always have Rabl-like organization. We find that this is likely a causal relationship because removing condensin II from human cells changes their organization

from chromosome territories to Rabl-like organization. An important feature of this Rabl-like organization is that certain parts of the chromosome, called centromeres, cluster together.

In **Chapter 4**, we examine the function of chromosome territories in human cells by investigating the effect of removing condensin II. Although chromosomes are organized completely differently in the cell nucleus, when we zoom in on the finer organization of DNA we find that there is little change. From this, we conclude that these different levels of genome organization largely operate independently of each other. We also find that removing condensin II only affects the expression of a small subset of genes, suggesting that chromosome territories are not that important for regulating gene expression.

Condensin II normally prevents the formation of Rabl-like organization and the clustering of centromeres. In **Chapter 5**, we try to understand how condensin II normally accomplishes this. We find that condensin II can exert this function specifically during or right after mitosis. This suggests that the organization of chromosomes in mitosis determines how the chromosomes organize themselves in the following interphase. Our data indicates that the specific role of condensin in shortening the chromosome axis is important for preventing centromere clusters. In **Chapter 6**, we discuss the findings from Chapters 3 to 5 in the context of the scientific literature and propose a model on how condensin II may control interphase organization.

After initially focusing on the effects of removing condensin II, we investigate the opposite in **Chapter 7**. In interphase, condensin II is inhibited by its negative regulator MCPH1. This regulator prevents condensin II from condensing DNA in interphase. When we remove MCPH1, interphase cells show clear condensation. In this chapter, we examine the biological consequences of this interphase condensation, with the aim of understanding what goes wrong when condensin II is activated prematurely. We find that cells without MCPH1 no longer evenly distribute their DNA during cell division. Normally, condensin II works together with a protein called topoisomerase 2 to untangle knots in DNA. We find that when MCPH1 is removed from the cell, this untangling process is affected.

Overall, this dissertation underlines the importance of balancing condensin II. We investigate the consequences of both loss of condensin II and over-activation of condensin II and find that both have far-reaching effects on the cell. We also identify vital functions of condensin II beyond the organization of chromosomes in mitosis. Apparently, condensin II is also a key determinant of chromosome organization in interphase. This dissertation hereby hopefully contributes to new, fundamental insights into condensin biology.

LIST OF PUBLICATIONS

Claire Hoencamp, Benjamin D. Rowland
Genome control by SMC complexes
Nature Reviews Molecular Cell Biology **24**, 633-650 (2023)

Claire Hoencamp[*], Olga Dudchenko[*], Ahmed M.O. Elbatsh[*], Sumitabha Brahmachari[*], Jonne A. Raaijmakers[§], Tom van Schaik[§], Ángela Sedeñ o Cacciatore[§], Vinicius Contessoto[§], Roy G.H.P. van Heesbeen[§], Bram van den Broek, Aditya N. Mhaskar, Hans Teunissen, Brian Glenn St Hilaire, David Weisz, Arina D. Omer, Melanie Pham, Zane Colaric, Zhenzhen Yang, Suhas S.P. Rao, Namita Mitra, Christopher Lui, Weijie Yao, Ruqayya Khan, Leonid L. Moroz, Andrea Kohn, Judy St. Leger, Alexandria Mena, Karen Holcroft, Maria Cristina Gambetta, Fabian Lim, Emma Farley, Nils Stein, Alexander Haddad, Daniel Chauss, Ayse Sena Mutlu, Meng C. Wang, Neil D. Young, Evin Hildebrandt, Hans H. Cheng, Christopher J. Knight, Theresa L.U. Burnham, Kevin A. Hovel, Andrew J. Beel, Pierre-Jean Mattei, Roger D. Kornberg, Wesley C. Warren, Gregory Cary, José Luis Gómez-Skarmeta, Veronica Hinman, Kerstin Lindblad-Toh, Federica di Palma, Kazuhiro Maeshima, Asha S. Multani, Sen Pathak, Liesl Nel-Themaat, Richard R. Behringer, Parwinder Kaur, René H. Medema, Bas van Steensel, Elzo de Wit, José N. Onuchic, Michele Di Pierro, Erez Lieberman Aiden, Benjamin D. Rowland
3D Genomics across the tree of life reveals condensin II as a determinant of architecture type
Science **372**, 984-989 (2021)

Linda Clijsters, **Claire Hoencamp**, Jorg J.A. Calis, Antonio Marzio, Shanna M Handgraaf, Maria C Cuitino, Brad R Rosenberg, Gustavo Leone, Michele Pagano
Cyclin F controls cell-cycle transcriptional outputs by directing the degradation of the three activator E2Fs
Molecular Cell **74**, 1264-1277 (2019)

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ACKNOWLEDGEMENTS

Here we are, at the end of this thesis and thus also at the end of my PhD. If you're reading this you've been a part of this journey in some way, and I would like to thank you for that. It was quite a rollercoaster and there have been many people whose support, mentorship, company and distraction were essential for the completion of this journey. It is thus apt to take some time to thank these amazing people for their efforts and presence during my PhD.

First, I would like to thank my promotor, **Benjamin**. From the first time I came to the NKI to interview for a Master internship, I was in awe of your enthusiasm for science. There are very little people that can bring across their scientific excitement as well as you can, spreading the love for SMC complexes everywhere you go. I'm grateful for the many hours you spent teaching me how to best present and write down the story of condensins. And I appreciate how you, when we did not agree on something, were always open to have a conversation and find a solution that we could both agree on. Very exciting that you now have been appointed professor at the TU Delft, and I'm curious to hear what fascinating scientific discoveries the lab will make next.

And then of course, my copromotor **Judith**. Though we never really worked on the same topic, you nonetheless were like a mentor for me. Your vast amount of scientific knowledge is indispensable for the group, and your lab-hacks have surely saved every single one of of us a lot of time. On top of that you're a wonderful and kind person, and it's been inspiring to see you fight for inclusivity within the institute with the D&I committee. I'm proud to see how you're carving your own path within the rigid existing scientific structures, and I am excited to see what the future will hold for you.

I would like to extent many thanks to the members of my reading committee and opposition: **Cees, Stan, Titia, Aniek and Jonne**. I would also like to thank my OOA supervisory committee, **René, Tassos, Bas** and **Kees** for their valuable insights during the yearly OOA evaluation and their supervision over the years.

My paranymphs – **Anoek** en **Roel**. As you have always been by my side during this PhD journey it is only fitting that you stand beside me during the day of my defense. Throughout the years, you were there to celebrate the highs and comfort me through the lows of my PhD. I could really not have imagined finishing this journey without you two, and I'm looking forward to many more games, drinks and laughs together, especially now that we all live in the same city! **Anoek**, your smile is contagious and your passion for pipetting admirable. Somehow you always have time for coffee and a heart-to-heart when it is needed, despite also doing thousands of experiments per day. This illustrates how devoted you are to your friends. I loved climbing mountains together (literally and figuratively) , and want to thank you for all your support over the years. It has been great to see you grow into such an amazing scientist, and I cannot wait to hear all about the accomplishments of the Friskes lab in the

future. **Roel**, my partner in crime in the Rowland lab. I'm thoroughly impressed by your creativity and your drive to solve (or make) even the most complicated puzzles – scientific or just for fun. Your mischievous jokes and little games have entertained me through a huge part of my PhD. Thank you for the many times that you were there to listen to me vent, and to put things into perspective. You're a great scientist, you have developed a profound understanding of SMC complexes and with this have been a valuable colleague to me and many others. Whichever direction you will take in the future; I am absolutely certain that you will succeed, and will make a party out of it along the way.

I might never find another group of colleagues as fun, kind and smart as my colleagues from the Rowland lab. **Marjon**, your sharp mind and eye for detail make you an excellent scientist and this combined with your positive attitude and kindness make you a wonderful colleague. I'm grateful for the many scientific adventures we went on together – visiting conferences at Cold Spring Harbor, in Vienna and in Montpellier. These weeks were always a perfect combination of nerding out together, encouraging one another to go network with that PI and fun nights in which we had drinks with other great scientists. I wish you all the best with your Postdoc and whatever comes after! **Alberto**, you are such a warm, generous and kind person. Thank you for always being interested in how I am doing. It's been a lot of fun being on team condensin together and figuring out how this complex works. Over the years I have really seen you grow both in your scientific knowledge as your professional skills, and it's exciting to see that you have now also almost finished that PhD journey! On top of that I'm grateful to have joined your beautiful wedding in Madrid, and to have watched your little family grow. I wish you lots of happiness back in Spain together with Clara, Andrés and Dutch. **Démi**, I love how we can nerd out together and chat at length about many of our shared interests – albeit D&D, Critical Role, or the U.S. or Dutch elections. Partying together on the music from our favorite emo bands was definitely worth repeating! You're a very thorough and disciplined scientist, with great insights and exciting projects. I'm convinced this will take you very far! I'm very grateful that you have succeeded me as B5 representative in the PhD Council, because I just know you will do a great job there. **Roan**, don't tell the others, but you were definitely my favorite student back in the day during the practical 'Van Molecuul tot Cel'. What an honor that after teaching you to pipet, I have been a small part of your future career as well. I'm very impressed with your passionate stance against injustice and applaud your effort to make the institute a better place, especially since this is not always easy. I really wish you all the best with the next step you'll be taking. **Beccy,** I'm so happy that I could convince you that team condensin was worth joining. Your energy, love for pipetting and mitosis, and exciting ideas are wonderful additions to the lab. Plus, leaving the NKI is a lot easier knowing that the condensin saga is continued by you! I'm very much looking forward to our future Jordaan tour, which surely will lead to many more great conversations, laughs and a lot of (Two Chefs?) beers. **Su**, what would we do without you? Thank you for keeping the lab running for the past years, and always being there to help when issues arise. You're such a kindhearted person, it's always great to chat with you. Thank you for your warmth and support over the past few years. **Klaudia**, I

enjoyed showing you the ropes in the a bit when you first arrived in the lab, and it has been a pleasure seeing you grow during your internship. The lab certainly is lucky that you have decided to stay for your PhD, which with your dedication will surely be very successful. You have a work hard, play hard attitude and you're great fun in the lab. Keep that up! **Tom**, it's been great to have you as my office-neighbor at the end of my PhD, as you were always up for a chat. I'm happy that you've continued working on the interesting relationship between condensin and MCPH1 and I'm excited to see what you'll find!

Of course, during my 5,5 years at the NKI, some members left the Rowland lab to continue their careers elsewhere. **Ahmed**, you are the one that taught me the ropes in the lab and enthused me to pursue condensin research. You were a great mentor in my internship and when I later started as a PhD. Thank you for sharing your knowledge, and your condensin projects, with me. **Ángela**, thanks for all your effort helping us understand bio-informatics, which surely was not always easy. It's been wonderful to see you pursue your passions outside of science, and I wish you lots of luck. **Laureen, Giorgio** and **Virginie**, my time in the lab would not have been the same without our chats, your kindness and the fun we had. Thank you for that! I wish you all the best in the careers you're now pursuing.

I have also had the pleasure to supervise multiple students during my PhD trajectory. **Aditya** and **Christa**, thank you very much for teaming up with me. Both of you taught me how to be a better teacher and a better scientist, and I wish you both the best for your PhD's!

In my PhD I had the pleasure to work at two different departments: B4 and B5. In B5, we shared meetings, benches and a lot of fun with the members of the Medema lab. **Dorine, Jonne, Lisa, Louise, Apostolos, Mila, Michael, Xabier, Marco, Rob and René**, thank you for this! **Dorine,** you're so positive and supportive, and you always bring a smile to my face. I loved partying together at DTRH, and hope to enjoy a lot more parties and borrels together! **Jonne**, I must have knocked on your door a thousand times over the years, to ask questions about condensin or topoisomerases, or to get some inside knowledge on a particular experiment. Thank you for all your help and advice over the years. I'm honored that you're in my committee and I'm looking forward an interesting scientific discussion during my defense! **Lisa**, chatting or having a drink with you is always fun. Very exciting that you're also about to defend your thesis, I'm looking forward to seeing where the future will take you! **Louise**, my condensin buddy. Thanks for always thinking along in my projects. **Apostolos**, you're always so kind and interested, thank you for that. **Mila,** it's been fun chatting every time I needed the sink. Your double minutes project is very promising, and I'm curious to see what interesting things you'll find. **Rob,** thank you for always taking the time to help when a machine has broken down. Thanks to you, (almost) everything runs smoothly at B5.

Of course I would also like to thank other B5 members for making this such a wonderful place to work: **Kees, Sravasti, Bram, Jeffrey, Arnoud, Piet**. **Kees** and **Sravasti**, thank you for your enthusiasm on our imaging screen ideas – hopefully someone else will take over

this exciting project in the future! **Bram**, you were an amazing help for everything image analysis related. Thank you for the many macros you build. **Marianne, Mariet,** thank you for arranging so many fun department activities.

I also have many fond memories of Wine & Cheese meetings, borrels and department outings with the members of B4. Thank you to all current and former members **van Steensel lab**, **Lenstra lab**, **de Wit lab** and **van Leeuwen lab** for these memories, and your help and input on my research. **Tom** and **Bas**, thank you for your work on the DamID in the condensin II paper. I specifically would like to thank Tom for patiently explaining the data analysis to me, I learned a lot from you. **Hans** and **Elzo**, thank you for your Hi-C contributions to the condensin II paper. **Stefano, Lise, Robin** and **Ninqing** (and **Anna** of course, though you're not a B4 member), you made the conference in Montpellier so much more fun!

There were also many other people all over the NKI who helped my during my PhD journey. I would like to also thank all members of **the lab of Jaqueline Jacobs** for their valuable input and interesting ideas put forward during our weekly Monday Morning meetings. **Abdel,** thank you for your help with everything screen-related. I really think we found some exciting things! **Henri,** thank you for your hard work and ensuring everything in the NKI runs smoothly. I also would like to thank the NKI facilities without whom this thesis would not have existed. Thank you **Bioimaging Facility**, **Flowcytometry facility, Cryogenic storage facility, Protein facility and the Genomics Core facility**. I specifically want to thank **Marjolijn Mertz** for the hours she spent helping me with the Imaris and the Spinning Disk Microscope. Every time I think of the spinning disk, I start singing that song from Russian Doll.

During my time at the NKI I was lucky enough to work together with many great people within **PhD Council**. I am very proud of all of you, and what we have achieved over these past years. I'd like to thank all of you for the fun times together, all useful meetings and your commitment to making the NKI an even better place for PhD students. I'm sure the future will hold a lot of bright things for you. **Iris**, thank you for your leadership and teaching me the ropes. **Marlize,** I love your commitment and insights, you're going to achieve great things. I always loved our in-depth discussions about how PhD student life could be better. **Daniela**, I'm proud of you, and I always really liked our coffee chats. **Živa**, you did such an amazing job advocating for better mental health for PhD students! **Mercedes,** it was great to welcome new PhD students to the institute side by side. Thank you for all your work and enthusiasm!

I also would like to thank the amazing people of the **Diversity & Inclusion committee** of the NKI: **Liam, Judith, Migaisa, Sara, Pia, Ana, Sravasti, Leila, Nicolaas, Roan, Kathy, Joana,** and all the other people that have joined the committee over the years. Thank you for the time you spend making the NKI a safer and more inclusive workplace. I enjoyed working alongside you, and I am inspired by your perseverance. Keep up the good work, despite it being difficult sometimes!

There are many more people in the institute that have made all the borrels and parties very memorable. **Antonio, Isabel, Mariana, Alessandra, João, Mar, Anna, Jeremy, Ronak, Simon, Clara, Soufiane, Ruben, Mathias, Christ, Nicolaas, Danielle,** thank you for all the good times and making the NKI an unforgettable workplace.

I would also like to thank the **Boehringer Ingelheim Fonds** for the great support during my PhD fellowship in the form of courses and the Hirschegg retreat.

Ook buiten het lab ben ik omringt met geweldige mensen die altijd bereid bleken te luisteren naar enthousiaste of minder enthousiaste PhD verhalen, en me vaak genoeg hebben geholpen om het lab even uit mijn hoofd te zetten.

Allereerst natuurlijk mijn **Hindes**. Mijn steun en toeverlaat, van hier tot aan Argentinië. Al ruim 10 jaar zetten we de boel op stelten op festivals, vakanties, in het theater en ver daarbuiten, en dat blijven we hopelijk nog heel erg veel jaren doen. **Eske,** ik waardeer hoe attent je bent, en je support door deze jaren heen. Of het nou politiek, literatuur of kunst is, we kunnen uren onder het genot van een wijntje elitair kletsen. Erg spannend dat je nu zelf aan je PhD journey bent begonnen, je kan het! **Janneke**, van samen studeren op Science Park en lab-stages lopen, zijn we toch een heel eind gekomen! Ik vind het inspirerend te zien hoe je je eigen pad hebt gevonden. Bedankt voor je luisterend oor en de gezellige dumky-dumky's. En het is heel bijzonder nu te zien hoe je een gezinnetje vormt met Marijn en Thijs en zo een heel nieuw avontuur aan gaat. **Josie,** ik kan met jou lachen tot ik buikpijn heb, of uren de diepte ingaan. Wat ben ik gelukkig dat we elkaar vaak zien bij allerlei leuke avondjes onder het genot van bubbels. Ik vind het zo knap dat je altijd tijd hebt voor vrienden, en tegelijk ook nu al zo'n indrukwekkende carrière hebt. Dankjewel dat je altijd betrokken bent bij hoe het met mij of mijn onderzoek gaat. **Lindsey,** ik kan niet geloven dat het al zo lang geleden is dat we onze 'perfecte zondag' hadden en het wilde plan opvatten op naar Hawaii te gaan! Wat was dat geweldig, en wat is er veel gebeurd in de tussentijd. Bedankt voor alle goede inzichten die je me hebt gegeven door de jaren heen, je gezelligheid en je empathische blik. **Marjolein**, van (semi-)buurtgenoten in Amsterdam tot stadsgenoten in Utrecht. Wat heb ik een geluk dat ik je altijd dichtbij hebt. Je positieve en lieve energie helpt altijd om de wereld weer een stukje zonniger te zien. Je bent zo begaan met de mensen om je heen, dat is echt prachtig. Ik vind het echt knap hoe je de afgelopen jaren bent gaan luisteren naar jezelf en hoe je meer rust hebt gevonden. Het is een plezier om samen het onderneem-avontuur aan te zijn gaan, en ik kijk uit naar nog heel veel wandelingetjes in ons stadsie. **Roos**, je bent uitgegroeid tot een van mijn meest waardevolle vriendinnen. Ik geniet ervan dat we heel erg veel samen kunnen nerden, of het nou in Brussel of Londen is voor Harry Potter activiteiten, of een weekend lang door de modder stampen en dobbelstenen kopen op Castlefest. We hebben samen al veel van de wereld gezien, en ik hoop zeker dat er nog veel mooie uitjes bij komen op de lijst! Ik duim dat je snel een fijn eigen plekje kan vinden, en kom dan graag langs in Leiden voor een paar goede glazen wijn.

Menig dinsdag kwam ik minder scherp op het lab door pubquiz avondjes met het meest walgelijke pubquizteam **Boudoir en de Blote Banjo's** bestaande uit de onovertreffelijke **Ruben, Kris, Josie, Sjoerd en Koen.** Of ik mijn PhD heb gehaald dankzij, of juist ondanks deze heerlijke maandagavonden laten we maar even in het midden. Hoe dan ook, geniet ik er altijd van als we samen zijn, ook nu we regelmatig afreizen naar Ankvist om de koning bij te staan (of te laten aftreden, het is maar net wie je het vraagt). En: komende februari gaan we natuurlijk weer voor een mooie 22e plek in Groningen, en wat droevige eierballen uit de muur om het te vieren. **Ruben**, ik kan me een mooie avond met goede gesprekken herinneren op het dakterras van een bar in New York. Ik denk dat hier onze vriendschap een nieuwe laag heeft gekregen. Bedankt voor de goede gesprekken, en de goede (en vaak ook foute) grappen waar je iedereen altijd mee opvrolijkt, ook al is er niet altijd iets om te lachen. **Kris**, de gelijkenissen met El Profesor zijn duidelijk, maar je bent zelf ook goed op weg om die felbegeerde professor-titel aan je naam toe te voegen. Erg fijn om iemand te hebben om het soms het even over het PhD-leven te hebben, maar vaak ook gewoon de bloemetjes mee buiten te zetten. **Sjoerd**, de rustige kracht in ons team die als een van de weinige de spelregels van King's Dilemma écht doorheeft. Je bent zo zorgzaam, altijd geïnteresseerd en je kennis van het menselijk lichaam en vliegtuigen is onmisbaar gebleken voor de successen van Boudoirs.

En vaak precies op het moment dat ik echt even niet meer aan mijn PhD kon denken, was daar het festivalseizoen. Ik ben de tel kwijt op hoeveel geweldige festival-weekenden ik heb gehuild van het lachen, gedanst tot in de vroege uurtjes en cocktails uit m'n handen heb laten vallen, maar ik weet wel dat er weinig andere weekenden zijn geweest waar ik even zo blij en onbezorgd was. **Anniek, Iris, Mirthe, Michelle, Xanne, Eske, Janneke, Josie, Lindsey, Marjolein, Roos, Leonie,** bedankt voor deze geweldige weekenden en de energie die ik van jullie allemaal kreeg. **Anniek**, je bent een topper. Bedankt voor je gelach en dat je veel met me meegedacht hebt over mijn toekomstige carrière! Pannenkoeken en het Mario Kart drinkspel is altijd een feest bij jou en **Iris** thuis, bedankt dat ik altijd welkom ben bij jullie. **Mirthe**, we gaan binnenkort wel echt die spelletjesavond doen samen met **Elmer** toch? Met jullie samen is het altijd gezellig!

Sinds kort mag ik mijn liefde voor boeken lekker etaleren in onze fijne **Boekenclub 'De Menselijke Conditie [overgeef-emoji, boek-emoji]'** met **Leonie, David, Jascha, Eske, Lindsey, Marjolein en Anniek.** Ik geniet van onze diepgaande discussies, en ben trots dat we samen de Himalaya beklommen hebben. **Leonie,** je blijft altijd mijn voorzitter, ik heb door de jaren heen zoveel van je mogen leren. Dankje voor je goede adviezen, je doorziet altijd hoe het nou écht met me gaat en bent zo betrokken bij wat ik doe. Het is een feest om bij jou en **David** thuis te komen en heerlijk te eten, zeker ook nu Mila ook in jullie midden is. **Jascha,** bedankt voor je filosofische inbreng in de discussies over boeken en fijne gesprekken.

Ultiem nerden met D&D is altijd een goede vorm van escapisme. Als je dat doet onder het genot van een smakelijk glas bubbels, en met heerlijke spelers die de die meest innovatieve

oplossingen vinden, is het helemaal een feest. **Roos, Josie, Sjoerd, Lindsey, Ruben en Rutger**, bedankt voor de avonden dat jullie met mij naar Phandalin zijn afgedaald, en ik kijk erg uit naar onze nieuwe avonturen in Exandria. **Rutger**, ik ben blij dat ik iemand heb gevonden die net zo'n grote D&D-nerd is als ik, met wie ik uren kan kletsen over onze karakters en werelden.

Sam, onze grote pepernoten smaaktest is mijn favoriete sinterklaas traditie! Maar ik geniet ook van de doordachte politieke discussies die we hebben, en ik bewonder de passie waarmee je voor je idealen opkomt.

Ik kan nog steeds niet helemaal geloven dat ik het geluk heb gehad om samen met mijn prachtige lustrumcommissie, **Jelle, Tijmen, See, Pelle, Jolijn,** een jaar lang zulke toffe activiteiten te organiseren. Het hoogtepunt was toch wel een weekend lang rondlopen met continu elkaars stem in onze oren op de WooHoo. Bedankt voor deze geweldige herinneringen, zowel tijdens het lustrum als de jaren erna, en voor het begrip dat ik niet altijd even veel tijd had vanwege mijn PhD of omdat ik in New York was.

Ooit, lang geleden begonnen we met z'n allen op Science Park als studentjes Bio-medische wetenschappen, en kijk ons nu eens. Wie had dat ooit gedacht tijdens die avonden in de Chups? **Anne, Daphne, Nina en Jelte** het is ontzettend leuk om te zien hoe jullie allemaal je eigen pad hebben gekozen na dat eerste jaar samen, en ik geniet van de etentjes, wandelingen en borrels die we ook nu nog soms hebben.

Simone, ondanks dat het toch al jaren geleden is dat we pubers waren op het Revius en op eindexamenreis gingen naar Blanes, blijft het altijd fijn te kletsen over het leven. Of het nou een dinertje met zijn tweeën is, of een spelletjesavond met jou, **Joost** en Koen, het voelt altijd als vanouds gezellig.

Hylke, wat bijzonder dat we elkaar na zoveel jaar weer terug hebben gevonden. Ooit begon het allemaal met ontzettend flauwe humor en veel plezier bij het schooltoneel. Tegenwoordig is geen Irish Pub of tramrails veilig als we de stad in gaan, en kunnen we uren praten over de serieuze en minder serieuze kanten van het leven. Laten we elkaar niet weer zoveel jaar uit het oog verliezen!

Megan, wat hebben we toch veel meegemaakt samen. Heel bijzonder hoe we gegaan zijn van elke avond samen hangen of tramponline springen in Amerongen, tot beiden een heel ander leven leiden. Gezellig dat jij, **Dion, Iain en Maeve** weer in de buurt wonen zodat we nog veel mooie nieuwe herinneringen kunnen maken samen.

Dan heb ik dankzij Koen ook nog het geluk een leuke groep vrienden in het Zuiden van het land te hebben. **Tjeu, Esther, Tom, Claudia, Jordy** en **Joyce,** oftewel 'de Nerdjes en Claudia' (al is het de vraag hoe accuraat die naam nog is?). Altijd in voor een lekker speciaalbiertje,

en soms ook voor de rare rookworst. Bedankt voor de gezellige weekendjes, het carnavallen in Eindhoven en alle andere leuke uitjes!

Ook heb ik het geluk een hele fijne schoonfamilie te hebben die me de afgelopen jaren gesteund hebben **Jan, Jeannette, Evy, Jeroen, Lynn, Job, Mark, Jolien, Fleur en Bart**, bedankt dat jullie me vanaf het begin welkom hebben geheten en kennis hebben laten maken met Brabantse gezelligheid. Ik heb veel mooie herinneringen aan de mooie reizen die we samen hebben gemaakt, en alle gezellige familie-uitjes en etentjes. Maar jullie staan ook altijd voor ons klaar als het nodig is, en dat waardeer ik ontzettend. Bedankt voor alles.

Britt, ondanks dat we heel wat jaartjes schelen, hebben we elkaar helemaal gevonden in Amsterdam! Ik vind het zo ontzettend leuk dat we, ondanks dat ik nu wel echt een jaartje ouder wordt, samen zo goed kunnen feesten en al heel wat festivals onveilig hebben gemaakt. Maar ook gewoon samen eten of een drankje doen is altijd gezellig, want we delen veel interesses. Bedankt dat je me altijd jonger laat voelen dan ik ben, en dat je altijd een feestje meeneemt waar je ook heen gaat. **George**, zo gezellig dat je sinds een paar jaar ook onderdeel van de familie bent. Bedankt voor je interesse, gezelligheid en interessante gesprekken.

Papa & Mama, zonder jullie had ik hier niet gestaan. Bedankt dat jullie me vroeger altijd aanmoedigde om te doen wat ik leuk vond, wat dat dan ook mocht zijn. En bedankt dat jullie me de kans gaven om mezelf te zijn, zodat ik uit kon groeien tot wie ik nu ben. Gezellig dat ik nu een stukje dichterbij ben komen wonen, zodat we elkaar wat makkelijker kunnen zien! **Mam,** bedankt voor de fijne gesprekken en je betrokkenheid. Door de jaren heen hebben we veel leuke dingen mogen doen samen, en ik hoop dat we dat volhouden. **Pap**, dankzij jouw hulp heb ik nu een heerlijk huis. Ontzettend bedankt voor al je hulp, je goede adviezen door de jaren heen, en je onvoorwaardelijke steun.

Momo & Jiji, mijn lieve kleine monstertjes. Wat ben ik blij dat ik Koen heb overtuigd om jullie te adopteren twee jaar geleden. Jullie knuffels en gekke capriolen hebben regelmatig een glimlach op mijn gezicht weten te toveren, en wat is het toch gezellig om thuis te komen als jullie beiden voor de deur staan. Thuis schrijven was een stuk leuker met jullie erbij.

Lieve **Koen,** bedankt dat je de afgelopen jaren er altijd voor me was. Je was er om de leuke momenten te vieren met een borrel, maar ook om de nodige frustraties aan te horen tijdens het avondeten. En als ik weer eens tot 's avonds laat in een Zoom-gesprek zat, had je daar begrip voor en verzorgde je eten, snacks en thee. Jouw rust en relativeringsvermogen hebben me door veel moeilijke moment heen geloodst. Ik geniet van ons leven samen, van onze mooie vakanties naar dichtbije of verre oorden, onze knusse bordspelavonden met speciaalbiertjes en onze culturele of culinaire uitjes. Ik vind het fijn hoe ons huis altijd open staat voor gezellige avonden met vrienden, en hoe we die dan als een perfect team hosten.

Ik kan niet wachten op de volgende hoofdstukken in ons leven samen, in onze nieuwe stad Utrecht en binnenkort als echtgenoten. Samen is alles beter. Ik hou van jou.

CURRICULUM VITAE

Claire Hoencamp was born on the 18th of January 1993, and grew up in Amerongen, The Netherlands. She completed her secondary education in 2011 at the Revius Lyceum Doorn with a focus on Biology, Chemistry, Physics and Math. During the last two years of secondary education she was selected for the Junior College program of the University Utrecht. This program focused on broadening and deepening the knowledge of the beta subjects, and included a short research project in the lab of Prof. Dr. Geert Kops.

In 2011 Claire started with the Bachelor Biomedical Sciences at the University of Amsterdam. During the final year of her Bachelor's programme she had the opportunity to work on cohesin regulator DDX11 in the lab of Dr. Rob Wolthuis, under the supervision or Dr. Job de Lange. During her Bachelor's, she was an active member in the Student Theater Association Amsterdam (STA!). In 2013/2014 she became a parttime board member of STA!, her role being Board Member External Relations and focusing on obtaining cultural grants and promoting the theatre plays. In 2014 she obtained her Bachelor's degree cum laude and with the completion of the honours programme. The next year, Claire took a gap year to teach practical courses to 1st and 2nd year Bachelor students for half a year and then travel for 5 months through South America.

She continued her study with the Master's Biomedical Sciences, specializing in Oncology, from which she graduated cum laude in 2018. During her Master's programme, Claire performed an internship with Dr. Benjamin Rowland, under the supervision of Dr. Ahmed Elbatsh, in which she studied potential regulators of the cohesin-removal factor Wapl. Her final internship was under the supervision of Dr. Linda Clijsters in the lab of Prof. Dr. Michele Pagano at New York University Medical Center. Here, she studied the effect of Cyclin F-mediated degradation of E2F2 and E2F3a on S-phase progression and apoptosis. After her time in New York she decided to return to the group of dr. Benjamin Rowland and start her PhD at the NKI. During the next 5,5 years, Claire studied the atypical functions of condensin II. This research accumulated in the thesis presented here. During her PhD, Claire also was the chair of the NKI PhD Council and a member of the NKI/AvL Diversity and Inclusion Committee.