Shaping mitotic chromosomes: From classical concepts to molecular mechanisms

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How eukaryotic genomes are packaged into compact cylindrical chromosomes in preparation for cell divisions has remained one of the major unsolved questions of cell biology. Novel approaches to study the topology of DNA helices inside the nuclei of intact cells, paired with computational modeling and precise biomechanical measurements of isolated chromosomes, have advanced our understanding of mitotic chromosome architecture. In this Review Essay, we discuss – in light of these recent insights – the role of chromatin architecture and the functions and possible mechanisms of SMC protein complexes and other molecular machines in the formation of mitotic chromosomes. Based on the information available, we propose a stepwise model of mitotic chromosome condensation that envisions the sequential generation of intra-chromosomal linkages by condensin complexes in the context of cohesin-mediated inter-chromosomal linkages, assisted by topoisomerase II. The described scenario results in rod-shaped metaphase chromosomes ready for their segregation to the cell poles.

Keywords:
chromosome condensation; chromosome segregation; cohesin; condensin; mitosis; SMC complex; topoisomerase II

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Abbreviations:
EM, electron microscopy; **NEBD,** nuclear envelope breakdown; **SAXS,** small angle x-ray scattering.

Introduction: Classical views of chromosome structure

The segregation of genetic information from mother to daughter cells during mitotic divisions is one of the most spectacular events of the cell division cycle. Successful segregation requires the extensive reorganization of chromatin fibers into compact cylindrical mitotic chromosomes. Although this "condensation" process has fascinated scientists since its first description at the end of the 19th century [1], the underlying molecular mechanisms have remained incompletely understood.

Mitotic chromosomes are thought to entail several levels of organization (reviewed in Ref. [2]). The first and best-understood level is the wrapping of 146 bp of DNA in 1.7 turns around an octamer of histone proteins to form a nucleosome – a structure that has been resolved to near-atomic resolution [3, 4] (Fig. 1A). Linear arrays of nucleosomes connected by spacer DNA regions give rise to chromatin fibers of ~11 nm diameter, which, when imaged by electron microscopy after chemical fixation in low salt conditions, appear as "beads on a string" [5, 6].

The next level of organization is thought to result from interactions between adjacent nucleosomes on the same DNA helix and binding of linker histone H1, creating a fiber of ~30 nm diameter. The 30 nm fiber can be readily observed when arrays of nucleosomes are reconstituted on particular DNA sequences in vitro [7]. Different hypotheses for the arrangements of nucleosomes in the fiber have been vividly discussed over the past years. Current versions include one-start interdigitated solenoid [8] and two-start zigzag models [9, 10] (Fig. 1A). Recent studies suggest that the choice between conformations depends on the lengths of the linker DNAs that connect the nucleosomes; hence different structures might co-exist [11]. Whether most of the chromatin inside a cell's nucleus folds into 30 nm fibers is, however, questionable. Support for the existence of repetitive structures of 30 nm in diameter in interphase chromosomes comes from electron microscopy (EM) images of chromosome fragments prepared from rat liver nuclei [12]

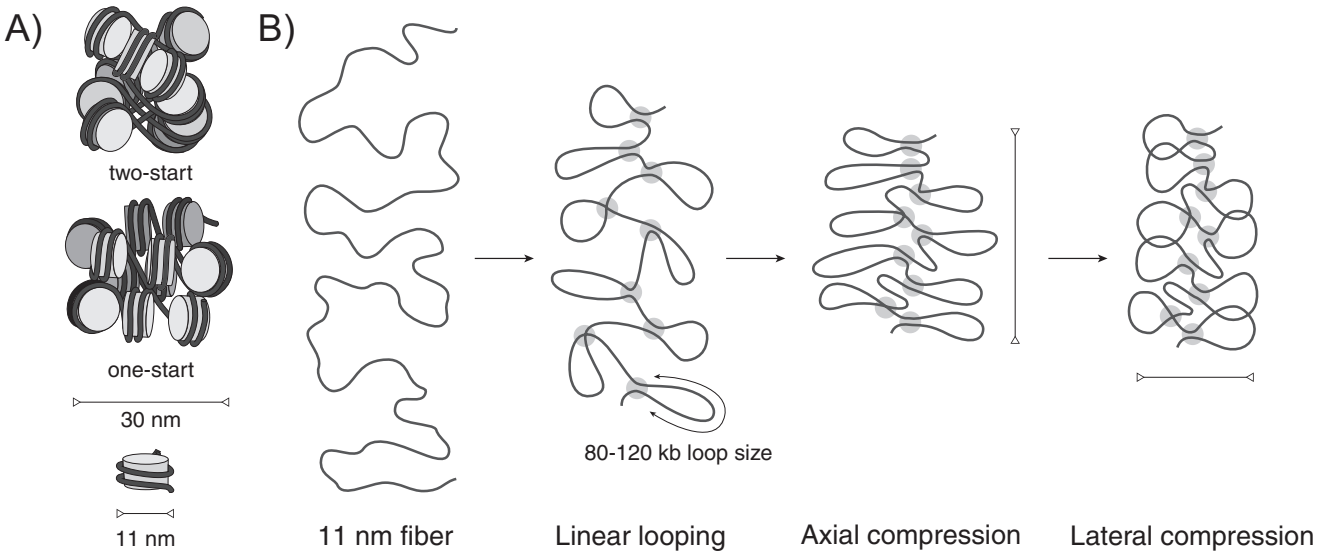

Figure 1. Levels of chromosome organization. **A:** Cartoon models of nucleosomes composed of DNA (dark gray tube) wrapped around an octamer of histones H2A, H2B, H3, and H4 (light gray cylinder) and of one-start (solenoid) and two-start (zigzag) 30 nm fibers. **B:** Proposed steps in folding an 11 nm nucleosome fiber into a mitotic chromosome by formation of loops of 80–120 kb in size, followed by compression along the longitudinal chromosome axis and reduction in chromosome diameter by lateral compression.

as well as from small angle x-ray scattering (SAXS) diffraction patterns and cryo-EM images of nucleated chicken erythrocytes [13, 14]. Evidence for the existence of regular 30 nm fibers in mitotic chromosomes remains, however, controversial (see below).

Our understanding of how chromatin fibers wind up into rod-shaped mitotic chromosomes in the next levels of organization is – at best – rudimentary. Based on the tube-like appearance of fibers of ~400 nm diameter obtained by spreading mitotic chromosomes isolated from cultured human cells after chemical fixation, Crick and colleagues proposed that 30 nm fibers might be laid out in a (super) solenoid [15]. Mitotic chromosomes might hence be formed by a hierarchical helical folding of chromatin fibers. A similar model of successive chromatin coiling was derived by Sedat and Manuelidis from light and electron microscopy images of isolated nuclei and mitotic chromosomes [16]. Based on electron micrographs of purified human metaphase chromosomes that had been fixed after depletion of histones or swelling by removal of divalent cations, Laemmli and colleagues instead suggested that chromatin fibers adhere to a central chromosome axis, from which they emerge as radial loops of several ten kilo-bases (kb) in length [17, 18]. In contrast to the hierarchical folding model, which could in principle be explained solely by nucleosome–nucleosome interactions, the radial loop model required a protein scaffold at the chromosome axis to anchor the bases of the chromatin loops. Remarkably, electron-dense material in the shape of a metaphase chromosome surrounded by a halo of DNA could indeed be visualized by EM [17].

Even though the two models substantially differ in the nature of the folding events that take place during the formation of mitotic chromosomes, both elegantly explain how chromosomes can fold into cylindrical – instead of spherical – shapes. In this Review Essay, we discuss how recent studies have given rise to new insights into the structure of mitotic chromosomes and explore the contributions of some of the major molecular machines involved in the chromosome condensation process.

New technologies to study chromatin architecture challenge classical models of chromosome folding

Hierarchical folding models posit the 30 nm fiber as the first step towards the formation of mitotic chromosomes. Indeed, x-ray diffraction patterns of isolated HeLa metaphase chromosomes after chemical fixation support the existence of 32 nm structures [19]. However, recent analyses of cryo-electron micrographs of HeLa cell nuclei or isolated mitotic chromosomes in a hydrated state showed merely a homogenous texture with no evidence for the presence of regular 30 nm fibers [20, 21]. SAXS experiments with the same chromosomes after incubation in buffers containing polyamines and EDTA gave likewise no indication of structures with a regular 30 nm periodicity, nor any larger regular arrangements [22]. The ~30 nm diffraction patterns, which were described for chromosomes that had not been incubated in these buffers, might have instead been caused by the aggregation of ribosomes on the chromosome surface [23]. Similarly, the 30 nm fibers observed by conventional EM might have resulted from isolating chromosomes in low Mg^{2+} conditions or from the fixation process. While it is still conceivable that small stretches of chromatin assemble into 30 nm arrays even within native mitotic chromosomes, these new data argue that the largest fraction of mitotic chromosomes consists of irregularly arranged chromatin fibers [24].

This arrangement might be best described as a dynamic fractal structure or "polymer melt," which can be explained by a shift from interactions between adjacent nucleosomes to an interdigitation of distant nucleosomes, caused by the high nucleosome concentrations in condensed mitotic chromosomes.

Further insights into the structure of mitotic chromosomes come from micromanipulation experiments. The bending and stretching elasticity of mitotic chromosomes isolated from amphibian cells are consistent with rods of a uniform cross-section [25] and argue against the notion of a rigid scaffold at the chromosome axes, which had previously been inferred from the high bending flexibility of chromatids assembled in *Xenopus* egg extracts [26]. The much higher stiffness of chromosomes isolated from *Xenopus* cells implies that chromosome assembly in extracts might not completely recapitulate the formation of mitotic chromosomes in vivo. Importantly, DNA cleavage within these isolated chromosomes rapidly eliminates their elasticity and eventually disintegrates them completely. Yet, chromosomes merely become softer but remain elastic after mild proteolysis. These findings suggest that the integrity of mitotic chromosomes is largely determined by the continuity of the DNA helix and not by a linear protein scaffold [27–29].

A completely different approach to study the structure of mitotic chromosomes was recently described by Dekker and colleagues based on chromosome conformation capture experiments (Hi-C and 5C) in combination with polymer simulations [30]. Hi-C and 5C rely on the identification by massive parallel sequencing of ligation products between distant genomic loci that have come into close physical proximity in 3D. In interphase human cells, these techniques identified long-range intra-chromosomal interactions within megabase-scale compartments and within smaller topologically associated domains (TADs). Remarkably, these patterns disappeared in cells arrested in a prometaphase-like state. The contact probabilities measured for mitotic chromosomes could not be described by polymer simulations of a hierarchical folding model, but instead matched the predictions made by models of consecutive loops of 80–120 kb in size, irrespective of whether the bases of the loops were constrained to a central axis or not. The size of the loops was consistent with the length of DNA loops observed previously in EM images of histone-depleted mitotic chromosomes [17, 31]. Simulation of homogenous axial compression following loop formation resulted in cylindrical shapes with similar dimensions as those measured for human mitotic chromosomes (Fig. 1B).

Can chromosome condensation be explained by changes in nucleosome interactions?

Can the changes in chromosome architecture that happen during mitosis be explained by changes in chromatin structure? Upon entry into mitosis, histone H3 is phosphorylated at serine residues 10 and 28 (H3S10 and H3S28) by Aurora kinases [32, 33] and at threonine residue 3 (H3T3) by Haspin kinase [34]. Since these phosphorylations generally coincide with chromosome condensation, they have been suggested as the driving factor for mitotic chromosome formation. Consistent with this notion are the findings that mutation of H3S10 to a non-phosphorylatable alanine residue results in chromosome segregation defects in fission yeast and mitotically dividing micronuclei of the ciliated protozoan *Tetrahymena thermophila* [35, 36]. Simultaneous mutations of H3S10 and H3S28 to alanine in budding yeast has, in contrast, no appreciable effect on chromosome segregation [32] or the step-wise conversion of the ribosomal RNA gene (rDNA) cluster into a loop-shaped structure [37, 38]. Moreover, incubation of human cultured cells with the phosphatase inhibitor okadaic acid results in H3S10 phosphorylation in the vast majority of cells, yet chromosomes condense only in a very small fraction of cells [39]. Finally, a *Drosophila* mutant of the Aurora B kinase-associated Borealin protein still shows a remarkable degree of chromosome condensation, even though H3S10 phosphorylation can no longer be detected in the mutant [40]. These observations imply that H3 phosphorylation cannot be the universal determinant of mitotic chromosome condensation. This conclusion is furthermore supported by the findings that in maize, H3S10 and H3S28 phosphorylation can only be detected on pericentric chromosome regions and only late during mitotic prophase, i.e. after mitotic chromosomes have already started to form [41]. It is, however, conceivable that, in plants, phosphorylation of H3T3 by Haspin kinase might compensate for a lack of H3S10 and H3S28 phosphorylation on chromosome arms (reviewed in [42]).

A recent report nevertheless proposed a specific role of H3 phosphorylation during chromosome condensation in yeast. Activation of photo-reactive residues introduced into histone H2A generated cross-links with the N-terminal tail of histone H4 [43]. The small fraction of cross-linked H4 observed in interphase cells increased ~threefold as cells entered mitosis, suggesting that the interaction between H2A and H4 might be related to chromosome condensation. Moreover, mutation of H3S10 to alanine prevented cross-linking and mitosis-specific deacetylation of histone H4 lysine residue 16 (H4K16). Based on these observations, the authors proposed that deacetylation of H4K16, as a result of phospho-H3S10-mediated recruitment of the deacetylase Hst2, promotes the interaction of the histone H4 tail with an acetic patch on histone H2A of neighboring nucleosomes, resulting in chromosome condensation [43]. However, the suggestion that this mechanism drives condensation in yeast is problematic for several reasons. As mentioned before, mutation of H3S10 to alanine does not notably affect cell division and rDNA condensation [32, 37] and results in only minimal defects in the condensation of a yeast fusion chromosome during anaphase [43]; significantly less than the defects caused, for example, by inactivation of condensin (see below) [44]. Likewise, deletion of the gene encoding the Hst2 deacetylase has no obvious effect on cell divisions and results in merely marginal condensation defects. Even when taking into account the difficulties in obtaining synchronous cell cycle populations by nocodazole washout, one would have expected to observe H4K16 deacetylation and H2A–H4 cross-linking when chromosomes condense as cells proceeded from the arrest into anaphase [45], which was not the case [43]. While it is likely that chromatin modifications such as acetylation and deacetylation play roles in regulating the mechanical properties of chromatin fibers during

segregation [46], the evidence that these modifications drive the formation of mitotic chromosomes remains limited.

In addition to phosphorylation of histone H3, several residues within the C terminus of linker histone H1 are phosphorylated by Cyclin-dependent kinase Cdk1 during prophase [47]. Yet, deletion of all genes encoding linker histone H1 proteins in cultured chicken cells has no apparent effect on the formation of mitotic chromosomes [48] and deletion of H1 genes in *Tetrahymena* only mildly increases chromosome volumes, without affecting chromosome segregation [49]. Likewise, deletion of the gene encoding a histone H1-like protein in budding yeast has no obvious consequences for cell division [50]. H1-type histones might nevertheless affect the mechanical properties of mitotic chromosomes, since depletion of linker histones from *Xenopus* egg extracts results in the conversion of non-replicated or replicated DNA substrates into more fragile chromatids or drastically elongated chromosomes, respectively [51, 52].

Do chromatin modifications influence the action of molecular machines that shape mitotic chromosomes?

Consistent with the notion that chromatin–chromatin associations alone cannot explain the formation of metaphase chromosomes, simulations of polymer models based on random nucleosome interactions result in spherical instead of cylindrical architectures [53]. Hence, other factors that determine the shape of mitotic chromosomes must exist. The recent years have seen the identification of several molecular machines that play central roles in the formation of mitotic chromosomes, including condensin (see Box 1), topoisomerase IIα (topo IIα; Box 2), and cohesin (Box 3). Yet, the function of these machines can nevertheless be controlled by the mitotic histone modifications described in the previous section.

Phosphorylation of H3S10 has, for example, been proposed to enhance the flexibility of the chromatin fiber, which might allow easier access of non-histone proteins such as condensin or topo IIα [54]. Alternatively, histone phosphorylation might create a direct binding platform for these proteins [35]. The discovery that preventing histone H3 phosphorylation by Aurora B kinase depletion or inhibition indeed reduces the chromosomal levels of total condensin in *Caenorhabditis elegans* or in cultured *Drosophila* cells [55–57] or specifically of condensin I in cultured human cells [58] is consistent with either hypothesis. Even though Aurora B kinase depletion also greatly reduces histone H3 phosphorylation in *Xenopus* egg extracts, its effects on the chromosomal association of condensin in this model system vary between different studies [59–61].

Another hypothesis suggests that phosphorylation of histones H1 and H3 releases constraints on the condensin- and

Box 1

Condensin complexes

Eukaryotic condensin complexes are composed of five subunits, including a heterodimer of Structural Maintenance of Chromosomes proteins (SMC2 and SMC4), a protein of the kleisin family that binds to the SMC ATPase head domains called CAP-H (or CAP-H2 for Condensin II), and two proteins called CAP-G (or CAP-G2 for Condensin II) and CAP-D2 (or CAP-D3 for Condensin II). The latter two proteins are largely composed of α-helical Huntingtin, Elongation factor 3, the A subunit of protein phosphatase 2A and TOR1 lipid kinase (HEAT) repeat motifs.

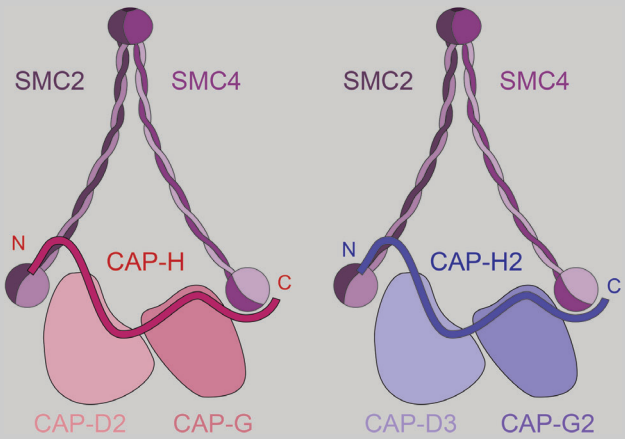

Figure. Architecture of condensin I and II complexes.

In vertebrates, condensin I localizes to the central axis of mitotic chromosomes in alternate patterns with condensin II [90] or topo II [91] and gains access to chromosomes only after NEBD; at a time when chromosomes have already condensed to a considerable degree [71, 72]. Condensin II, in contrast, can be detected in the nucleus throughout the cell cycle and shows little or no turnover once it accumulates on chromosomes at the beginning of prophase [96]. Condensin I, II, and topo IIα associate with chromosomes independently of one another [71]. Depletion of condensin I or both condensin complexes from M phase *Xenopus* egg extracts prevents the transformation of added sperm chromatin into the shapes of mitotic chromosomes, suggesting that condensin complexes are essential for chromosome condensation [72, 114]. Depletion of condensin I and/or II in other model systems has, however, varying effects on the dynamics and levels of chromosome condensation (reviewed in [115]). Nevertheless, such chromosomes quickly loose their rod-shaped appearance under physical strains, for example when stretched out onto glass slides or under hypotonic conditions, and invariably fail to segregate during anaphase [70, 71, 90, 96, 98]. How condensin complexes contribute to the formation of mitotic chromosomes is not yet understood. Condensin I purified from *Xenopus* egg extracts can bind DNA substrates, influence the changes in DNA topology that are introduced by topoisomerases, and compact DNA helices in an ATP-dependent manner in magnetic tweezers experiments [63, 116, 117].

Box 2

Topoisomerase IIα

Type II topoisomerases change the topology of DNA substrates by introducing a double-strand break into one DNA helix, passing a second helix through the break site, and re-sealing the initial break. Strand passage is stimulated by hydrolysis of two bound molecules of ATP (reviewed in [118]). Since eukaryotic type II topoisomerases display, unlike prokaryotic type-II gyrases, no preference for the handedness of the strand passage, they can equally well introduce as they can remove topological DNA linkages. The directionality of the reaction towards the latter is, however, essential for the resolution of sister chromatid catenanes during mitosis.

The genomes of vertebrates encode two topoisomerase II isoforms, alpha and beta. Topoisomerase IIα (topo IIα) localizes to the chromosome axis of mitotic chromosomes [69, 91, 119, 120], but can decorate complete chromosome arms when present in vast excess [121]. The rapid turnover of topo IIα on mitotic mammalian chromosomes [94, 122, 123] and the finding that topo IIα can be extracted even at low salt concentrations from chromosomes assembled in *Xenopus* egg extracts without notably affecting axial chromosome structures [93] suggest that topo IIα is not part of a stable "chromosome scaffold."

Evidence that topo II nevertheless plays a central role during mitotic chromosome condensation comes from the observation of extended and unresolved metaphase chromosomes after inactivation of topo II in fission yeast cells [124]. Moreover, *Xenopus* egg extracts depleted of topo IIα can no longer support the conversion of sperm chromatin or chicken erythrocyte nuclei, either of which contain little topo II, into well-structured chromosomes [93, 125].

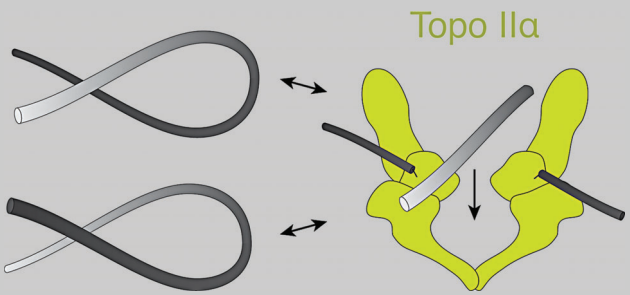

Figure. Change of the handedness of DNA crossings by type II topoisomerases. The two ends of the double-strand break in one segment the DNA helix (dark gray) remain covalently attached to the active site tyrosine residues of the topo II dimer while the second segment (light gray) passes through the gap in an ATP-dependent reaction (arrow), followed by re-ligation of the cleaved DNA helix. If both segments are of the same DNA, this reaction changes the handedness of DNA crossings.

topo IIα-mediated conversion of nucleosome linker DNA crossings from a negatively to a positively supercoiled state [62]. This model is based on the discovery that condensin complexes promote the introduction of positive supercoils into plasmid DNA [57, 63–65]. Positive superhelical torsion might be introduced by condensin complexes, presumably at the short nucleosome-free regions condensin preferentially binds to [66]. Torsion could then spread along the chromatin fiber by topo IIα-mediated conversion of the handedness of nucleosomal linker DNA crossings until over-coiling results in the formation of plectoneme loops that compact chromosomes [62]. Binding of histone H1 to linker DNA and the nucleosome dyad, close to the histone H3 tail, might limit the propagation of torsion, and hence H1 might need to be removed from chromosomes upon its phosphorylation. Whether phosphorylation of histone H1 indeed loosens the protein's association with chromatin is, however, not yet clear. The reports that a non-phosphorylatable version of histone H1 exhibits decreased binding to *Xenopus* chromosomes [67] and that a phospho-mimicking mutation of a single Aurora B kinase target site within the N terminus of the human histone variant H1.4 results in lower H1.4 turnover on mitotic chromosomes [68] argue rather against this hypothesis.

Having ruled out changes in chromatin architecture as the sole determinant of mitotic chromosome architecture, we now focus on the mechanisms of action of condensin, topo IIα, cohesin, and other components of the chromosome condensation machinery. In light of the newly available data derived from the analysis of chromatin topology in mitotic chromosomes by molecular and structural biology, biophysical, and polymer modeling approaches discussed above, we will attempt to synthesize a three-step framework for the formation of mitotic chromosomes.

A three-step model for the formation of mitotic chromosomes

Step 1: Linear chromatin looping

Linear chromatin structures become first visible by light microscopy at the beginning of mitotic prophase [69]. Knockdown of condensin SMC subunits in *C. elegans* embryos or in cultured chicken cells significantly delays the initial appearance of such thread-like structures [55, 57, 70], which suggests that condensin complexes must play a role in this process. Depletion of condensin II subunits, but not of condensin I subunits, causes the same effects in cultured human cells [71–73]. Unusual chromatin structures can also be observed during prophase after deletion of the condensin II kleisin subunit in mouse neuronal stem cells, while chromosome morphology is not notably affected by deletion of the condensin I kleisin subunit [74]. These observations suggest that condensin II is essential for initiating the formation of individualized chromosomes early during prophase.

How condensin II is activated in early prophase is still incompletely understood. Activation is most likely initiated by

Box 3
Cohesin complexes

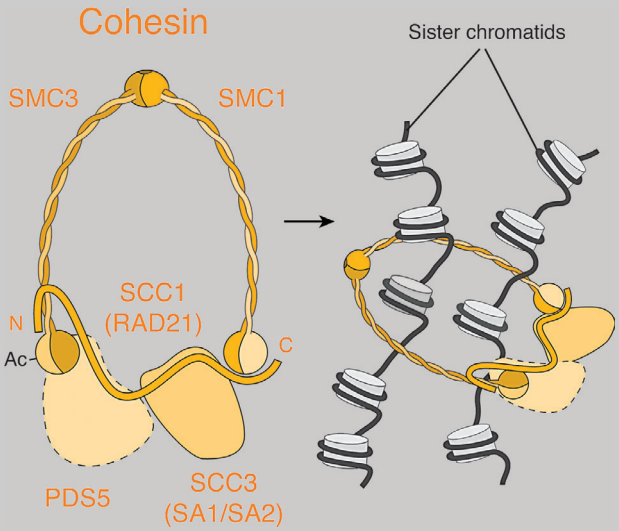

Figure. Architecture of the cohesin complex and mechanism of sister chromatid cohesion. "Ac" indicates acetylation of the SMC3 ATPase domain.

Cohesin complexes are similar in shape and subunit composition to condensin complexes. They are thought to hold together replicated sister chromatids by entrapping both sister DNAs within the ring-shaped architecture created by their Structural Maintenance of Chromosomes (called SMC1 and SMC3) and kleisin (called RAD21 or SCC1) subunits (reviewed in [88, 126, 127]). Alternative models suggest that two or more cohesin rings, each encircling a single DNA helix, interact (reviewed in [128]). Similar to condensin, the kleisin subunit binds to HEAT-repeat subunits called SCC3 or SA1/SA2 and PDS5.

Cohesin rings lock around the sister DNAs at the nascent replication fork in a manner that depends on the stable interaction of the N terminus of the kleisin subunit with the SMC3 ATPase domain. This interaction is regulated by acetylation of the latter. In metazoan cells, most cohesin complexes dissociate from chromosome arms by opening of this interface. Cohesin complexes at centromeric regions and residual cohesin at chromosome arms are then removed at anaphase onset through cleavage of the kleisin subunit by separase protease, which triggers the segregation of chromatids to the cell poles.

phosphorylation of the CAP-D3 subunit by Cdk1, which then results in the recruitment of polo-like kinase Plk1 for further phosphorylation of all condensin II non-SMC subunits [75]. In addition, condensin II association with chromosomes has been reported to be controlled by a direct interaction with protein phosphatase 2A (PP2A) [76] and by the human microcephalin protein MCPH1 [77]. Untimely loading of condensin II onto chromosomes might hence be responsible for the premature chromosome condensation phenotype described in MCPH1 patient cells [78].

In light of the polymer simulations discussed before [30], the formation of first linear structures is consistent with the tethering of adjacent sites in a chromatin fiber to form consecutive loops of 80–120 kb in length (Fig. 1B). Since condensin II is required for the initiation of chromosome condensation during early prophase, it is tempting to speculate that condensin II is responsible for loop formation, for example by acting as a chromatin linker. One way one could imagine such linkages are generated is by entrapment of two 11 nm chromatin fibers within condensin's large ring-shaped structure [79]. Instead of multimerizing into a continuous protein scaffold [31], condensin complexes might create individual pair-wise links (Fig. 2A, B). This notion is consistent with the proposal of a network of protein linkages connecting chromatin fibers inferred from the micromechanical properties of mitotic chromosomes [27].

In this scenario, how might linkage by condensin II create consecutive loops of 80–120 kb in size? During early prophase, frequent connections between sister chromatid arms formed by cohesin complexes might allow condensin II to create linkages only within regions between two cohesin binding sites (Fig. 2B). Such a mechanism would imply that loop sizes are determined by the genomic distances of the tens of thousands cohesin bindings sites in human cells [80] and could explain why cohesin limits the association of condensin II with chromosomes [81]. However, it is equally well possible that loop sizes are determined by intrinsic properties of condensin II or additional factors. One challenge of this model is that condensin II would need to generate linkages only within the same chromatid and not between different chromosomes. It might, for example, be conceivable that condensin complexes were able to track along the chromatin fiber while holding on to their original binding sites and thereby protrude a loop of chromatin [82, 83]. Fastening of such loops would ensure that condensin connected two segments of the same chromatid. Alternatively, the generation of condensin links might favor particular intra-chromosomal topologies, for example DNA crossings of a specific handedness. The latter hypothesis is consistent with the topoisomerase-mediated changes in the topology of plasmid DNA substrates in the presence of condensin complexes (see Boxes 1 and 2). Insights into the nature of the abilities of condensin complexes to arrange chromatin fibers will presumably need to await the in vitro reconstitution of their association with chromatin templates.

Step 2: Axial compression

In addition to loop formation, polymer models require a second step to accurately simulate the formation of cylindrical chromosomes: compression along the longitudinal chromosome axis [30]. Quantitative imaging experiments of fluorescently labeled histones are indeed consistent with

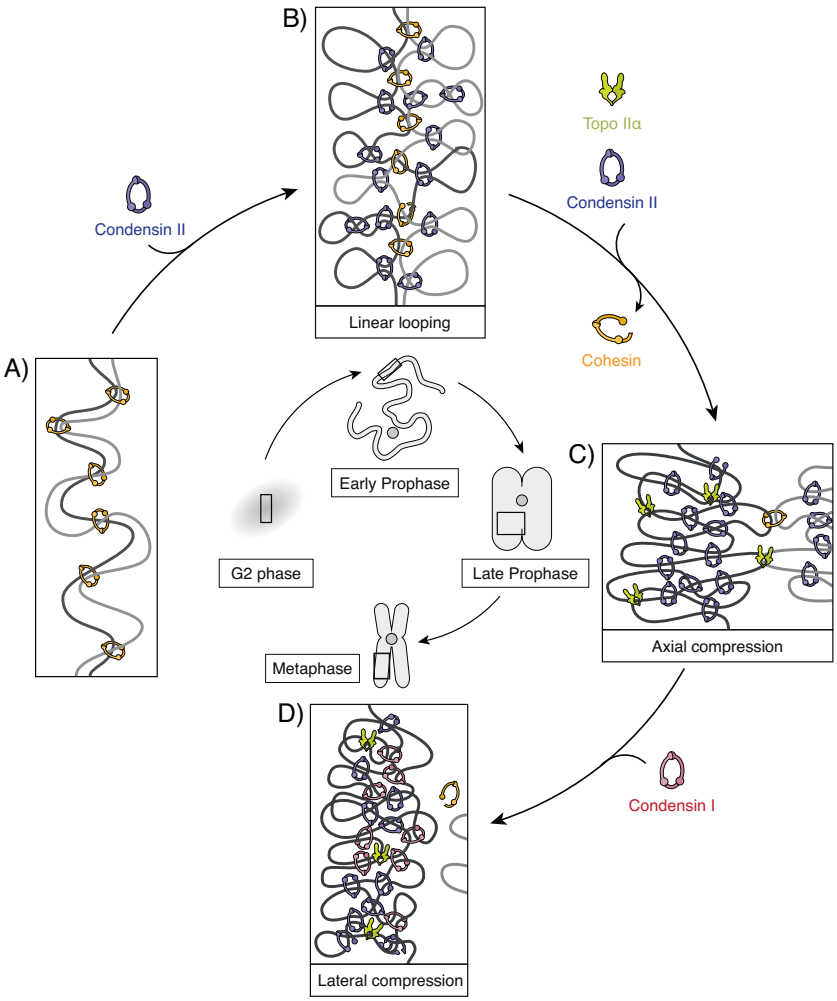

Figure 2. A three-step linkage model for the condensation of a mammalian chromosome. Sister chromatids (light and dark gray lines) held together by their entrapment within cohesin rings (yellow) are organized into linear arrays of loops by condensin II (purple). Loop sizes might be limited to 80–120 kb by restricted action of condensin II within the region between two cohesin binding sites. Release of the bulk of cohesin from chromosome arms by the prophase pathway allows the generation of linkages between different loops by condensin II, resulting in a linear compression along the longitudinal chromosome axis. Topo IIα (green) might help in this process by catalyzing intra-chromatid strand passage. Binding of condensin I (red) after NEBD results in lateral compression by fastening loops that protrude from the chromosome and thereby assists in removal of residual arm cohesion and directs inter-chromatid decatenation by topo IIα.

condensation proceeding in two distinct steps [84]. The second step takes place between late prophase and early prometaphase [85] and coincides with the release of cohesin from chromosomes through the action of the "prophase pathway." Together with the removal of inter-sister catenations by topo IIα, this step results in the resolution of sister chromatid arms (reviewed in [86–88]). One possibility is that release of cohesin might now enable condensin II to link more distant sites in the genome and thereby compress chromatin fibers in the direction of the chromosome axis (Fig. 2C).

Consistent with a role of condensin II in the axial compression step are the findings that metaphase chromosomes appear longer and/or curly after depletion of condensin II subunits in cultured human or chicken cells, or when assembled in *Xenopus* egg extracts in which condensin II has been depleted or prevented to load onto chromosomes [77, 89, 90]. In contrast, normal-shaped prometaphase chromosomes can sometimes be observed in human cells depleted of condensin II subunits by RNA interference [71]. In these cases, it might be possible that residual amounts of condensin II, which have escaped depletion, are sufficient for the first two steps of chromosome condensation. Aberrant condensin II loading onto chromosomes might in return result in the shorter and thicker metaphase chromosomes observed in MCPH1 patient cells [77].

Consistent with a contribution of topo IIα to the axial compression step is the observation that the enzyme accumulates on the chromatid axes in cultured human cells during prophase [91]. Knockdown of topo IIα in cultured chicken cells results in longer and thinner metaphase chromosomes [92] and depletion of topo IIα in *Xenopus* egg extracts blocks the transformation of sperm chromatin into M phase chromosomes [93]. Even though depletion or inhibition of topo IIα in human cells increases the fraction of partially condensed chromosomes, normal-shaped metaphase chromosomes form eventually [94]. Likewise, inhibition of topo IIα with an antibody or a small molecule inhibitor has no effect on mitotic chromosomes once they have been assembled in *Xenopus* egg extracts [93]. These findings suggest that topo IIα might only play a role for chromosome assembly during prophase, but not for the later maintenance of mitotic chromosome structure. Since topo II is required for the resolution of sister chromatid catenations [95], it is conceivable that sister chromatid intertwinings might restrict the formation of long-distance linkages by condensin II in a similar manner as sister chromatid linkages mediated by cohesin might do. Cohesion between sister chromatids, whether mediated by cohesin or by catenations, therefore needs to be resolved to allow the proper formation of metaphase chromosomes. Since topo IIα depletion also affects the transformation into mitotic-like chromosomes of unreplicated chromatids [93], topo IIα might still be required to catalyze intra-chromatid strand passage during the formation of chromosomes (Fig. 2C, D).

Step 3: Lateral compression

If most chromosome condensation has been completed even before the bulk of condensin I gains access to chromosomes [71, 85, 96], what role does condensin I have for the

formation of mitotic chromosomes? Chromosomes assembled in *Xenopus* egg extracts contain approximately five times the amount of condensin I compared to condensin II [90]. Reducing the proportion of condensin I results in the formation of shorter and wider chromosomes [81]. Condensin I has hence been suggested to reduce the chromosome diameter. Support for this proposal also comes from the report that the caspase-dependent degradation of the kleisin subunit of condensin I in human cells, arrested by spindle poisons over long periods of time, results in an increase in chromosome width [97].

Yet, the width of human chromosome arms does not appear to change once condensin I binds after NEBD [96], suggesting that a lateral compaction step might occur in some cell types but not in others. However, it might still be conceivable that, even in HeLa cells, condensin I is essential to "reel in" individual chromatin fibers that protrude from the chromatid cylinder, for example those that are still connected to the other sister chromatid by cohesin complexes that escaped the prophase pathway (Fig. 2D). Such a scenario could explain how condensin I contributes to the complete removal of cohesin from chromosome arms in these cells [71]. In addition, condensin I binding is thought to mechanically stabilize the chromatids, since centromeric regions that come under tension by their attachment to spindle microtubules stretch extensively after depletion of condensin I in fly or human cells [96, 98] or after knockdown of a condensin SMC subunit in cultured chicken cells [99]. The stabilization by condensin complexes is presumably essential not only at centromeres but throughout the entire chromosome, since chromosome arms frequently fail to follow the centromeres to the cell poles after inactivation of the single condensin complex present in yeast [100, 101].

Assuming that condensin I also functioned as a chromatin linker similar to condensin II, how might its action direct lateral instead of axial compression? If, by the time condensin I arrived on chromosomes, condensin II had already generated a saturating number of chromatin linkages along the chromosome axis (Fig. 2C), binding of condensin I would not result in further chromosome shortening in the longitudinal direction. The only possible conformational change that could still take place is, instead, in the lateral direction (Fig. 2D). Under certain circumstances, however, condensin I might be able to induce a further shortening of the chromosome axis: When human cultured cells are arrested with spindle poisons such as nocodazole, chromosomes shorten by an additional third of their length. This additional shortening is considerably reduced after condensin I depletion [71]. Condensin I might also be able to take over part of the function of condensin II in the first two condensation steps, since compaction is merely delayed in cells depleted for condensin II [71, 81, 84, 96].

Another protein that might contribute to the lateral compression step is the chromokinesin KIF4A. KIF4A contains a microtubule plus-end directed motor domain at its N terminus, followed by a coiled coil dimerization domain and a C-terminal tail domain that binds to chromatin (reviewed in Ref. [102]). During mitosis, KIF4A localizes to the axes of chromosome arms in human and chicken cells [89, 92, 103], probably via recruitment by PP2A [76]. Remarkably, human or chicken chromosomes of cells arrested in prometaphase or metaphase, respectively, become shorter and wider after depletion of KIF4A [92, 103]. KIF4A might therefore either counteract condensin II-mediated axial compression or contribute to the condensin I-mediated lateral compression step. Yet, the phenotype of KIF4A depletion cannot be solely explained by a decrease in chromosome-bound condensin, since chromosome architecture is affected more drastically by co-depletion of KIF4A and SMC2 than by depletion of either protein alone [92].

Chromosome condensation proceeds beyond metaphase

The three condensation steps – linear chromatin looping, axial compression, and lateral compression – describe a scenario that accumulates in rod-shaped metaphase chromosomes ready for their segregation to the cell poles. Remarkably, chromosomes have not yet reached their maximum compaction at this point. Measurements of chromosome volumes in live mammalian cultured cells shows that compaction is highest a few minutes after anaphase onset [85]. The additional compaction during anaphase is due to axial chromosome shortening and depends on the activities of the chromokinesin Kid [104] and Aurora B kinase [85]. Similarly, the budding yeast Aurora kinase is essential for the maintenance of a compact linear arrangement of the rDNA cluster in cells arrested after anaphase onset [38]; as is condensin, which accumulates at the rDNA during anaphase in a manner that depends on the Cdc14 phosphatase early anaphase release (FEAR) network [105, 106]. In addition to rDNA compaction, further shortening of chromosome arms during anaphase can be observed in budding [44] and fission yeast [107]. Since the extent of arm shortening increases for an extra-long fusion chromosome, it was suggested that cells possess an Aurora kinase-mediated "chromosome ruler" mechanism that adjusts the degree of anaphase condensation to chromosome length [44]. The purpose for the additional shortening of chromosome arms during their segregation might be to promote their clearance from the cell mid-plane before the onset of cytokinesis and/or the packaging of all chromosomes into a single nucleus upon nuclear envelope reformation.

Do condensin complexes act as structural linkers or chromosome remodelers?

One could think of two fundamentally different mechanisms how condensin complexes could drive the formation of mitotic chromosomes: condensins might either actively reconfigure chromosome topology [62, 108] or act as static linkers that mechanically stabilize the chromatin fiber by bridging distant sites within the same fiber [79]. The findings that condensin complexes are able to constrain supercoils in circular DNA substrates in vitro (see Box 1) support the former hypothesis.

However, changes in DNA superhelicity might be caused by the manner condensins bind the DNA helix rather than be the result of an enzymatic activity. Electron spectroscopic imaging of complexes between in vitro-assembled *Xenopus* condensin complexes and DNA suggest that the double helix is wrapped in two tight turns, possibly around the SMC ATPase head domains [109]. Condensins might also preferentially bind to sites of DNA crossings, consistent with their higher binding affinity for structured DNA substrates [63, 110].

If condensin merely promoted topo IIα-mediated changes in chromosome conformation, its activity were most likely no longer required for the maintenance of a folded chromosome. However, when condensin is inactivated in yeast cells only after mitotic chromosomes have formed, such chromosomes nonetheless fail to segregate [38, 111]. The energy for any active change in chromatin topology would probably need to come from ATP hydrolysis by the SMC subunits. Compared to motor proteins such as chromokinesins, the ATPase activities that have been measured for condensins or their SMC dimers are lower by one or two orders of magnitude [63, 66, 112]. Even though we cannot rule out that the optimal conditions for condensin's ATPase activation have not yet been found, the data available suggest that the ATP binding and hydrolysis reactions might act rather as a conformational switch than as a motor. For these reasons, we favor the hypothesis that condensin complexes instead function as structural linkers. The idea of chromosome stabilization by a network of condensin-mediated linkages is furthermore consistent with the micromechanical properties of isolated mitotic chromosomes [27, 29] and with models based on chromosome conformation capture data [30]. Such linkages would not need to be static but could be generated and dissolved in a dynamic equilibrium, as suggested by the high turnover measured for condensin I [96].

Conclusions and future perspectives

The structure of mitotic chromosomes has been the topic of controversial discussions over the past decades. New approaches, from precise measurements of the mechanical properties of isolated chromosomes to next generation DNA sequencing-based techniques used to determine the conformation of chromatin fibers inside the cell's nucleus, have provided important new insights into chromosome architecture. In combination with computational modeling and ways to manipulate key chromosomal proteins, including condensin, cohesin, topo IIα, and KIF4A, these novel approaches have the potential to revolutionize our understanding of one of the cell's largest macromolecular assemblies. Future studies could involve the specific removal of major structural components from mitotic chromosomes during micromanipulation measurements or the coordination of the position of condensin complexes with genomic proximity information from Hi-C experiments during the different steps of chromosome condensation, ideally in single cells. Additional technological advances, for example in super-resolution microscopy, might at some point enable in vivo tracking the chromatin fiber in mitotic chromosomes [113].

However, understanding the formation of mitotic chromosomes will not only require the generation of a three-dimensional map of the chromatin fiber, but will depend on in-depth knowledge of the mechanisms behind the molecular machines that generate these topologies and their interplay with the chromatin fiber. Insights from biochemical and structural studies will be crucial to appreciate the action of condensin complexes and to unravel the roles of other recently identified proteins involved in chromosome condensation. The condensin linker model, if correct, poses challenging questions that need to be addressed: what causes condensins to accumulate at the axes of the chromosome cylinder, how do condensin complexes specifically link two DNA helices from the same chromatid, and what determines that condensin II specifically creates links that shorten the chromosome axis, while condensin I specifically creates links that decrease the chromatid diameter? The coming years promise to be some of the most exciting in the history of chromosome research.

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