Kinetic control of eukaryotic chromatin structure by recursive topological restraints

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SUMMARY

Chromatin structure undergoes many changes during the cell cycle and in response to regulatory events. A basic unit of chromatin organization is the nucleosome core particle. However, very little is known about how nucleosomes are arranged into higher-order structures *in vivo*, even though the efficiency and precision of cell division imply high levels of structural organization. We propose abandoning the current paradigm of chromatin organization based on thermodynamics of the lowest energy state and replace it with the idea of a topologically restrained, high-energy structure. We propose that DNA is subject to a recursive topological restraint, and is anchored by hemicatenates that are part of the chromosomal scaffold. Long-distance *cis*-regulation of transcription is a natural consequence of recursive topological restraint. This new theory of

chromatin structure has a multitude of consequences for key aspects of cellular biology.

INTRODUCTION

In all higher Eukaryota, mitotic cell division proceeds through virtually the same steps at the macroscopic level and in terms of the proteins involved (Francis, 2007). Decades of studies have established the basic facts about chromatin: DNA being wrapped around the nucleosome cores (Luger et al., 1997), multiple origins of replication (Costa and Blow, 2007), lack of any interpretable nucleosomal arrangements when using *in situ* electron microscopy (Maeshima and Eltsov, 2008), and, nevertheless, chromatids being organized with a single DNA sequence advancing along their axis. The very regularity of large-scale structures during mitosis and lack of entanglement between DNA chains require underlying, robust organization, which has escaped elucidation until now (Belmont, 2006).

The discussion of chromatin structure (Maeshima and Eltsov, 2008; Tremethick, 2007; van Holde and Zlatanova, 2007) has been so far dominated by an implicit assumption that higher-order structure of chromatin is formed by the thermodynamically-driven association of nucleosome core particles and other chromosomal proteins. In this view, which we refer to as the associative paradigm, chromatin is in a lowest energy state, which is inherently stable unless conditions are severely perturbed. By definition, such thermodynamic states have no memory of how they were achieved, while epigenetics requires a form of such

memory to exist. Epigenetic mechanisms discussed in the literature involve covalent modification, either of DNA by methylation or of histones by many known modifications. Both routes create memory routes that have serious limitations or faults. DNA methylation patterns are not conserved even in Metazoa (Mandrioli and Borsatti, 2006), so they cannot be the principal driver of highly conserved epigenetic mechanisms, e.g. cell differentiation. Single nucleosomes are unstable even on the timescale of one second (van Holde and Zlatanova, 2006), so memory of histone modifications requires extremely stable higher-order structures that would stabilize individual nucleosomes. Here we arrive at the underlying paradox in the current understanding of chromatin structure: cellular processes require a high level of chromatin organization, while the associative paradigm of chromatin assembly would make such structures stable and easy to observe, which is not the case (Supplemental Discussion 1 and 2).

Another unsolved problem is how the dynamic and flexible chromatin structure avoids DNA entanglement without any observed barriers between chromatids (Branco and Pombo, 2007). Avoiding entanglement cannot be driven thermodynamically, since tangled and unmixed states can differ by just minimal structural changes, without significantly affecting their energy. As for chromatin protein complexes, even the largest are much smaller than the scale of the chromosome, and so lack the ability to identify the necessary spatial reference on that scale. Also, non-equilibrium sources of motion, e.g. ATP-driven translocases (Flaus et al., 2006), lack both spatial and sequence landmarks that

could be used to avoid intermingling of chromatids by designating a particular DNA fragment to its segregated localization. In higher Eukaryota such landmarks cannot be invoked, because if they existed, they would have a very strong genetic signature, and such a signal has never been observed. The conclusion is that, within the existing paradigm of chromatin organization, there is no route to avoid entanglement.

It has been recently recognized that ongoing studies of chromatin structure increase the number of contradictions and that even the most basic assumptions need to be re-analyzed (Belmont, 2006). In this spirit, the main idea we pose here is the need to abandon the associative paradigm of chromatin organization, and to replace it with kinetically-imposed structural restraints. The flexibility of such topological restraints allows the structure to be dynamic and to have many geometric forms. This change of paradigm allows the constrained structure to be a high-energy state that is defined by the history of its construction and rebuilding. In contrast to structures subject to the associative paradigm of assembly, our proposed basic structure unravels after only a single double strand DNA (dsDNA) break, and for this reason may not have been noticed in typical chromatin experiments.

Two main questions need to be addressed: 1) what types of structural restraints organize chromatin structure and 2) how are such topological restraints imposed and maintained with very low error level? Many types of topological restraints have been already considered as possibly existing in eukaryotic chromosomes (Supplemental Discussion 3), but they are insufficient to explain the higher-order

chromatin structure and observed chromatin dynamics. Any explanation has to consider that individualization and resolution of chromatids are fast and essentially error-free, with even the seemingly inconsequential error of sister chromatid exchange happening at the rate of only 10^{-10} per base pair (Gutierrez et al., 1983).

The main idea we introduce defines how nucleosomes are packed into a basic linear structure, which we shall call a chromosome filament. One argument for the postulated filament structure comes from observations of long-distance *cis*-transcriptional-regulation in Eukaryota. Such regulation requires a continuous DNA chain between a regulatory element and the start of transcription. In the associative paradigm, the only approach to explain *cis*-regulation involved mechanistically *trans*, through-space interactions. The only *cis* aspect of that explanation is the local concentration increase of the *cis*-acting transcription factors, which fits poorly with their observed low sequence-specificity. In contrast, we propose a structure with intrinsic structural interdependencies resulting in long-range *cis* effects. Subsequently, we explain how the filaments are controlled by their end structures, and we make several arguments why hemicatenates (Gaillard and Strauss, 2006) are involved there. Finally, we discuss the association of filaments into a large-scale chromosomal structure and how it may be responsible for the observed cell cycle dynamics.

RESULTS

INTRODUCTION OF THE RECURSIVE TOPOLOGICAL RESTRAINT

We consider a novel type of dsDNA-based topological restraint that involves recursion and that can be created without breaking or unwinding the double DNA helix, and so does not directly involve topoisomerases. This restraint is based on extruding a dsDNA loop through one of previously made dsDNA loops, in a manner analogous to knitting or crocheting (Fig. 1). The stability of such structures is based on linear recursion: the topological stability of one loop is a consequence of its being extruded through another topologically stable loop. The starting and ending points of the recursion involve other types of topological or metastable restraints, which anchor the entire structure. Notably, such topologically restrained structures allow for internal rearrangements redistributing any tension. Crocheted or knitted fabrics have an obvious (but hard to formalize) property that allow them to emerge from a washing machine as individual pieces, in contrast to bundles of unknitted yarn, which would become a tangled mass. Since spatial separation requires cohesive organization of an object and not necessarily its maximum compaction, our model considers individualization and condensation as separate events.

FORMATION OF THE RECURSIVE TOPOLOGICAL RESTRAINT

Chromatin remodeling complexes containing SWI2/SNF2-like ATPase domain (Flaus et al., 2006), work by extruding DNA loops out of nucleosomes (Lia et al., 2006; Strohner et al., 2005; Zhang et al., 2006; Zofall et al., 2006). To create a knitted structure, it is sufficient to spatially and temporally coordinate this activity, so that loops are extruded one through another in the proper order. Multi-subunit chromatin remodeling complexes (Aalfs and Kingston, 2000) can coordinate loop

extrusions by covalently modifying and structurally entangling histone tails. The size of these complexes, in excess of 20 nm, allows for the extrusion of a loop to be coordinated with structural features in its closest environment. Repeating the extrusion of a loop through a loop made earlier establishes a recursive restraint. In this process many possible patterns could be created, analogous to these of knitted fabrics, but it is not yet possible to assign particular patterns to different types of chromatin. One of the simplest possible arrangements with 22 nm width that agrees with EM data (Caravaca et al., 2005; DuPraw, 1965; Rattner and Hamkalo, 1978), consists of two rows of nucleosomes with DNA strands wrapped around each nucleosome extruded through a dsDNA loop made two nucleosomes earlier in the sequence (Fig. 2).

In traditional associative models, a chromatin sub-structure could locally unfold randomly, due to thermal and mechanical movements. Such unfolded structures could associate with other chromatin strands and not necessarily fold back to their original conformation. In addition, any opening of higher chromatin structures during mechanical stretching would make a single unit of dsDNA the weakest link. Conversely, the proposed recursively restrained structure has relatively high resistance to mechanical stretching, since external tension is redistributed along the intertwined dsDNA strands (Fig. 2C) wrapped around nucleosomes, and relaxing the tension allows the structure to revert to its original state. Thus, a thermodynamically driven randomization process is not possible in the structure that we propose, obviating the problem of intermingling nucleosomal strands.

To create the proposed knitted structure, one ATP molecule is hydrolyzed for every one to three DNA base pairs of the extruded loop (Hopfner and Michaelis, 2007). This amount of free energy is high to create tight dsDNA bends (Fig. 2C). Due to the energy stored in these bends, the knitted structure has significantly higher energy than a topologically unrestrained one. As long as the end restraints hold and the dsDNA is continuous, this energy will be stored indefinitely. When the restraint is relaxed, the stored energy will drive the unwinding process needed for replication, transcription and repair. Likewise, a dsDNA break will also result in unwinding the structure on a scale limited by the end restraints, with huge consequences for interpreting experimental data.

A structure formed by a recursive restraint can be unraveled by reversing the operations that created it. If the structure is symmetrical with respect to both its ends, it can unravel from either end (Fig. 2), whereas in asymmetrical structures the unraveling would have to proceed in the direction opposite to the way the structure was formed. Many biological functions may require the unraveling of topological restraint and asymmetry would introduce additional steps in otherwise unidirectional processes such as transcription. Because we cannot find evidence for such additional steps, we propose the simplest symmetrical structure of a recursive restraint (Fig. 2C).

Since even a single dsDNA break in the filament would result in uncontrolled unwinding of the structure, we expect the length of the filament to be limited by end restraints to a size consistent with the repair mechanism capabilities. In our

model, the hemicatenate-based end restraints defining the filament size belong to the next, higher level of chromatin organization.

HIGHER LEVELS OF CHROMATIN ORGANIZATION

One of the previously proposed higher levels of chromatin organization involves chromosomal loop structures 100-1000 nucleosomes long (Paulson and Laemmli, 1977). The observed properties of eukaryotic replication suggest that these loops may be replication units (Dijkwel et al., 1979). It has been proposed that they are joined at their bases into a chromosomal scaffold (Adolph et al., 1977), the structure and dynamics of which still remain enigmatic (Belmont, 2006). Our description of such a structure expands the idea of hemicatenates (Fig. 3) as DNA-based topological restraints involved in the formation of the chromosome scaffold (Gaillard and Strauss, 2006), together with scaffold-bound proteins (Mirkovitch et al., 1988).

We anticipate a particular form of hemicatenates acting as topological end anchors for the chromatin filaments and, with some additional level of organization, forming the chromosome scaffold. A combination of loop extrusion with hemicatenates allows us to explain the spatial coordination of strand differentiation within the DNA duplex during replication. In one of the possible arrangements, which creates a topological differentiation of strands within dsDNA, either the first or the last extruded loop of dsDNA in the filament inserts itself between two strands of straight DNA belonging to the same chromatid (Fig. 3B). In another possible structure, the straight dsDNA fragment plays the role of

an insert in the extruded loop of dsDNA (Fig. 3C). In our view, these hemicatenate-based DNA loop structures, spatially coordinated, form the long-sought chromosomal scaffold, the properties of which are regulated by large protein complexes (ORIs, cohesin, lamins, HMGB1, topoisomerase II, p53, Polycomb-group, SATB1) known to recognize topologically distinctive structures of DNA (Zlatanova and van Holde, 1998). These hemicatenate-loop structures are very likely to be nuclear matrix attachment regions (MARs). MARs are located at the base of chromosomal loops and have high base-unpairing potential (Bode et al., 2006) correlated with high AT content (Liebich et al., 2002), without, however, any other characteristic sequence motifs found in higher Eukaryota. There are indications that an unknown, structural component of the chromosome controls MARs, ORI (Gilbert, 2004) and transcription initiation, and we propose the hemicatenate-based topological construction of the chromosomal scaffold for this role.

So far, the most studied form of dsDNA-based topological restraints are dsDNA knots observed in plasmids, viruses and phages (Arsuaga et al., 2005). Knots were not discussed in the context of eukaryotic chromatin, since the problem of keeping them small enough to be recognized and resolved by topoisomerase II prior to replication was never addressed, and without it, highly complex catenates would form, which would then be hard to unravel during the chromatid separation. However, strain inherent in the proposed chromatin filament could provide a force that would keep dsDNA knots tight enough to be recognized by protein complexes. We cannot entirely exclude the possibility of dsDNA knots

being involved in organizing a specific form of chromatin, but we do not expect chromatin to be primarily organized by them. Hemicatenates have a number of advantages over dsDNA knots as topological constraints organizing chromatin. Bases around a hemicatenate are paired, keeping its structure tight, so it can be recognized as a special form of DNA. Hemicatenates can migrate when a force provided by the chromatin remodeling complex is applied; without it, however, they should stay in a particular area of DNA sequence, characterized by high unpairing potential. Replication of a hemicatenate without its prior unraveling by a topoisomerase would create a simple catenate between sister chromatids, which may be a desired outcome, as catenates are known to keep sister chromatids together (Nasmyth, 2001).

The highest level of the chromatin compaction process during mitosis is the spiralization of chromatids as observed using light microscopy (Boy de la Tour and Laemmli, 1988; Ohnuki, 1968). Along with spiralization and the topologically restrained chromosomal scaffold, another level of organization must exist to explain striking changes in chromatin structure, which occur during transcriptional regulation and the cell cycle. In our model, this third level of chromatin organization is defined by chromosomal loops folding dynamically into more complex structures through the side-by-side association of basic filaments (Fig. 4B). We expect that at least two different schemes of filament-to-filament association exist: one, which involves side-by-side association of consecutive chromosomal loops, and a second, analogous to the Greek-key motif in protein folding (Fig. 4B). We interpret the changes of chromatin organization during

mitosis as predominantly involving transformations between these two organizational schemes. From the beginning to the middle of prophase, side-by-side association of consecutive loops dominates, creating long tubular structures. Each chromatid would create one such structure, with cohesin molecules and DNA catenates densely connecting the sister chromatids along their axes before spiralization. After passing the G2/M checkpoint, the majority of these connections are broken, allowing the chromatids to undergo a change into a large-scale corkscrew shape, leading to metaphase chromosomes. The later compaction into a final mitotic chromosome involves the loss of highly regular, middle-of-prophase organization of chromosomal loops.

We postulate that the filament-to-filament association is stabilized by a novel, protein-based topological restraint, in which threading the histone tail through the nearby nucleosome (Fig. 5) stabilizes association of filaments or a turn within a filament. In this model, histone tails are caught in holes between dsDNA and the histone octamer of another nucleosome during chromatin formation or remodeling, with a covalent modification trapping the tail on the other side of the hole (Fig. 5). Due to their size, ubiquitination and SUMOylation are natural candidates for anchoring tails in another nucleosome. Protein tail-based kind of topological restraints have not been considered to date, in part because it is known that nucleosomes in arrays are not very stable (van Holde and Zlatanova, 2006). In our model, a recursively restrained filament can provide a substantial, internal tension that keeps nucleosomes stable, at least until ionic strength and divalent ions are present in sufficient amounts, allowing covalent modifications of

chromatin proteins to be used as the third type of topological restraint, after knitting and hemicatenates.

DISCUSSION

REPLICATION IN THE CONTEXT OF THE PROPOSED MODEL

Semi-conservative replication has a mechanistic explanation in eubacteria, which start their replication from a single origin (ORI), with chromosomal segregation assisted by a strand-specific sequence signal (Wang et al., 2005). However, the mechanism of semi-conservative replication in Eukaryota and Crenarchaeota, which use multiple ORIs, has not been explained. In these cases, the spatial destination of each of the original strands at all replication forks in the chromosome must be coordinated, so that each original strand paired with a newly synthesized, complementary one, will end up as a distinct entity. To achieve the coordination, the same DNA strand needs to be identified simultaneously in all the ORIs, even if the two DNA strands in a chromatid are wrapped around each other millions of times. We can deduce how this happens without sequence-specific signals from the classic observations of diplochromosomes.

Many experiments of replication mechanism have been performed on cells inhibited so that they could not separate and segregate chromosomes. Such cells passed through another cell cycle and during the next mitosis formed well-ordered diplochromosomes with four chromatids (Fig. 6). The connection network between chromatids created after the first of the two replications prevented the

unwinding of new chromatid pairs created during the second replication. This indicates that unwinding is not used during the initial resolution of sister chromatid pairs. The individualization and resolution without unwinding to facilitate them indicates that during replication the newly synthesized chromatids are moved to their territories in a coordinated manner, sideways relative to their axes (Manuelidis and Chen, 1990).

The spatial pattern of diplochromosomes has 2-fold symmetry, with four chromatids parallel to each other forming only side-by-side interactions. Each of the chromatid pairs related by the 2-fold symmetry originates from the duplication of a chromatid during the last DNA replication. If all four chromatids were identical in a physical sense, the connections by catenation would form either between all possible six pairs or between none of them. The presence of only three groups of connections (Goyanes and Schvartzman, 1981) between the neighboring chromatids of diplochromosomes indicates that chromatids with the same sequence possess characteristics that differentiate them. A distinguishing characteristic of newly synthesized pairs of chromatids is that they are copies of complementary DNA strands, i.e. strands with different sequences. However, introducing labeled nucleotides into the medium during the first cell cycle, but not the second, resulted in the two outward (Fig. 6) chromatids being labeled (Goyanes and Schvartzman, 1981; Schwarzacher and Schnedl, 1966), proving that sequence difference is not the explanation for the segregation mechanism. Irrespectively of which DNA strand was used as a template for the last-cycle

replication, its position was symmetrically equivalent to the position of the other strand (Fig. 6).

While this pattern of labeling was known 40 years ago, the state of knowledge about chromatin did not allow for its interpretation, and later the pattern was assumed to be a rule of nature, without much discussion of the mechanics behind it. These astonishing observations clearly point to the existence of a memory mechanism that identifies which DNA strand in a duplex was synthesized during the last replication, and which one was synthesized earlier. This information is then used during the subsequent DNA replication to spatially coordinate chromatid segregation at multiple replication forks.

Our chromatin model provides a basis for a mechanistic explanation of many experimental observations regarding replication. First, it identifies the hemicatenate-containing structural element of DNA as defining the origins of replication. In higher Eukaryota, the ORIs are spread through the genome and defined in a controlled fashion not directly determined by the sequence (Costa and Blow, 2007; Gilbert, 2004; Remus et al., 2004). The structure of the hemicatenate involves the formation of single-stranded DNA, which facilitates the creation of a pre-priming complex. Proteins known to bind to hemicatenates, e.g. p53 and HMGB1, are also known to interact with ORIs (Stros et al., 2004). Plasmid (Lucas and Hyrien, 2000) and viral (Laurie et al., 1998) replication in Eukaryota involves the formation of hemicatenates.

It is well established that eukaryotic replication has more common features with Archaea than with Eubacteria. Like Eukaryota, the most closely related archeal organisms, Crenarchaeota, have to solve the problem of coordinated segregation of multiple replication bubbles. Remarkably, it has been recently discovered that the ORI structures in *Sulfolobus solfataricus* contain a hemicatenate (Robinson et al., 2007), suggesting that the hemicatenate mechanism is evolutionarily conserved.

INDIVIDUALIZATION AND DNA CONTINUITY AT G2/M CHECKPOINT

The mechanistic explanation of individualization in Eukaryota has been approached from three directions: as a consequence of condensation (Marko and Siggia, 1997), of condensation combined with helical winding (Swedlow and Hirano, 2003) and, in a toy model, as a consequence of threading DNA through condensin-based complexes (Nasmyth, 2001). All three proposed models start from an assumption that is not valid for eukaryotic cells. Condensation is completed after the individualization stage (Leblond and El-Alfy, 1998), so it cannot be the primary factor driving individualization. Individualization errors are subject to strong selection pressure, because consequences of chromosomal translocations are highly negative. If condensation drove the individualization, the selection pressure would be toward maximizing the impact of condensation and so it would be completed by the time of individualization, which is not the case. Helical winding cannot explain individualization because in Eukaryota, unlike in Prokaryotes, the required gyrase activity is not present (Ullsperger and Cozzarelli, 1996). In the third case, a condensin-based complex was proposed in

an illustrative model, accompanied by a discussion why it was a poor candidate (Nasmyth, 2001). Neither condensation nor helical winding can sufficiently explain the extremely low level of errors during individualization. A major difficulty for any explanation of individualization lies in the fact that both light and EM *in situ* observations of chromatin show a lack of separation between the chromatin strands of neighboring chromosomes during interphase (Branco and Pombo, 2006). However, it is obvious that different chromosomes are topologically distinct, even in the presence of erroneous catenates between them. Connections between chromatids do not prevent individualization and resolution: the initial resolution of sister chromatids occurs in spite of the presence of a large number of catenates between them, and diplochromosomes form even if their parent chromatids are joined by catenates (Goyanes and Schvartzman, 1981). Individualization and resolution in the presence of catenates is so difficult to account for in the traditional, associative models of the chromosome, that the issue has not been even discussed.

The literature lacks an explanation of a mechanistic link between dsDNA continuity defined on a protein-size scale, and the observed continuity of individualized chromatids on a scale almost a thousand times larger. The recursive topological restraint is a unique mechanism of propagating continuity conditions between these scales by the recursive nature of the restraint rather than its exact topology. Our model of chromatin organization emphasizes dsDNA continuity, as this is necessary to preserve the topological restraint. The self-threading filament structure would make the entanglement of chromatin

originating from two different chromosomes much less likely, due to the filament's smoothness and elasticity preventing the formation of tension knots. The tubular arrangement created by simultaneous, side-by-side association of chromosomal loops, proposed to form during the G2/M checkpoint, explains the distributive character and the consequent speed of the individualization.

After passing the G2/M checkpoint, the cell makes an irrevocable commitment to go through the rest of mitosis (Mikhailov et al., 2005). The breaking of the majority of connections before the final spiralization of chromatids is an irreversible operation, involving the decatenation of DNA linkages between sister chromatids and displacement of cohesin molecules coupled to the chromosome's structural reorganization, resulting in partial separation of chromatids by hundreds of nanometers. We propose that the formation of individualized and initially resolved chromatids is a necessary and sufficient condition for the G2/M checkpoint, in which the topology of chromatin is verified mainly by a dsDNA continuity check. In a topologically restrained chromatin filament structure, any dsDNA break would release energy stored in this structure, leading to immediate unwinding of the filament up to the point of its end restraint. The unwinding would both trigger of the dsDNA repair mechanisms, causing the delay of the checkpoint, and prevent the loops attached to the chromosome scaffold from organizing into the observed regular, tubular structures.

TOPOLOGICAL CONTROL IN EPIGENETICS AND TRANSCRIPTION

Chromatin structure is central to transcriptional regulation, developmental control and epigenetics; however, the three-dimensional geometry involved in controlling them remains enigmatic. One of the unexplained phenomena is the mechanism of action and inheritance of *cis*-acting elements. In the existing view of chromatin structure, it is difficult to understand how *cis*-acting elements execute their action when separated from the transcription start by thousands or even a million of base pairs (Lettice et al., 2002) and how they can be inherited, given highly dynamic nature of protein factors. The most striking and yet unexplained example of *cis*-control is co-linearity of chromosomal location of genes critical for development and of their expression positions along anterior-posterior axis (Burke et al., 1995). The fundamental departure of the recursive topological restraint idea from the associative proposition is that recursion propagates control of the three-dimensional state to the end anchors of the restrained structure. That means that protein factors associated with the end anchors can exercise their control over the whole topologically restrained segment without any direct interactions with its interior. Since these end anchors are presumed to involve hemicatenates, they are inherently stable even though their associated protein factors are dynamic. Moreover, the inheritance of such topological restraints at anchor points during DNA replication is mechanistically plausible given the complexity of ORI mechanism. *Cis*-control at a distance is then a natural consequence of recursive topological restraint. Within that idea, the developmental control would require only a mechanism of precise repositioning of end anchor restraint at an appropriate moment. Repositioning would involve a

synchronized action of chromatin-remodeling translocases and topoisomerase(s), for which there are many possible candidates. Repeated repositioning of such a restraint in the same direction during serial formation of metamers in chordates would explain the co-linearity between chromosomal location of Hox genes and their activation in metamers. The proposed hemicatenate-based boundaries of transcriptionally active chromatin would correspond to chromatin insulators, which have many of the characteristics agreeing with hemicatenate properties in our model, e.g. DNAse I hypersensitivity, scaffold-like organization and interactions with nuclear lamina in MAR-like fashion (Brasset and Vaury, 2005; Capelson and Corces, 2004).

In vertebrates, Hox genes form strongly conserved clusters, featuring not only spatial but also temporal co-linearity (Crawford, 2003; Kmita and Duboule, 2003), which suggests the involvement of not yet defined biological clock mechanisms. We propose the existence of a novel type of clock mechanism, based on repositioning of the transcription control elements, most directly indicated by metameric oscillation that controls Hox genes (e.g. in chick embryos, the period of metameric oscillation is 90 minutes and the oscillation lasts for about three days (Pourquie, 2004)). Co-linearity of Hox gene controls has many independent manifestations: it shows simultaneously in four clusters, with homologous genes being synchronized (Crawford, 2003); the genes are switched on in groups that correspond to a linear segment in the genome; the genes are transcribed from the same strand of DNA; the position of the first gene to be switched on in a

segment is advancing linearly with the consecutive formation of metamers, creating patterns of temporal and spatial co-linearity.

The simultaneous appearance of all these co-linearities and their strong preservation in evolution has a natural explanation in what we propose here: that it is driven by unidirectional repositioning of a particular hemicatenate, which is the master Hox control element. Metameric oscillation drives the repositioning of hemicatenates controlling Hox genes involved in the development of anterior-posterior axis, and afterwards the control advances at much more sporadic times. The repositioning may happen either by a unit of uniform length or by advancing to a new sequence landmark. Temporal co-linearity is also observed for many other developmental gene clusters and typically involves long time periods. What is still unknown is what triggers the putative repositioning of a transcription control in such cases. We speculate that it may involve multi-level chromatin timers, where advancing a timing element in one region of the genome to its end triggers two actions: a rebooting of that timer and an advance of a different, higher-scale timer by one clock unit. Such timers could cascade providing the ability to measure arbitrarily long periods of time.

Transcription has to involve other levels of control, probably associated with different stages of chromatin topological organization. The repositioning of hemicatenates explains only the relocation of insulator elements, without addressing the mechanism of unravelling the structure between insulators. We propose the likely possibilities for some of the steps that occur during transcription by RNA polymerase II.

An early step in enabling transcription requires the removal of DNA topological linkage between the insulator and the filament knit. At this stage the filament is not yet unravelled due to some other, presumably protein-based anchoring. This first step could be accomplished with topoisomerase II (Ju et al., 2006), without changing the structure of the hemicatenate. To prevent DNA from tangling, we expect that the filament unravels only just prior to the passage of RNA polymerase II, and it is recreated afterwards (Andersson et al., 1982). All these steps presumably involve the chromatin-remodelling complexes also destroying and forming the previously described protein-based topological restraints (Fig. 5). This view agrees with the start of transcription by eukaryotic RNA polymerase II involving complex chromatin structure changes (Li et al., 2007) and requiring many more steps than the equivalent process in prokaryotes.

EVOLUTIONARY CONSIDERATIONS

Chromatin structure and cell cycle transformations of chromosomes are remarkably similar in plants and animals. Considering that they diverged very close to the last common eukaryotic ancestor (Cavalier-Smith, 2002), the chromatin structure of higher Eukaryota must be the ancestral state, even for organisms in which chromatin is differently organized today (Livolant and Bouligand, 1978). For example, in budding yeast a number of cell cycle simplifications occurred, with concurrent reduction of genome and chromosome size, with the largest chromosome having only 1.5 Mb. In many organisms, including mammals, sperm cells lost nucleosomes in their highly regularly arranged chromatin. We still expect that such chromatin has all the levels of

topological restraints present, since it can easily revert to normal chromatin upon fertilization. Dinoflagellates evolved hierarchically coiled chromosomes, which lack nucleosomes during the whole cell cycle. Long-known EM images of their striking chromosomal pattern inspired the extension of hierarchical coiling idea to other Eukaryota. Based on the precise large-scale organization of dinoflagellate chromosomes, we expect them to be built around multilevel topological restraints as well.

SUMMARY

The body of observations on individualization has provided us with the main assertions leading to the proposed hierarchy of chromatin structure. Any proposed mechanism of individualization must explain how erroneous mixing between DNA fragments, either from different chromatids or from distant parts of the same chromatid, is avoided. Traditional, implicitly associative models do not resolve this problem at any level of structural hierarchy. Our model solves the problem of entanglement at the lowest level by invoking a recursive topological restraint of the basic chromatin filament to prevent the association of different DNA strands. The springy character of the filament reduces the formation of tension knots that could potentially stop individualization. At a higher level of the structural hierarchy, we address the unexplained appearance of chromatids as long, tubular structures during the initial stage of prophase. To explain how the interphase chromatin proceeds to such shapes, we propose that filaments are organized by additional topological restraints. The second level of restraint is based on hemicatenates, which anchor loops created from the basic filament

along the chromatid axis. At the third level of restraint, we propose that filament loops interact side-by-side, with the possible involvement of additional non-scaffold hemicatenates (Fig. 3A). Highly regular side-by-side interactions of loops create long tubular structures that undergo spiralization after the G2/M checkpoint, when most regular aspects of side-by-side interactions are lost in order to accomplish maximum chromatin compaction. This last stage is achieved with the help of protein factors, in particular condensin and histone H1; however, experiments show that such protein factors are not necessary for individualization, even if they improve its efficiency (Supplemental Discussion 3). The involvement of hemicatenates is needed not only to form the chromosome axis; without them it would be hard to explain the observed order of diplochromosomes and the synchronized, distributive character of sister-chromatid resolution.

SUPPLEMENTAL DATA

Supplemental Data include Supplemental Discussion and Supplemental References.

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FIGURE LEGENDS

Figure 1. The simplest form of recursive topological restraint based on dsDNA. Starting from unrestrained dsDNA loops (left), the second loop is extruded through the first loop. Extruding the third loop through the second one and then the fourth loop through the third one leads to a crocheted structure. For clarity, the dsDNA wrapping around histone cores is not shown in the first three views. The right view shows a structure that includes histone cores. The figure depicts a recursive topological restraint simpler than the structure we expect to exist.

Figure 2. The chromatin filament structure. (A) dsDNA-based recursive topological restraint of a knitted type and its schematic representation showing twofold symmetry. (B) The simplest form of symmetric structures with two rows of loops threaded through each other. We show a schematic representation of the topology of such a basic chromatin filament. Nucleosomes in neighboring rows are translated 1/2 repeat along the filament axis. Color is used only to visualize which strand in a crossing pair is above and which is below. The structure is fivefold intertwined, with two up and down loops contributing four units, and the fifth being the connecting segment (shown in orange). (C) The dimensions of the basic filament are shown with the nucleosomes present. The real structure is expected to be more compact that the one visualized in this panel, with the periodic unit of one nucleosome and its linkers being about 200 base pairs. The DNA strands were loosened here to better show the details of topology.

Figure 3. Hemicatenates as structures differentiating strands within dsDNA. (A) The simplest form of hemicatenate, in which two fragments of dsDNA separated in sequence are interlocked. The single DNA strands from both fragments that are interlocked (pink) are topologically differentiated from strands that do not create catenates (blue). (B) A second type of hemicatenate. If we consider only the structure of hemicatenate on the right side of the dotted line, it cannot differentiate the strands within dsDNA, as both blue and pink strands can potentially have the same interactions with a fragment of dsDNA passing between them. However, introducing an unknot, as presented on the left side of the dotted line, differentiates single DNA strands involved in this structure. Additionally, the hemicatenate stabilizes the structure of unknot, since the unknot structure unravels without topoisomerase involvement in the absence of hemicatenate. (C) An alternative form of interaction between the hemicatenate and the unknot, with the same consequences as in (B).

Figure 4. Higher levels of chromatin folding. (A) Chromosome scaffold with loops built from the basic filament. (B) Two possible modes of loops associating into tubular structures, which are further compacted into higher-level structures. The cross-sections of possible folds (roll structure, Greek key structure or comb-like structure) are presented for the one tube mode or double-roll structure for the two- tube mode. (C) Spiralization of a squeezed tube structure into the final chromatid. Note that these pictures are not drawn to scale. In particular, we expect consecutive loops in (B) to be very close to each other, even as close as when nucleosomes are placed on top of each other.

Figure 5. The third, protein-based level of topological restraint in chromatin organization. (A) Nucleosome structure (PDB code: 1KX5) is presented in two views, together with ubiquitin (PDB code: 2ZCC) and SUMO1 (PDB code: 2UYZ), known to covalently modify histone H2B tail. Histone H3 is shown in red, histone H4 is shown in green, histone H2A is shown in blue, and histone H2B is shown in yellow. (B) Some holes between histone core and DNA strands present shown in detail. These particular holes extend through both dsDNA strands wrapped around the nucleosome core. Other types of holes are harder to visualize, as they bend around one of the dsDNA helices. Both types of holes are large enough to accommodate an extended polypeptide conformation. (C) How a protein modification may lock two nucleosomes together with the histone H2B tail being modified.

Figure 6. Formation of diplochromosomes. Complementary strands within chromatids are labeled A and B, with the subscript corresponding to the replication cycle when the strand is synthesized and with the asterisk signifying labeling. Diplochromosomes are formed when cells proceed through spiralization of chromatids (A1*B0 and A0B1*) without detaching the chromatid pairs, and do not proceed through anaphase and cytokinesis. After the first replication, the cell doubles its ploidy level, with every chromatid being labeled on one strand. This cell can undergo another round of replication without segregation of chromatids, resulting in chromosomal quadruplexes. In quadruplexes connections exist only between three pairs of chromatids, creating the characteristic, planar pattern with two-fold symmetry that is observed both in plants and animals. If the label is not present in the medium during the second replication, the resulting pattern will contain two labeled chromatids in outside positions of the quadruplex. This pattern indicates the existence of a mechanism of coordinated differentiation of strands within chromatids.

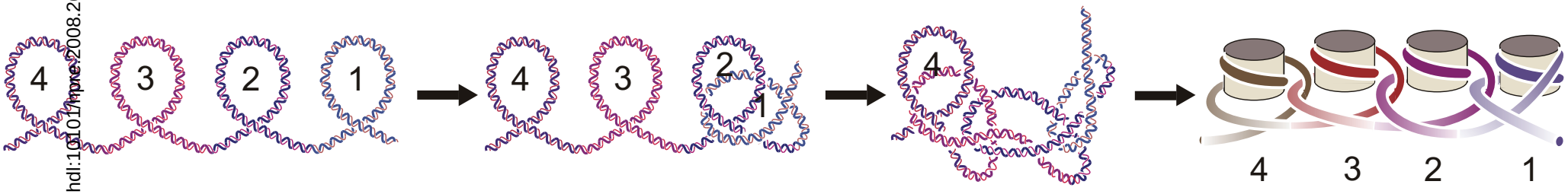


Figure 1

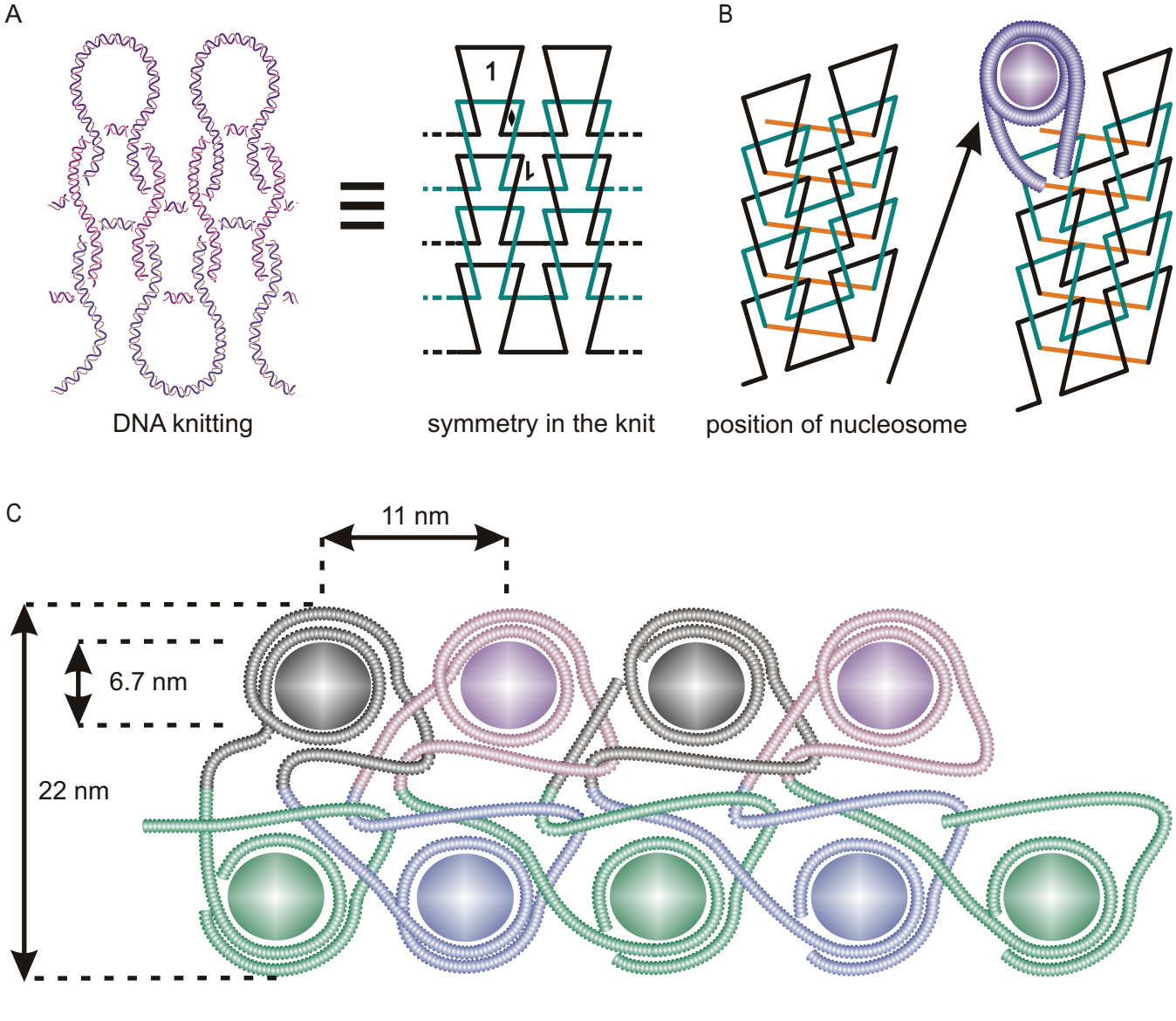


Figure 2

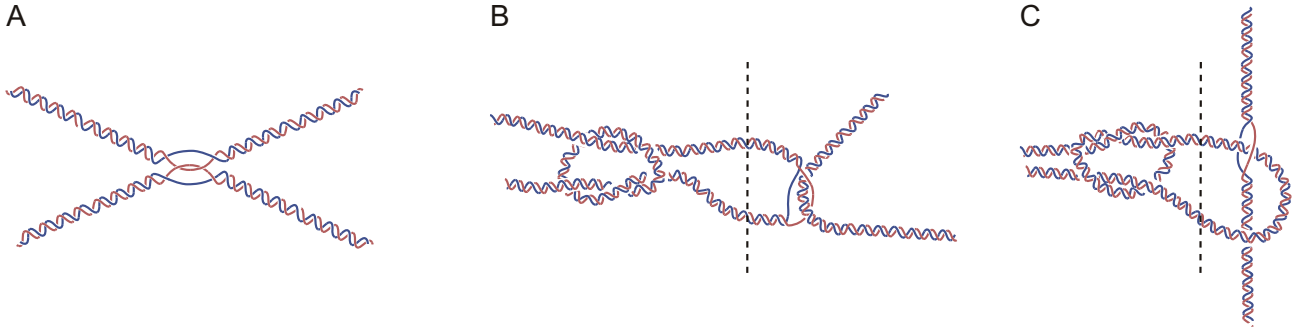


Figure 3

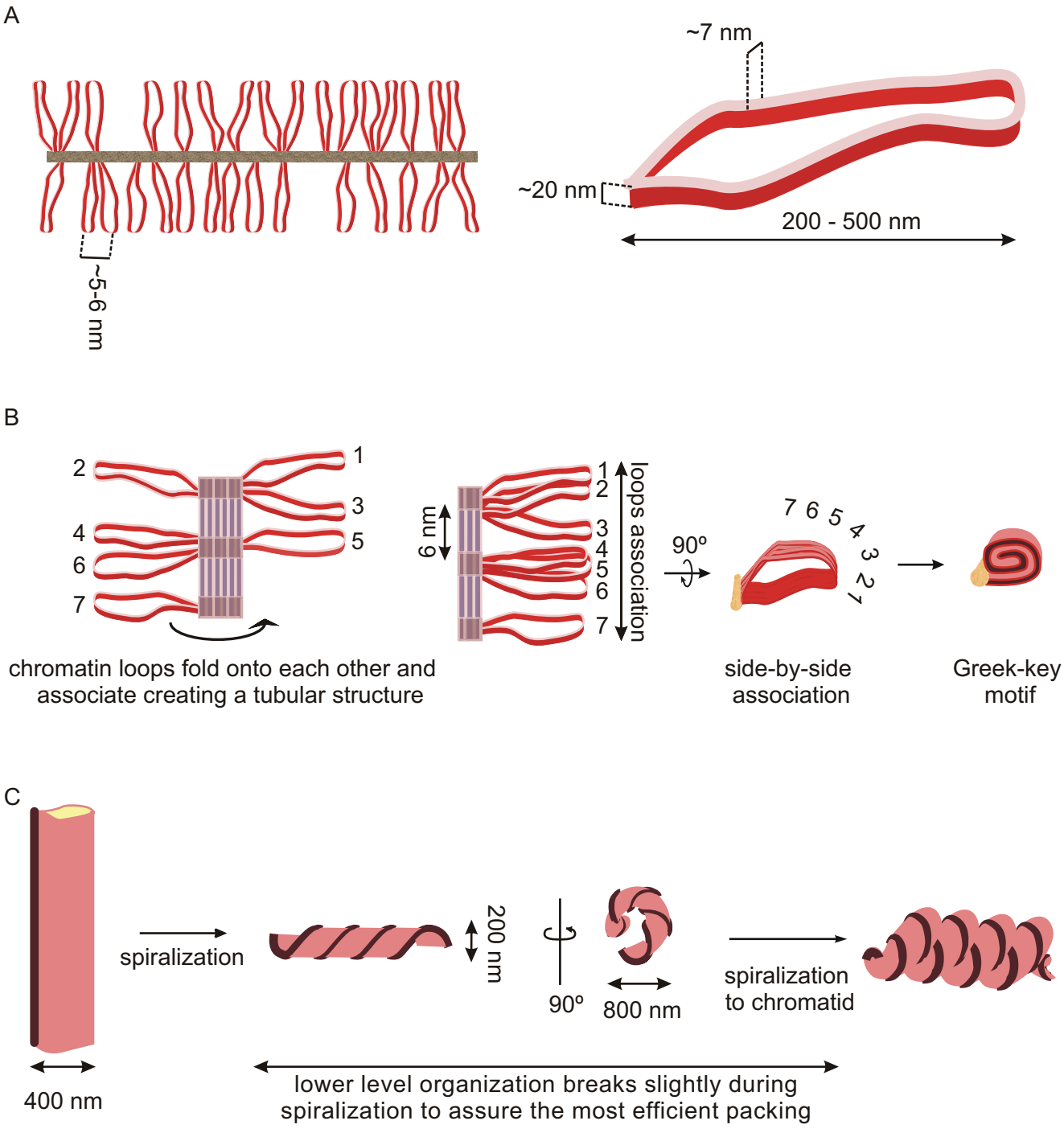
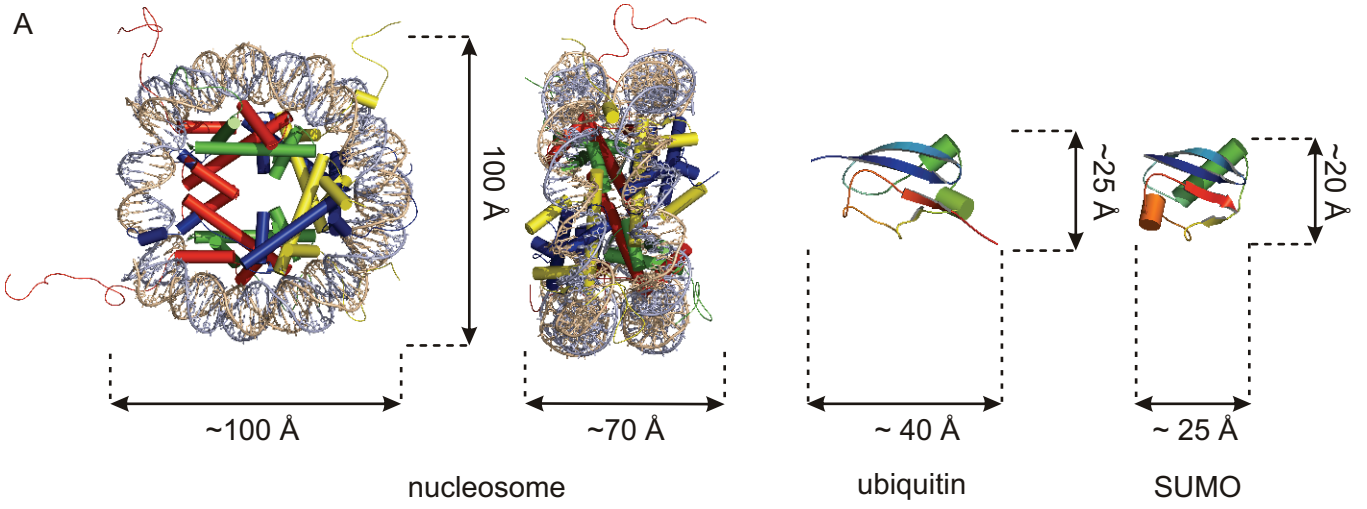
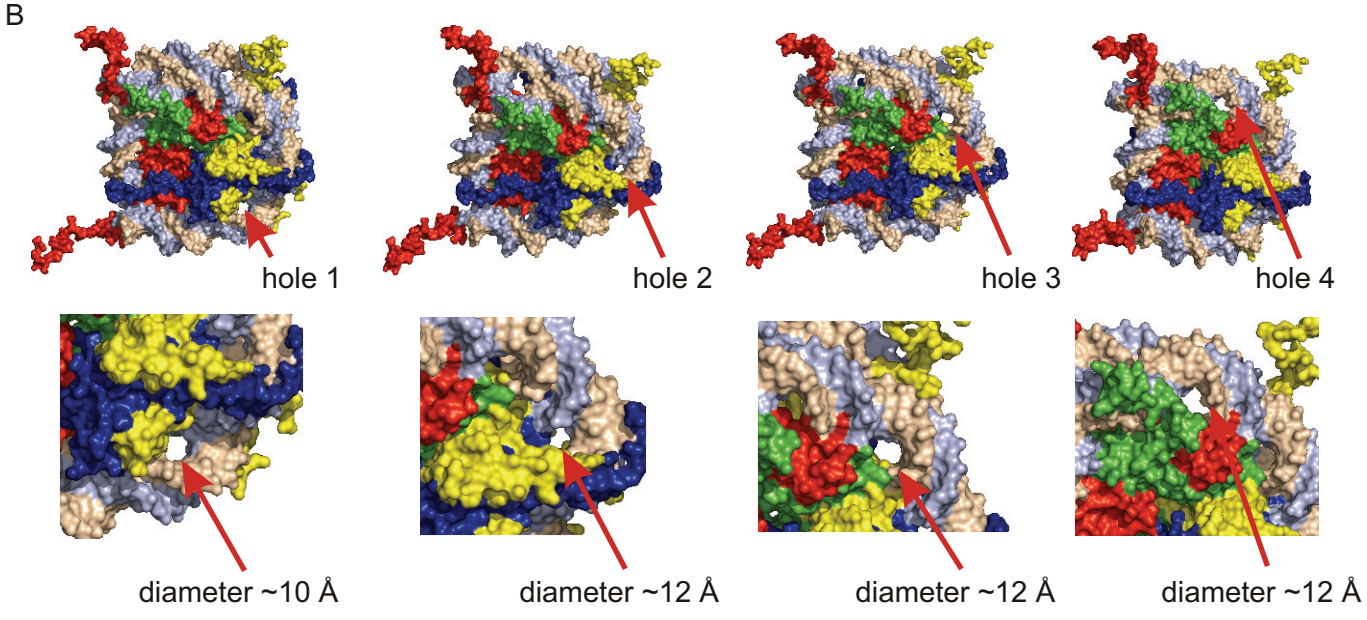
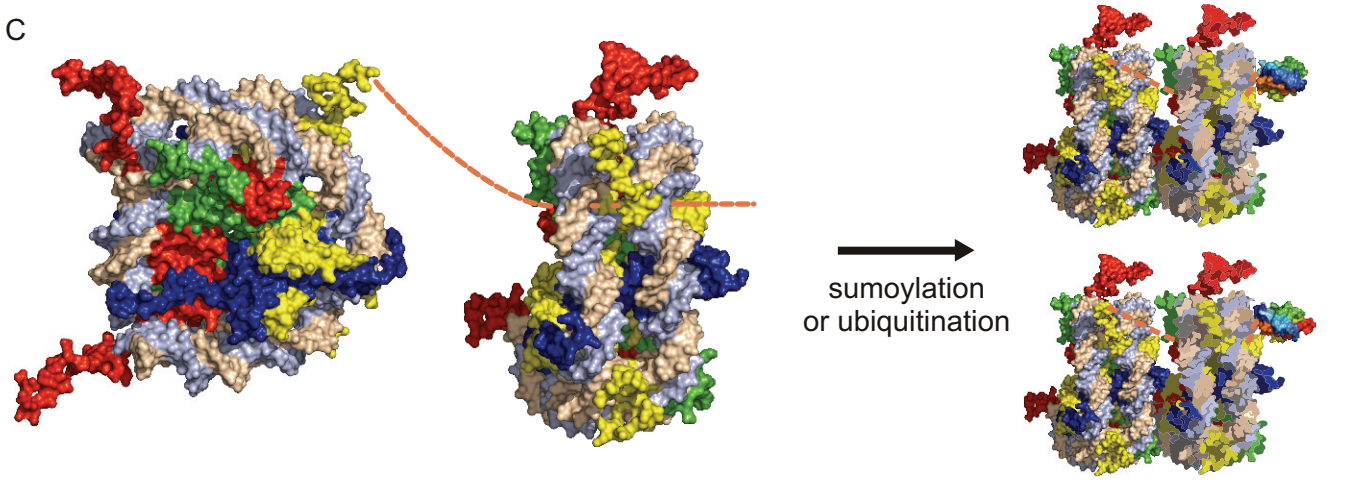


Figure 4







the tail of one of the nucleosomes treads through the hole in another nucleosome

the modifications of tail treaded through hole in neighboring histone restrain the orientation of two histones

Figure 5

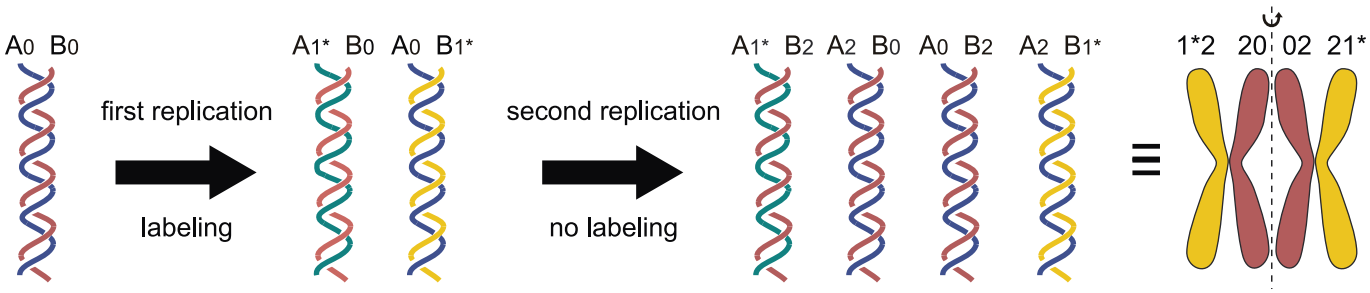


Figure 6

Supplemental Data

Kinetic control of eukaryotic chromatin structure by recursive topological restraints

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SUPPLEMENTAL DISCUSSION

S1. PREVIOUS MODELS OF METAPHASE CHROMATIN STRUCTURE

The basic features of all currently discussed models were introduced by DuPraw in 1965 (DuPraw, 1965). The chromatid structure in this model was built from a single 23 nm fibril, coiled irregularly into elongated chromosomes. At that time, the nature of the 23 nm fibril could not be even addressed, as the nucleosome structure was not known. With time, the DuPraw's model got refined and modified in a number of directions, to include newly acquired microscopic, biochemical and genetic data, and the fibril width changed to 30 nm. However, the models still imply the association between nucleosomal units as the main source of chromatin organization. Three categories (Daban, 2000) of

chromosome structure models have been discussed: models containing loops, hierarchical helical models, and models built from irregular substructures. In these models, the basic nucleosomal strand folds into 30-nm fiber of helical symmetry, repeating a single nucleosomal unit in a solenoid (simple helical fiber) model (Finch and Klug, 1976) or a double-start helical fiber model (Worcel et al., 1981), or repeating a double nucleosomal unit in a two-start twisted model (Williams et al., 1986). Some authors considered zigzag-type models of basic fiber (Bednar et al., 1998), but barely addressed the issue of what could keep such loose conformation organized and densely packed (Adkins et al., 2004).

The next, intermediate level of chromatin organization is where the structure has been described only in the most schematic terms, so it is not easy to classify the proposed models. We shall consider the differences between them in terms of symmetry of geometric arrangements. Some of the models assume helical symmetry at the intermediate levels, with basic 30-nm fiber helically coiled into thicker helical fiber in radial-array model (Manuelidis and Chen, 1990) or hierarchically coiled in the rosettes model (Filipski et al., 1990). Similar concepts were described by other authors using different schematic pictures (Cook, 1995; Pienta and Coffey, 1984). Other models follow the approach of DuPraw and assume asymmetrical, not well-defined assemblies of loops, either organized by anchoring in a core, scaffold structure in the radial loop model (Marsden and Laemmli, 1979; Sheval and Polyakov, 2006) or only in general terms stating hierarchical folding of chromosomes (Belmont et al., 1989; Kireeva et al., 2004; Wanner and Formanek, 2000). The last stage of organization, supercoiling into a

metaphase chromatid, is either an explicit part of, or at least can be made consistent with, all the above models.

However, all these models were results of interpreting particular types of experiments rather than an attempt to propose a structure that would explain all, or at least a majority, of the published data. One of the main problems with these models is that the structures proposed were either vague, starting with the original DuPraw model, or, being superficially specific, like the rosettes, they lacked support in EM images. These models attempted only to describe the geometry of chromatin, barely addressing the mechanisms that would produce the described structures. Regular, high-order structures, postulated in some models, were never observed by electron microscopy, and no explanation was provided of how irregular, intermediate-level structures can fold into well-defined shapes of mitotic chromosomes.

S2. METAANALYSIS OF EXPERIMENTAL DATA ON CHROMATIN STRUCTURE

High interest in the determination of chromatin structure resulted in a large number of observations by electron microscopy published over more than 50 years. There was a significant effort to study chromatin under native conditions *in situ*, but it did not result in an interpretation of nucleosomal arrangements. Alternatively, the partially unfolded chromatin was employed to gain insight into its possible substructures. Many aspects of chromatin preparation can impact such observations: the continuity of the DNA in chromatids, the pH of solutions used and their ionic strength, with particular significance of divalent ions concentrations (Giannasca et al., 1993), the use of cross-linking agents (Goyanes et al., 1980), protein denaturing fixatives, intercalators, RNAses, DNAses (Abuelo and Moore, 1969) and proteases (Abuelo and Moore, 1969; Goyanes et al., 1980). Moreover, the chromatin structure organization changes during the cell cycle, from the least organized in S-phase to the most organized in prophase, and the most studied – and most condensed – in metaphase. Additionally, some cell types incapable of division are clearly unique in terms of having pronounced, fibrous chromatin substructures. Together, all these factors contributed to an overwhelming lack of consistency in the interpretation of visualized substructures. Among others, the following substructures were described: multi-stranded (multineme) chromosomes (Callan, 1992), fibers of widely varying size and regularity (Abuelo and Moore, 1969; Adolph, 1980; Barnicot, 1967; Belmont et al., 1989; DuPraw, 1965; Goyanes et al., 1980;

Laughlin et al., 1982; Rattner, 1986; Rattner and Hamkalo, 1978b; Ris, 1966; Stubblefield and Wray, 1971; Wanner et al., 1991; Zentgraf and Franke, 1984), tubular structures (Bak et al., 1977; Earnshaw and Laemmli, 1983; Stubblefield and Wray, 1971), plates (Caravaca et al., 2005), rosettes (Okada and Comings, 1979) and granules (Hozier et al., 1977; Lafontaine and Chouinard, 1963; Zentgraf and Franke, 1984). Due to the great variety of results, the starting point for metaanalysis are the observations of chromatin studied under the most native-like conditions.

The least disturbed structure is the one observed by electron microscopy performed on cryo-vitrified, intact, unstained cells (Bouchet-Marquis et al., 2006; Konig et al., 2007; McDowall et al., 1986; McEwen et al., 1998). These studies, conducted over more than 20 years, including the use of tomography with nucleosome-level resolution, concluded only that chromatin has a mottled or finely granular appearance. The most recent research described chromatin in prematurely condensed chromosomes as forming "aligned arrays" (Konig et al., 2007), without, however, visualizing the DNA connecting nucleosomal particles, which prevented a detailed interpretation. The aligned arrays seen on a larger scale can be also described as having mottled appearance, consistent with previous observations. No helical substructures on the scale of tens of nanometers were observed, even though they should have been easily recognized at the data resolution. Together, these studies have not produced a “positive result”, i.e. a model of how chromatin threads through space, so they left little impact on the wider perception of the problem.

A very broad group of electron microscopic observations are *in situ* experiments involving fixation and staining of cells. They typically show a grainy image of chromatin that could also be labeled as having mottled appearance, without prominent features pointing out to any type of chromosomal arrays at the scale below a hundred of nanometers. Lack of resolution is definitely not the limitation in these experiments, since very regular chromatin structure with periodicity of 4.8 nm has been observed for condensed bacterial chromosomes (Frenkiel-Krispin et al., 2001). Densely packed chromatin in cells not capable of division, and thus never undergoing individualization, has been observed to have highly regular structure. In sperm cells that have densely packed DNA, very regular arrays of chromofilaments with dimensions around 7 nm and spaced with 18 nm periodicity were already observed in 1957 (Yasuzumi and Ishida, 1957). Regular, columnar chromatin structures with spacing of 36 nm were also observed in erythrocytes (Zentgraf and Franke, 1984), a type of cells disposing of the nucleus in their development. However, even for those cells, DNA could not have been traced in these structures.

As *in situ* experiments did not provide the hoped for information, another approach applied was to study isolated chromosomes. To loosen the chromosome structure, low salt conditions were typically used. The experiments show bunches of intertwined, twisted and coiled fibrils ranging from 3 to 50 nm, with the most common range of 10 to 20 nm (Barnicot, 1967; Ris, 1966). Most remarkably, after partial trypsin digestion, a 20 nm thread gave an impression that it contained four parallel dsDNA-sized chains (Ris, 1966). These studies

have been followed by a large number of their variants in the following decades. Originally, a strong impression of multi-chain (multineme) construction of chromatid was perceived (Abuelo and Moore, 1969; Barnicot, 1967; Konig et al., 2007; Ris, 1966; Stubblefield and Wray, 1971), even if subsequent experiments and genome sequencing proved that the chromatid consists of a single dsDNA chain. The observation of multi-strand substructures pointed to questions essentially ignored later: whether the observed fibrous chromatin substructures consist of one contiguous DNA fragment or a group of DNA segments separated in sequence, and whether DNA threads back and forth through the fibril rather than advancing in a helical fashion (Ris, 1966).

Another approach to studying the isolated chromosome was to remove all proteins from it using high salt concentration. Such chromosomes formed a characteristic pattern of dsDNA chains winding back and forth over a large area of grid, clearly preserving some aspects of original chromosomal organization, and gave rise to the radial loop models (Paulson and Laemmli, 1977). In some of such spreads particular care was taken to avoid single and double strand breaks in DNA (Mullinger and Johnson, 1979, 1980) and these show many signs of topological restraint presence in chromosomes, among others a strikingly clear visualization of the chromosomal axis represented by the presence of about 30 parallel dsDNA chains (Figures 2, 4 and 17 in (Mullinger and Johnson, 1980)). The bundles of these DNA chains pass through a series of constriction points, where it is impossible to trace the continuity of a particular chain. The constriction points are also the place from which a large number of long side loops originates.

Our interpretation is that DNA chain segments between the constriction points are either part or extension of the matrix attachment region (MAR), with constriction points being the location of the putative hemicatenate restraints. Interestingly, chromosomal loops become much shorter close to telomeres. The peritelomeric regions are also characterized by a thousand times higher sister chromatid exchange (SCE) rates (Rudd et al., 2007). The combination of higher rates of SCE and shorter loops indicates that scaffold elements need to be stabilized by adjoining elements, and telomeres are clearly deficient in this role.

Some observations of isolated chromatin were so peculiar that they left little impact on subsequent discussions of chromatin structure, and yet they are among the strongest indicators of the nature of chromatin organization. These publications were mostly passed by because they didn't fit into the existing paradigm. In a series of articles, a very consistent picture of *Drosophila* and human chromosome, built of long, apparently hollow tubular structures. was presented (Bak et al., 1977; Bak et al., 1979; Zeuthen et al., 1979). The publications did not mention that these structures rarely appeared in preparations of mitotically arrested cells, the point made only in a personal letter of F. Crick to A. Klug (Crick, 1977). We interpret these tubular structures as images of prophase chromosomes, since the same letter states that the cells were not well synchronized, so low-level contamination with prophase cells was to be expected. The peculiarity of these structures raised a suspicion of them being a contaminant; however, we have observed structures of similar, elongated shape,

which are clearly chromosomes, as they stain with a number of DNA-specific dyes.

Another remarkable result was published recently, where plates and other structures were observed in metaphase chromosomes obtained under close-to-physiological ionic strength conditions (Caravaca Guasch, 2004; Caravaca et al., 2005). These plates were only 6-7 nm thick, so they could only be formed by nucleosomes lying flat, in side-by-side arrangement. Also in this work, thin, flat filaments of the same 22 nm width as recursively restrained chromatin filament that we propose, were observed. Some of these filaments were seen to bend smoothly, which would strongly indicate that mechanical tension is being redistributed by a topological restraint over distances on a scale of hundreds of nanometers. We interpret Caravaca's plates as being made by side-by-side filaments association rather than, as suggested in the article, a geometrically impossible proposition of these 6.7 nm thick plates (Caravaca et al., 2005) being built from granules of 35 nm thickness (Bartolome et al., 1994; Caravaca et al., 2005).

Experiments with mechanically disrupted cells showed the linear, ribbon-like arrangements of tightly packed nucleosomes, in which single nucleosomes could be recognized (Rattner and Hamkalo, 1978a, b, c, 1979). The linear arrangements have the width of 20-30 nm; the images suggested side-by-side interactions of 2 to 3 nucleosomes, depending on the position within the ribbon. The authors stated that the observed structures do not agree with either helical fiber-based models or the models assuming hierarchical associations. These

images can be interpreted as the proposed nucleosomal filament that lost some of its nucleosomes, lying flat on the EM grid. It is less clear, and it may vary from image to image, if the recursive restraint is present in the visualized structures.

To loosen the structure of isolated chromosomes, solutions with relatively low ionic power were often used. Such treatment resulted in partial decondensation, unraveling substructures potentially relevant to chromatin organization. These substructures predominantly contained groupings of irregular loops, radiating from the center of a chromatid. In particular, such arrangements were observed in perpendicular slices through the chromosome (Adolph, 1980; Marsden and Laemmli, 1979). The width of the strand forming the loops was reported as 20 nm (Adolph, 1980) or in 20 to 30 nm range (Marsden and Laemmli, 1979). The dimensions 20 - 30 nm were considered in good enough agreement with the dimensions of either short chromatin fragments, purified after nuclease treatment, or reconstituted nucleosomal aggregates; so all these structures were assumed to be the same.

This assumption is, however, troublesome on a number of grounds. The nucleosomal arrays observed *in vitro* forming “30 nm (helical) fiber” (Finch and Klug, 1976; Robinson et al., 2006) have not been seen *in situ* in eukaryotic cells capable of division (Tremethick, 2007; van Holde and Zlatanova, 1995). The fibrils observed in chromatin loosened *in situ* or in isolated chromosomes tended to be closer to 20 nm in diameter, and the reconstituted nucleosomal arrays had diameters 30 to 40 nm (Giannasca et al., 1993; Robinson et al., 2006) and sometimes more. As a compromise, the notion of “30 nm fiber” became popular

(Finch and Klug, 1976), with almost any observed property, e.g. size, diameter, pitch *etc.* of such a structure being highly uncertain (van Holde and Zlatanova, 2007). The only property not questioned was the helical nature of the fiber, made from a single chain of dsDNA, which implied that the ends of the fiber are the most distant in DNA sequence. The assumption of helical symmetry of the 30 nm fiber was restricted to two possibilities: a helix built from one-nucleosome repeat, and a helix built from two-nucleosomes repeat (Robinson et al., 2006). The validation of the previous interpretations was limited to comparing the individual, non-reproducible (in terms of EM image-averaging methods) projection images to a small group of theoretical models. The discussed models ignored any possible arrangements allowing for dsDNA intermingling, even if such geometry were equally consistent with the electron microscopy data. Models that intermingle two distinct dsDNA chains would require only minor adjustment to the positions of DNA linkers compared to a single-chained fiber, and it is known that the lengths of DNA linkers in chromatin are quite variable (Compton et al., 1976). Another ignored possibility was that of a fiber built from a nucleosomal-array hairpin squeezed in a corkscrew shape, with DNA linkers positioned in essentially the same place as in the case of helical fiber made of mixed dsDNA chains. The structures proposed in our topologically restrained model cannot be considered with respect to these data, as reconstitution conditions do not lead to recursive topological restraint and digestion conditions destroy it.

So far, none of the microscopic observations has **directly** provided enough information to create a trustworthy model of chromatin structural hierarchy.

However, both microscopic and biochemical data present patterns indicating the nature of this hierarchy. In many experiments, chromosomes show remarkable resilience to harsh treatment (e.g. boiling in 50% acetic acid (Engelhardt, 2000)), with their global architecture preserved. Experiments with trypsin and proteinase K showed that even if elastic response of chromosomes changed, their overall structure was preserved (Poirier and Marko, 2002a, b; Pope et al., 2006). One interesting feature of all microscopic observations is that if separate nucleosomal particles can be observed in the chromatin substructures, the higher levels of structural hierarchy cannot be identified and, conversely, if higher levels of hierarchy can be seen, the nucleosomes are not visible as separate entities. It is clear that this generally observed pattern is not due to resolution limitations of electron microscopy methods. In our model, such correlation can be explained. We assume that in the native filament nucleosomes are packed so tightly that they cannot be resolved by EM. Potentially there are two aspects impacting the transformation of the proposed filament into structures with visible nucleosomes: one possibility is the loss of a small fraction of histone cores, without losing the recursive restraint; another is the loss of topological restraint. In both cases we expect looser packing of nucleosomes and their repositioning resulting in the detachment of the protein complexes anchored in nucleosomal holes, consequently making the nucleosomes visible.

Another common feature of many electron microscopic experiments is the presence of fibrous structures of various widths, often in parallel bunches (Kume and Maruyama, 1986; Rattner, 1986; Wanner and Formanek, 2000; Wanner et

al., 1991). These structures do not show any signs of internal, on 30 nm or similar scale, helical substructures and, if seen as part of higher-level helical-like arrangements, they appear as a bunch of parallel fibrils coiling up together. We interpret these fibrous structures as bunches of parallel filaments; such bunching could result in observations of thick fibers of essentially any width, unlike hierarchical coiling models that would produce large-scale structures of a particular geometry.

Our model explains why *in situ* observations did not lead to structural models: following the path of DNA in the recursively restrained filament requires higher resolution than available in electron microscopy and most of the higher-level structures that we propose lack sufficient regularity to be recognized without any expectation of what to look for.

One of the aspects of chromatin structural hierarchy is the question how centimeters long dsDNA molecule packs into a micron size chromosome. In post-prophase, nucleosome density in chromosomes is quite high, and it was already noticed that the past chromatin models have problems in achieving the observed density due to complex angular arrangements of nucleosomes generating gaps in spatial packing (Daban, 2003). An advantage of our model comes from the ease of efficiently packing flat ribbon filaments, both side-by-side and on top of each other. In the middle of prophase, chromatid is built from regular arrangements of filament loops related to tubes observed by Bak *et al.* (Bak et al., 1977) This regular prophase structure is twisted during spiralization, with twisting generating a wringer effect. It leads to squeezing out empty spaces and

destroying rather than creating regularity in the resulting structure. High-density packing in general does not require a regular structure, which means that highly condensed state of chromatin can be based on structures without well-defined, visible, regular pattern at the EM resolution.

S3. THE LEVEL OF COHESIN, CONDENSIN, TOPOISOMERASE II AND HISTONE H1 INVOLVEMENT

Some forms of DNA-based topological restraints have already been discussed in the literature, i.e. helical winding, hemicatenates, knots and catenates. Condensin, cohesin and topoisomerase II may create a temporary topological restraint by holding together two distal DNA fragments from the same or different chromatids. A similar restraint can also be created by complexes of multi-strand DNA with proteins (Zlatanova and van Holde, 1998)

All those protein-based structural restraints mechanically stabilize the structure of chromatin, but do not have by themselves activities allowing them to hierarchically organize it (Belmont, 2006). However, the proposed, topological restraints based on DNA can introduce such hierarchy, defining the sites of attachment for topoisomerase II and condensin, with these proteins dynamically modulating the three-dimensional structure of chromosomes during the cell cycle.

Cohesin, condensin and topoisomerase II clearly contribute to the individualization and segregation processes, but the question is: to what extent? Cohesin is a protein complex that forms a ring joining two dsDNA chains of sister chromatids. This bridging structure is created during DNA replication, so recognition of which strands should be kept together by cohesin is determined by the replication complex (Uhlmann and Nasmyth, 1998). The cohesin involvement explains how the chromatids are kept together, but it is not enough to explain how chromatids self-organize into well-defined structures. Topoisomerase II has

three distinct activities: topoisomerase II knotting-unknotting activity, topoisomerase I-like activity that is very efficient in relaxing helical tension (Salceda et al., 2006), and the third one - holding two different DNA strands together (Bojanowski et al., 1998; Earnshaw and Heck, 1985). This last activity is analogous to the functions of cohesin and condensin. Both condensin and topoisomerase II can keep DNA strands together; however, the activity telling them that a particular pair of DNA strands should be kept together, as opposed to a large number of possible but counterproductive connections, has not been identified. Considering the number of broad genetic screens, such activity, were it specific to the cell cycle, should have already been recognized. In particular, we do not expect that any chromosomal proteins (Uchiyama et al., 2005) with unknown functions could be responsible for this critical aspect of individualization. Experimental data show that both topoisomerase II (Carpenter and Porter, 2004) and condensin (Hudson et al., 2003; Vagnarelli et al., 2006) are dispensable for the process of mitotic condensation. Condensin localizes to the chromosome axis after individualization (Kireeva et al., 2004). In individualization, topoisomerase II is required only to perform decatenation, both in the process of the separation of sister chromatids and in the correcting of erroneous connections between chromatids of different chromosomes (Wood and Earnshaw, 1990). Nevertheless, topoisomerase II is a critical protein, localized at the bases of chromosomal loops (Earnshaw and Heck, 1985), involved in transcription and potentially other processes (Zlatanova and van Holde, 1992).

We have not found any indications that histone H1 is involved in the formation of the recursive topological restraint. Histone H1 is often implicated in the organization of chromatin; however, its function is very different from core histones. In some lower eukaryotes, histone H1 is involved in DNA repair and is dispensable for cell division (Downs et al., 2003; Harvey and Downs, 2004); in *Xenopus,* depletion of H1 resulted in individualized, but not spiralized, chromosomes (Maresca et al., 2005). We presume that histone H1 involvement in the late stages of chromatin condensation is due to its role as molecular glue, stabilizing gaps and bends in densely packed filaments. Such a non-orthodox view agrees very well with the observed stabilization of irregular chromatin fibers by histone H1, studied in reconstituted chromatin, and with histone H1 compacting chromatin that have lost topological restraint, and so helping to repair the damaged DNA. Histone H1 role as a molecular glue also explains how such a fast evolving, mostly low-complexity protein can play an important role in the highly conserved process of chromatin organization (Kasinsky et al., 2001). Since we postulate a different role for histone H1 than so far assumed, in this article we do not differentiate the terms ‘nucleosome’ and ‘nucleosome core particle’ and use simply ‘nucleosome’.

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