Mitotic Chromosome Assembly In Vitro: Functional Cross Talk between Nucleosomes and Condensins

KEISHI SHINTOMI AND TATSUYA HIRANO

Chromosome Dynamics Laboratory, RIKEN, Wako, Saitama 351-0198, Japan

Correspondence: hiranot@riken.jp

The mitotic chromosome is a macromolecular assembly that ensures error-free transmission of the genome during cell division. It has long been a big mystery how long stretches of DNA might be folded into rod-shaped chromosomes or how such an elaborate process might be accomplished at a mechanistic level. Cell-free extracts made from frog eggs offer a unique opportunity to address these questions by enabling mitotic chromosomes to be assembled in a test tube. Moreover, the core part of the chromosome assembly reaction can now be reconstituted with a limited number of purified factors. A combination of these in vitro assays makes it possible not only to prepare a complete list of proteins required for chromosome assembly but also to dissect functions of individual proteins and their cooperation with unparalleled clarity. Emerging lines of evidence underscore the paramount importance of condensins in building mitotic chromosomes and shed new light on the functional cross talk between nucleosomes and condensins in this process.

In eukaryotic cells, chromatin undergoes a series of dynamic structural changes throughout the cell cycle, which culminate in the assembly of chromosomes during mitosis. The dramatic transformation of an amorphous mass of chromatin into a discrete set of rod-shaped chromosomes is thought to be an essential process for faithful segregation of the genome during cell division. Among various experimental systems for studying mitotic chromosome organization, the cell-free system derived from *Xenopus* egg extracts is unique in the sense that the whole process of mitotic chromosome assembly is recapitulated in a test tube (e.g., Hirano and Mitchison 1993). Remarkably, at least the core part of the chromosome assembly reaction achieved in the cell-free system can now be reconstituted using purified factors in vitro (Shintomi et al. 2015). We will summarize what we have learned from these in vitro assays and what we will need to learn by extending these efforts.

IDENTIFICATION OF CONDENSINS USING *XENOPUS* EGG EXTRACTS

The cell cycle of unfertilized eggs of the frog *Xenopus laevis* is naturally arrested at metaphase of meiosis II, where the activity of cyclin B–Cdk1 is kept at a high level. Upon fertilization, a transient increase of cytosolic calcium ions (Ca^{2+}) triggers the degradation of cyclin B, resulting in mitotic exit. Therefore, when the eggs are crushed by centrifugation in a buffer containing the Ca^{2+} -chelator EGTA, a metaphase egg extract can be obtained (Lohka and Maller 1985; Murray 1991). When demembranated *Xenopus* sperm nuclei are incubated with this metaphase extract, they quickly swell and then turn into a cluster of fibrous structures. These chromatin fibers

progressively get thickened and individualized and are eventually converted into rod-shaped chromatids (Fig. 1A; Hirano and Mitchison 1993). Because the chromatin does not undergo DNA replication in this experimental setup, the resulting structures are composed of “single” chromatids. Isolation of these chromatids from the reaction mixture by single-step centrifugation led to the identification of their major proteinaceous components, collectively referred to as *Xenopus* chromosome-associated polypeptides (XCAPs). The composition of XCAPs turned out to be surprisingly simple, being composed of the core and linker histones, topoisomerase II (topo II), and five subunits of condensin I (Hirano and Mitchison 1994; Hirano et al. 1997). Following these pioneering studies, a second condensin complex, termed condensin II, was identified in *Xenopus* egg extracts although it was less abundant than condensin I (Ono et al. 2003). It is now known that the two condensin complexes distribute widely in many if not all eukaryotic cells (Hirano 2016; Uhlmann 2016; Kalitsis et al. 2017).

One of the most powerful applications of this cell-free system is the so-called immunodepletion assay. In this assay, a protein of interest is depleted from the extracts using its specific antibody, and then substrate nuclei are added to test how loss of that particular protein affects the chromatid assembly reaction. This in vitro immunodepletion approach contrasts various in vivo depletion methods (e.g., siRNA-mediated and drug-induced transcriptional repression), often producing “clean” defective phenotypes that enable researchers to make their straightforward interpretations and to draw solid conclusions. For example, when sperm nuclei were incubated with egg extracts depleted of both condensin I and condensin II, they completely failed to be transformed into mitotic chromatids, resulting in amorphous cloud-like masses of chroma-

Figure 1. Two different cell-free assays for chromosome assembly in *Xenopus* egg extracts. (A) When demembrated *Xenopus* sperm nuclei are directly incubated with a mitotic extract, they are transformed into single chromatids without the process of DNA replication. (B) Sperm nuclei are first incubated with an interphase extract to allow nuclear envelope formation and DNA replication. Cyclin B is then added to convert the cell cycle state of the extract into mitosis, resulting in the assembly of duplicated chromosomes. Temporal orders of chromatin binding of condensins I and II in each assay are shown at the bottom of each panel.

tin (Hirano et al. 1997; Wignall et al. 2003; Takemoto et al. 2006; Shintomi and Hirano 2011). This result convincingly demonstrated that condensins I and II are indispensable for chromatid assembly during mitosis. Furthermore, individual depletion experiments showed that condensins I and II play nonoverlapping functions although the contribution of condensin I is dominant over that of condensin II in this cell-free system (Ono et al. 2003; Shintomi and Hirano 2011).

BALANCING ACTS OF CONDENSINS I AND II

Xenopus egg cell-free extracts can also be used to assemble “duplicated” chromosomes, in which a pair of sister chromatids are juxtaposed with each other. To this end, unfertilized eggs are treated with a calcium ionophore to mimic fertilization, and they are crushed by centrifugation to prepare an “interphase” extract (Shintomi and Hirano 2017). When sperm nuclei are incubated with this interphase extract, the nuclear envelope is assembled around them, in which a single round of DNA replication takes place and the physical linkage between replicated chromatids (i.e., cohesion) is created by the action of cohesin (Fig. 1B). Addition of cyclin B into the reaction mixture then triggers entry into mitosis, where bulk cohesin is dissociated from chromosome arms and condensin II accumulates on each chromatid axis. Upon nuclear envelope breakdown, condensin I gains access to chromatin and promotes the assembly of fully compacted chromosomes (Shintomi and Hirano 2011). This spatiotemporal regulation of condensins is reminiscent of that observed in mammalian tissue culture cells (Hirota et al. 2004; Ono et al. 2004; Gerlich et al. 2006). Thus, the duplicated chromo-

some assembly assay represents a more physiologically relevant reaction than the single chromatid assembly assay where the ordered action of condensins I and II cannot be assured (Fig. 1A).

Immunodepletion experiments clearly demonstrated that condensin I makes a greater contribution to chromosome assembly compared with condensin II in the duplicated chromosome assembly assay, as had been shown in the single chromatid assembly assay (Shintomi and Hirano 2011). To gain further insight into the functional differences between the two complexes, a protocol was devised that made it possible to precisely manipulate the levels of condensins I and II in the egg extracts. For example, when the original ratio of condensin I to II (5:1) in the extracts was changed to 1:1, shorter and thicker chromatids were assembled. This series of manipulation experiments indicated that condensin II primarily acts to promote axial shortening of chromatids, whereas condensin I acts to compact them laterally. Thus, the shape of chromosomes is determined by an exquisite balance between condensins I and II. This conclusion has been supported by a subsequent *in vivo* depletion study using chicken DT40 cells (Green et al. 2012).

CONDENSIN-MEDIATED CHROMATID AXIS FORMATION

How do condensins contribute to the assembly of rod-shaped chromatids? Condensins I and II were enriched along the central axes of chromatids (Ono et al. 2003), and perturbation of condensin functions by antibody addition converted rod-shaped chromatids into random-coiled chromatin masses (Hirano and Mitchison 1994). These

Figure 2. Reconstitution of mitotic chromatids with six purified factors. Deduced roles for each factor in the whole reaction and the resulting (intermediate) structures of chromatin are indicated. In short, nucleoplasm evicts sperm-specific proteins (SPs) from sperm chromatin, and Nap1 loads histone H2A–H2B onto preexisting histone H3–H4. FACT destabilizes nucleosomes to support subsequent actions of topo II and condensin I, the latter of which needs to be phosphorylated by cyclin B–Cdk1.

observations suggested that the formation of condensin-positive axes is a key step for linear organization of mitotic chromatids. This notion was addressed by a recent study using a panel of recombinant condensin I complexes in the single chromatid assembly assay (Kinoshita et al. 2015). Although the wild-type holocomplex efficiently rescued defective phenotypes observed in an extract depleted of endogenous condensins, ATPase mutant complexes failed to do so, demonstrating that the ATP-binding and hydrolysis cycle is essential for proper actions of condensin I. Interestingly, a mutant “subcomplex” lacking CAP-G, one of the two HEAT-repeat subunits, produced highly characteristic shape of chromatids, in which a discrete axis positive for this subcomplex was surrounded by hazy chromatin loops. In contrast, another subcomplex lacking CAP-D2 produced a poorly organized chromatin structure with no axes. Together with other results, it was proposed that the CAP-G and CAP-D2 subunits of condensin I have seemingly antagonistic impacts on chromatid axis formation, and that balancing actions of these two subunits support proper assembly of mitotic chromatids. More recent experiments under a different setup have demonstrated that condensin II also participates in chromatid axis assembly by collaborating with condensin I (Shintomi et al. 2017), details of which will be discussed later.

Topo II was also enriched along chromatid axes. Although depletion of topo II impaired the early step of the single chromatid assembly process, it was possible to remove this protein from chromatids without disrupting their overall morphology once their assembly was complete (Hirano and Mitchison 1993). Moreover, although budding yeast topo II could functionally rescue a *Xenopus* egg extract depleted of endogenous topo II, it failed to display continuous, axial localization along chromatids (Shintomi et al. 2015). Thus, although topo II plays a vital role in ongoing chromatid assembly process (most likely by catalyzing removal of entanglements between chromatids), its contribution to the structural maintenance of chromatid axes remains not fully understood.

RECONSTITUTION OF MITOTIC CHROMATIDS WITH PURIFIED FACTORS

As mentioned above, the single chromatids assembled in mitotic egg extracts had exhibited a very simple protein composition (Hirano and Mitchison 1994). Importantly, no factor other than condensin I or topo II had been shown to be required for chromatid assembly in this cell-free

system. It should be also noted that the linker histone B4 was found to be dispensable for such a reaction (Ohsumi et al. 1993; Maresca et al. 2005). These pieces of information prompted us to recapitulate the assembly reaction using a limited number of purified factors in vitro. *Xenopus* sperm nuclei contained core histone H3–H4 and highly basic, sperm-specific proteins (SPs) (Shechter et al. 2009b). Previous studies had shown that nucleoplasm removes SPs from sperm nuclei (Ohsumi and Katagiri 1991; Philpott and Leno 1992) and that the histone chaperone Nap1 loads core histone H2A–H2B onto preexisting histone H3–H4 to assemble octameric nucleosomes (Shintomi et al. 2005). We therefore initiated our attempt by mixing *Xenopus* sperm nuclei with a cocktail of five factors (H2A–H2B, nucleoplasm, Nap1, topo II, and condensin I), which turned out to be unsuccessful. We then biochemically fractionated egg extracts and identified the missing factor as another histone chaperone FACT (Shintomi et al. 2015), thereby establishing a protocol in which mitotic chromatids could be reconstituted in vitro with only six purified factors (Fig. 2).

The reconstitution system was instrumental in further addressing cell-cycle regulation of mitotic chromatid assembly. Five out of the six factors used were recombinant proteins, and no considerations had been given for their cell cycle-dependent modifications. The only exception was condensin I, which had been purified as a phosphorylated form from mitotic egg extracts. When a nonphosphorylated form of condensin I purified from interphase egg extracts was used instead, chromatids failed to be reconstituted. Remarkably, addition of the mitotic kinase cyclin B–Cdk1 fully restored the reconstitution reaction (Shintomi et al. 2015), demonstrating that the Cdk1 phosphorylation of condensin I is the sole posttranslational modification important for chromatid assembly, at least in the current setup. Cdk1 phosphorylation had been shown to stimulate the positive supercoiling activity of condensin I in vitro (Kimura et al. 1998; St-Pierre et al. 2009), implicating that condensin I-mediated manipulation of DNA topology could underlie large-scale mitotic chromatid assembly.

NUCLEOSOME DYNAMICS DURING CHROMATID ASSEMBLY

Although the core histones account for approximately half the weight of the whole protein components of mitotic chromatids (Hirano and Mitchison 1994; Ohta et al. 2010),

to what extent they might directly contribute to large-scale chromatid assembly had largely been unknown. This was mainly because conventional experimental systems had not allowed researchers to manipulate the level of histones or nucleosomes at will. By using the chromatid reconstitution assays, however, it became possible to deposit any pairs of recombinant H2A and H2B (variants and mutants) along the entire genome. It was found that, among the histone H2A–H2B dimers tested, only the combination of amino-terminally truncated versions of H2A.X-F (an embryo-specific variant of H2A; Shechter et al. 2009a) and canonical H2B supported successful chromatid reconstitution (Shintomi et al. 2015). This result provided us with two implications. First, deleting the amino termini of H2A and H2B might bypass potential requirements for posttranslational modifications of the corresponding regions in the current setup. Second, unique characteristics of H2A.X-F (e.g., its extended, acidic carboxyl terminus) could weaken or destabilize interactions between histones and DNA, consequently facilitating the productive action of topo II and/or condensin I on nucleosome arrays.

A similar argument may also be applied to the action of the histone chaperone FACT, as recent structural studies suggested that FACT facilitates nucleosome reorganization by partially disrupting histone–DNA interactions (Hondele et al. 2013; Kemble et al. 2015; Tsunaka et al. 2016; Valieva et al. 2016). It is therefore reasonable to speculate that FACT confers structural flexibility on nucleosomes in the chromatid reconstitution reaction. Collectively, large-scale assembly of mitotic chromatids is likely to be dependent on the dynamic nature of the nucleosomes.

CHROMATID AXIS ASSEMBLY WITHOUT NUCLEOSOMES

Although the chromatid reconstitution system had revealed the hitherto underappreciated importance of nucleosome dynamics in large-scale chromatid assembly, it remained unknown whether nucleosome assembly per se is an essential prerequisite for this process. To address this question, we made a simple modification to the single chromatid assembly assay using the *Xenopus* egg cell-free extracts; mouse sperm nuclei, which barely contain all core histones (Brykczynska et al. 2010), were used as a substrate instead of *Xenopus* sperm nuclei. It was first confirmed that mitotic extracts have the ability to support H3–H4 deposition on mouse sperm DNAs, followed by full nucleosome assembly, further converting them into a cluster of rod-shaped single chromatids (Shintomi et al. 2017).

The use of mouse sperm nuclei allowed us to block the whole process of nucleosome assembly by depleting the histone chaperone Asf1 from the egg extracts (Ray-Gallet et al. 2007). Although virtually no nucleosome was assembled on mouse sperm DNA as expected, we were surprised to find that mitotic chromatid-like structures were built under this condition (Shintomi et al. 2017). The resultant “nucleosome-depleted” chromatids were composed of DAPI-dense central axes and fuzzy “loop” regions surrounding them (Fig. 3, DAPI). The axes were positive for topo II and condensins, and the overall structures were sparser and more fragile than normal nucleosome-containing chromatids. No protamine was detectable in the nucleosome-depleted chromatids as well as in the control chromatids (Fig. 3, Protamine-1), excluding the

Figure 3. Immunofluorescence analysis with an antibody against protamine. Demembranated mouse sperm nuclei were incubated with mock-depleted and Asf1-depleted egg extracts for 3 h at 22°C to assemble nucleosome-containing chromatids (Δ mock) and nucleosome-depleted chromatids (Δ Asf1), respectively. The resultant chromatids, along with the original sperm nuclei (right column), were labeled with anti-protamine-1 (HAL Technologies, Mab-001; bottom row). DNA was counterstained with DAPI (upper row). The results clearly show that protamine present in sperm nuclei is completely displaced from DNA during the chromatid assembly reactions, regardless of the presence or absence of nucleosomes.

possibility that residual protamine contributed to the assembly of this unique structure.

It is intriguing to note that the nucleosome-depleted chromatids assembled in the *Xenopus* egg cell-free extracts were reminiscent, in terms of both their morphology and protein compositions, of the “chromosome scaffold,” a substructure obtained after chemical treatments of mitotic chromosomes (Laemmli et al. 1978; Earnshaw et al. 1985; Saitoh et al. 1994; Shintomi et al. 2017). Close comparison of the two structures prepared under completely different conditions will be of great help to get deep insights into the roles of condensins and topo II in chromatid axis formation. The peculiar morphology of the nucleosome-depleted chromosomes was also in good accordance with Kruitwagen et al. (2015), who used yeast genetics to address functional cross talk between nucleosomes and condensin. Taken together, these results converge on the very simple view that nucleosomes compact chromatin, whereas condensins shape chromosomes.

FUNCTIONAL CROSS TALK BETWEEN NUCLEOSOMES AND CONDENSINS

To gain deep insight into functional cross talk between nucleosomes and the two different condensin complexes, Asf1 depletion was combined with condensin I depletion or condensin II depletion (Shintomi et al. 2017). Each reaction produced characteristic morphology of chromatids and condensins’ anomalous localization, as summarized in Fig. 4. In short, double depletion of Asf1 and condensin I did not significantly affect the axial distribution of condensin II, whereas depletion of Asf1 and condensin II severely impaired the action of condensin I. These observations clearly showed that nucleosome

Figure 4. Chromatids assembled from mouse sperm nuclei with egg extracts depleted of condensins and/or Asf1 in different combinations. Their presumed structures, along with the localization of condensin I (yellow ovals) and condensin II (blue ovals), are depicted. The thick and thin lines indicate nucleosome and non-nucleosome fibers, respectively.

Figure 5. Schematic presentation of the proposed functional cross talk between nucleosomes and condensins. Details are described in the text.

assembly is an essential prerequisite for condensin I-mediated compaction of chromatin loops, whereas condensin II’s ability to form chromatid axes is largely independent on nucleosomes (Fig. 5). This scheme is also consistent with the results from the reconstitution assay demonstrating that the productive action of condensin I relies on proper assembly of nucleosomes (Shintomi et al. 2015).

The functional cross talk described above raises a pair of new questions. First, why does condensin I appear to function more efficiently on nucleosomal DNA than on non-nucleosomal DNA? Although early studies proposed the model that the nucleosome itself serves as a “receptor” for targeting condensins to chromatin (Liu et al. 2010; Tada et al. 2011), accumulating lines of recent evidence from diverse experimental approaches support the idea that condensins’ binding to chromosomal DNA is nucleosome-independent (Piazza et al. 2014; Zierhut et al. 2014; Shintomi et al. 2015, 2017; Toselli-Mollereau et al. 2016). One of the plausible scenarios is that condensin I first gets targeted to nucleosome-free regions of chromosomal DNA and initiates looping of their surrounding nucleosomal regions, possibly by imposing positive superhelical torsion (Kimura and Hirano 1997; Hirano 2014) or through an extrusion mechanism (Goloborodko et al. 2016). In either case, mechanical responses to the proposed action of condensin I would be substantially different between nucleosomal and nonnucleosomal DNAs.

Second, does condensin II indeed have an ability to form chromatid axes independently of nucleosomes and, if so, how? It has been proposed that formation of consecutive loops could be sufficient to bring distantly located condensins together and to accumulate them in axial regions (Goloborodko et al. 2016). An alternative, but not mutually exclusive, possibility is that intermolecular attractions between multiple condensin II complexes, possibly through their HEAT-repeat subunits, help to generate crowded environment at the central part of chromosomes, leading to the formation and stabilization of chromatid axes (Yoshimura and Hirano 2016). Given the amphiphilic and highly flexible nature of the HEAT-repeat subunits, it will be of great interest to consider potential mechanistic parallels between chromatid axes and phase-separated organelles made by intrinsically disordered proteins and other factors (Hyman et al. 2014).

CONCLUSION AND FUTURE CHALLENGES

In the current manuscript, we have summarized the history and development of the *in vitro* assays for mitotic chromosome assembly. Recent major breakthroughs in this approach include the successful reconstitution of mitotic chromatids using purified factors *in vitro* (Shintomi et al. 2015) and the demonstration that nucleosomes can be dispensable for building chromosome-like structures under certain conditions (Shintomi et al. 2017). These studies strongly suggest that the mechanism of mitotic chromosome assembly may be much simpler than previously thought and further emphasize the central importance of condensins in this process. Despite the exciting progress, a number of outstanding questions remain ahead of us.

It has become increasingly clear that the condensin complexes are very elaborate molecular machines. As discussed in the previous section, understanding how condensins might work at a mechanistic level remains one of the most important challenges in the years to come. Could condensins act as a putative loop extruder (Goloborodko et al. 2016)? How could gigantic protein complexes such as condensins function in a crowded environment of the interior of chromosomes (Hihara et al. 2012; Yoshimura and Hirano 2016)? To address these questions, it is worth testing the functions of recombinant condensin II and its mutants in the cell-free add-back assay and in the chromatid reconstitution assay. Also, there is much to be learned about the exact function of topo II in the process of building mitotic chromosomes. For instance, we need to keep in mind that we still know very little about how topo II acts on nucleosomal DNA (Salceda et al. 2006) or how it collaborates with condensins (Baxter et al. 2011).

There remains room for further modification and refinement of the first-generation chromatid reconstitution system (Shintomi et al. 2015). It will be possible to further advance the reaction by supplementing it with additional factors such as linker histones, chromatin remodeling factors, and condensin II. Equally important, some agents that mimic macromolecular crowding in crude extracts could further improve the reconstitution reaction (Hancock 2012). Moreover, we must admit that we are still largely ignorant of ion atmospheres (Mg^{2+} , Ca^{2+} , Na^+ , and K^+) that contribute to large-scale chromosome assembly *in vivo* (Mathieson and Olayemi 1975; Strick et al. 2001; Hudson et al. 2003; Phengchat et al. 2016; Ono et al. 2017). The reconstitution system will be instrumental in sharply addressing this issue with an unprecedented precision. In addition, the use of mouse sperm nuclei as a substrate will allow us to engineer H3–H4 as well as H2A–H2B in the reconstitution assays and provide us with an opportunity to learn much more about nucleosome-mediated regulation of chromatid assembly.

Finally, it should be noted that the current *in vitro* assays rely solely on the morphology of chromosomes as judged by light microscopy. Only limited efforts have been made to analyze the *in vitro* assembled chromosomes by electron microscopy (Konig et al. 2007). It will be of great interest to analyze them, for instance, by recently developed electron microscopy (EM) tomography, known as

ChromEMT (Ou et al. 2017). There is no doubt that genome-based approaches such as Hi-C techniques (Nau-mova et al. 2013; Kakui et al. 2017; Lazar-Stefanita et al. 2017; Schalbetter et al. 2017) and mechanical stretching approaches (Almagro et al. 2004; Yan et al. 2007; Xiao et al. 2012) will also provide valuable information regarding the architecture and physical properties of the *in vitro* assembled chromosomes. Powerful combinations of the sophisticated *in vitro* assays and the emerging analytical technologies will provide us with a key to unlock one of the most important questions in biology: How is a centimeters-long genomic DNA folded into a micrometers-long chromosome?

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Keishi Shintomi and Tatsuya Hirano

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