Biochemical Journal (2022) **479** 2153–2173
https://doi.org/10.1042/BCJ20210140

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Review Article

Regulation of the mitotic chromosome folding machines

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Over the last several years enormous progress has been made in identifying the molecular machines, including condensins and topoisomerases that fold mitotic chromosomes. The discovery that condensins generate chromatin loops through loop extrusion has revolutionized, and energized, the field of chromosome folding. To understand how these machines fold chromosomes with the appropriate dimensions, while disentangling sister chromatids, it needs to be determined how they are regulated and deployed. Here, we outline the current understanding of how these machines and factors are regulated through cell cycle dependent expression, chromatin localization, activation and inactivation through post-translational modifications, and through associations with each other, with other factors and with the chromatin template itself. There are still many open questions about how condensins and topoisomerases are regulated but given the pace of progress in the chromosome folding field, it seems likely that many of these will be answered in the years ahead.

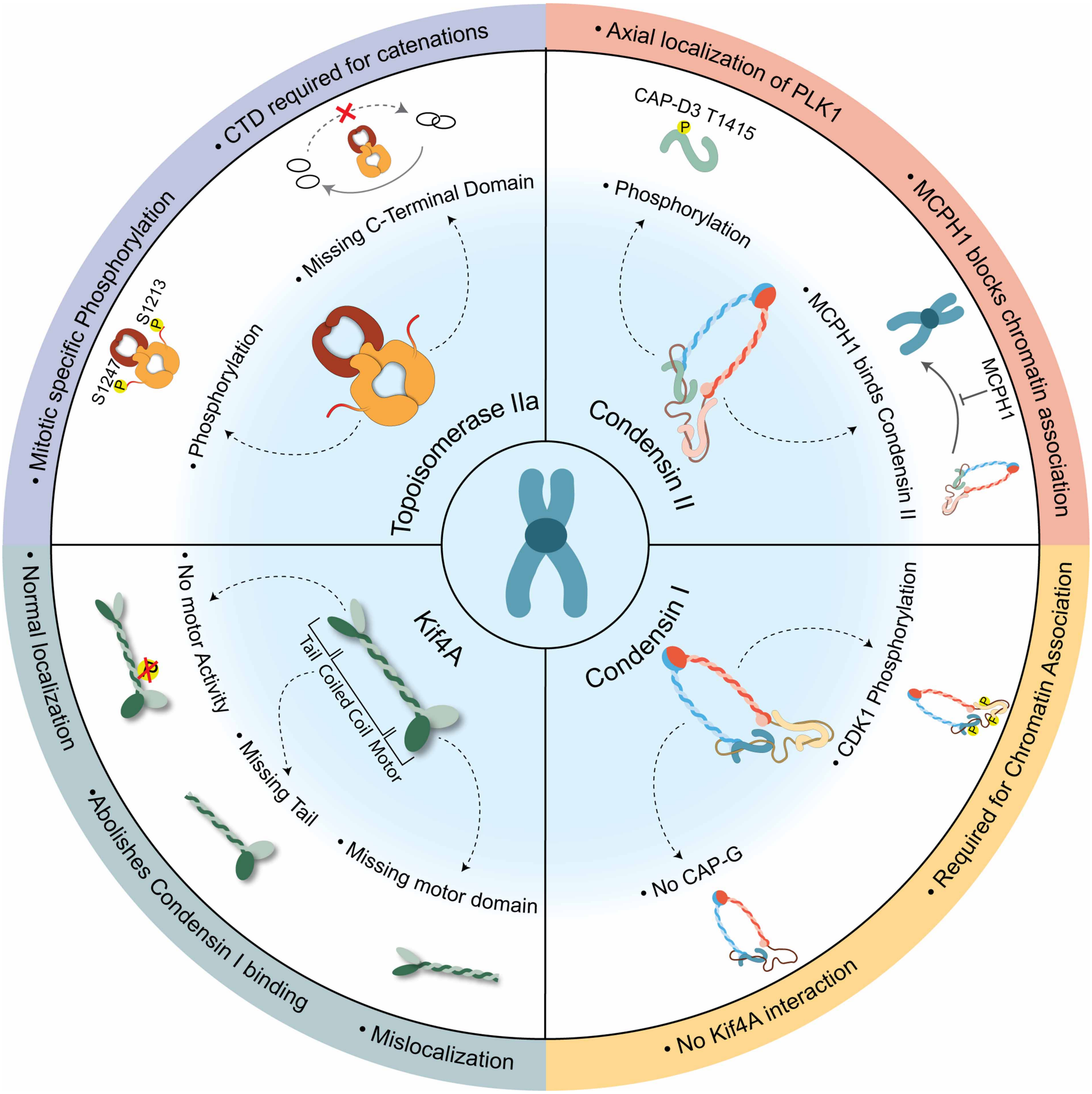
Introduction

Mitotic cells fold their chromosomes into compact rod-shaped structures for their faithful segregation to daughter cells. By late prometaphase, each chromosome is composed of a pair of sister chromatids that are mostly separate from each other but that continue to run side-by-side along their length. Connections between sister chromatids remain, most prominently so at their centromeres but also at points of contact all along their arms. During anaphase these remaining inter-sister connections are removed as the spindle pulls the sister chromatids towards opposite poles for their segregation to daughter cells.

Mitotic chromosomes have fascinated cell biologists for over a century. Ever since Flemming's microscopic observations of mitotic chromosomes in the late nineteenth century [1], and Sutton and Boveri's deduction that they represent the physical carriers of genetic information [2,3], have they been the topic of intense studies and debate. It was known early on that mitotic chromosomes are transient structures: for most of the cell cycle (interphase) chromosomes are decondensed, apparently amorphous, and hardly visible as distinct entities, but as cells enter mitosis chromosomes rapidly transform onto the well-defined compact rod-shaped mitotic chromosomes. As cells exit mitosis chromosomes return to their decondensed state. This classical observation of dynamics of chromosome organization has led to important questions in the field of chromosome biology: first, what is the three-dimensional structure of the genome within mitotic chromosomes, and within the interphase nucleus; second, what molecular machines and biophysical processes fold mitotic and interphase chromosomes, and by what mechanism(s); and third, how is the process of dynamical refolding chromosomes during the cell cycle regulated? Here we will focus on the folding of mitotic chromosomes, the machines that fold them, and especially how these machines might be regulated (Figure 1).

Received: 9 February 2022
Revised: 30 September 2022
Accepted: 3 October 2022

Version of Record published:
21 October 2022


• CTD required for catenations
• Mitotic specific Phosphorylation
S1213
S1247
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• Missing C-Terminal Domain
• Phosphorylation
Topoisomerase IIa
• Axial localization of PLK1
CAP-D3 T1415
P
• Phosphorylation
• MCPH1 blocks chromatin association
• MCPH1 binds Condensin II
MCPH1
Condensin II
• Normal localization
• Abolishes Condensin I binding
• Mislocalization
• No motor Activity
• Missing Tail
• Missing motor domain
Kif4A
Tail
Coiled Coil
Motor
P
Condensin I
• CDK1 Phosphorylation
• No CAP-G
• Required for Chromatin Association
• No Kif4A interaction
P
P

Figure 1. Regulation of the four major complexes for mitotic chromosome folding.

Overview of various regulatory processes influencing machinery assembling the mitotic chromosome. Importance of Topoisomerase IIa C-Terminal Domain (CTD) as it affects its function and mitotic specific phosphorylation sites within the CTD [4–6]. Interaction between MCPH1 and condensin II can prevent condensin II function, similar to the loss of CAP-D3 T1415 phosphorylation site [7,8]. Requirement of CDK1 phosphorylation for condensin activity and the importance of CAP-G subunit to interact with Kif4A. Kif4A requires its motor domain rather than its activity for localization [9–11].

The structure of mitotic chromosomes

Early microscopic observations, before the structure of DNA was known, led to the proposal that the basic structural unit of mitotic chromosomes was a filament, a chromonema, that during mitosis becomes shortened and compacted through a series of helical coiling steps. Over the years, up until the 1990s several related models based on hierarchical coiling have been proposed [12,13]. DuPraw proposed a different model, where a chromonema was randomly folded within rod-shaped chromatids [14]. However, the model that has survived the test of time best and that has been confirmed with observations made by more recent microscopic methods and increasingly genomic technologies (3C-based data), is the radial loop model first proposed by Uli Laemmli [15,16]. Laemmli and co-workers showed in a series of classic publications that mitotic chromosomes are formed as linear arrays of chromatin loops that emanate from a central core composed of non-histone proteins that runs through the center of each chromatid [15–17]. For human cells, each of these loops was found to be ~80–100 kb, and loops were found to be arranged radially around the central scaffold. More recently, 5C and Hi-C data obtained with mitotic chromosomes, combined with polymer simulations allowed direct testing of several models of mitotic chromosome folding, including hierarchical coiling models and radial loop models [18]. The radial loop model described the chromatin interaction data best. Furthermore, Hi-C data obtained with chicken and human prometaphase chromosomes revealed a striking periodicity in long-range interactions: loci separated by 8–12 Mb showed elevated interactions. It was found that this could be explained when loops project from the central scaffold in a non-random way so that the bases of sequential loops rotate around the central scaffold in a helical fashion [19,20]. Interestingly, such arrangement of protruding loops gives mitotic chromosomes an intrinsic helical internal organization, while the overall morphology is a straight rod. The helical positioning of radial loops may explain why under some fixation conditions chromosomes can appear coiled [21]. The helical arrangement of loops is apparent in population-averaged Hi-C data, but in individual chromosomes the helical path may be quite variable. This would explain why imaging experiments did not detect a regular helical arrangement of condensins in individual chromosomes [22]. In a recent study, a different path of the loop array was proposed, where the handedness of the axis changes handedness each half turn [23]. This study employed microscopy to image single chromosomes, whereas the Hi-C-based studies mentioned above studied cell populations. Population-based studies cannot discern variation, e.g. in handedness, among individual chromatids in their helical arrangement. Future work may shed more light on the precise path of the loop array, and how its helical properties, including handedness, may change along individual chromatids.

From the chromosome scaffold to mitotic chromosome folding machines

When histones are extracted from mitotic chromosomes, a proteinaceous but non-histone scaffold is observed in the electron microscope [16]. From this scaffold DNA loops emanate. This classic observation has been foundational to the radial loop model but had initially been contentious for many years due to concerns that the scaffold structure could be an experimental artifact. However, the concept of a central core of non-histone proteins is now well supported by many lines of evidence. We refer to a recent in-depth review by Paulson et al. for a recent evaluation of this issue [12].

The microscopic observation of a scaffold might suggest a contiguous framework of proteins, leading to a rather rigid structure. This interpretation was ruled out by elegant experiments on purified mitotic chromosomes by Poirier and Marko [24]. These investigators performed micromechanical force-extension measurements and found that mitotic chromosomes are highly elastic: they can be reversibly extended 5-fold. Importantly, nuclease treatment dissolves the chromosomes including any scaffold structures, providing strong evidence that the scaffold is not a contiguous proteinaceous structure. Consistent with a much more dynamic structure are observations that components of the scaffold (see below) show frequent turnover as assessed by fluorescence recovery after photobleaching (FRAP) [9,25–27].

Several components that make up the mitotic scaffold have been described. One of the first proteins that was identified is Topoisomerase Type IIa (Topoisomerase IIa) [28,29], initially referred to as scaffold component 1 (sc1) [30]. Topoisomerase II is a homodimeric enzyme that can perform strand passage reactions where one DNA strand passes through another strand thereby changing the topology of DNA molecules. Strand passage involves cleavage of one strand, passing through a second strand which is then followed by resealing the cleaved DNA molecule. Strand passage can occur within one DNA molecule or between different molecules. Given that the enzyme has no ability to assess the higher order arrangement of the DNA molecules, its local strand passage activity can lead to catenation or decatenation. Topoisomerase IIa activity is critical for decatenating sister chromatids that after replication are at least initially catenated with each other [31]. In addition to its role in resolving topological problems along and between sister chromatids, Topoisomerase IIa also plays a direct, though poorly understood, structural role in maintaining mitotic chromosome compaction during prometaphase [9,32].

The second major scaffold component was initially named scII [30], and was later identified to be a subunit of condensin (SMC2) [33]. Independently, two subunits of the condensin complex, XCAP-C and XCAP-E (now known to be SMC4 and 2) were purified from reconstituted chromosomes using Xenopus egg extracts and shown to be required for mitotic chromosome formation and maintenance [34]. A 13S condensin complex was subsequently purified from Xenopus egg extracts and shown to include three more subunits, including the Barren homolog XCAP-H, XCAP-D and XCAP-G [35]). Subsequent work showed that there are two types of condensin complexes, condensin I and II, that differ in subunit composition [35,36]. Condensin complexes are composed of two Structural Maintenance of Chromosomes (SMC) proteins, SMC2 and SMC4, a kleisin protein CAP-H (condensin I) or CAP-H2 (condensin II), and two HEAT repeat containing proteins CAP-D2 (condensin I) or CAP-D3 (condensin II) and CAP-G (condensin I) or CAP-G2 (condensin II).

The final known scaffold component is KIF4A, a chromokinesin dimer with DNA binding domains [37,38]. Kif4A has the ability to transport cargo in neurons, in line with it being a kinesin [39]. In mitosis, Kif4A associates with condensin I and has been shown to be involved in correctly localizing condensin I complexes to the chromosome scaffold. As a kinesin it also associates with mitotic spindles [40–42].

Imaging of the localization of scaffold components has provided further evidence that although these proteins are mostly located near the central axis of the rod-shaped chromosomes, there is no rigid contiguous scaffold. First, staining for condensin and Topoisomerase IIa reveals discontinuous patches that can sometimes alternate along the axis. This pattern has been described as a ‘barber pole’ arrangement [43,44]. Second, when condensins are imaged on chromosomes that are stretched through micromechanical pulling, they form a series of well separated dots and not a continuous staining pattern indicating that stretches of DNA connect different condensin complexes [43,45].

All scaffold components have been directly implicated in mitotic chromosome formation: depletion of any of them can change the morphology of chromosomes or prevent mitotic chromosome formation entirely [9,31,35,46,47]. The Hirano lab recently demonstrated that, for the Xenopus system, in addition to core histones and histone chaperones, purified Topoisomerase IIa and condensin I are sufficient to reconstitute mitotic chromosomes *in vitro* [48]. Condensin II was not tested in this initial study, but a more recent study from the Hirano lab shows that in more complex Xenopus egg extracts condensin II can generate compact chromosomes without condensin I [49]. Other studies, in chicken and mammalian cells, have shown that compacted rod-shaped chromosomes, somewhat resembling mitotic chromosomes, can be generated when only condensin I, or only condensin II is present, although the morphologies of the chromosomes clearly differ from wild type chromosomes pointing to the presence of folding defects [19,36,50]. These experiments suggest that the two condensin complexes play related yet distinct roles in mitotic chromosome formation. As described below, it is now clear that condensin I and II are ATP-dependent machines that generate loops, but that each have distinct processivities and stabilities leading to loops of different sizes (see below). The fact that major components of the chromosome scaffold are machines that generate loops is again strong evidence in support of the radial loop model for mitotic chromosome folding.

We note that cohesin complexes, related to condensin complexes, also associate with mitotic chromosomes. Cohesin complexes generate loops during most of interphase, and mediate sister chromatid cohesion from S-phase till late mitosis. We refer the reader to several excellent reviews on cohesin complexes and their many functions [51,52]. Here we focus on condensins, topoisomerase IIa and KIF4A as the main machines that fold and organize mitotic chromosomes but acknowledge that cohesin is also contributing through mediating connections between sister chromatids. Below we outline relatively briefly the main enzymatic activities of these complexes, which have been reviewed in detail recently elsewhere [53,54], and then focus mostly on how their localization and activity is regulated as cells enter and then progress through mitosis.

Mechanisms of chromosome folding machines

We will briefly discuss what is known about the molecular mechanisms of condensin I, II, topoisomerase II and KIF4a, and then focus the rest of the review on how these complexes are regulated.

Condensin complexes are ATP-dependent loop extrusion machines

The *in vitro* activity of condensin complexes on DNA has been studied extensively. Initial studies showed that condensin’s association with DNA, in the presence of topoisomerase, can alter the topology of circular molecules [55–57]. This led to models where DNA wraps around the condensin complexes [13]. In the context of the radial loop model, it has long been hypothesized that condensins somehow mediate loop formation, but how their effect on DNA topology would facilitate loop formation has been unknown. Given the resemblance with other motor proteins such as myosins and kinesins (that share coiled-coil and ATPase domains), it had been speculated that the complexes have motor activities and that these could be involved in loop formation [58,59]. Over the years various models have been proposed how SMC complexes may encircle or entrap and extrude DNA [53]. In a major experimental breakthrough, Ganji and co-workers demonstrated using single molecule experiments that purified yeast condensin complexes can associate with a tethered DNA molecule, and then extrude a DNA loop in an ATP-dependent manner [60]. In quick succession, similar experiments were done with purified mammalian complexes and in Xenopus in egg extracts [61,62]. It is now well established that condensins and cohesins are ATP-dependent loop extrusion machines.

The precise molecular mechanism of loop extrusion remains unclear. There is ongoing debate on whether extrusion occurs in a uni-directional or bi-directional manner. Experimental evidence shows that yeast condensin strictly extrudes in one direction [60], while polymer simulations predict that to generate a compact mitotic chromosome in vertebrates, gaps between loops need to be minimized which would require at least some loop extruding machines to act bi-directionally [63]. Detection of loop extrusion in Xenopus mitotic extracts suggests that a fraction of the extruders (~20%) present in the extract indeed can act bi-directionally [61,62]. Possibly this involves condensin dimers, and/or a specific condensin complex that as a monomer can extrude in bi-directional manner (possibly condensin II), or a condensin complex that is modified and regulated in a specific manner. Recent structural studies are starting to shed light on how the complexes bind DNA, and the full cycle of extrusion, which is ATP-binding and hydrolysis driven, and may involve large-scale changes in the conformation of the complex is starting to be understood [64]. We refer to recent reviews that cover the current insights in detail [53,54,65–67]. Here we simply will assume condensins are machines that generate mitotic loops, the key structural feature of mitotic chromosomes, and below we outline our current understanding of how these machines are regulated.

Topoisomerase IIa decatenates sister chromatids, but may catenate DNA within chromatids

After DNA replication, pairs of sister chromatids are initially entangled all along their length. During mitosis each sister chromatid does not only need to be compacted, through condensin-driven loop array formation, but sister chromatids also need to be separated from each other. Sister separation requires the resolution of any entanglements between them. Topoisomerase IIa is required for disentangling sister chromatids. When topoisomerase IIa is blocked, e.g. through topoisomerase poisons [32,68], cells arrest after replication with a G2 DNA content, and any cells that progress through anaphase display frequent anaphase bridges between sister chromatids, a hallmark of unresolved inter-sister catenations. As outlined above, topoisomerase IIa can both entangle or disentangle dependent on which strand crosses through which other strand. Condensin driven compaction, which is strictly in cis, i.e. along single chromatids, will pull sisters away from each other so that any strand passage that results in entanglement will be disfavored, while events that facilitate disentanglement can still readily occur. Therefore, disentanglement of sister chromatids is thought to be favored [69]. Experimental evidence that shows that condensin-mediated compaction is continuously required to maintain disentanglement of sister chromatids comes from studies in Drosophila: depleting condensin I late in mitosis (late prometaphase), when sister chromatids are largely disentangled, resulted in re-catenating them [70]. Recently, several groups have proposed that loop extrusion by condensins (or cohesins) can provide directionality to the topoisomerase II reaction and lead preferentially to unlinking and topological simplification [71–75].

Topoisomerase IIa appears to also mediate intra-chromatid catenations and this may contribute to compaction and chromosome stiffness [4,9,76]. When mitotic chromosomes are reconstituted with purified factors, it was found that topoisomerase IIa is required for late-stage chromatid thickening that may be driven by formation intra-chromatid entanglements or catenations. However, to our knowledge, catenations of mitotic loops have not been directly experimentally observed. Acute depletion of topoisomerase IIa at prometaphase leads to reduced chromosome compaction, pointing to possible roles of the enzyme in maintaining mitotic chromosome structure [32]. Clearly, topoisomerase IIa has several functions during mitosis: unlinking of sister chromatids, possibly catenating loops within chromatids, and playing structural roles in maintaining compact chromosomes during late stages of mitosis. In this review we focus on roles of topoisomerase IIa along the arms of mitotic chromosomes, but we note that topoisomerase IIa has possibly specialized roles at centromeres (mentioned briefly below), and in mediating resolution of sister chromatids at the rDNA loci [77].

KIF4a is an ATP-dependent motor protein

KIF4a, a chromokinesin, is also an ATP-dependent motor protein, but whether its role in mitotic chromosome formation involves its motor activity in unclear. At the centromere Kif4A plays specific roles that require the presence of its motor domain [9]. On the mitotic arms KIF4A is the least characterized of the scaffold proteins. It is known to physically associate with condensin I, and its depletion results in reduced chromosome association, mis-localization of the remaining condensin I and altered chromosome morphologies [9,10,38] (Figure 2). Axial localization of KIF4a as well as condensin I along chromosome arms was disrupted when the ATP-binding site of the motor domains of KIF4a was mutated, suggesting that ATP binding/hydrolysis, and thus likely its motor activity, is involved [10]. Below we will focus on the role of KIF4a along arms specifically.

Regulation of mitotic chromosome folding machines

Condensins, and likely their loop extrusion activities, are widely conserved across the tree of life. Interestingly, how, when and where these machines are deployed varies, producing mitotic chromosomes that can differ in their final shape and dimensions [4]. For instance, while in vertebrates condensins are required for chromosome compaction all along chromosomes, in the budding yeast *Saccharomyces cerevisiae* condensin acts mostly

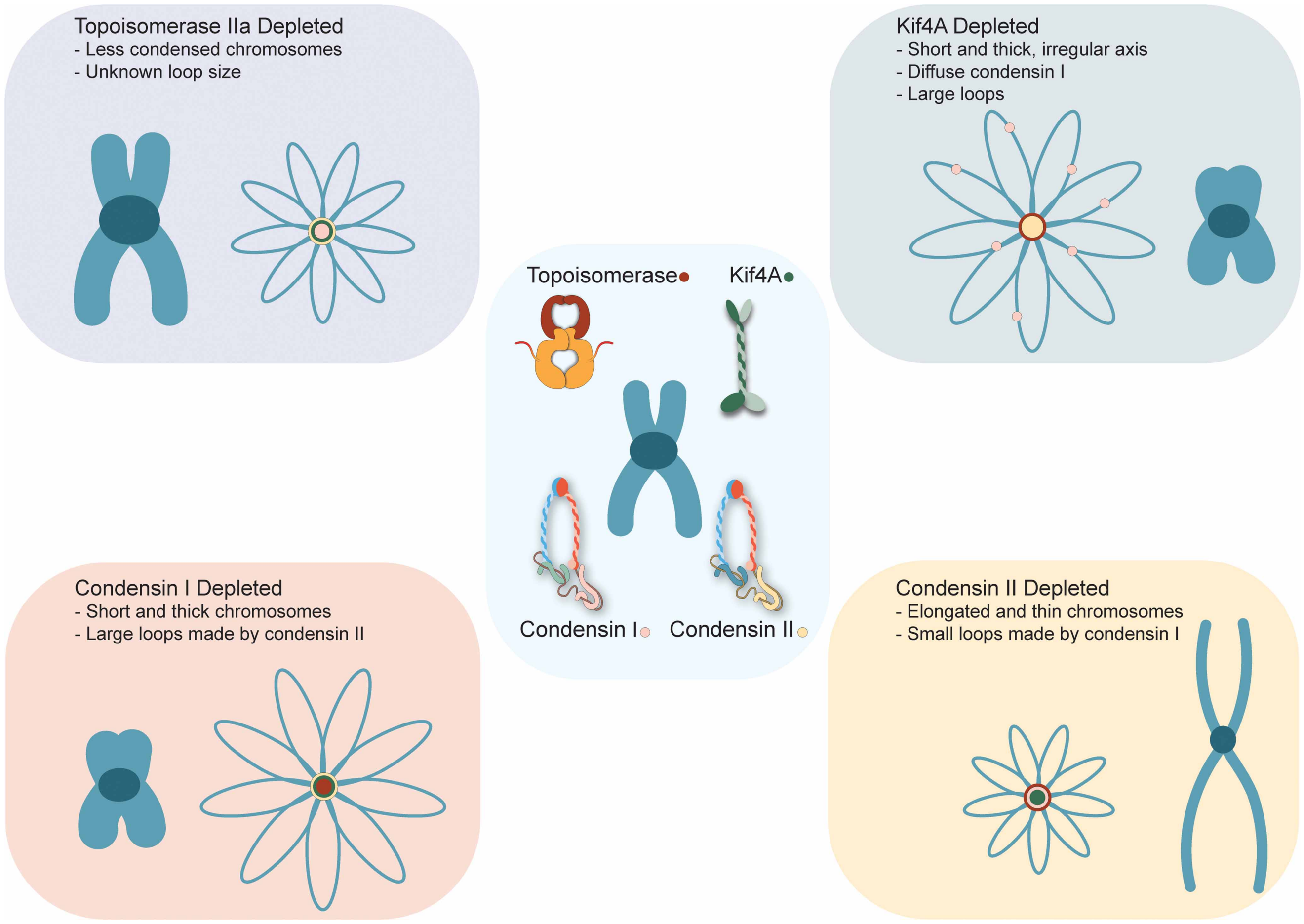
Topoisomerase IIa Depleted
- Less condensed chromosomes
- Unknown loop size
Kif4A Depleted
- Short and thick, irregular axis
- Diffuse condensin I
- Large loops
Topoisomerase●
Kif4A●
Condensin I●
Condensin II●
Condensin I Depleted
- Short and thick chromosomes
- Large loops made by condensin II
Condensin II Depleted
- Elongated and thin chromosomes
- Small loops made by condensin I

Figure 2. Differences in Chromosome morphology by different folding machinery.
Chromosome morphology changes when folding machineries are depleted. Topoisomerase IIa depleted chromosome are less condensed with unknown impact on loops (top right corner) [9,32]. Depletion of Kif4A results in smaller and thicker chromosomes with larger loops and a diffuse condensin I pattern (top left corner) [9,11]. Condensin I depletion has similar phenotype as Kif4A depletion, with large loops and wide chromosomes (bottom left corner) [19,36,46]. Condensin II depletion results in thin and elongated chromosomes with small loops [19,36,46] (bottom left corner). Color legend: Kif4A: green, Topoisomerase: red, Condensin I: pink, Condensin II: yellow.

at centromeres and the rDNA. In this organism, cohesin mediates loop formation along mitotic chromosome arms [78], while other cohesin complexes mediate sister chromatin cohesion as shown by the recently developed SisterC method [79]. In vertebrates condensin I and II compact mitotic chromosomes. Intriguingly, the relative reliance on one or both complexes varies between species. In chicken DT40 cells, condensin II is essential for mitosis, while some human cell lines, e.g. HAP1 cells, progress through mitosis relatively normally even in the absence of condensin II [46,80]. Furthermore, mitotic chromosomes reconstituted in *Xenopus* egg extracts in which condensin II is depleted, show relatively few defects [81].

Condensin I and II act at different times during mitosis. Condensin II is localized to the nucleus throughout the cell cycle and starts compacting chromosomes as soon as cells enter prophase. Condensin I is localized in the cytoplasm during interphase and only gains access to the chromatin after condensin II when pores lose their barrier function and therefore acts mostly during prometaphase [45]. The two condensin complexes both generate loops, but they differ in residence time on chromatin. Condensin II complexes remain chromatin associated for a relatively long time and hardly turn over during the time window of a normal mitosis (tens of minutes), as shown by FRAP, while condensin I complexes dissociate and re-associate at the time scale of minutes [25]. As a result, condensin II has more time to extrude and generate larger loops than condensin I [19]. What determines and regulates residence time and loop extrusion processivity is not known. The combined action of the two distinct condensin complexes, their different timing of chromatin association and their differences in residence time, leads to the formation of nested loops. In DT40 cells large 300–400 kb loops generated by stably associated condensin II complexes are split into smaller more dynamic loops generated by action of condensin I complexes that continuously and rapidly associate and dissociate from chromatin [19,22,25]. How the interplay between these complexes is regulated is not known. Finally, different ratios of condensin II and condensin I activities produce chromosomes that are either long and thin or short and fat [46,81] (Figure 2). When condensin I dominates, the chromosomes are long and thin, possibly because the loops are small. When condensin II dominates chromosomes are shorter and wider, possibly because loops are much larger. The ratio of the activities of these complexes can be regulated at different levels, e.g. expression level, chromatin association level, extrusion activity and so on. Interestingly, mitotic chromosomes change their dimension during development. For instance, in *Xenopus*, mitotic chromosomes are long and thin during the early cleavage stages, but they become progressively shorter and wider as differentiation proceeds [82–84]. Possibly the ratio of condensin I and II activities and/or their chromatin association is developmentally regulated. Finally, condensin complexes, and topoisomerase IIa, are all phosphoproteins, and their cell cycle stage-dependent phosphorylation is essential for their activity [85] (Figure 1, see below). From these observations it is clear that the activity of the machines and complexes that mediate mitotic chromosome formation are strictly regulated at the level of expression, sub-cellular localization, chromatin association and dissociation, and processivity. Here we will describe what is currently known about how these complexes and their activities are regulated to produce mitotic chromosomes of the appropriate species- and cell type-specific shape and dimensions.

Entry into mitosis is controlled by the main mitotic kinase Cdk1. Activation of Cdk1 is a precisely controlled process [86] that ensures that cells normally only enter mitosis when any DNA damage has been repaired. Cdk1 is activated through association with cyclins (Cyclin B or A, dependent on the cell system/organism) and through the antagonistic action of the Myt1/Wee1 kinases and the Cdc25 phosphatase. Wee1 phosphorylates the so-called T-loop domain in Cdk1 which then inhibits its kinase activity. Cdc25 dephosphorylates this site, activating the kinase. A variety of signals can shift the balance between Wee1 or Cdc25, dependent on the organism. Cdk1 activation leads to phosphorylation of as many as a thousand proteins, and these are involved in the many different cellular events that need to occur as cells proceed through mitosis including reorganizing the cytoskeleton, building the mitotic spindle, dissolving the nuclear lamina and nuclear envelope, and folding mitotic chromosomes [85,87].

Besides Cdk1, several other kinases are important for mitotic progression, e.g. the aurora kinases and the polo kinases. Their activation depends in part on Cdk1, and they phosphorylate additional targets. At the same time phosphatases are inhibited. The major phosphatases are PP2A and PP1. Some of these are actively repressed during mitosis. For instance, PP2A is inactivated through binding of its inhibitor ENSA following ENSA phosphorylation by Greatwall kinase that is activated by CDK1, whereas PP1 activity is suppressed by CDk1 phosphorylation and binding of inhibitor-I (Inh1) [88,89].

To exit mitosis, when the spindle checkpoint is satisfied and cells proceed into anaphase, telophase and cytokinesis, rapid depletion of cyclin B through ubiquitin mediated proteolysis controlled by the APC/Cdc20

Biochemical Journal (2022) **479** 2153–2173
https://doi.org/10.1042/BCJ20210140

complex inactivates Cdk1. At the same time phosphatases are activated leading to removal of the phosphorylation of the many proteins involved in cellular and chromosomal events required for mitosis.

Here we focus on how regulatory mechanisms involving cell cycle-regulated gene expression, phosphorylation/dephosphorylation, ubiquitination and other modifications, and associations with other complexes, regulate the machinery that folds mitotic chromosomes as cells enter, progress, and exit mitosis (Figure 1). We focus on the machineries that organize chromosomal arms and refer the reader to several other reviews that cover the control of centromeric chromosome structure [90,91].

Regulation of condensin complexes

The localization and activity of complexes can be regulated at the level of gene expression, sub-cellular localization, protein modification, protein stability, and association with other complexes including the chromatin template itself. Condensin complexes are regulated at all these levels, but mostly through control of sub-cellular localization, protein modifications such as phosphorylation, and chromatin association.

Cycle cell stage dependent expression

Condensin complex components such as SMC2 and SMC4 can be detected throughout the cell cycle [92], but some subunits show cell cycle stage dependent differences in their levels. For instance, in *Saccharomyces cerevisiae* the condensin subunit Ysg1 (CAP-G), and in Drosophila the condensin II subunit CAP-H2 are down-regulated in interphase, likely through proteasome-mediated degradation [93–97]. It is increasingly appreciated that some condensins, in some organisms, play roles outside of mitosis, which would explain the presence of (nuclear) condensins throughout the cell cycle, and the limited control of condensins through cell-cycle stage dependent expression. For instance, condensin II may play roles in compacting chromatin in senescent cells, a role observed in yeast as well [98–100]. However, there is some evidence that a distinct shorter CAP-H2 isoform partakes in senescence, with the full-length protein's activity limited to mitosis [101]. In addition, in flies condensin II plays roles in chromosome territory formation and homolog pairing during interphase [102,103]. Focusing on the main mitotic function of condensin, all subunits are required to produce normal mitotic chromosomes [104]. Kleisin levels have been proposed to be a limiting factor for condensins [22], giving cells the potential to regulate condensin ratios, an important aspect for building mitotic chromosomes [81].

Sub-cellular localization

The condensin I complex is localized in the cytoplasm during interphase, and only gains access to chromosomes after pores lose barrier function at the prophase-prometaphase transition [45]. Nuclear exclusion provides a straightforward mechanism to ensure that condensin I does not act on chromatin during interphase.

Nuclear localization enables the condensin II complex to start chromosome compaction prior to NEBD (i.e. in prophase). Spatial segregation of condensin I and II will automatically lead to temporally staggered activity of the two complexes. It is not known whether sequential, and not simultaneous, initiation of condensin II and condensin I — driven loop formation is important for correctly folding mitotic chromosomes. Simulations indicate that nested loop formation can occur even when the two complexes initiate loops formation simultaneously, as long as their residence times are distinct, and there is sufficient time for condensin I complexes to turn over [19].

Activation of condensin complexes

Condensin II is present in the nucleus throughout interphase, which directly points to the need for mechanisms to prevent condensin II from compacting chromosomes during G1, S and G2, as well as mechanisms that trigger its activation as cells enter prophase. Across subunits of both condensin I and II, many potential sites of post-translational modifications (PTMs) have been identified. The majority of PTMs are phosphorylation events, consistent with mitotic entry being controlled by massive proteome-wide phosphorylation driven by a cascade of kinases starting with CDK1 (see above). Early experiments have demonstrated the importance of phosphorylation for condensin activity: purified condensin I from interphase can bind DNA *in vitro* but did not induce supercoiling, whereas phosphorylated condensin I purified from mitotic extracts, or phosphorylated *in vitro* by CDK1 did induce supercoiling. CDK1-mediated phosphorylation is also required for condensin-dependent mitotic chromosome formation in Xenopus egg extracts [105,106]. Other kinases also phosphorylate condensins, including PLK1, Aurora B Kinases, and Caseine Kinase II [97,107–109].

Given that condensin II is the first complex to start acting on chromatin as cells enter prophase, we will first discuss its PTMs, and how they may control chromatin binding and loop extrusion activities. Condensin II is initially phosphorylated by Cdk1 at the CAP-D3 subunit (at residue T1415; [7]). Cdk1-dependent phosphorylation of CAP-D3 is required for subsequent hyperphosphorylation at several condensin II subunits by PLK1 (Figure 1). Ablation of the putative Cdk1 phosphorylation site on CAP-D3 in Hela cells leads to a failure of PLK1 to localize along mitotic chromosome arms [7]. In agreement, the same phosphorylation site is required in prophase for condensin II activation [110]. However, in chicken DT40 cells ablation of the corresponding phosphorylation site leads to increased loading of condensin II, reduced loading of condensin I, and hyper condensation of mitotic chromosomes [111]. This intriguing observation shows that cells may be able to, through phosphorylation, balance the activity of the two condensin complexes and thereby control chromosome condensation [81]. It should be noted that differences between effects of phosphorylation events/ablations between cell lines and organisms have been observed, pointing to potentially alternative strategies to appropriately activate condensins in different organisms or cell types. Other phosphorylation events on condensin II include the phosphorylation at S492 of CAP-H2 by the MPS1 kinase. This phosphorylation has been shown to be essential for the loading of condensin II on chromosomes [96].

Condensins are also acetylated and sumoylated [112]. Acetylation targeted proteomics identified multiple acetylation sites on both SMC2 and SMC4 [113]. Although these subunits are shared by condensin I and II, this study did not allow the determination of which complex, or both are acetylated. The functional relevance of condensin acetylation is not known. Acetylation of the related cohesin complex leads to increased residence time of this complex on chromatin and larger loop formation[114,115]. This observation raises the possibility that in mitosis condensin's residency time may be similarly regulated by acetylation. Given that condensin II is much more stably associated with chromatin compared with condensin I [22,25], despite sharing the same SMC subunits, it is possible that condensin II displays higher acetylation levels. However, it is currently not known whether the two complexes differ in acetylation, or whether acetylation is cell cycle regulated.

Condensin I has been studied more extensively, in part because it is the dominant condensin complex in the widely used Xenopus egg extract system used for reconstitution of mitotic chromosomes. Phosphorylation of condensin I by Cdk1 is essential: phosphorylation, likely of at least CAP-D2, is required for DNA supercoiling activity in *in vitro* assays whereas disruption of CAP-G phosphorylation sites affect chromatin association [106,116]. In addition, assays reconstituting mitotic chromosomes with purified complexes require phosphorylation of condensin I [48]. Aurora B also phosphorylates condensin I. Inhibiting Aurora B during mitosis reduces condensin I's association with chromatin [109]. This suggests that maintenance of phosphorylation levels throughout mitosis is important for appropriate chromosome folding. It is therefore somewhat puzzling that during anaphase, when Cdk1 is inactivated, loading of condensin on chromosomes increases [22,25]. Interestingly, the first real-time loop extrusion experiments used purified overexpressed complexes from asynchronous yeast populations, and used naked DNA [60], suggesting condensin may have extrusion activity throughout the cell cycle. Alternatively, the extrusion observed in these experiments may be driven by condensins derived from the subpopulation of mitotic cells. Loop extrusion experiments performed with interphase or mitotic extracts, depleted for H3 and H4, from Xenopus showed that condensin activity is only observed in mitotic extracts [61]. Differences can be explained by differences in condensin activity in yeast and Xenopus, or the very different biochemical conditions under which these experiments were performed.

Inactivation of condensins

As cells exit mitosis, condensins dissociate from chromatin [22,117,118]. This is likely driven by net dephosphorylation due to inactivation of mitotic kinases, and activity of global phosphatases such as PP2A and PP1. In addition, during interphase Casein Kinase 2 phosphorylates condensins, and these modifications inactivate their supercoiling activity [107].

It seems likely that much remains to be discovered about the different kinases, acetylases, phosphatases etc., that modify subunits of these complexes, and modification of which residues results in either activation or inhibition of their activities. Furthermore, the molecular mechanism of how PTMs determine condensin activity is not known. Possibly, these PTMs serve as binding sites for other regulatory factors, as briefly discussed in the next section. An ongoing focus on systematically and comprehensively mapping all PTMs on condensins is warranted. The large sizes of these proteins make this goal challenging, but we feel it will lead to important new insights into the regulation of these essential folding machines.

Modulation of condensin activity through interplay with other complexes

It is likely that the activity, chromosomal association, and location of condensin complexes is at least in part controlled by regulatory factors. This is the case for the related cohesin complex: cohesin extrudes chromatin loops until it is blocked by sites bound by the CTCF protein. This blocking is mediated through specific protein–protein interactions between the N-terminus of CTCF and STAG1 and RAD21 cohesin subunits [119–121]. Blocking leads to accumulation of cohesin at CTCF sites, and the positioning of loops anchored at these sites. The WAPL/MAU3A proteins can unload cohesin complexes, while acetylation of cohesin and association with sororin protects cohesin from WAPL-mediated unloading [114]. It is currently not known whether related processes, and factors, control condensin loading, extrusion or unloading. In recent studies MCPH1 was identified as a factor that prevents condensin from acting during interphase [122–124]. Loss of MCPH1 leads to premature chromosome condensation in interphase cells. MCPH1 interacts with CAP-G2 and may unload condensin in a manner related to how WAPL unloads cohesin: by dissociating the kleisin (CAP-H2) from one of the SMC proteins (SMC2) [8,124]. Phosphorylation within the central domain of MCPH1 disrupts condensin II binding. Overexpression of MCPH1 in meiotic cells or supplementation of the protein in Xenopus Egg extracts can prevent condensin II's association with chromosomes despite the presence of active CDK1 that is capable of phosphorylating MCPH1's central domain. The interaction and regulation of condensin and MCPH1 may involve more than a single phosphorylation site. The N-terminal domain of MCPH1 is of interest given that it is capable by itself to rescue part of the premature condensed chromosome phenotype in interphase [124], suggesting it may by itself be able to release at least some condensin from chromatin. It remains unclear if the retained condensin complexes observed on prematurely condensed chromosomes are remaining from the prior mitosis, or if turnover and de novo condensin II association establishes these condensed chromosomes. Acute MPCH1 deletion would reveal more about the nature of this interaction with condensin II and the importance of phosphorylation/dephosphorylation. Importantly, in mouse and xenopus, MCPH1 had no effect on condensin II, reinforcing the concept of organism specific condensin II regulation [124].

There are likely additional factors that control loading and activity of condensins, including topoisomerase IIa (see above and below), and the different condensin and cohesin complexes themselves. First, Kif4A directly interacts with condensin I and their association with mitotic chromatin appears interdependent, as discussed in more detail in the section on regulation of Kif4A below. Second, there appears to be extensive interplay between the different SMC complexes as they actively extrude and encounter one another along chromosomes. As mentioned above, altered phosphorylation of condensin II in DT40 cells not only increases condensin II loading on chromatin, but also leads to reduced condensin I association [111]. How the two complexes interact is not known. Third, inadequate removal of cohesin complexes upon mitotic entry affects the association of condensin II with chromatin suggesting that there is some incompatibility between these complexes [81]. Fourth, the relative levels of condensin I and II are different between organisms and this ratio determines the relative dimensions of mitotic chromosomes [81] (Figure 2). Although the latter observation does not directly require physical interplay between the complexes, it does indicate that their activities may be co-ordinated or even inter-dependent. Finally, as described below, the chromatin template itself plays roles in recruiting condensins to chromosomes.

Regulation of Topoisomerase IIa

After replication sister chromatids are entangled, i.e. they are wound around each other [125]. Sisters need to become mostly decatenated by late prophase through topoisomerase IIa activity and to become completely decatenated during anaphase. SMC-driven loop formation and stiffening and shortening contributes to topoisomerase-driven disentangling sister chromatids [73]. Topoisomerase IIa is also important as a structural component of the mitotic chromosome and is required for maintaining the fully compacted state in late mitosis [9,32,47]. Thus, there is an increased demand for topoisomerase II activity from S to late M-phase. The expression of Topoisomerase IIa is cell cycle regulated, and its levels increase from S, and G2 and into mitosis. Topoisomerase IIa activity is further modulated by several PTMs, and by association with regulatory factors, as outlined below. Vertebrates have a second Topoisomerase II type enzyme, the β variant. The two enzymes both perform the typical topoisomerase II strand passage reactions but are not entirely redundant. The two Topoisomerases have distinct C-terminal domains (CTD) that play a key role in regulating their activity as will be discussed below. Topoisomerase IIa is the main topoisomerase involved in mitotic chromosome formation.

Cycle cell stage-dependent expression and nuclear localization

Topoisomerase IIa is often used as a marker for proliferation given its strong cell cycle dependent expression. Topoisomerase IIa levels start to accumulate through S-phase, peak in G2 and early mitosis, and then decline during later mitotic stages, likely mediated through ubiquitination and proteasome-mediated degradation [126–129].

Topoisomerase IIa is mostly nuclear, even before entry into mitosis. A nuclear localization sequence (NLS) is located towards the end of the C-terminal domain (1454–1497). In addition, two nuclear export signals (NES) have been found within the catalytic domains, but it is unclear to what extent these are used [130,131]. Mutations within the NLS region result in cytoplasmic localization of Topoisomerase IIa. Such cells can still enter mitosis, which may be due to activity of Topoisomerase IIb. After NEBD, Topoisomerase IIa can still become correctly localized on chromosomes [132], indicating that association is highly dynamic consistent with FRAP experiments [26,27].

Activation of Topoisomerase IIa complexes

Topoisomerase IIa is most likely active even prior to mitosis. As cells progress through S-phase and G2, some decatenation of sister chromatids is probably already happening. Blocking topoisomerase activity with the ICRF-193 inhibitor leads to accumulation of cells with a G2 DNA content, and for some cell types it was shown that cells did not enter prophase [68]. A number of PTMs have been detected on the enzyme, some ascribed to Cdk1 activity suggesting that at least some of these PTMs are mitosis-specific.

Topoisomerase IIa is heavily phosphorylated with most post-translational modification sites on the C-terminal domain (CTD). The CTD is required for appropriate chromosomal localization [4,133], and appears to influence the activity of topoisomerase in peculiar ways. Topoisomerase IIa with the CTD deleted can still perform strand passage reactions (at least at low salt concentrations), but the enzyme now appears to be defective in catenating DNA molecules *in vitro*, while it is still capable of decatenating interlinked circular DNA molecules. Hirano and co-workers proposed that the CTD is required for generating intra-chromatid entanglements possible near the condensin core of the chromatids, while without a CTD it can still decatenate sister chromatids [4]. The mechanism of CTD action is unclear, but this domain may play roles in localizing Topoisomerase IIa to the chromatin-dense core of mitotic chromatids.

Within the C-terminal domain there is a nuclear localization signal (NLS) located at 1454–1497, disruption within this region at 1490–1492 impairs nuclear import leaving topoisomerase II cytosolic (for the human protein) [132,134]. K1492 can be SUMOylated, which prevents nuclear import. The functional relevance of this modification beyond influencing nuclear localization of Topoisomerase IIa is not known [132,135]. Mass spectrometry experiments have identified many phosphorylation sites within the CTD [136]. So far, only one of these sites, S1496, is known to be phosphorylated in mitosis. Phosphorylation can be observed *in vitro* with Caseine Kinase I and this phosphorylated site is recognized by mitosis-specific antibodies [137]. However, the functional relevance of this phosphorylation site remains unclear.

Another region of importance within the CTD is the so-called C-terminal regulatory domain (CRD). This domain was first identified in Top2B and a similar domain has more recently been found in Topoisomerase IIa [138]. This region is responsible for binding RNA, which in its turn inhibits Topoisomerase IIa's activity [139]. The RNA binding region was defined in rat Topoisomerase IIa to be between amino acids 1192–1289 [140]. The importance of this region was further highlighted in human Topoisomerase IIa where the region between 1193–1217 has an important role in the strand passage process [141]. Furthermore, incremental deletions of Topoisomerase IIa's CTD indicates that the region between amino acids 1212–1320 is essential for accurate localization of Topoisomerase IIa on mitotic chromosomes [5]. All these deletions have one major phosphorylation site in common: S1213. This phosphorylation site is located in the linker that connects the core of the enzyme and the CTD [5]. Topoisomerase IIa phosphorylated at this residue is found enriched at centromeric chromatin [6], but it is not known which kinase phosphorylates this residue, or what the functional effect of the modification is.

Finally, at the extreme C-terminal end of the enzyme is the so-called 'chromatin tethering domain' (ChT) [132]. Deletion of this domain abolishes localization of Topoisomerase IIa to mitotic chromosomes, likely through loss of interactions with H3. This domain has been shown to interact with methylated H3 tails but not with H3 phosphorylated at S28, a marker for mitotic chromosomes. At the edge of the ChT domain is S1525, a site phosphorylated throughout the cell cycle [142]. Phosphorylation of this site has been proposed to be part of a decatenation checkpoint through binding of MCD1 (mediator of DNA damage checkpoint protein-1). In the presence of any DNA damage, this interaction is lost [143]. Multiple kinases are linked to this phosphorylation site, including Caseine Kinase II, PLK1 and P38y [142,144,145]. Besides S1525, PLk1 also targets S1337. Cells expressing Topoisomerase IIa with double mutations at both sites arrest in G1, and the mutated Topoisomerase IIa displayed reduced decatenation activity [144]. This effect could be due to the mutation of S1337, as conflicting reports indicate no change in Topoisomerase IIa decatenation activity upon mutating S1525 [143]. A lysine residue within the ChT, K1520, is SUMOylated by NSE2 [146]. Interestingly, NSE2 is a subunit of the SMC5/6 complex and is required for Topoisomerase IIa localization in mitosis [147]. Similar to the CRD other potential modification sites are present in the ChT domain [136], yet no detailed functions have been reported for these. Other phosphorylation sites have been identified outside the CTD. Phosphorylation of S29 is cell cycle dependent and increases during G2/M. Phosphorylation of S29 increases the catalytic activity of Topoisomerase IIa [148,149]. Phosphorylation of S1106, located within the catalytic domain, decreases Topoisomerase IIa activity [150].

Many phosphorylation sites are either found through phospho-proteomics or by *in vitro* assays and for many phosphorylation events it remains unclear how they affect Topoisomerase IIa regulation at different cell cycle stages, or which kinases are involved. Casein Kinase II is suggested to phosphorylate three sites, all residing within the CTD: T1343, S1377, and S1525. None of these phosphorylation events are cell cycle stage-specific [6,142]. Other sites were suggested to be targeted by Proline-directed kinases, like Cdk1 and MAPK [151]. Two of those sites are cell cycle stage (G2/M) specific (S1213 and S1247), and three others are modified through the cell cycle (S1354, S1361, and S1392). Recently, S1247 has been suggested to be modified by Cdk1. Phosphorylation of S1247 together with T1244 affect SUMOylation efficiency at the neighboring K1240 [5]. Sumoylation of K1240 is important for centromeric Topoisomerase IIa localization.

Soon after topoisomerase II was identified as a target for SUMOylation, the role for SUMOylation in sister separation became apparent [152–154]. Sister chromatid segregation at the metaphase-anaphase transition was hindered after disruption of SUMO conjugation. Both RANBP2 and PIASy have been linked to SUMOylation of Topoisomerase IIa but in different species. In Xenopus Egg extract, PIASy drives Topoisomerase IIa SUMOylation with no role for RANBP2 [155,156]. In mice, deficiency of RANBP2 causes loss of SUMOylated Topoisomerase IIa and sister chromatid segregation defects, while there was no defect after PIASy deletion [157]. SUMOylation at the catalytic domain inactivates Topoisomerase IIa (K660), which may play a role in maintaining inter-sister interlinks at centromeres [158]. SUMOylation at the C-terminal domain did not affect catalytic activity, but it did provide a binding site for Haspin, a key protein involved in sister chromatid cohesion at the centromeres and downstream spindle assembly checkpoints [159–161].

Other Topoisomerase IIa modifications include acetylation and ubiquitination. It is likely that ubiquitination is important for proteasome-mediated degradation, e.g. during mitotic exit when Topoisomerase IIa levels decline. Acetylation of Topoisomerase IIa has not been studied in detail. Recent mass spectrometry studies revealed acetylated sites within the catalytic domains [136]. Within the CTD only K1240 was found acetylated, the same site that has been found to be SUMOylated (see above) [5]. Lastly, Histone deacetylases (HDACs) interact with Topoisomerase IIa and this interaction affects its activity *in vitro* [162]. Although no further data has suggested a mechanism for such interaction, other HDACs are known to deacetylate mitotic proteins in mitosis, i.e. HDAC8 deacetylates cohesin [163,164]. However, broad range HDAC inhibitors like VPA did not lead to obvious changes in mitotic chromosome morphology or their mechanical properties [165].

Interplay with other factors

Relatively little is known about other factors that facilitate or regulate localization and activity of Topoisomerase IIa. As indicated above, the NSE2 subunit of the SMC5/6 complex influences Topoisomerase IIa localization [146]. Topoisomerase IIa is strongly enriched at the centromeres, and at the central core all along chromosomal arms. Centromeric localization is based on modifications that serve to recruit other proteins (see above). Topoisomerase IIa localization along arms depends on condensin: loss of condensin leads to more diffuse localization of Topoisomerase IIa [166,167]. However, this can be an indirect effect of the altered path of the chromosomal scaffold. As described in more detail below, the chromatin template itself also plays roles in recruiting Topoisomerase IIa to mitotic chromosomes.

Regulation of Kif4A

Relatively little is known about the role of Kif4A in mitotic chromosome formation. In the absence of Kif4A mitotic chromosomes become shorter and thicker, and the chromosomes lose their structural integrity as shown using the 'intrinsic metaphase structure' assay [9,38], a phenotype similar to that observed after condensin I depletion (Figure 2). Kif4A acts at the spindle, centromere and along chromosome arms. Here we focus on regulation of its activity along chromosome arms. Kif4A is a chromokinesin. Chromokinesins are a special subset of the kinesins that in addition to their motor domains and microtubule binding domains, contain DNA binding domains. Kif4A interacts with condensin I and localizes to the mitotic chromosome scaffold [10,168]. In the absence of Kif4A, condensin I is mislocalized and chromosomes become shorter and fatter, indicating that one important function of Kif4A is controlling or participating in the condensin I-mediated loop extrusion process. Kif4A has a motor domain, but whether that domain's activity is involved in some form of loop extrusion by itself, or in combination with condensins, is not known.

Kif4B is a highly similar paralog of Kif4A and shares the same domain organization and phosphorylation sites (Figure 2). Both proteins form dimers, but as far as we are aware, it is not known whether they form homo- or heterodimers or both. Heterodimerization seems likely, given that knockdown of Kif4B knockdown produces very similar mitotic defects as loss of Kif4A [169].

Localization of Kif4A

Kif4A is localized in the nucleus throughout interphase, with potential roles in chromatin structure and DNA repair [170,171]. During mitosis Kif4A is associated with spindles and with the chromosomes. Here we focus on the pool of Kif4A that is chromosomally localized. As expected, the DNA binding domain of Kif4A is important for Kif4A to associate with chromosomes [9,11,172]. Kif4A binds AT-rich regions, which is interesting given early models proposed by Laemmli and co-workers that AT-rich sequences form the core of the mitotic loop array [171,173,174]. The ability of the DNA binding domain to mediate chromosome binding appears to be cell cycle stage-dependent: in anaphase Kif4A no longer binds chromatin [11]. It is still unclear to what extent the motor activity of Kif4A is required for its function on chromosomes. On the one hand, Aurora-regulated motor activity of Kif4A is not required as morphologically normal mitotic chromosome can still form when the residues modified by Aurora kinases are abrogated, while leaving the motor domain otherwise intact [11]. On the other hand, deletion of the motor domain as well as part of the N-terminal coiled coil domain, led to a more disorganized Kif4A organization on mitotic chromosomes [9]. Consistently, subsequent studies deleted (part of) the motor domain leading to defects mitotic chromosomal structure [10,11]. Altogether, the motor domain is required, but whether normal Aurora-dependent motor function is required as well is still unclear. The roles of physical interactions between condensin I and Kif4A for their appropriate localization to mitotic chromosomes is not entirely clear and apparently contradictory results have been reported. Mutating the binding site on Kif4A for condensin I leads to loss of Kif4A from chromosomes, suggesting its chromosomal localization does depend on its interaction with condensin I [11]. However, depletion of condensin I does allow Kif4A to localize on the mitotic chromosome [46]. Both within the coiled-coil and tail of Kif4A various domains have been identified that disrupt localization with the DNA and therefore interaction with condensin I [11,172].

Modifications of Kif4A

Kif4A is phosphorylated at S1186 and T1161, and this modification is essential for its interaction with the CAP-G subunit of condensin I and chromosomal localization [175,176]. Both phosphorylation events are CDK1-dependent. As with many other domains on Kif4A, if localization on the chromatin is compromised the interaction with condensin I is lost. For T1161 such effect seems true, as a non-phosphorylatable mutant has a diffuse localization pattern. This required the presence of the motor domain, highlighting once more the importance of the motor domain of Kif4A. Additional Aurora kinase-dependent phosphorylation sites are located outside the C-terminal domain. These phosphorylation events are important for metaphase alignment and segregation, possibly through activities the motor function of Kif4A and the spindle, but do not affect chromosome morphology [11]. The interaction between condensin I and Kif4A is independent of this Aurora phosphorylation [11,177]. Kif4A located at spindles is SUMOylated, an important mark for progression at cytokinesis [178]. There are additional PTMs for Kif4A, identified through mass spectrometry experiments, but their roles remain uncharacterized [113,179,180].

Interactions with other factors

As discussed above, Kif4A interacts with condensin I (Figure 1). The C-terminus of Kif4A interacts directly with the CAP-G subunit of condensin I. It is not known how this interaction contributes to mitotic chromosome formation. Takahashi et al. proposed that a KIF4A homodimer holds two condensin complexes at the CAP-G subunit with Kif4A acting as a motor protein for extrusion [10]. It is now well established that condensin I is a loop extrusion factor itself [60,63,181]. Condensin I appears to acts as a one-sided loop extrusion factor, extruding only on one side, while holding on to the locus at the side of the growing loop [60]. However, loop extrusion simulations have indicated that in order to fully compact chromosomes at least some loop extrusion has to be two-sided [63]. Possibly, condensin II can act as two-sided loop extrusion factor [61]. An interesting additional hypothesis is that Kif4A generates condensin I dimers, that by dimerization could effectively produce two-sided loop extrusion. The shorter residence time of Kif4A compared with an already dynamic condensin I would be hard to unify with stable dimers. Contrary, if Kif4A were to act as a loader of condensin this would raise many questions such as what it motor domain is for, and why condensin I can load without KIF4a in *in vitro* experiments using naked DNA.

Kif4A interacts with multiple additional proteins during mitosis, including plant homeodomain finger protein 14 (PHF14), which belongs to the PHD finger family. PHD domain-containing proteins typically bind various modified histones. For PHF14 interactions with epigenetic marks are not known. Depletion of PHF14 shows similar phenotypes as Kif4A depletion, including misalignment and anaphase bridges [182]. Chromosome misalignment suggests a function with Kif4A with respect to its activity along spindles, while the detection of chromosome bridges suggest additional defects in chromosome condensation.

Kif4A and condensin I also interact with DNMT3b [183], an enzyme that methylates DNA. Mitotic chromosomes isolated from cells lacking Dnmt1/3a/3b are less compact [184]. This could be the result of altered DNA methylation levels itself, or through defective Kif4A/Condensin I localization, or both. Interestingly, Dnmt3 is enriched at heterochromatic regions [185] that are known to recruit more condensin than euchromatic regions [186]. Localization of Dnmt3b at heterochromatic regions could be one explanation why regions with the heterochromatic mark H3K9me3 display increased condensin occupancy (see below)

The role of the chromatin template in regulating condensin and topoisomerase loading

Where and when condensins and topoisomerase IIa act is not only regulated by their modifications and interaction partners, as decribed above. Several observations indicate that the composition and local structure of the chromatin fiber itself also contribute to recruitment and possibly activity of Topoisomerase IIa and condensin complexes. This is a topic that has not been extensively explored, and here we just mention several observations that suggest the chromatin composition and the presence of histone modifications can influence condensin and topoisomerase II loading onto the chromatin template. First, linker histones have been shown to negatively influence topoisomerase IIa and condensin recruitment in Xenopus extracts. Depletion of H1.8 from Xenopus egg extracts increases the amount of Topoisomerase IIa, condensin I and condensin II associated with mitotic chromosomes. Due to the increased condensin loading, loops become on average smaller, and chromosomes longer and thinner [187]. Thus, H1.8 indirectly controls the formation of mitotic chromosomes, at least *in vitro*, by modulating the amount of Topoisomerase IIa, condensin I and condensin II that becomes chromatin bound. Second, XPB, a subunit of TFIIH, was shown to control condensin I and condensin II but not topoisomerase IIa's association with chromatin in Xenopus extracts [188]. Possibly the TFIIH complex changes local chromatin organization, e.g. creating open sites, necessary for condensin association similar to H1 or H3/4 depletion [187,188]. Changes in local chromatin might be important as work demonstrated various chromatin configurations, e.g. supercoils, could favor condensin association [189–191]. Third, domains enriched in H3K9Me3 recruit higher levels of condensin in mammalian cells [186], and this results again in smaller loops and thinner mitotic chromatids at these locations. Fourth, in yeast, condensin specifically acts on centromeric and rDNA loci, suggesting the presence of specific factors, including histone variants and modifications, involved in recruiting condensins to these locations. Fifth, in mouse meiotic cells during meiosis I, the two condensin complexes get recruited to distinct chromosomal domains: condensin II localizes on the arms and condensin I localizes to centromeric regions, while at later stages they both localize axially [78,192]. What determines complex-specific recruitment is not known, but we speculate chromatin state, including chromatin composition and modifications may play roles. Furthermore, whether condensin-mediated loop extrusion (e.g. processivity, extrusion speed etc.), or Topoisomerase IIa-mediated strand passage are also modulated by the local structure and composition of the chromatin template remains to be determined.

Future perspective

One of the most surprising findings over the last several years is that it requires only a small number of protein complexes to generate mitotic chromosomes [48]. Going forward, a major challenge for the field is to identify all cell cycle-regulated modifications of the mitotic chromosome folding machines, and how these modifications contribute to appropriate temporal and spatial (along chromosomes) deployment of condensins and topoisomerases. We anticipate that future studies will lead to the discovery of additional factors that control the recruitment, activity (extrusion processivity and speed), and timely inactivation of condensins and Topoisomerase IIa. Local variation of chromatin composition along chromosomes may demand and control local differences in how the folding machines are deployed. Together, these studies may reveal how a relatively small set of machines can fold heterogeneous chromatin fibers into uniformly packed mitotic chromosome.

Competing Interests

The authors declare that there are no competing interests associated with the manuscript.

CRediT Author Contribution

Job Dekker: Conceptualization, Resources, Supervision, Writing — original draft, Writing — review and editing.
Bastiaan Dekker: Conceptualization, Investigation, Writing — original draft, Writing — review and editing.

Abbreviations

CRD, C-terminal regulatory domain; CTD, C-terminal domains; FRAP, fluorescence recovery after photobleaching; HDACs, histone deacetylases; NLS, nuclear localization sequence; PHF14, plant homeodomain finger protein 14; PTMs, post-translational modifications; SMC, structural maintenance of chromosomes.

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