Helical metaphase chromatid coiling is conserved

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Summary
The higher-order metaphase chromosome organization has been under controversial discussion already for 140 years. Classical light and electron microscopy proposed chromatids to be composed of helically organized chromatin fibers, so-called chromonemata. More recently also non-helical models were suggested. We studied the chromosome organization in barley by interdisciplinary cutting-edge approaches, such as chromosome sorting, chromosome conformation capture, oligonucleotide-fluorescence *in situ* hybridization, base analog incorporation, super-resolution microscopy, and polymer simulation to elucidate the arrangement of chromatids of large mitotic metaphase chromosomes. Our data provide cumulative evidence for the presence of a helically arranged 400 nm chromatin fiber representing the chromonema within the chromatid arms. The number of turns is positively correlated with the arm length. Turn size and chromatin density decrease towards the telomeres. Due to the specialized functions of centromeres and nucleolus-organizing regions, the helical organization is interrupted at these regions, which display several thinners and straight chromatin fibers. Based on our findings and re-analyzing previously published data from other plant and non-plant species we conclude that the helical turning of metaphase chromatid arms is a conserved feature of large eukaryotic chromosomes.

Keywords: chromatin condensation, chromonema, chromosome conformation capture sequencing (Hi-C), flow cytometric sorting, helical chromatid structure, *Hordeum vulgare*, mitotic metaphase chromosome, oligo-fluorescence in situ hybridization (oligo-FISH), polymer simulation, sister chromatid exchange (SCE), structured illumination microscopy (SIM)

Introduction
Cell growth, division and differentiation are accompanied by drastic changes in the chromatin structure to facilitate DNA replication, repair, transcription and segregation. The most obvious changes are observed during mitosis and meiosis when the chromatin of interphase chromosome domains becomes compacted ~1000-fold to form rod-like structures. After replication during S phase of the cell cycle, the chromosomes consist of a pair of genetically identical chromatids (Paulson and Laemmli, 1977; Marsden and Laemmli, 1979; Earnshaw and Laemmli, 1983; Maeshima et al., 2005). Individual chromosomes can most easily be addressed during nuclear division. Except for species with holocentric chromosomes (containing elongated centromeres), most eukaryotes have monocentric chromosomes, characterized by a distinct primary constriction at which spindle fibers attach for proper chromatid segregation during anaphase of nuclear division (Musacchio and Desai, 2017; Schubert et al., 2020). Besides, some chromosomes harbor a 45S rDNA cluster, the nucleolus organizer region (NOR), recognizable as a less conspicuous secondary constriction on metaphase chromosomes (McClintock, 1934; Schöfer and Weipoltshammer, 2018). The long-standing questions, how DNA is compacted into rod-shaped mitotic and meiotic chromosomes, what are the principles of folding, and whether there are differences between species in this respect, will be addressed in this paper.
So far, analyses of the chromatin structure in interphase nuclei revealed a 10 nm nucleosome fiber formed by ~147 bp of DNA wound around the octamer of nucleosome histones. The nucleosomes are connected by linker DNA to form a fiber named “beads-on-a-string” (Thoma et al., 1979). However, little is known about how the nucleosome fiber folds into a higher-order structure. In experiments with purified 10 nm fibers Finch and Klug (1976) observed the formation of a 30 nm chromatin fiber, the so-called solenoid. Nevertheless, its existence in native chromatin has not

been confirmed and there is growing evidence that the 30 nm fiber forms *in vitro* only in solutions with low salt concentration. Several studies across various eukaryotes demonstrated that the higher-order structure of chromatin is formed by an irregularly folded 10-nm fiber (Grigoryev et al., 2016; Maeshima et al., 2010a, b, 2016, 2018, 2019; Nishino et al., 2012). The periodical arrangement of chromatin has been questioned as well. In the study of Wako et al. (2020), the chromatin fibers of different compactness forming human metaphase chromosomes have been analyzed using beam/scanning electron microscopy. Thick 30 nm fibers were rare and thus were excluded as structural units. In contrast to several existing models (Finch and Klug, 1976; Manuelidis and Chen, 1990), no periodical structure was found. Thus, several researchers accept that a 10 nm fiber basically organizes the metaphase chromosome, but the higher-order organization remains obscure.

Based on data collated by a range of molecular and microscopic methods, several models have been proposed to explain the higher-order structure of metaphase chromosomes (Beseda et al., 2020), which can be categorized as helical and non-helical. The helical models assume the arrangement of chromatin as a helix in each sister chromatid at metaphase (Manton, 1950; Ohnuki, 1965, 1968; Iino, 1971; Bak, et al. 1977; Sedat and Manuelidis, 1978; Marsden and Laemmli, 1979; Ris, 1981; Rattner and Lin, 1985a; Nokkala and Nokkala, 1986; Boy de la Tour and Laemmli, 1988; Saitoh and Laemmli, 1994; Sumner, 1998; Inaga et al., 2002; Gibcus et al., 2018; Schloissnig, et al. 2021). On the contrary, non-helical models suggest folding of chromatin within the chromatids without forming a helix (Paulson and Laemmli, 1977; Belmont et al., 1987, 1989; Wanner and Formanek, 2000; Poirier and Marko, 2002; Strukov et al., 2003; Kireeva et al., 2004; Marko, 2011; Naumova, et al. 2013; Daban, 2015; Chu et al., 2020).

The helical structure of chromosomes was first observed in the plant *Tradescantia virginica* L. by Baranetzky (1880) and later confirmed by Ruch (1949). A similar chromatin organization called chromonema (from chromo- + Greek nēma thread) was discovered in other species of diverse plant families after observing native, physically and/or chemically treated chromosomes using light microscopy (Nebel, 1932a, 1939; Manton, 1950; Schvartzman et al., 1978; Nokkala and Nokkala, 1985). Interestingly, such chromatin coiling was not found in *Drosophila* (Strukov et al., 2011) and although Ohnuki et al. (1968) observed helical coiling in mitotic chromosomes of stimulated human leukocytes, such a structure was not observed later by other authors in mammalian cell lines (Strukov and Belmont, 2009; Chu et al., 2020). Furthermore, the helical chromatin arrangement in mitotic chromatids was not identified in three human cell lines using the chromatin conformation capture (3C) methods 5C and Hi-C, which quantify genome-wide chromatin contact frequencies (Naumova et al., 2013). On the contrary, Hi-C data obtained from mitotic cells of chicken (Gibcus et al., 2018) and axolotl (Schloissnig et al., 2021) suggested a helical chromosome organization. After reanalyzing mitotic HeLa cell Hi-C data of Naumova et al. (2013) in more detail by deeper sequencing, Gibcus et al. (2018) revealed a helical structure and suggested that the helical arrangement might be a common feature among vertebrates.

To decipher the higher-order chromatin organization of chromosomes, in addition to classical light and electron microscopy, advanced microscopical, cytological, and molecular techniques can be applied to provide the required sensitivity and resolution to reveal the spatial (3D) chromatin organization of mitotic chromosomes. The application of fluorescence dyes to label specific DNA segments and proteins allowed the development of super-resolution microscopy techniques such as structured illumination microscopy (SIM), a sub-diffraction imaging method bridging the resolution gap between light and electron microscopy (Rego et al., 2012). These nanoscopic methods were applied successfully in cell biology (Fornasiero and Opazo, 2015) and allowed the

discovery of new structures within plant chromatin (Schubert, 2017; Baroux and Schubert, 2018; Schubert et al., 2020).

Fluorescence *in situ* hybridization (FISH) using oligonucleotide probes (oligo-FISH) is an efficient method to identify whole chromosomes and specific regions to investigate karyotype structure, meiotic chromosome behavior, and chromosome evolution via comparative cytogenetic mapping (Aurich-Costa et al., 2007; Han et al., 2015; Braz et al., 2018; Tang et al., 2018; Albert et al., 2019; Bi et al., 2019; Jiang, 2019; Šimoníková et al., 2019; Liu et al., 2020a,b; Xin et al., 2020; Li et al., 2020; Hoang et al., 2021; Rosin et al., 2021). The application of super-resolution microscopy techniques in combination with oligo-FISH enabled the characterization of sub-chromosomal structures (Szabo et al., 2018). To analyze the arrangement of chromatin in sister chromatids spontaneously occurring sister chromatid exchanges (SCEs) can be studied through differential incorporation of DNA base analogs, such as 5-bromodeoxyuridine (BrdU) (Latt, 1973) or 5-ethynyl-2′-deoxyuridine (EdU) (Schubert et al., 2011), into chromosomes during replication (Schvartzman, 1987; Wilson and Thompson, 2007; Schubert et al., 2016b). The method that leads to differential labeling of sister chromatids was called harlequin staining and can be combined with FISH (Perry and Wolff, 1974; Rachel et al., 1991; Jordan et al., 1999).

Recently, 3C techniques were used to determine contact probabilities of genomic loci with different distances in a linear sequence (Lieberman-Aiden et al., 2009; van Berkum et al., 2010; Kempfer and Pombo, 2019; Oluwadare et al., 2019), and uncovered the hierarchical chromatin organization (Oluwadare et al., 2019; Szabo et al., 2019). Together with polymer simulations Gibcus et al. (2018) determined the helical organization of chicken late prometaphase chromosomes with turns of 12 Mb of a 200 nm thick fiber.

To decipher the higher-order structure of plant mitotic chromosomes, we used barley (*Hordeum vulgare* L.; 2n=14; 1C=4.88 Gb) (Doležel et al., 2018), possessing large chromosomes as a model. From a combination of metaphase chromosome-derived Hi-C data, oligo-FISH, sister chromatid exchange detection, super-resolution microscopy, and polymer simulation we conclude that sister chromatids are formed by chromatin helices of identical handedness. The helical turns cover 20-38 Mb creating a ~400 nm thick fiber. Only centromere and NOR regions are free of the helical higher-order organization. Thus, we confirm experimentally the classical findings of a helical organization of somatic chromosomes for a plant species with large chromosomes.

Results

Hi-C indicates that mitotic chromatids are organized as a helix with variable turn lengths

To analyze the organization of condensed chromosomes, metaphase chromosomes of barley were purified by flow cytometric sorting and the sequenced contact pairs obtained by Hi-C analysis were aligned against the barley cv. Morex genome assembly version 2 (Morex v2) (Monat et al., 2019). The Hi-C contact matrix for metaphase chromosomes showed a second diagonal parallel to the main diagonal along the length of the chromosome, which faded in the vicinity of the primary and the secondary constrictions and the telomeres (Figures 1A, S1). The additional diagonal was unique to the metaphase chromosomes and was not observed in interphase chromatin, which only showed a perpendicular diagonal indicative of the Rabl conformation (Mascher et al., 2017).

The differences in chromatin conformation between interphase and metaphase chromosomes were also reflected by the relationship of the Hi-C contact probabilities of loci pairs and their distance in the linear genome (*d*) (Figures 1B, S2). The contact probabilities in interphase showed a uniform

exponential decay (~d^{-1}) from 100 kb to 100 Mb distance (Figures 1B, S2A, B, E). This pattern possibly relates to the reminiscent Rabl conformation present in anaphase.

In metaphase, the distance-dependent-decay rates vary more, and its derivative shows two regions with increased contacts, ~500 kb and 30 Mb (Figures S2C, D, E). Because we expect that the mitotic chromosomes become compacted by chromatin loops (Gibcus et al., 2018), we suggest that the broader peak at ~500 kb arises from an arrangement of nested loops, where the largest loops, around 3 Mb, appear only in metaphase, as indicated by the first peak in the contact probability ratio between metaphase and interphase (Figure S2F). The highest peak in this contact probability ratio, around 30 Mb, the only one with a positive derivative, corresponds to the second diagonal in the contact matrix (Figure 1B).

Gibcus et al. (2018) proposed that this pattern reflects the arrangement of the chromatids into a helix, whose turn length corresponds to the position of the "bump" in Figure 1B. The arrangement into helical turns is schematically presented in Figure 1C, where the spatial distance between genomic loci is proportional to the contact probability. This observation suggested that the helical coiling could be observed along the chromosome axis when differentially labeled (Figure 1D). We estimated that depending on its size, a condensed barley chromosome consists of 18-23 helical turns Figure 1E, Table S1).

Closer inspection of the metaphase contact matrix suggested that the distance between the main and the secondary diagonal is variable. We analyzed turn sizes along the chromosome lengths based on Hi-C using a sliding window approach and considering only contacts spanning a moving focal point. We observed different local patterns. The characteristic bump of the contact probability shifted (Figures S3, S4; Movie 1) as the focal point moved along the chromosome. These different placements of the bump indicate that the turn length varies along the chromosome length.

The turn size decreased smoothly, but non-linearly, from the centromere towards the telomere, with the turn sizes ranging from 20 to 38 Mb. Moreover, the turn length decreased sharply towards the primary and secondary chromosome constrictions (Figure 1E).

Oligo-FISH confirms the helical organization of mitotic chromatids

We employed FISH with pooled oligonucleotide (oligo) probes to validate the results obtained by Hi-C. A search for unique sequences suitable for the probe design by scanning the barley cv. Morex genome assembly version 1 (Morex v1) (Beier et al., 2017; Mascher et al., 2017) with a 40 Mb window revealed a continuous region of ~157 Mb with a high density of single-copy sequences on the long arm of chromosome 5H (Figures 2A, S5). FISH probes comprising pools of ~45 bp oligos were designed to label selected parts of this region and the pooled probes were named Stork, Eagle, Ostrich, Rhea, Moa, and Flamingo for simplicity. The examples of Stork and Moa probes are shown in Figure S6. Based on the Hi-C data and the barley genome v1, Stork and Eagle covered half helical turns, whereas Ostrich, Rhea, and Moa each represented one full helical turn. The Flamingo probe was located at the chromosome terminus, just before subtelomeric and telomeric repeats (Figures 2B, C). The density of single-copy sequences increased towards the telomere (Figure S7) in agreement with the reported higher density of genic and single-copy sequences in distal regions (Mascher et al., 2017).

Subsequent to our probe design, an improved assembly of the barley genome (Morex v2) was published (Monat et al., 2019), resulting in a change of the probe coordinates (Table S2), without noticeably impacting the oligo probe distribution and specificity, but the lengths of the painted intervals changed.

The completeness of the assayed interval, located between 442 and 599 Mb in the Morex v2 5H pseudomolecule, was validated by a genome-wide optical map of barley cv. Morex (Mascher et al., 2021). The interval was covered by three optical map contigs (1, 55, and 17), which were concordant with the sequence (Figure S8). A single discrepancy within the interval was a missing sequence of ~403 kb around the position 491 Mb, detected as unaligned ends of the optical map contigs 1 and 55. This indicates that the real length of the DNA segment labeled by the Ostrich probe is 34.8 Mb.

Three-dimensional structured illumination microscopy (3D-SIM) of chromosomes after FISH with pooled oligo probes (Figure 2D; Movies 2, 3) showed that the chromosome diameter was decreasing towards their termini. This corresponded with the changes in turn size along the chromosome derived from Hi-C analysis. After the Morex v2 assembly was available, we re-estimated the turn coverage of the probes. Stork and Eagle cover ~0.6 of a turn (in a region where the turn size is 29.6 Mb), Ostrich, Rhea and Moa ~1.5 turns (in a region where the turn size is 22.5 Mb), and Flamingo represents a region of ~2.3 decreasing turns (in a region where the turn size is 8.3 Mb). Accordingly, the probes cover less turns in regions where the turn sizes are larger. (Figures 2F, G; Table S2).

The signals of the oligo-FISH probes Stork and Eagle showed an average height of 380 nm. Although the three FISH probes (Ostrich, Rhea, and Moa) represented more than one full helical turn and were labeled by fluorescence dyes delivering a different SIM resolution (laterally ~120 nm for Alexa488 and ~140 nm Atto594) we determined similar dimensions for all three probes (Figures 2H, S9A). On average, the height of the signals of these oligo probes was ~460-480 nm. As Hi-C predicted 21 turns for chromosome 5H (Figure 1E), this corresponds to a chromosome length of 9.0-9.4 µm, which is consistent with our chromosome length measurements (Figure S9A).

Based on these observations, we term the helically arranged chromatin fiber of 400 nm chromonema. A partial intermingling of smaller chromatin loops inside the chromonema was identifiable between neighboring turns of the helix (Figures 2D, S10; Movie 4). This implied an arrangement of chromatin into lower-order loops within the chromonema, and the intertwining may be caused by the dynamic properties of the loops. Remarkably, the orientation of the FISH signals was always identical in both chromatids, indicating that both chromonemata were turned with the same handedness (Figure S11).

The volumes of the different oligo-FISH signals and the whole chromosome 5H were determined after surface rendering of the 3D-SIM image stacks (Figures 2I, 6C, S9B; Movie 5). In order to avoid an artificial flatting of chromosomes after preparation, isolated chromosomes were embedded in polyacrylamide and imaged by 3D-SIM after DAPI staining. These spatially preserved chromosomes showed ~1.2fold higher volumes than those dried on slides for subsequent FISH (Table S3). The volume of an embedded 5H chromatid (~599 Mb) was ~24.75 μm^3, corresponding to ~24.2 Mb/μm^3, i.e., 1 Mb chromatin is folded into ~0.041 μm^3. In the Flamingo-positive chromosome region, the Hi-C data indicated a smaller turn contact pattern (Figures 2F, G). The volume of the Flamingo signal, the only one bigger than expected for a uniformly dense chromosome (Figures 2I) suggested a lower chromatin density (DNA amount per μm^3). Compared to Flamingo (19.5 Mb/μm^3), the regions of the Stork (32.0 Mb/μm^3), Eagle (34.3 Mb/μm^3), Ostrich (38.2 Mb/μm^3), Rhea (34.0 Mb/μm^3), and Moa (32.0 Mb/μm^3) probes were ~1.7 times more condensed. Thus, the terminal Flamingo region was characterized by lower chromatin density and smaller turn size. The flexibility of the chromatin arrangement at the chromosome ends was also visible after FISH with subtelomeric and telomeric probes. Their positions varied at the

chromosomal termini, and the subtelomeres showed ring-like shapes as already described by Schubert et al. (2016a) (Figure 2E; Movie 6).
In short, the cytological observations suggest that both metaphase sister chromatids are formed by a ~400 nm thick chromatin fiber arranged as a helix of identical handedness with decreasing turn size towards the telomeres. The chromosome ends are less compact, possibly allowing a higher chromatin fiber flexibility than in interstitial arm regions.

Sister chromatid exchanges (SCEs) confirm the helical 400-nm chromonema

The harlequin staining pattern after differential labeling of sister chromatids with base analogs is caused by SCEs and allows the detecting of chromatin segment exchanges even smaller than the width of a chromatid. Based on this observation, it was inferred that metaphase chromatids are formed by a chromatin helix in *Allium* (Schvartzman and Cortés, 1977; Schvartzman et al., 1978). We applied the same approach to confirm the spatial chromatin organization of mitotic barley chromosomes.
The extension of the exchanged chromatid segments differed in height and width, measurements parallel and perpendicular to the chromatid axis the chromatid (Figure 3). Although both dimensions were affected by the degree of chromosome condensation, all exchanged regions shared the following patterns. The height never dropped below 300 nm, the minimum size that may be exchanged. The maximum height was restricted by the chromosome arm length. Importantly, the height of the exchanged part never exceeded 300-550 nm before the exchanged part spanned the full chromatid width (Figure 3B). These findings agreed with the helical chromatin arrangement as determined by Hi-C and oligo-FISH.
Then, we combined SCE detection and FISH with the Stork, Eagle, Rhea, and Flamingo oligo-probes to relate the exchanged chromatid segments to the FISH-painted regions. As shown in Figure 4 and Movies 7, 8 both 5H homologs have one small SCE segment close to a Rhea-positive region and label the adjacent regions not painted by FISH with the Moa and Ostrich probes. The shape and heights (~400 nm) of the exchanged chromatid segments and FISH signals were similar and partially replaced each other. These findings agree again with the helical chromatin arrangement of metaphase chromatids.
In contrast to the majority of the chromatid, no helical organization was identified in the centromeric regions and secondary constrictions (NORs). Instead, straight chromatin fibers were observed (Figure 3C).

The 400 nm chromonema is sub-structured by loops of ~80 nm chromatin fibers without forming large cavities

In addition to the ongoing discussion about the helical versus non-helical higher-order metaphase chromosome structure, it remained an open question, whether the observation of chromatin-free 'cavities' within the chromatids along their entire length, as found in several electron microscopy investigations in plants and human (Hao et al., 1988, 1990; Hamano et al., 2014; Kuznetsova et al., 2017), reflects a real phenomenon or simply a preparation artifact. Thus, we stained barley chromosomes with DAPI to reach a practical 3D-SIM resolution of ~130 nm (Kubalová et al., 2021) for reliable spatial ultrastructure observation (Figure 5). Large cavities were never identified within complete 3D-SIM image stacks of the metaphase chromosomes. Instead, a network of looped ~80 nm chromatin fibers (n=36; mean 77.2±7.7) containing accumulations was found, and in between, chromatin-free regions not larger than ~120×220 nm are evident (Movies 9, 10). The 80 nm fibers form the 400 nm chromonemata.

At centromeres and NORs, looped and also straight ~80 nm fibers occur, apparently as a constriction-specific substructure of the chromonema, instead of the missing helical arrangement of the chromonema at these regions.

Polymer modeling confirms the helical chromatid architecture

We constructed polymer models using the calculated turn sizes to reproduce the bump position in the contact probabilities of mitotic chromosome Hi-C data and the arrangement of the chromosome regions highlighted by oligo-FISH (Figure 6). The models were built with equilibrium molecular dynamics simulation according to the bottle-brush model proposed by Gibcus et al. (2018). The models were created by large side-by-side loops, whose bases followed a helical path. The large loops consisted of minor side-by-side loops which filled the space surrounding the helical path, both internal and external to it. The loops formed turns of chromatin that stack along the chromatid axis (Figure S12D).

To compare the polymer models with the Hi-C contact probability, we first checked whether our simulation reproduced the contact probability of chicken chromosome 1 at prometaphase, as described by Gibcus et al. (2018) (Figure S12). For this purpose, we simulated only 5 turns of the helical arrangement (with constant turn lengths) using a model of the 10-nm chromatin fiber, where each monomer represents one nucleosome.

The simulated model for chicken closely followed the experimental contact probability. To reproduce the bump that we observed in the Hi-C contact probabilities of the barley chromosomes, we set the turn length to 30 Mb and the turn height to 400 nm. However, our barley models 1-4 did not reproduce the contact probability as good as our model using the data coming from chicken chromosomes (Figure S12A-C). Therefore, we considered a model (5) where the chromatin loops were larger, leading to a dispersed helical protein scaffold (Figure S12E). This chromatin loop arrangement follows the Hi-C data suggesting on average large loops of 3 Mb and small loops of 500 kb (Figure S2E, F). Accordingly, we suspect that the loop arrangement could differ in barley compared to other species, possibly in the density and size of loops surrounding the helical path.

To consider a half-helical arrangement of chromatids as suggested by Chu et al. (2020) we simulated a 10 Mb region (for low computational costs) with coarse-grained models of the 10 nm chromatin fiber. In this model, the helical path changes the turn handedness (left to right) every half turn. The turn length was set to 2 Mb (making 10 half-turns in total), but instead of a lower contact probability at the half-turn distance (1 Mb), characteristic of helical arrangements, we observed a plateau. Sites separated in the genome sequence by half a turn length may be spatially far or close, but in the helical arrangement, they are always far apart. The irregularity of half-turn contacts broadens the bump for inter-turn contacts. We expect that other irregular structural features, like turns with different lengths and perversions between half-turns as suggested by Chu et al. (2020), would only broaden and not sharpen the contact probability peak for distances in the range of a turn length (Figure S13).

To achieve a model matching with our cytological observations, we simulated a 600 Mb chromatid with a coarse-grained model, where each monomer represents 2 kb (Figure 6D; Movie 11). The bases of the loops were constricted to a helical arrangement, with turn lengths as calculated from Hi-C data (Figure 1E). The resulting model for chromosome 5H has 7 turns in the short arm and 14 in the long arm, which fits with the estimation derived from the cytogenetic observations. Concordantly, regions labeled by oligo-FISH and representing successive helical turns appear as stacked layers, consistent with our cytological observations. The Stork and Eagle oligo probes (encompassing less than one turn) present lower heights (corresponding to one-turn height) and do

not fully occupy the width of the chromatid (depending on the viewpoint). Ostrich, Rhea, Moa, and Flaming oligo probes (encompassing more than one turn) have higher heights and always occupy the entire width of the chromatid. The variable turn lengths in the model reproduce the cylindrical shape of the chromosome, with decreasing radius towards telomeres and constrictions. Replicates with different and randomly chosen loop sizes (see Materials and Methods) introduced variability into the model, where the labeled regions appear with slightly different shapes (Figure S14). Other experimentally observed features could not be reproduced by the simulated model. Subtelomeric and telomeric regions mainly absent in the genome assembly, may be less dense than simulated, as indicated by volume measurements (Figures 2I, S9B). Sequences of the primary and secondary constrictions are also lacking in the genome assembly and the less clear organization of centromeres and NORs precludes modeling of their conformations.

Discussion

Helical metaphase chromosome organization – a conserved feature?

A helical structure of plant chromosomes was identified in living tissues, but became also visible after physical treatments and the application of a wide range of agents on fixed chromosomes. In early publications, the coiled chromatin fiber forming the chromatids was called chromonema. It has been observed in mitosis and meiosis of species of different plant families. The coiling of mitotic chromosomes may vary from loose and irregular to the formation of a regular compact helix. Originally, various authors described two chromonemata per chromatid (Nebel, 1932a, 1939; Sax, 1935; Matsuura, 1935, 1938; Manton, 1950). However, Ruch (1950) clarified in *Tradescantia virginica* that these observations were based on optical artifacts and consequently postulated only one chromatin helix per chromatid. Our investigations based on molecular, cytological, and super-resolution microscopy data provide independent evidence that one helically coiled 400 nm chromatin fiber forms a barley metaphase chromatid, obviously corresponding to the classically named chromonema in plants. Similarly, via electron microscopy, a helical chromatid organization was visualized in large-genome plants like onion (Hao et al., 1990), barley (Iwano et al., 1997), and faba bean (Döbel and Schubert, 1982). Besides, the helical structure was observed in chromosomes of animals, such as frog (Seto, 1972), axolotl (Schloissnig et al., 2021), mouse (Sumner, 1998; Rattner and Lin, 1985a), Chinese hamster (Ris, 1981), and also in human (Ohnuki, 1965, 1968; Utsumi, 1982; Rattner and Lin, 1985a; Boy de la Tour and Laemmli, 1988; Sumner, 1991, 1998; Phengchat et al., 2019; Hayashida et al. 2021).

The available data suggest that longer and thicker chromosomes of species with larger genomes contain more and bigger chromonema turns and also loops of larger lengths inside (Table 1). In human the number of turns is positively related to the chromosome length (Boy de la Tour and Laemmli, 1988). If we consider the 15 turns Ohnuki (1968) observed in chromosome 1 and the molecular size of the chromosome of 248.96 Mb (*Homo sapiens* GRCh38.p13 genome assembly), the average size of one turn should be 16.6 Mb. This is almost similar to 12 Mb and 10 Mb determined by Gibcus et al. (2018) using Hi-C for the helical turns in chicken and human chromosomes. In contrast to the macrochromosomes of chicken, the chromatids of the microchromosomes of this species, which are smaller than 12 Mb do not form turns (Gibcus et al., 2018). The diameter of the helically coiled chromatin fiber forming mouse chromatids was estimated to be 200-300 nm (Rattner and Lin, 1985a). A single turn of the helically organized 28 mitotic chromosomes of the giant axolotl genome (32 Gb/1C) comprises ~35 Mb DNA (Schloissnig et al. 2021).

The number of turns was reported to vary from 16-46 in mitotic and from 3-23 in the more condensed meiotic metaphase chromosomes in species of the plant genera *Fritillaria, Osmunda, Rhoeo, Sagittaria*, *Tradescantia* and *Trillium* (Manton, 1950). We found, that depending on chromosome length and position, 18-23 turns of 20-38 Mb per barley chromosome, ranging in sizes from 522 to 675 Mb, occur along the arms. Towards the chromosomal termini, the turns become smaller and the chromatin is less densely packed. Different positions of subtelomeres and telomeres in both sister chromatids indicate the flexibility of the lower-order chromatin structural organization. It is worth noting that using FISH on longitudinally stretched barley chromosome 1H, Valárik et al. (2004) found that telomeric sequences do not appear to be localized at the very end of the chromosome. Ring-like subtelomeric repeat arrangements (Schubert et al., 2016) may reflect the helical higher-order chromatin structure at the chromosome termini. Possibly due to lower chromatin density and higher flexibility, decondensation of barley telophase chromosomes starts at the chromosome ends (Wanner and Formanek, 2000).

In the same species, the chromosome length does not affect the average turn length. After comparing the contact probability of condensed chicken (Gibcus et al., 2018) and barley chromosomes we found that the turn length differs between both species but not intra-specifically (Figure S15). This is more obvious for chicken, whose chromosome sizes range from 0.6 to 196 Mb. Only chromosomes smaller than a turn length (12 Mb) lack the characteristic bump in the contact probability diagram. A comparison between the contact probability derivatives of chicken and barley suggests that the chromatin loop arrangement within the chromonema can also differ between species, but is similar between the chromosomes of the same species, so chicken shows smaller chromatin loops than barley.

The second parallel diagonal in the Hi-C contact matrix, and the respective bump in the contact probability, that relate to the turn length, are key features explainable only by a helical arrangement of mitotic chromatids (Figure 1). Other arrangements such as equilibrium globule, fractal globule and linearly compacted loops, modeled by polymer simulation (Mirny, 2011; Naumova et al, 2013), resulted in different contact patterns (Figure S13). In our work we simulated also a half-helical model, where the handedness changes every half turn, similar to the model proposed by Chu et al. (2020). But in this case, half-turn distances are not always apart in space, and full-turn distances are not always close in space. Thus, this model has to be rejected, as the contact probability lacks the characteristic bump between half-turn and full-turn distances (Figure S13).

With bottle-brush polymer models (Gibcus et al., 2018), following a helical arrangement, we successfully reproduced the chicken contact probability. Our model for barley mitotic chromosomes, with a turn length of 30 Mb, correctly positioned the bump in the contact probability, confirming that the bump originates from the helical arrangement and is related to the lengths of the turns. Assuming a height of 400 nm, as detected cytologically for the barley chromonema, we further approximated the model to the experimental observation. By varying the lengths of major and minor loops fitting to the experimental Hi-C data, we induced a dispersed helical scaffold (Figure S12E) and improved the contact probability. The various chromonema helix turns lengths and heights in barley and other species (Table 1) suggest a different mitotic coiling of the major chromatin loops. Due to the different minor loop sizes in these species, the major chromatin loops may attain different widths, resulting in different turn heights. The extent of these differences and how the chromonema coils remains to be elucidated.

Paulson and Laemmli (1977) suggested a 'scaffold model' based on histone-depleted human chromosomes with a non-histone protein backbone responsible for chromatin looping and chromosome condensation. Topoisomerase IIα and the Structural Maintenance of Chromosomes

(SMC) complexes condensin I and II are components of the scaffold in *Drosophila* (Sureka et al., 2018), chicken (Ohta et al., 2019) and human (Maeshima and Laemmli, 2003; Fukui and Uchiyama 2007; Maeshima et al., 2018; Walther et al., 2018), and are also evident in *Arabidopsis* and barley (Kubalová et al., 2021; Municio et al., 2021). In human HeLa cells the chromatid axes appear as isolated compaction centers rather than forming a continuous scaffold (Sun et al 2018), and appear to consist of a helical structure that serves to organize chromatin loops into the metaphase chromatid (Phengchat et al., 2019). The network-like distribution of Topoisomerase IIα we found in barley metaphase chromosomes (Kubalová et al. ,2021) fits to the predicted Hi-C data-based dispersed helical scaffold (Figure S12E).
The chromosome compaction by condensin II during mitosis seems to be a conserved mechanism influencing the arrangement of chromosomes in interphase nuclei (Hoencamp et al., 2021), such as the Rabl configuration (Rabl, 1885) present in barley (Jasencakova et al., 2001; this work). Further studies on localization and quantification of scaffold proteins may allow establishing a functional model of condensed barley chromosomes including lower-level chromatin organization. Altogether, we are tempted to conclude that a helical arrangement is evolutionary conserved in large (>12 Mb) metaphase chromosomes of plants and animals.

The chromatid helix turning direction is random

In the oligo-FISH labeled regions of both mitotic barley 5H chromatids we observed always the turning of the chromonema in identical directions. Sister chromatids of human HeLa chromosomes have predominantly opposite (mirrored) helical handedness (Boy de la Tour and Laemmli, 1988). Because the separation of sister chromatids starts already at the beginning of prophase Nagasaka et al. (2016) the turning direction has to be determined already after replication during G2 phase, but seems not to be related to the left-handedness of chromatin fibers as reported for the salamander *Necturus* (Williams et al., 1986). Based on investigations in *Drosophila* polytene chromosomes Sorsa (1986) suggests that the size and accumulation of chromomeric loops bends the chromonema to form a helix.
In the plant genera *Trillium*, *Rhoeo*, *Osmunda*, and *Vici*a it was found that the turning direction can change at the centromere and in different regions along the arms, so that there seems to be no uniform control mechanism acting throughout a whole chromosome. Mostly, the ratio of left-handed to right-handed turning is approximately at random. Only in certain *Tradescantia* genotypes, an excess of right-handed segments suggests a special genetic control (Nebel, 1932a, b; Sax and Humphrey, 1934; Nebel and Ruttle, 1936a, b; Manton, 1950; Manton and Smiles, 1943). In short, it seems that the higher-order helical turning direction of chromonemata is flexible rather than strictly determined.

Why is the chromatid helix interrupted by straight and less condensed chromatin at centromeres and NORs?

At the monocentromeres and secondary constrictions of barley lacking the helical chromonema organization, we detected via super-resolution microscopy thinner straight chromatin fibers. This finding is in line with the observations achieved by scanning electron, helium ion, and classical light microscopy in barley (Seto, 1972; Iwano et al., 1997; Wanner et al., 1991; Wanner and Formanek, 2000; Schroeder-Reiter and Wanner, 2009; Schroeder-Reiter et al., 2012; Sartsanga et al., 2021), faba bean (Maruyama, 1983; Schroeder-Reiter and Wanner, 2009), animals, such as frog (Seto, 1972), Indian muntiac (Rattner, 1987), wallaby, and also in human (Utsumi, 1982; Schroeder-Reiter and Wanner, 2009). The 200-300 nm chromonema of mouse, consisting of a

coiled 100 nm sub-fiber appears straight in the centromere region (Rattner and Lin, 1985, 1987). Even at elongated pea centromeres (Neumann et al., 2012) and along holocentric *Luzula nivea* chromosomes parallel chromatin fibers are evident (Schroeder-Reiter and Wanner, 2009).

DNA-specific dyes stain primary and secondary constrictions weaker than chromosome arms, which may reflect a lower chromatin density. Electron microscopy revealed that the amount of looped chromatin fibers and 'knobby' chromatin, abundantly visible along the chromosome arms, is reduced in barley centromeres, displaying less DNA but more proteins than chromosome arms (Wanner et al., 1991; Iwano et al., 1997; Wanner and Formanek, 2000; Schroeder-Reiter and Wanner, 2009). Bailey et al. (2013) showed in HeLa cells that epigenetic modifications of the N-terminal tail of the centromeric histone variant CENH3 influence the physical properties of chromatin fibers in such a way that the centromere chromatin organization may be altered. Transcription of centromeric DNA during mitosis (Liu et al., 2021) could additionally contribute to this alteration.

Features of active rDNA might be maintained during mitosis, preventing a helical chromonema structure at NORs. Not only microscopic but also our Hi-C data indicated a peculiar non-helical chromatin architecture around the NORs and centromeres, manifested as regions without periodical contact patterns in Hi-C contact maps. We hypothesize that the local loss of long-range contacts is prevented by proteins associated with NORs and centromeres. Data obtained by chromatin conformation capture and analysis of nucleolus-associated chromatin domains in *Arabidopsis* support this hypothesis for the NOR (Feng et al., 2014; Pontvianne et al., 2016) and the centromere (Grob and Grossniklaus, 2017). An alternative explanation for the missing contacts in the Hi-C matrices might be the absence of centromeric and rDNA sequences in the reference genome of barley and/or the highly repetitive nature of surrounding regions, which does not allow unique contact mapping.

Overall, we speculate that the missing helical arrangement and the relaxed chromatin may allow kinetochore establishment at centromeres and maintain structures of transcriptionally active NORs. The observation of several parallel ~80 nm chromatin fibers within both constrictions suggests a specific arrangement of the chromonema which remains to be elucidated.

Lower-level chromatin organization – a network of chromatin globules and looped fibers

Without specific labeling the tight compaction of metaphase chromosomes makes individual helical turns invisible. The intermingling of lower-order chromatin fibers between adjacent turns of the chromonema visualized by oligo-FISH in barley explains this observation. Super-resolution microscopy revealed a network of globular (clustered) and fibrous chromatin structures within and at the surface of barley metaphase chromatids, similar to the fibers and 'knobby' structures, possibly corresponding to chromomeres (coiled solenoids) (Serra, 1947; Zatsepina et al., 1983) observed by scanning electron microscopy at the surface of barley (Wanner et al., 1991; Iwano et al., 1997; Wanner and Formanek, 2000; Sartsanga et al., 2021), Chinese hamster (Zatsepina et al., 1983), and human chromosomes (Sumner, 1991). Because our Hi-C contact matrices from barley inter- and metaphase chromosomes, analyzed at 1Mb resolution, do not show TADs or compartments they could not be related to the observed globular structures.

Depending on the species analyzed and the methods used, different levels of chromatin organization were observed (reviewed in Kusnetzova and Sheval, 2016). Several studies showed that in vivo chromatin is basically organized in 10 nm fibers and not 30 nm fibers as found after fixation (reviewed in Maeshima et al., 2019). We measured a mean lower-order chromatin fiber diameter of ~80 nm in DAPI labeled barley metaphase chromosomes while scanning electron

microscopy yielded 15-150 nm (Wanner et al., 1991), helium ion microscopy (with improved chromosome preparations preventing artifacts) 11.6 nm (Sartsanga et al., 2021), and human metaphase chromosomes contain reticulate chromatin fibers with a diameter of up to 30 nm (Wako et al., 2020). Due to the visualization of fluorescence signals via a point spread function the signal thickness measurements, such as that from DAPI, may cause, depending on the settings, an overestimation. Consequently, electron microscopy-based measurements are regarded to be more reliable.

Within the chromatin fiber network, we found minor chromatin-free regions of ~120×220 nm, but never larger cavities as previously observed by electron microscopy in plant and human chromosomes (Hao et al., 1988, 1990; Hamano et al., 2014; Kuznetsova et al., 2017). Hamano et al. (2014) noted that the cavities were not visible when an ionic liquid method, keeping the chromosomes wet, was used. Later, Chen et al. (2017) did also not identify large cavities in human chromosomes. Thus, minor chromatin-free spaces occur within the condensed chromatin of mitotic chromosomes, while the observed larger cavities may rather be preparation artifacts (Beseda et al., 2020).

Finally, we conclude that sister chromatids of large metaphase chromosomes in eukaryotes are formed by a helically arranged chromonema consisting of looped minor chromatin fibers which may form clusters and small chromatin-free regions between them.

Material and Methods

Plant material

Seeds of barley (*Hordeum vulgare* L.) cv. Morex were obtained from the Gene Bank of the Leibniz Institute of Plant Genetics and Crop Plant Research, Gatersleben, Germany.

Preparation and sequencing of chromosome Hi-C libraries

Suspensions of barley metaphase chromosomes were prepared from root tip meristems after cell cycle synchronization as described in Lysák et al. (1999) with the following modifications: first, barley seeds were germinated for two days at 4°C, then placed at 25°C for three days. Second, the roots were fixed in 2% formaldehyde in 1×PBS buffer for 12 min at 5°C. Fixed metaphase chromosomes were released by mechanical homogenization into LB01 buffer (Doležel et al., 1989) and stained by 4',6-diamidino-2-phenylindole (DAPI) at final concentration of 2 µg/ml. The chromosomes were purified by sorting using the FACSAria SORP flow cytometer (Becton Dickinson, San Jose, USA). The initial gating was performed on FSC-A vs. DAPI-A parameters. To discriminate doublets, the chromosome gate was drawn on DAPI-A vs. DAPI-W scatter-plot (Figure S16).

Three replicates of chromosome Hi-C libraries were prepared. For each, five million chromosomes were flow sorted into a 15-ml Falcon tube with 2 ml LB01, centrifuged at 500 g for 30 min at 4°C and the supernatant was removed except for 20 µl. The pelleted chromosomes were gently resuspended in the remaining supernatant, diluted with 8 ml ddH_20 and spun down at 500 g for 30 min at 4°C. The supernatant was removed except for 20 µl. The pelleted chromosomes were gently resuspended and the entire volume was carried over to 0.2-ml Eppendorf tube. Further steps were carried out with Arima Genomics Hi-C kit (Arima Genomics, San Diego, USA) according to the manufacturer's protocol. The sequencing libraries were prepared with NEBNext Ultra II DNA library preparation kit (NEB, Ipswitch, USA) with 10 cycles of PCR amplification. Libraries were

sequenced on Illumina NovaSeq 6000 instrument (Illumina, San Diego, USA) in 150-bp paired end mode. Parameters of the generated Hi-C data are summarized in (Table S4).
The data for chromosome model simulation were generated by an alternative protocol. Eight million chromosomes were sorted into a 15-ml Falcon tube with 2 ml isolation buffer (Šimková et al., 2003), centrifuged at 500 g for 30 min at 4°C and the supernatant was removed except for 45 µl left in the tube. Pelleted chromosomes were gently resuspended in the remaining supernatant and mixed with low-gelling agarose to create a 1% agarose plug of 90 µl. The plug was washed twice for 30 min in 2 ml TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH 8) on ice. Subsequent Hi-C library preparation steps, including DNA digestion, biotin fill-in, ligation, and chromatin release from the plug, were performed according to the "In situ Hi-C in agar plugs protocol" of Rao et al. (2014) with minor modifications: the DNA was digested by 800 U HindIII restriction enzyme and biotin-14-dATP was replaced by biotin-14-dCTP. The finished Hi-C library was sequenced on HiSeq2500 in 100-bp paired end mode.
Hi-C reads were mapped against the barley cv. Morex genome version 2 (v2; Monat et al., 2019) using the computational pipeline previously described (Beier et al., 2017; Monat et al., 2019). Contact matrices were visualized in the R statistical environment (R Core Team, 2020).

Contact probabilities from Hi-C data

All contacting pairs from Hi-C data were counted and mapped to the Morex genome version 2 (v2), as a function of the genomic distance between them (d). All values of distance d were split into bins in a logarithmic scale from 100 kb to 1 Gb. The contact probability of each bin was calculated as the sum of all observed pairs within the distance range of this bin, divided by the number of all genomic loci within the same distance range.

Quantification of Hi-C local contacts in metaphase chromosomes

The chromosomes were divided into 5 Mb-long non-overlapping regions. For each region, we considered only Hi-C pairs with at least one side of the contacting pair inside of this region or with both sides of this pair spanning this region (Figure S3). For each region, the considered pairs are counted according to the genomic distance between them (d). The counted pairs as a function of d are well described by the sum of an exponential and a Gaussian distribution. The Gaussian distribution coincides with the bump characteristic of a helical arrangement (Figure 1B-D), and its center indicates the turn length of the helical arrangement around the analyzed region. The center of the Gaussian distribution was assigned as the turn length for each region and it was used to build the graphics in Figure 1E.

Building of the helical path with changing radius and estimation of the turn numbers

The monomers selected as basis of the loops were constrained to a helical path in the Cartesian space. A turn length (in base pairs) I_i is assigned to each monomer i, according to the values calculated from the Hi-C data. With this value we calculate how many monomers (n_i) from the helical path would fit in this turn, with the monomer size (in base pairs) m and the average major loop size (in monomers) I_o (equation 1). Each monomer is then assigned a height z_i and an angle θ_i, relative to the previous monomer. The height z_i is equal to the constant turn height (400 nm) divided by n_i, and the angle θ_i is the full turn (360°) divided by n_i (equation 2). The radius r_i is calculated from the turn length (n_i multiplied by the distance d between monomers) and the turn height as in equation 3, from Pythagoras' theorem. The cartesian coordinates are then retrieved by these cylindrical coordinates.

$$n_i = l_i/m/l_o \quad (1)$$
$$z_i = z_{i-1} + 400/n_i \qquad \theta_i = \theta_{i-1} + 360/n_i \quad (2)$$
$$(dn_i)^2 = (2\pi r_i)^2 + 400^2 \quad (3)$$

The number of turns is then the number of cycles in the function *cos(θ)*, using 1 Mb as the monomer size and 1 monomer as average major loop size.

Growth and treatment for FISH

Barley seeds were incubated for three days in the dark at room temperature. Roots were collected into cold tap water and incubated on ice for 24 hours (h). Next, roots were transferred into an ethanol:acetic acid (3:1) fixation solution and placed into a vacuum for 5 min. Afterwards, roots were kept in the fixation at room temperature (RT) overnight.

Growth and treatment for EdU labeling of sister chromatid exchanges

Barley seeds were incubated for three days in the dark followed by a treatment in 20 µM EdU in Hoagland medium for 17 h at RT. Then, the seedlings were transferred into a new Hoagland medium for 19 h in dark at RT (recovery phase). Subsequently, roots were cut from seedlings and put into ice-cold water for an additional 24 h. Finally, roots were fixed in ethanol:acetic acid (3:1), first 5 min in a vacuum, then overnight at RT.

Chromosome spread preparation

Selected roots were washed twice in ice water for 5 min, and once in a citric buffer for 5 min. Subsequently, an enzyme mixture [1 % Pectolyase (Sigma); 1 % Cytohelicase (Sigma); 0.7 % Cellulase R-10 (Duchefa), and 0.7 % Cellulase (Calbiochem) in 0.1 M citric buffer] was added and the roots were incubated at 37°C for 45 min. After enzymatic digestion, roots were washed twice in ice-cold water for 5 min and twice in ice-cold ethanol for 5 min. Root tips without root caps were collected into the tube containing ethanol:acetic acid (3:1) and mashed. 7 µl of this suspension was dropped onto cold wet slides, transferred to a hot plate (55°C), and fixed with 25 µl of ethanol:acetic acid (3:1). Slides were allowed to dry at least for 1 h at RT.

To preserve the native volume, metaphase chromosomes were flow-sorted into 1×meiocyte buffer A (1×buffer A salts, 0.32 M sorbitol, 1×DTT, 1×polyamines) and subsequently embedded into a 5% polyacrylamide gel as described (Bass et al. 1997; Howe et al. 2013) with minor modifications (Němečková et al. 2020).

FISH

To detect half and full helix turns within chromosome arm 5HL by fluorescence microscopy, oligo-FISH probes were designed against the chromosome-scale sequence assembly of the barley cv. Morex genome assembly version1 (Morex v1; Beier et al., 2017; Mascher et al., 2017) using Abor Biosciences' proprietary software. Briefly, target sequences were cut into 43–47 nucleotides-long overlapping probe candidate sequences that were compared to the rest of the genome sequence to exclude any candidates with potential cross-hybridization based on a predicted *Tm* of hybridization. Non-overlapping target-specific oligonucleotides were selected for the final sets and synthesized as myTAGs® Labeled Libraries (Arbor Bioscience, Ann Arbor, USA).

To validate the lengths of intervals covered by the particular oligo-FISH probes, we verified the completeness of the Morex v2 genome assembly in the analyzed region of 5H by an optical map

constructed from barley cv. Morex on the Saphyr platform of Bionano Genomics (Figure S8). The optical map contigs were aligned to the selected interval 442-599 Mb in the chromosome 5H pseudomolecule using RefAligner, version 9232 (Bionano Genomics). Query-to-anchor comparison was performed with default parameters and a p-Value threshold of 1e−10. The alignment was visualized in Bionano Access, version 1.5. The coordinates from the original design were uplifted and are now based on the improved Morex assembly v2 (Monat et al., 2019). The labeled region starts at the position of 442.1 Mb. For simplification, the designed probes were named according to bird species, and labeled in different colors: Stork-Atto647N, Eagle-Alexa488, Ostrich-Atto594, Rhea-Alexa488, Moa-Atto594, and Flamingo-Alexa488 (Figure 2C; Figures S6, S7; Table S2).
The subtelomeres and telomeres were labeled by the HvT01 oligo-probe (Belostotsky and Ananiev, 1990; Schubert et al., 1998) using TexasRed and the *Arabidopsis* telomere-type oligo probe (Richards and Ausubel, 1988) using Cy5, respectively. The centromeres were labeled by the probe for the centromeric repeats (GA)15 (Zhang et al., 2019) using FAM, the NORs by the 45S rDNA probe using the clone pTa71 and Alexa488 (Gerlach and Bedbrook, 1979).
For FISH the slides were incubated in 45% acetic acid at RT for 10 min and then washed in 2×SSC at RT for 10 min. In the case of strong background caused by cytoplasm 50 µl of pepsin solution (0.1% in 0.01M HCl) was added onto the slides, covered with parafilm, and incubated in a wet chamber at 37°C for 10 min. Afterwards, slides were washed twice in 2×SSC for 5 min and post-fixed with 4% formaldehyde in 2×SSC at RT for 10 min. Next, slides were washed 3-times in 2×SSC at RT for 4 min and immediately dehydrated in an ethanol gradient (70%, 85% and 100%, each step 2 min). Slides were air-dried at least for one hour. In the meantime, all selected oligo probes were pooled into a microtube and let evaporate using a SpeedVac concentrator (Eppendorf). Probes were reconstituted in 1 µl of ddH_2O and 19 µl of hybridization mixture (50% formamide; 10% dextran sulfate; 10% salmon sperm DNA; 2×SSC). The entire volume of the reconstituted probe was added on the slide, covered with a coverslip, and sealed with rubber cement. The slides were incubated for 20 min at 37°C and then denatured on a hot plate (70°C) for 3 min. Finally, slides were directly placed into a wet chamber and hybridized for 22 h at 37°C. Post-hybridization washing was carried out as follow: slides were briefly washed in 2×SSC at RT to remove the coverslip, then washed under shaking condition at 58°C for 20 min, for 5 min in 2×SSC at RT, dehydrated in an ethanol series (70%, 90% and 96%, each step 2 min), air-dried in the dark and counterstained with 8 µl DAPI (2 µg/ml in Vectashield).

Combination of EdU labeling and FISH

Seedling growth and treatment were identical as for the EdU labeling experiment. Slides containing metaphase spreads with EdU labeled sister chromatid exchanges were pre-selected and used for oligo-FISH. Depending on cytoplasm background, slides were incubated in 45% acetic acid at RT for 10 min, then washed in 2×SSC at RT for 10 min, or directly post-fixed with 4% formaldehyde in 2×SSC at RT for 10 min. The following steps were identical to the FISH procedure described above.

Super-resolution microscopy and FISH signal volume measurements

To detect the ultrastructural chromatin organization of chromosomes at a resolution of ~120 nm (super-resolution achieved with a 488 nm laser excitation) spatial structured illumination microscopy (3D-SIM) was performed with a 63×/1.4 Oil Plan-Apochromat objective of an Elyra PS.1 microscope system and the software ZENBlack (Carl Zeiss GmbH). Images were captured

separately for each fluorochrome using the 642, 561, 488, and 405 nm laser lines for excitation and appropriate emission filters (Weisshart et al., 2016). Maximum intensity projections of whole cells were calculated via the ZENBlack software. Zoom-in sections were presented as single slices to indicate the subnuclear chromatin structures at the super-resolution level. 3D rendering to produce spatial animations was done based on SIM image stacks using the Imaris 9.6 (Bitplane) software. The FISH signal and DAPI-labeled whole chromosome volumes were generated and measured via the Imaris tool 'Surface'.

Polymer simulation

Similar to Gibcus et al. (2018) we performed a simulation for the barley metaphase chromatin organization based on a 10 nm chromatin fiber and the bottle-brush model of chicken containing a protein scaffold. Furthermore, we assume that the individual 400 nm fiber turns consist of bigger and smaller flexible loops. Therefore, a partial intermingling of the minor loops originating from adjacent turns occur (Figure S10).

In all simulations, the chromatin was modeled as a beads-on-string homo-polymer. The Molecular Dynamics simulations (MD) were performed using the OpenMM Python application programming interface (Eastman et al. 2017) and the library Openmm-lib (https://github.com/mirnylab/). The motion of the polymer was simulated as in Langevin dynamics, with the temperature at 300 K, a collision frequency of 0.001 ps^{-1}, and a variable time step (Gibcus et al., 2018). Three internal forces were applied to the polymer: (1) a harmonic force covalently binding two neighboring beads; (2) a harmonic angular force between three sequential beads with a spring constant of 1 k_BT/rad^2; and (3) a polynomial repulsive force allowing the crossing of the fiber by setting an energy truncation value of 1.5 k_BT, when the distance between two non-bonded beads is zero. In the bottle-brush model, the entire polymer is organized into side-by-side major loops, divided into side-by-side minor loops as proposed by Gibcus et al. (2018). The lengths of both types of loops are randomly chosen following an exponential distribution. Two external forces were applied to constraint the polymer in a cylindrical bottle-brush model: (1) the monomers forming the base of major loops are tethered to a helical path by a harmonic potential with a spring constant of 4 k_BT/nm^2; and (2) this helical path is at the center of a cylinder, which boundaries are defined by a harmonically increasing potential with a spring constant of 10 k_BT/nm^2. Apart from these characteristics, the bottle-brush model is defined by four parameters: (1) the average length of major loops; (2) the average length of minor loops; (3) the turn height, and (4) the turn length of the helical path. These parameters are explicitly mentioned for each model we tested (Figure S12C). All simulations start with a conformation where the base of the major loops follow a calculated helical path, and the loops emerge radially. All monomers in the helical path are assigned cylindrical coordinates (angle, radius, and height). These coordinates are calculated so that the monomers are equally separated given the specified total number of major loops and the turn height, and then converted to Cartesian coordinates. The simulation runs until the contact probability is equilibrated.

To reproduce the contact probability of chicken chromosome 1 at prometaphase (30 min after release of G2 arrest), as described by Gibcus et al. (2018), with major loops of 400 kb average length, minor loops of 80 kb average length, 150 nm turn height, and 8 Mb turn length, we used a coarse-grained model where each monomer represents one nucleosome. Covalent bonds were separated by 10 nm average distance and 1 nm wiggle distance, cylindrical confinement secured a volume of 11^3 nm^3 per monomer, and the monomers in the helical path were separated by 50 nm. With the same parameters, we built four models to reproduce the contact probability of metaphase

barley chromosome 5H varying the average length of major and minor loops, the turn height, and the turn length. These models simulate a region with 5 turns of the helical path (Figures S12D, E). For the comparison between helical and half-helical arrangements we used the same coarse-grained model as above but simulating a 10 Mb region with 2 Mb turn length, 100 nm turn height, 100 kb major loops, and 10 kb minor loops. The radius of the helical path was set to 100 nm and no cylindrical confinement was set. The half helical arrangement differed from the helical only in the position of the major loops' basis – only the absolute values of the x-coordinate were considered in the conversion from cylindrical to Cartesian coordinates. Ten replicates of each model were used to calculate the contact probability.
To observe the changing turn length, we simulated the ~600 Mb of chromosome 5H with a coarse-grained model where each monomer represents 2 kb. In agreement with the volume measured for the entire chromosome (~43^3 nm^3/2 kb), we set covalent bonds which were separated by a 40 nm average distance and 4 nm wiggle distance. The cylindrical confinement was set separately for each major loop, 800 nm in height around the position of the loop basis and radius proportional to the turn length, being minimum 500 nm and maximum 900 nm following our chromatid width measurements after EdU labeling (Movie 12). Major and minor loops were 1 Mb and 400 kb long, respectively. Monomers in the helical path were separated by 50 nm. The helical path was calculated with a constant turn height of 350 nm and a radius proportional to the local turn length, as previously calculated from our Hi-C data. At the constriction regions, where no helical arrangement occurs, the position of the major loops' basis follows a straight line in the z-axis and minor loops are only 40 kb long, a value that approximately mimics the observed size of the primary constriction.

Contact probability of polymer models

To measure the contact probabilities of the equilibrated models, the final conformation of each simulation was calculated by counting all monomers spatially close to each other by no more than 51 nm, as in (Gibcus et al., 2018). These pairs of monomers are then grouped according to their distance in the linear genome. The sum of observed contacts in each group was divided by the sum of all possible contacts between two monomers separated by this group distance in the linear genome. The contact probability as a function of the linear genomic distance was directly compared to the experimental data, obtained by Hi-C experiments, upon normalization of all probability functions to be equal to 1 for 100 kb.

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Figure legends

Figure 1. Periodical Hi-C contacts indicate the helical organization of somatic barley metaphase chromosomes. A) Hi-C contact matrix of barley chromosome 5H in inter- and metaphase. The anti-parallel diagonal in interphase (green arrow) indicates the Rabl configuration, while metaphase chromosomes show a parallel diagonal (blue arrow), indicating a periodical contact pattern missing around the centromere and the NOR (black arrows). **B)** Average Hi-C contact probabilities of all chromosomes as a function of the distance between two contacting sites in meta- and interphase. The characteristic bump (solid arrow) relates to the second diagonal observed in the Hi-C metaphase contact matrix (blue arrow in **(A)**. The bump follows a decrease at half of its position (dashed arrow). This pattern indicates an arrangement into turns, where sites distant by one turn (solid arrow) are more likely to contact than sites half-turn apart (dashed arrow). The bump position indicates the turn length Intra-turn contacts below 10-Mb distance (grey area) indicate the chromatin lower-order organization, where nested loops are packed within each turn (Gibcus et al., 2018). **C)** The three contact types observed with different contact probability patterns **(B)** are represented in a helical arrangement. Sites distant by one turn are closer to each other (solid arrow, inter-turn contacts) than sites distant by half-turn (dashed arrow, half-turn contacts), and contacts between adjacent loops (inset) constitute a lower-order organization with higher contact probability, (grey region). **D)** Schema of a metaphase chromosome arranged into turns as inferred from the Hi-C matrix **(A)** and the contact probability **(B)**. The color gradient along the turns relates to the linear genomic sequence. **E)** Turn length along all chromosomes based on the local contacts observed in the Hi-C experiments (Table S1). Positions of primary (dashed arrows) and secondary (solid arrows) chromosome constrictions can be recognized by zero turn length. All interstitial regions show similar turn lengths, decreasing towards the telomeres. Abrupt peaks, such as in chromosome 7H at 160, 195 and 330 Mb, relate to gaps or assembly errors in the genome sequence. The total turn number per chromosome is indicated.

Figure 2. FISH using oligo probes reveals the helical organization of barley metaphase chromosomes. (A) Density of predicted single copy oligo probes along chromosome 5H. The distal 157 Mb-long region of the long arm of chromosome 5H is enriched in single-copy sequences suitable for the detection by oligo-FISH. **(B)** Karyogram of barley indicating the distribution of applied centromere-, NOR-, subtelomere- and telomere-specific FISH probes. **(C)** Design of the oligo-FISH probes (named according to birds for simplicity) covering the 157 Mb-long region of chromosome 5HL. Precise probe coordinates are given in Table S2. Stork and Eagle cover together ~1.2 turn, Ostrich, Rhea and Moa ~1.5 turn. Flamingo covers the subterminal part of the long arm. **(D)** Metaphase chromosome spread labeled with the FISH probes shown in **(B)**. The enlarged oligo-painted region of 5HL (asterisks) shows the chromatin arrangement in both sister chromatids as predicted by the Hi-C based helical chromatin arrangement model (Figure 1D). Due to chromosome tilting, Moa shows in a top-side view the spiral organization of the ~430 nm thick chromatin fiber (marked with yellow lines). **(E)** Positions of telomeres and subtelomeres vary at both chromosome termini of different chromosomes. Subtelomeres form ring-like structures (right, arrows). Chromatin was counterstained with DAPI (blue).

Figure 2 continued. (F) Turn lengths (blue line), calculated from the Hi-C data, across the 300 Mb region of 5HL encompassing the designed oligo-FISH probes. The regions covered by each probe are colored according to the probe color. **(G)** Helical arrangement of the target region illustrating the changes of the turn lengths according to the region shown in (F). The model is

compatible with a helical arrangement with similar turn height and volumes (see H), and varying turn length. **(H, I)** Box-plots showing the relative (absolute values are visualized in Figure S9) measured signal heights **(H)** and volumes **(I)** in percent per whole chromosome of the oligo-labeled half (Stork and Eagle)- and full-turn regions. The numbers of measured chromosomes per oligo probe are in parentheses. The boxplots show the mean/median (cross/yellow line), lower and upper quartiles with explicit values. Whiskers extend to 150 % of the interquartile region below and above the box (Ross 2004). The high variability within each probe relates to signal detection difficulties. The black dots in the right diagram **(I)** are the expected chromosome volume in percent for each probe if the chromatin density would be uniform along the entire chromosome. For all probes, this volume is higher than the observed mean reflecting an underestimation in the measurements. The exception is the Flamingo probe, the only one with a higher observed than expected mean volume. This indicates that the Flamingo region is less dense than the other measured chromosome regions. The signal heights were measured within SIM image stacks by the ZENBlack software, and the volumes (see Figure 6C) using the surface rendering tool of Imaris 9.6.

Figure 3. Sister chromatid exchanges (SCEs) confirm the helical organization of barley metaphase chromosomes. (a) Metaphase chromosome spread labeled with EdU to detect SCEs creating a harlequin pattern. Some exchanged chromatin regions appear as thin bands with a minimum height of ~400 nm (enlarged regions * and ** in dashed rectangles). These regions correspond in size to the ~430 nm chromatin fiber (chromonema) detected by the oligo-FISH probes (Figure 2D, H, S9A) reflecting a helix within the chromatid arms. **(B)** Scatter plot of 257 measured SCEs of different height (along the chromosome longitudinal axis) and width (in the chromatid). The color code indicates how much of the chromatid width is covered by the SCE, dark green means complete covering. The schematic chromosomes around the diagram show examples of measured SCEs. The lack of values in the dashed region indicates that SCEs higher than ~400 nm and not covering the complete chromatid width never occurred. The black line indicates the median height (~380 nm) of SCEs with incomplete width (less than chromatid width), and the grey area spans lower and upper quartiles. The observed possible configurations of SCEs imply that the chromatids are formed by ~400 nm high chromatin units. They are compatible with a helical arrangement, as illustrated in the scheme below. The small SCEs in the centromeric, NOR and telomeric regions were not considered. **(C)** At centromeres (top) the EdU labeling indicates a straight thin connection between the ~400 nm high fibers. Immediately next to the centromere, due to chromatin tilting, a ~400 nm thick turn is visible in the pericentromeric region (arrow). At the NOR (bottom) also thin chromatin fibers occur. **(D)** SCE units of ~400 nm are also evident at the chromosomal termini (arrows). Chromatin was counterstained by DAPI (blue/white).

Figure 4. Combination of oligo-FISH and EdU labeled SCEs endorse the helical chromatin organization. Metaphase chromosome spread (top) showing all barley chromosomes after EdU and oligo-FISH labeling. In both homologous oligo-FISH painted 5HL regions two thin exchanged regions occurred (bottom). The left one does not span the entire chromatid width, but the right one does (insets in the merged images). The SCEs are present at the transition from the Rhea region to the not labeled Moa (left, red arrow) and Ostrich (right, white arrow) regions. The heights of the SCEs, oligo-FISH probe and EdU-free regions are similar (~380-450 nm).

Figure 5. Primary (centromere) and secondary (NORs) chromosome constrictions are not organized as a helix. Instead, these regions (marked by red and blue dashed rectangles) contain parallel chromatin fibers (red and white arrows) (see also Figure 3C). At chromosome arms, the DAPI-labeled chromatin (white) occurs as a network of looped fibers within the ~400 nm fiber. The fibers contain chromatin clusters (yellow arrows), and in between them, small chromatin-free regions (green arrows). No large continuous cavities were observed as demonstrated also in Movies 9, 10 representing a whole chromosome volume.

Figure 6. Simulated Hi-C data-based polymer chromosome model according to cytological findings. (A) Metaphase chromosome 5H labeled by different FISH probes (marked by an asterisk in Figure 2D). The chromatids are separated by the dashed line. The ortho-view (Movie 3) **(B)** and the surface rendering (Movie 5) **(C)** of the same chromosome prove that the FISH signals are present throughout the whole chromosome volume. **(D)** A polymer model of a single chromatid based on the turn lengths calculated from the experimental Hi-C data (Figure S14). The simulated model, built as a bottle brush, is formed by large loops, whose bases follow a helical arrangement (right). The large loops are further divided into minor loops, which fill internally and externally the space surrounding the helical arrangement. Thus arranged, the loops form thicker helical turns of chromatin. Each monomer in the model represents ten nucleosomes (~2 kb) The entire polymer has 300,000 monomers corresponding to ~600 Mb. The multi-colored region follows the design of the oligo-FISH probes (Figure 2C). The model is applicable to illustrate the number and changing sizes of the turns along the chromatid arms, but has to be adapted according to the observed FISH-labeling pattern, e. g. the NOR appears larger via FISH labeling than as simulated based on the Hi-C data.

Figure 7. Helical model of the higher-order chromatid organization of a somatic plant metaphase chromosome based on data from barley.
Both chromatids, the highest level of chromatin organization, are formed by a ~400 nm thick chromatin fiber (chromonema) arranged in a helix of identical handedness (Figure S11). The chromatids are completely filled with chromatin without large cavities. Adjacent chromonema turns intermingle due to the flexibility of the smaller 80 nm fibers present within the chromonema. The helical order is interrupted at centromeres and secondary constrictions displaying mainly straight 80 nm chromatin fibers. The chromosomal termini contain less condensed, more flexible chromatin. Due to this flexibility the telomeres may be embedded into the subtelomeric chromatin and not appear at the very end of the chromatid. The left long arm chromatid is shown as a stretched helix representing their higher-order chromatin folding based on oligo-FISH labeling. The different colors represent half (white+green) and complete (red+green) chromonema turns, respectively. The chromonema is formed by looped thinner 80 nm chromatin fibers. This lower-order chromatin arrangement (colorless inset) is still under debate. According to previous findings (**Thoma et al. 1979**), at the lowest hierarchical level, 147 bp of left-handed DNA is wrapped around a nucleosome core. Nucleosomes, with DNA linkers in between them, form the 10 nm chromatin fiber. Several nucleosomes associate into thicker 80 nm chromatin fibers which form loops within the chromonema.

Figure S1. Hi-C contact matrices of all barley chromosomes (except 5H shown in Figure 1A) at interphase and metaphase from the first replicate of the Hi-C experiment. The anti-parallel diagonal in interphase (green arrows) indicates the Rabl configuration, while metaphase chromosomes show a parallel diagonal (blue arrows), indicating a periodical contact pattern missing at the NOR of 6H and the centromeres (black arrows).

Figure S2. Contact probabilities calculated from the three Hi-C experiment replicates of interphase chromatin (A, B) and metaphase (C, D) chromosomes. In all graphics, each line represents one chromosome and each color one replicate. In **(A)** and **(C)** the probabilities are normalized to have the same value at 100 kb. In **(B)** and **(D)** the probabilities are exponentially normalized to have the same values at 100 kb and 30 Mb. In **(D)** the exponential normalization withdraws experimental variability and highlights the position of the bump characteristic to the metaphase contact probability. (**E**) Derivative of the contact probability log comparing metaphase **(D)** and interphase **(B).** (**F**) Contact probability ratio between metaphase **(D)** and interphase **(B)**. The dashed lines in **(E, F)** correspond to the observed peaks. In interphase both peaks relate to the Rabl conformation reminiscent of anaphase. In metaphase the first peak corresponds to the size of chromatin loops and the second one to the size of helical turns. In the ratio of metaphase/interphase contact probabilities, the peaks highlight the differences between metaphase and interphase conformations.

Figure S3. Quantification of Hi-C local contacts in metaphase chromosomes. The chromosomes were divided into 5 Mb-long non-overlapping regions. All Hi-C pairs within or spanning each region **(A)** were analyzed as a function of the distance between the contacting sites. For each region, such a function is plotted as the number of contacts distant by 1 Mb, 2 Mb, ... until 100 Mb **(B)**. Normalized, it can be interpreted as a local contact probability (similar to Figure 1B). This function is well described by the sum of an exponential and a Gaussian distribution. The Gaussian distribution coincides with the bump characteristic of a helical arrangement (see Figure 1). Its centre indicates the turn length of the helical arrangement around the analysed region and it was used to build the graphics in Figure 1C.

Figure S4. Local contact amounts at nine different positions (100-550 Mb) of chromosome 5H retrieved from experimental Hi-C data. At all positions, a prominent bump is present, with the center at slightly different positions. The bump represents a contact increase followed by a decrease at half of its position. This pattern indicates the chromatin arrangement into turns, at which the turn length corresponds to the center of the bump. The bump position at any region along chromosome 5H has been calculated as described in Figure S3. The placement of the bump for all 5 Mb regions (119), separated by 5 Mb along the 5H sequence is visualized in Movie 1.

Figure S5. Predicted oligo density along all barley chromosomes (except 5H shown in Figure 2A) used to design the oligo-FISH experiment. The x-axis shows the position of the oligos on the chromosome in the Morex v2 genome. The y-axis represents the number of oligos within 40 Mb bins.

Figure S6. Graphical representation of the oligo density based on the barley cv. Morex v2 assembly covering the Stork and Moa regions applied for oligo-FISH. (A) The 33,987 oligos of 45 nucleotides on average of the Stork probe cover homogeneously a 0.6-turn of the helix with minor gaps arising from the lack of unique sequences. **(B)** The 108,831 oligos of 45 nucleotides on average of the Moa probe cover homogeneously a 1.6-turn of the helix without significant gaps. The orange color marks a threshold of a minimum of three oligos per kilobase. The green color denotes a sufficient number of oligos for FISH in the selected region.

Figure S7. Graphical representation of the oligo density of all designed probes at the long arm of chromosome 5H. The lower diagram shows the region covered by the oligo probes enlarged.

Figure S8. Validation of the chromosome 5H interval 442-599 Mb in the Morex V2 genome assembly by optical mapping. The selected sequence segment (green bars) shows a good alignment to the optical map contigs 1, 55 and 17 (blue bars), indicating overall correctness and completeness of the sequence (top), except for a region around 491 Mb where non-aligned overhangs of contigs 1 and 55 indicate a missing sequence of at least 403 kb (bottom).

Figure S9. Box-plots showing the measured signal heights (A) and volumes (B) of the oligo-labeled regions on chromosome 5HL.
The variable heights of the probes are consistent with the different turn lengths along the mitotic chromosome (Figure 2) and the different metaphase chromosome condensation degrees. Volumes were measured after surface rendering using Imaris 9.6, and the values correspond to a single chromatid. The numbers of measured chromosomes per oligo probe are in parentheses.

Figure S10. Adjacent helix-turns are partially intermingled. The differentially oligo-FISH labeled turns at chromosome arm 5HL intermingle. Smaller looped chromatin fibers forming the turned chromonema (indicated by dashed lines) and marked by the different colors invade into each other (arrows in the right enlarged image). Chromatin was counterstained with DAPI (blue).

Figure S11. In both chromatids, the 400 nm fibers turn in the same direction. (A) The oligo-FISH probes (here two examples of Stork, Eagle and Ostrich) reveal that the sister chromatids coil in the same direction, and not in a mirrored manner. In the merged image the sister chromatids are divided by a dashed line. **(B)** The schemata show the observed (top and middle) and not occurring (bottom) arrangements.

Figure S12. Comparison between applied bottle brush models for condensed barley and chicken chromosomes. (A) Contact probability of a barley polymer model generated with settings of Gibcus et al. (2018) compared with the Hi-C contact probability for chromosome 1 of chicken in prometaphase (Gibcus et al., 2018). **(B)** Contact probabilities of four barley polymer models, with the same settings as in **(A)**, but with varying helical arrangement parameters as shown in the table **(C)**. The models are compared with the Hi-C contact probability of barley chromosome 5H in metaphase. All probabilities are normalized to be equal to 1 at 100 kb. **(D, E)** Conformations of barley models 3 **(D)** and 5 **(E)**. The 30 Mb turns are coloured alternating in red and green. From

left to right: the bases of major loops form the helical path; the bases of the minor loops are in white; a single major loop is divided into minor loops; all loops together complete the model. In **(D)** the bases of major loops (model 3) follow a helical path where the monomers are ~10 nm apart. In **(E)** the bases of the major loops (model 5) follow the same path, but the monomers are ~100 nm apart. Thus, the helical structure is not as constrained as in **(D)**. Consequently, in **(D)** a backbone scaffold is formed. Instead in **(E)** the scaffold appears dispersed.

Figure S13. Contact probabilities of polymer models support a helical chromatid organization. The diagram shows the contact probabilities of different polymer models predicted for equilibrium and fractal globules, for simulated helical organization as suggested by Gibcus et al. (2018) and simulated half-helical arrangement with changing handedness as suggested by Chu et al. (2020). In all spatial models the polymer is coloured as shown for the unfolded polymer. For the helical and half-helical organizations, the polymer is transparent, and the basis of the loops appear as white spheres arranged in turns. Only the helical model creates the contact probability bump.

Figure S14. Nine replicates of the simulated Hi-C data-based polymer chromosome model. The replicates were the basis for establishing the cytologically adapted model shown in Figure 6D. The simulations were based on the turn lengths calculated from the experimental Hi-C data. The different patterns arose from the varying loop distributions. Their positions and sizes were chosen randomly, according to an exponential distribution to reach the same average loop size.

Figure S15. Comparison between chicken and barley Hi-C based contact probabilities of mitotic chromosomes. (A) Contact probabilities of barley chromosome 5H at metaphase and chicken chromosome 1 at prometaphase (Gibcus et al., 2018). The right diagram shows the derivative of the log of the same contact probabilities indicating the bump position distinctly. The dashed line marks the average size of the nested loops **(B)** Contact probabilities of all barley chromosomes at metaphase and **(C)** of all chicken chromosomes at prometaphase (Gibcus et al., 2018). All probabilities are normalized to be equal to 1 at 100 kb.

Figure S16: Flow sorting of barley metaphase chromosomes. Flow karyotype of barley cv. Morex. Initial gating was set-on relative DAPI fluorescence vs. forward scatter parameters (P0), dependent sorting gate was drawn on DAPI-area vs. DAPI-width scatter plot (P1).

Tables

Table 1: Parameters of helically organized somatic metaphase chromosomes of barley compared to other species. Chromatid helix turn sizes and nested loop sizes are based on Hi-C data and a bottle brush-like model except for mouse and human.

Table S1: Values of local turn length of each chromosome calculated for 5 Mb bins, as described in supplementary materials and Figure S4. These values are graphically shown in Figure 1E. The three first lines display the sequence length of each chromosome, and the positions of the primary

(centromere) and secondary constriction (NOR) at the short arms of 5H and 6H, as predicted from Hi-C data.

Table S2: Coordinates of the oligo-FISH probes (named according to birds) adjacent to the 5HL subtelomere based on the barley cv. Morex v2 genome assembly.

Table S3: Volumes of the barley satellite chromosomes 5H and 6H (identified based on their secondary constriction and centromere position) and NOR-free (1H-4H, 7H) chromosomes. The measurements were done on polyacrylamide embedded spatially preserved chromosomes.

Table S4: Statistics of the metaphase Hi-C datasets. Seven libraries were analyzed: three replicates from interphase libraries, three replicates from metaphase libraries and one more metaphase library generated with an alternative protocol, which yields less unspecific contacts, for comparison with polymer modelling. For each library we computed: total number of contact reads (all reads), percentage of PCR duplicated reads (duplicated), number of non-duplicated reads with a mapping quality (MAPQ) of at least 10 (high-quality reads), number of intra-chromosome contacts separated by more than 1 kb (1 kb separation), intra-chromosome contacts separated by more than 1 Mb (1 Mb separation), and number of inter-chromosome contacts (inter-chromosome). * percentage of all reads: ** percentage relative to number high-quality reads; *** percentage of the reads separated by more than 1 kb.

Movies

Movie 1. Bump position along chromosome 5H indicating the turn sizes. Placement of the bump calculated for 119 different positions in the sequence of chromosome 5H separated by 5 Mb.

Movie 2. The 14 somatic barley metaphase chromosomes labeled by FISH as described in (Figures 2B-D). The movie shows clearly that the oligo-FISH labeled 5HL region is also labelled inside of the chromosome arms.

Movie 3. The slices of a chromosome 5H SIM image stack shown in ortho view (Figure 6B) indicate the complete FISH labelling of the inner chromatin.

Movie 4: Spatial minor chromatin fiber intermingling of adjacent chromonema turns at chromosome arm 5HL labelled in different colors by oligo-FISH (Figure S10).

Movie 5. The spatial surface rendering of chromosome 5H (Figure 6C) displays that the FISH signals are present within the whole chromosome volume.

Movie 6. Spatial arrangements of telomeres and subtelomeres (Figure 6E).

Movie 7. Spatial arrangement of the oligo-FISH and EdU labeled region of the left 5H chromosome (*) shown in (Figure 4).

Movie 8. Spatial arrangement of the oligo-FISH and EdU labeled region of the right 5H chromosome (**) shown in (Figure 4).

Movie 9. The single SIM image stack slices of a DAPI-labelled 6H chromosome show a network of minor ~80 nm looped chromatin fibers free of large cavities throughout the chromosome arms Within centromere and NOR straight ~80 nm chromatin fibers occur.

Movie 10. Spatial organization of the same 6H chromosome shown in Movie 9.

Movie 11. Animation of the polymere simulation model shown in (Figure 6D) for a single FISH labelled 5H chromatid.

Movie 12. Polymere simulation of two helical chromonema half-turns (red and green) of a metaphase chromatid within a cylinder.

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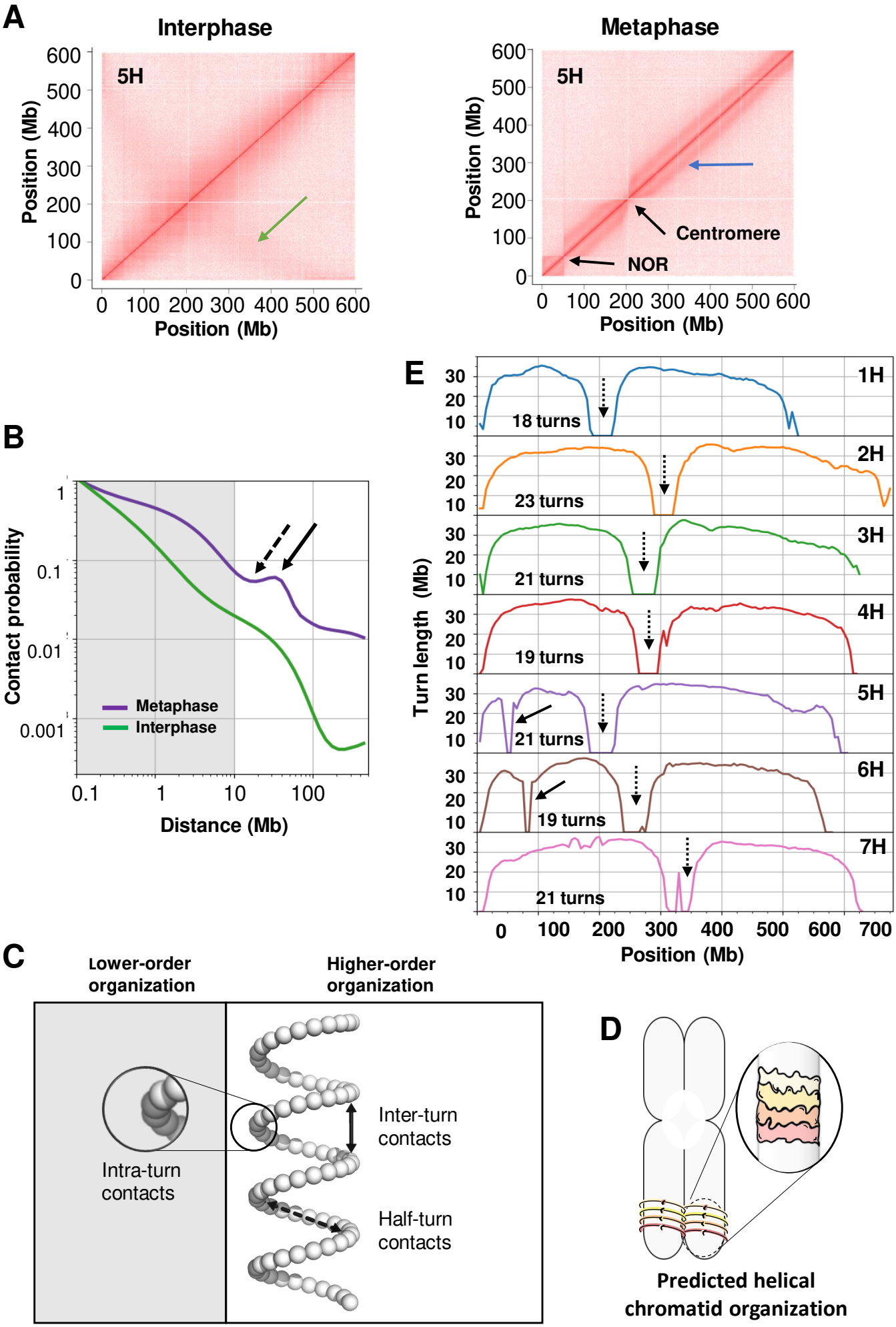

A
Interphase
Metaphase
5H
5H
Position (Mb)
Position (Mb)
Centromere
NOR
B
Contact probability
Distance (Mb)
Metaphase
Interphase
E
Turn length (Mb)
Position (Mb)
1H
18 turns
2H
23 turns
3H
21 turns
4H
19 turns
5H
21 turns
6H
19 turns
7H
21 turns
C
Lower-order organization
Higher-order organization
Intra-turn contacts
Inter-turn contacts
Half-turn contacts
D
Predicted helical chromatid organization

Figure 1

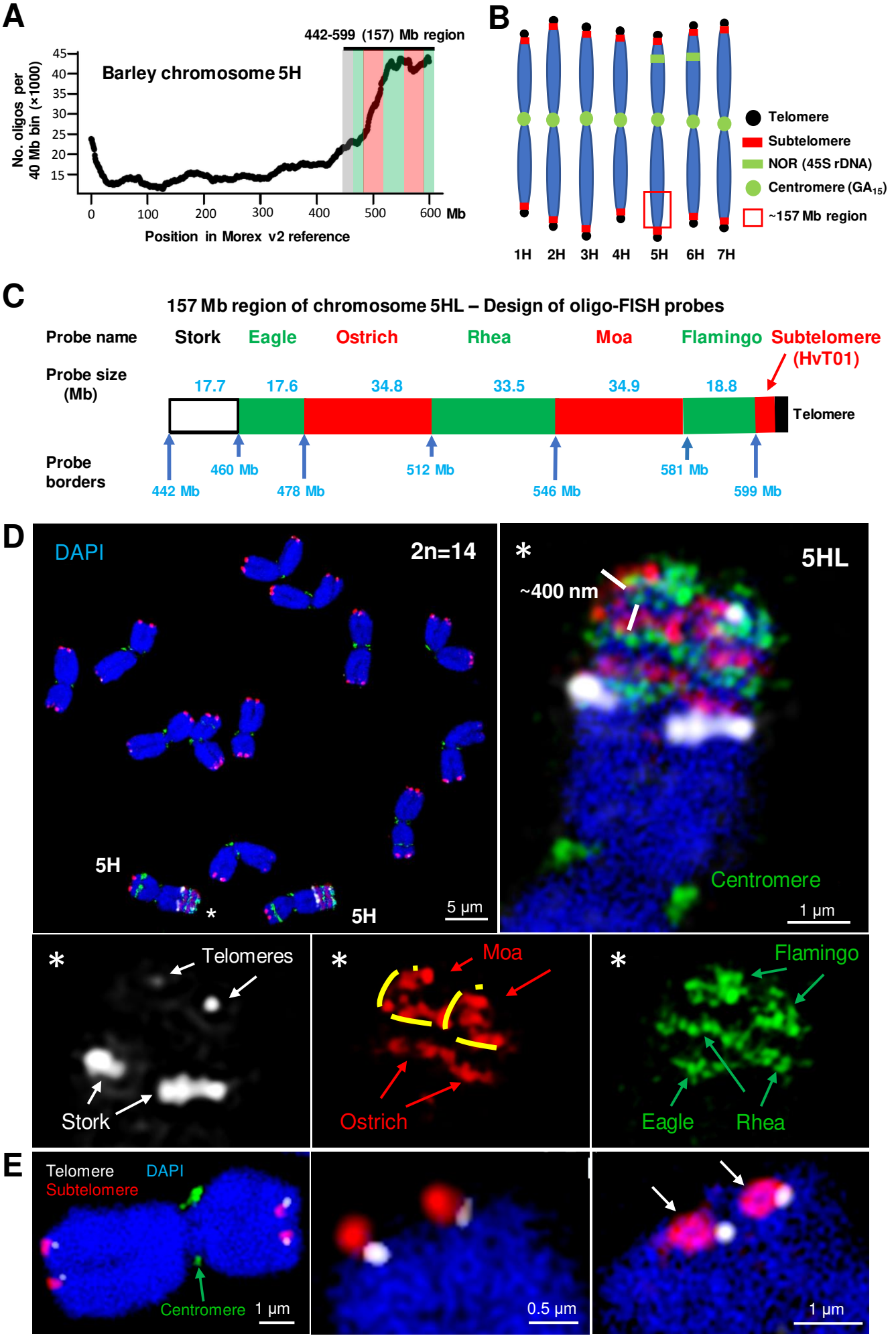
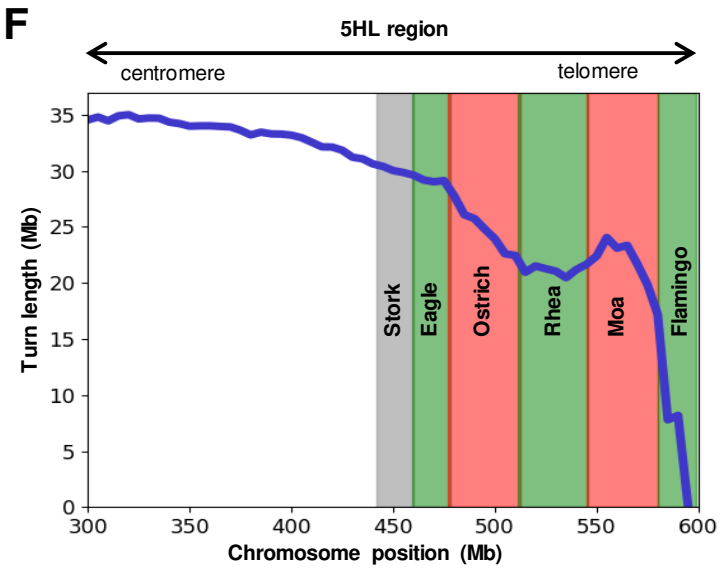
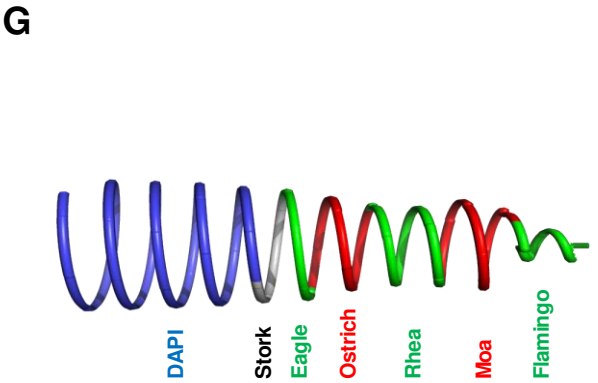
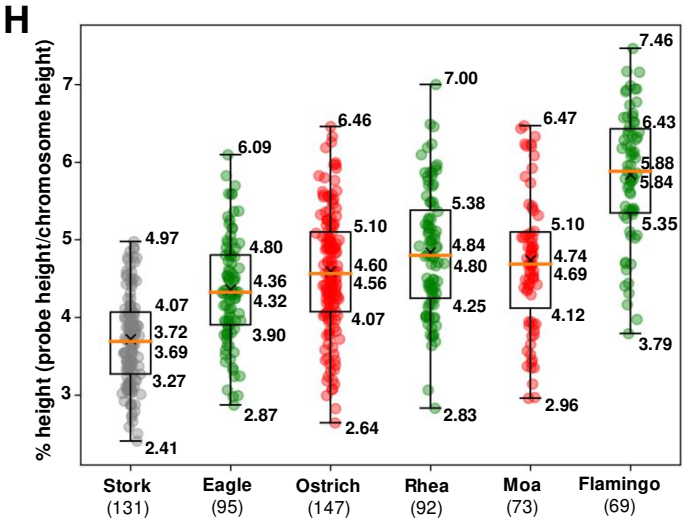

A
442-599 (157) Mb region
Barley chromosome 5H
No. oligos per 40 Mb bin (×1000)
45
40
35
30
25
20
15
0
100
200
300
400
500
600 Mb
Position in Morex v2 reference
B
Telomere
Subtelomere
NOR (45S rDNA)
Centromere (GA15)
~157 Mb region
1H
2H
3H
4H
5H
6H
7H
C
157 Mb region of chromosome 5HL – Design of oligo-FISH probes
Probe name
Stork
Eagle
Ostrich
Rhea
Moa
Flamingo
Subtelomere (HvT01)
Probe size (Mb)
17.7
17.6
34.8
33.5
34.9
18.8
Telomere
Probe borders
442 Mb
460 Mb
478 Mb
512 Mb
546 Mb
581 Mb
599 Mb
D
DAPI
2n=14
5H
5H
5 µm
*
5HL
~400 nm
Centromere
1 µm
*
Telomeres
Stork
*
Moa
Ostrich
*
Flamingo
Eagle
Rhea
E
Telomere
DAPI
Subtelomere
Centromere
1 µm
0.5 µm
1 µm

Figure 2







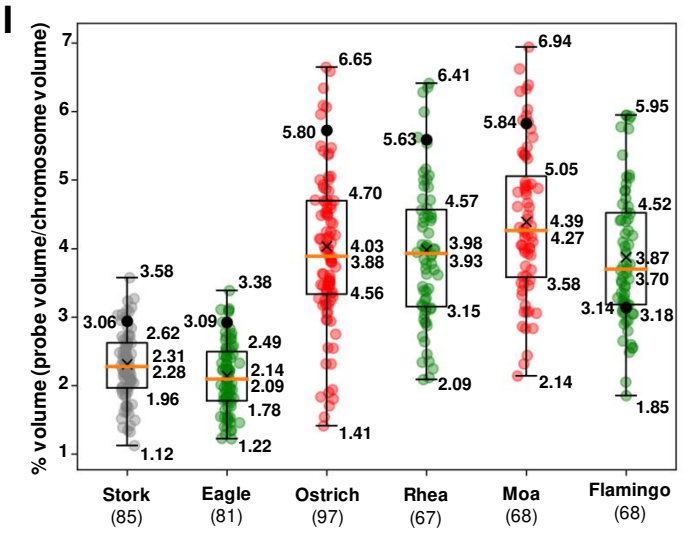


Figure 2 continued

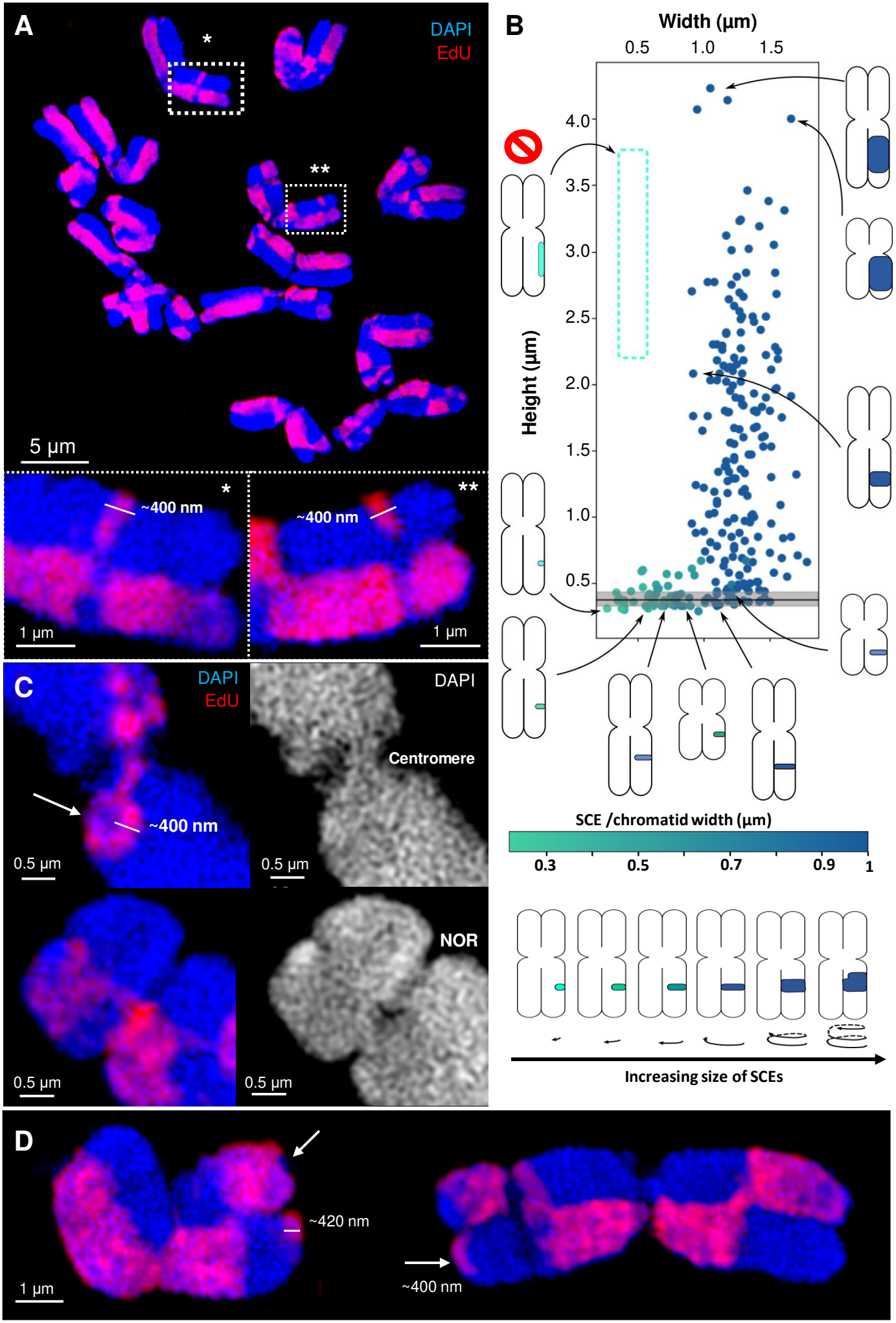

A
DAPI
EdU
*
**
5 µm
~400 nm
1 µm
B
Width (µm)
0.5
1.0
1.5
Height (µm)
4.0
3.5
3.0
2.5
2.0
1.5
1.0
0.5
SCE /chromatid width (µm)
0.3
0.5
0.7
0.9
1
Increasing size of SCEs
C
Centromere
NOR
0.5 µm
D
~420 nm
~400 nm

Figure 3

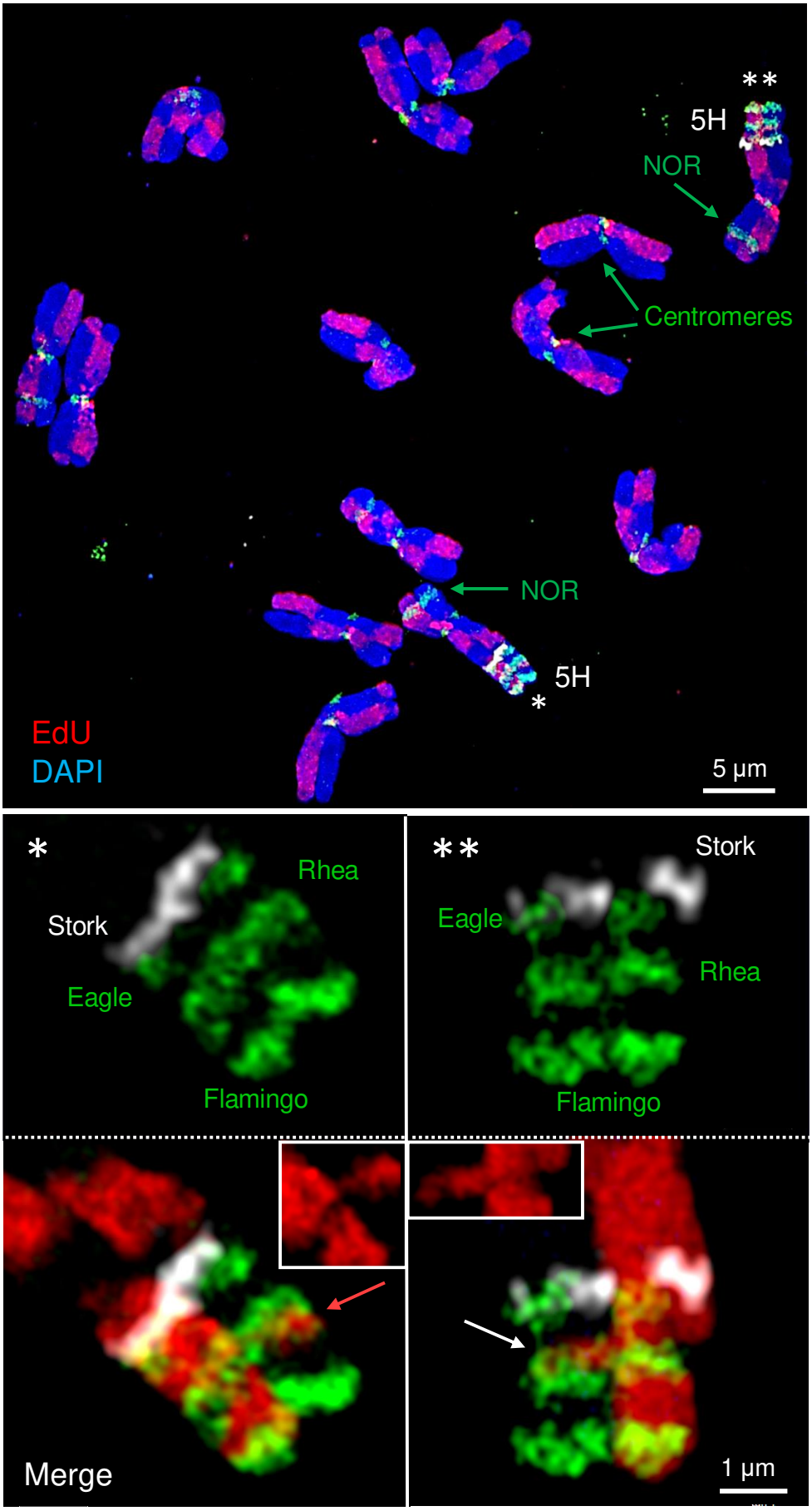

**
5H
NOR
Centromeres
NOR
5H
*
EdU
DAPI
5 µm
*
Rhea
Stork
Eagle
Flamingo
**
Stork
Eagle
Rhea
Flamingo
Merge
1 µm

Figure 4

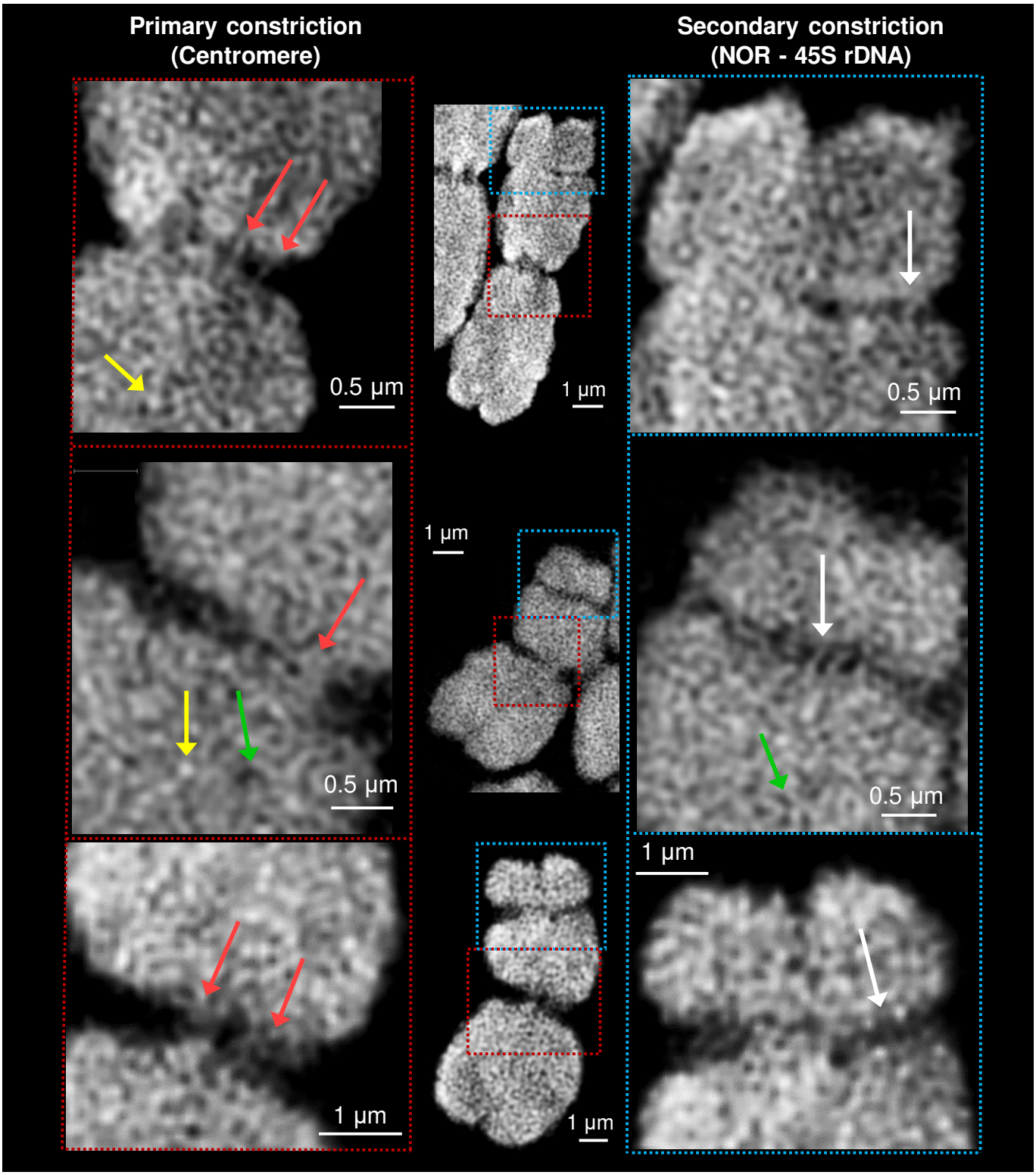

Primary constriction
(Centromere)
Secondary constriction
(NOR - 45S rDNA)
0.5 µm
1 µm
0.5 µm
1 µm
0.5 µm
1 µm
1 µm
1 µm

Figure 5

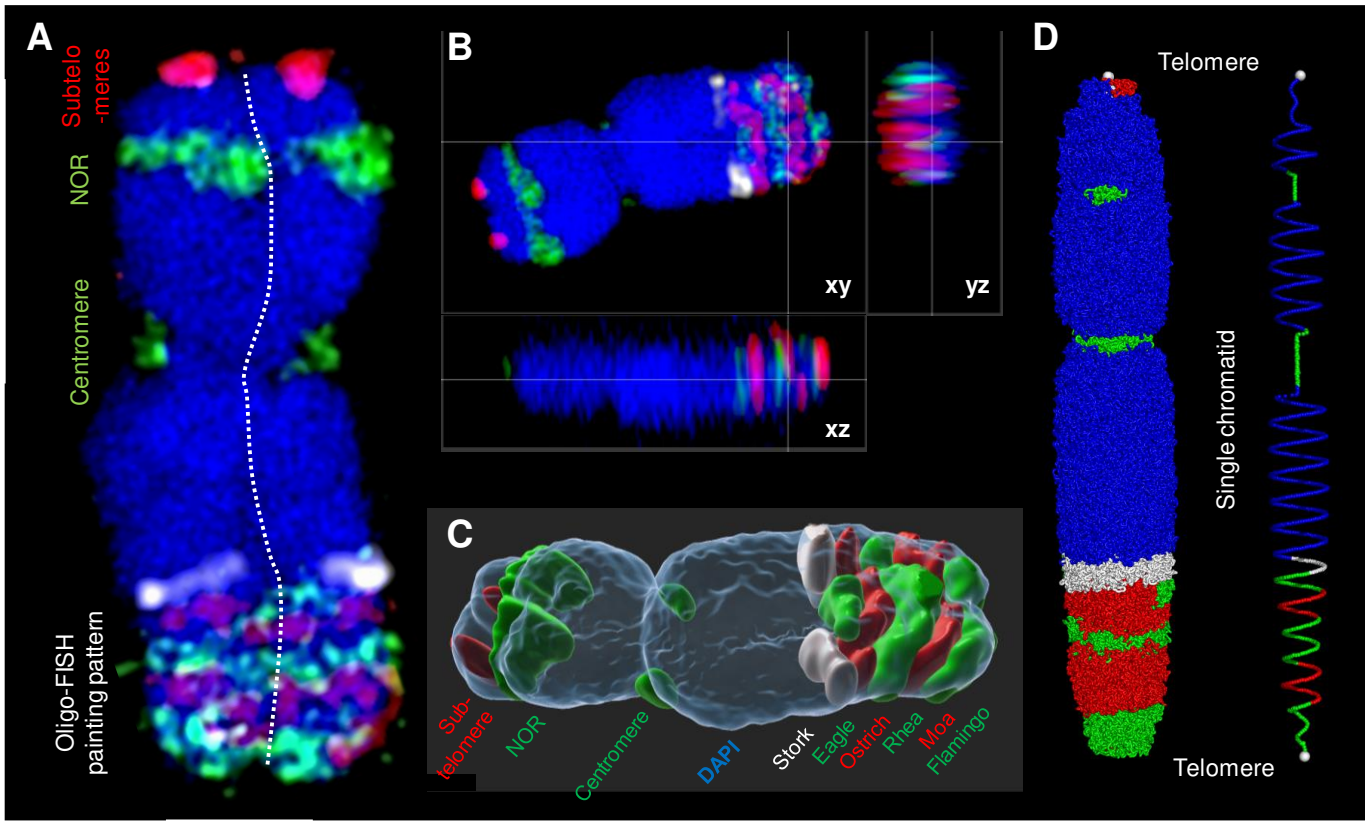

A
Subtelo
-meres
NOR
Centromere
Oligo-FISH
painting pattern
B
xy
yz
xz
C
Sub-
telomere
NOR
Centromere
DAPI
Stork
Eagle
Ostrich
Rhea
Moa
Flamingo
D
Telomere
Single chromatid
Telomere

Figure 6

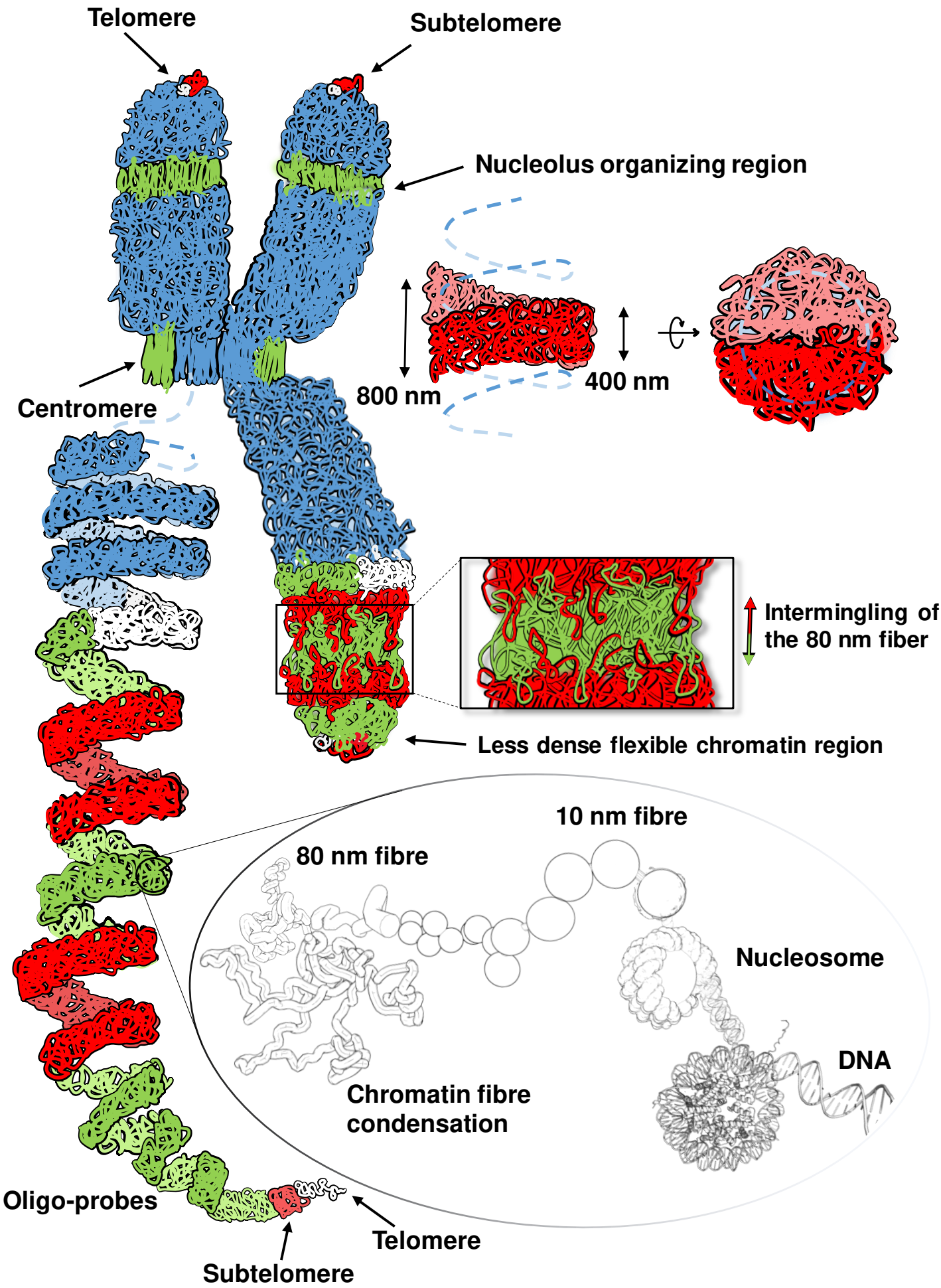

Telomere
Subtelomere
Nucleolus organizing region
800 nm
400 nm
Centromere
Intermingling of the 80 nm fiber
Less dense flexible chromatin region
10 nm fibre
80 nm fibre
Nucleosome
DNA
Chromatin fibre condensation
Oligo-probes
Telomere
Subtelomere

Figure 7

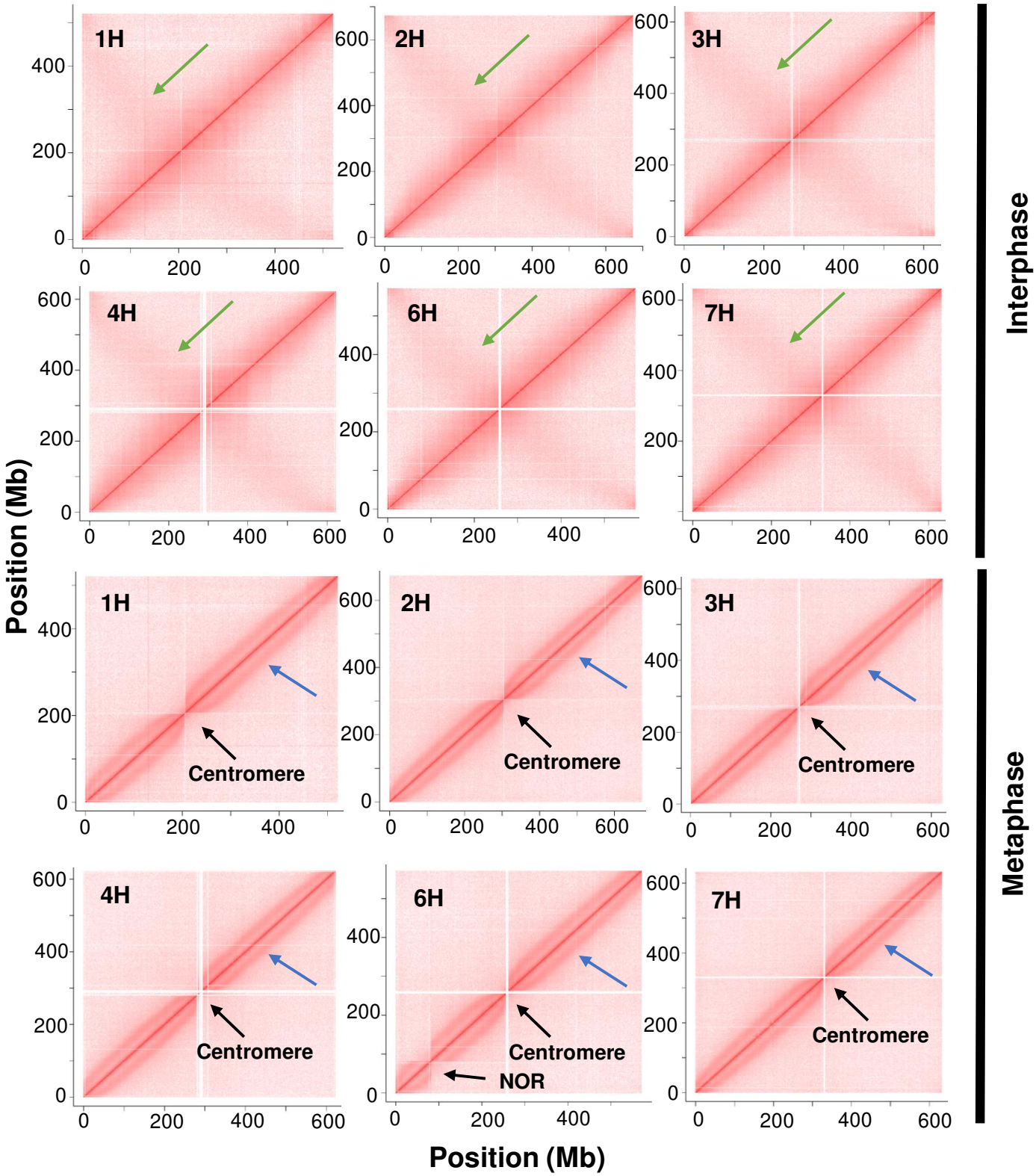

1H
2H
3H
4H
6H
7H
Interphase
Metaphase
Centromere
NOR
Position (Mb)
Position (Mb)

Figure S1

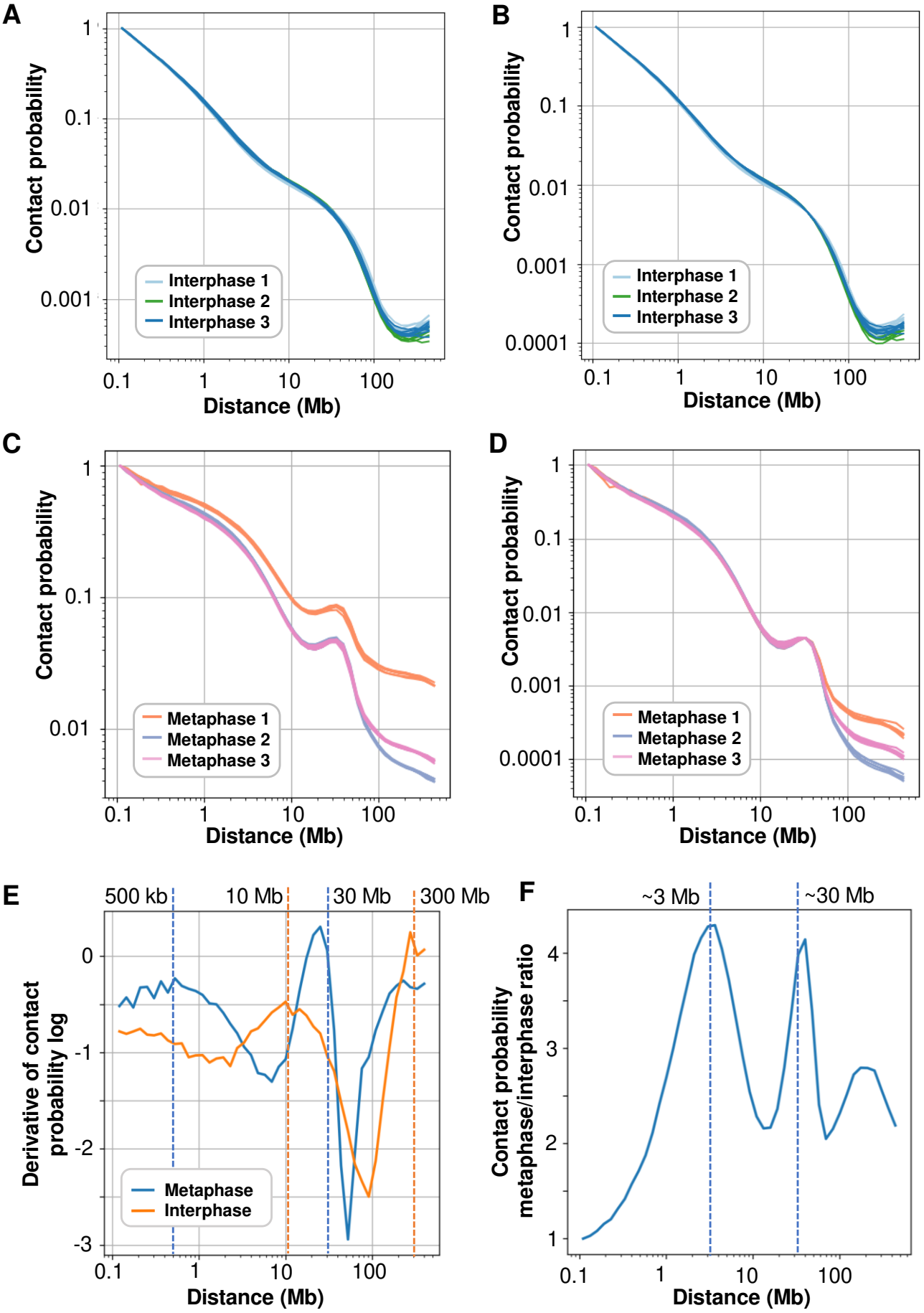

A
Contact probability
Distance (Mb)
Interphase 1
Interphase 2
Interphase 3
B
Contact probability
Distance (Mb)
Interphase 1
Interphase 2
Interphase 3
C
Contact probability
Distance (Mb)
Metaphase 1
Metaphase 2
Metaphase 3
D
Contact probability
Distance (Mb)
Metaphase 1
Metaphase 2
Metaphase 3
E
500 kb
10 Mb
30 Mb
300 Mb
Derivative of contact probability log
Distance (Mb)
Metaphase
Interphase
F
~3 Mb
~30 Mb
Contact probability metaphase/interphase ratio
Distance (Mb)

Figure S2

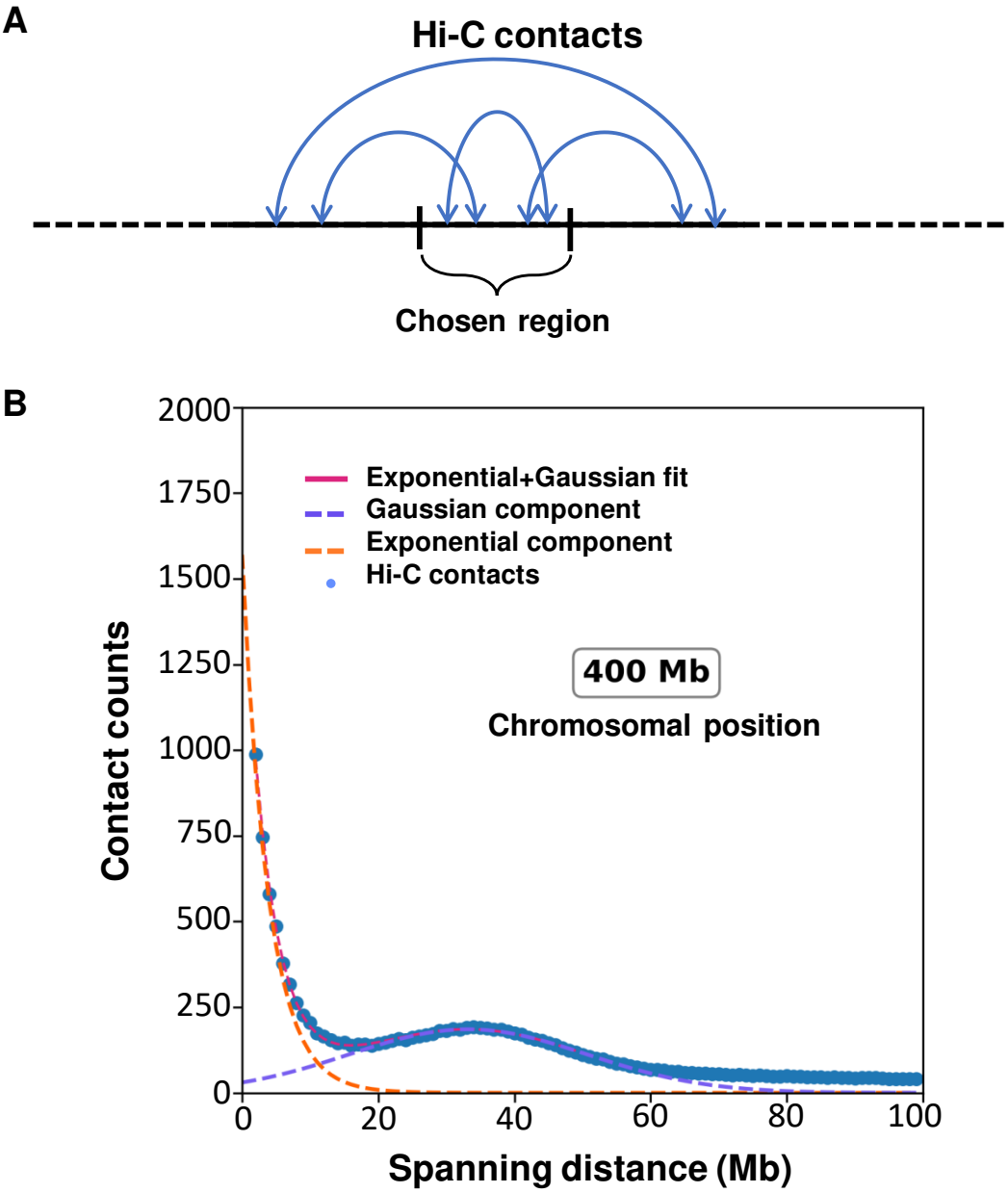

A
Hi-C contacts
Chosen region
B
Exponential+Gaussian fit
Gaussian component
Exponential component
Hi-C contacts
400 Mb
Chromosomal position
Contact counts
Spanning distance (Mb)
0
250
500
750
1000
1250
1500
1750
2000
0
20
40
60
80
100

Figure S3

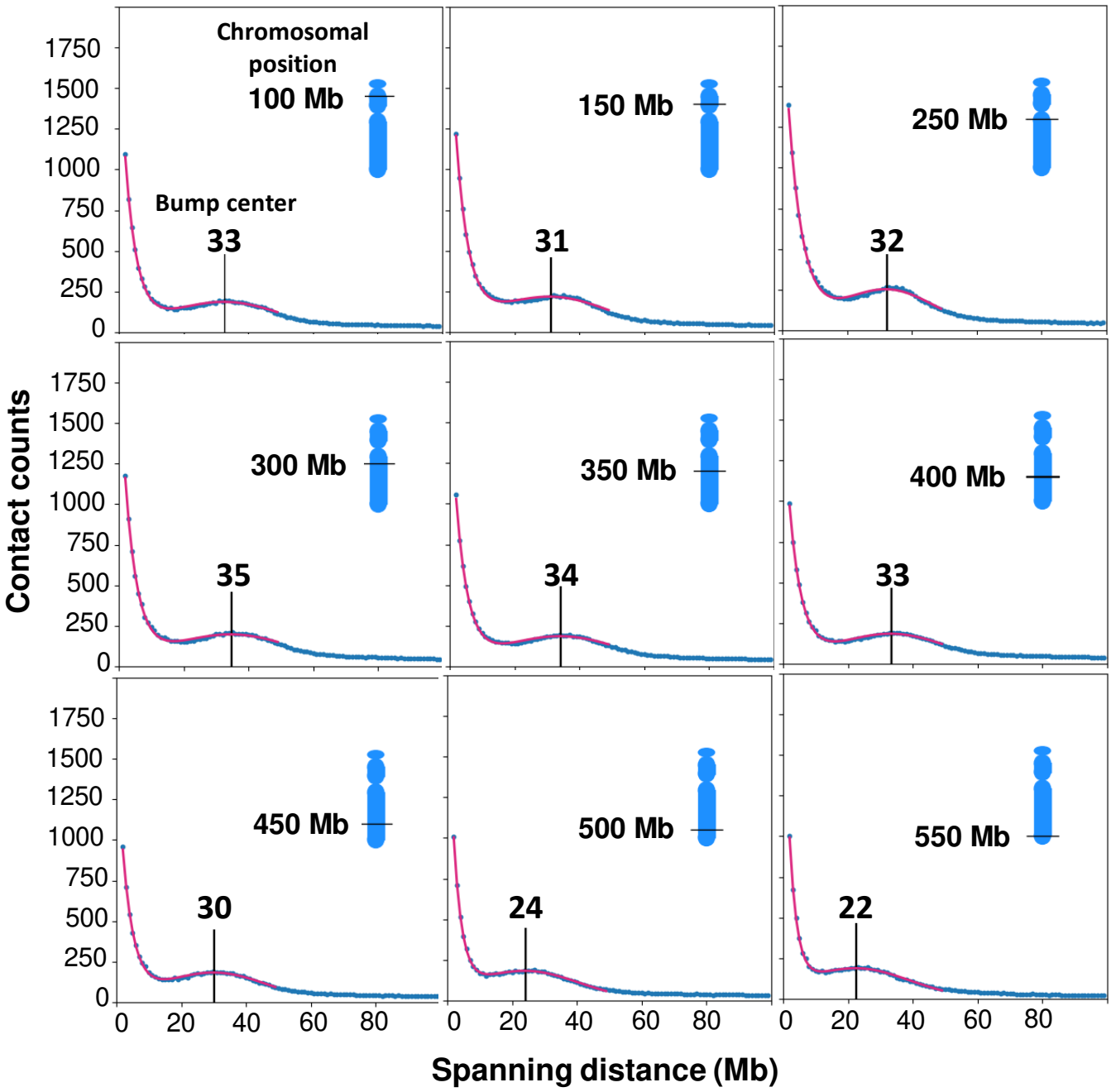

Chromosomal position
100 Mb
Bump center
33
150 Mb
31
250 Mb
32
300 Mb
35
350 Mb
34
400 Mb
33
450 Mb
30
500 Mb
24
550 Mb
22
Contact counts
Spanning distance (Mb)
0
250
500
750
1000
1250
1500
1750
0
20
40
60
80

Figure S4

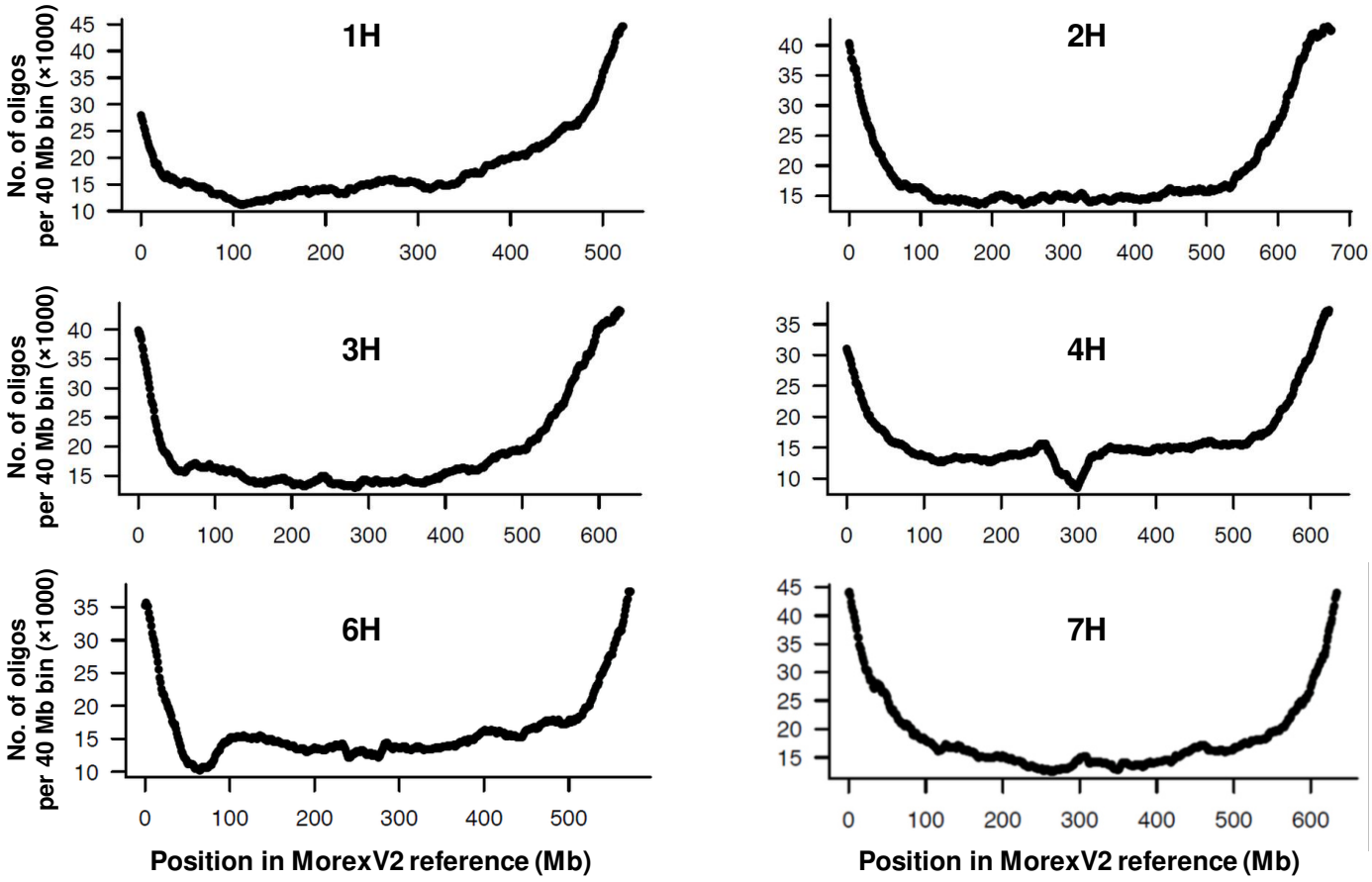
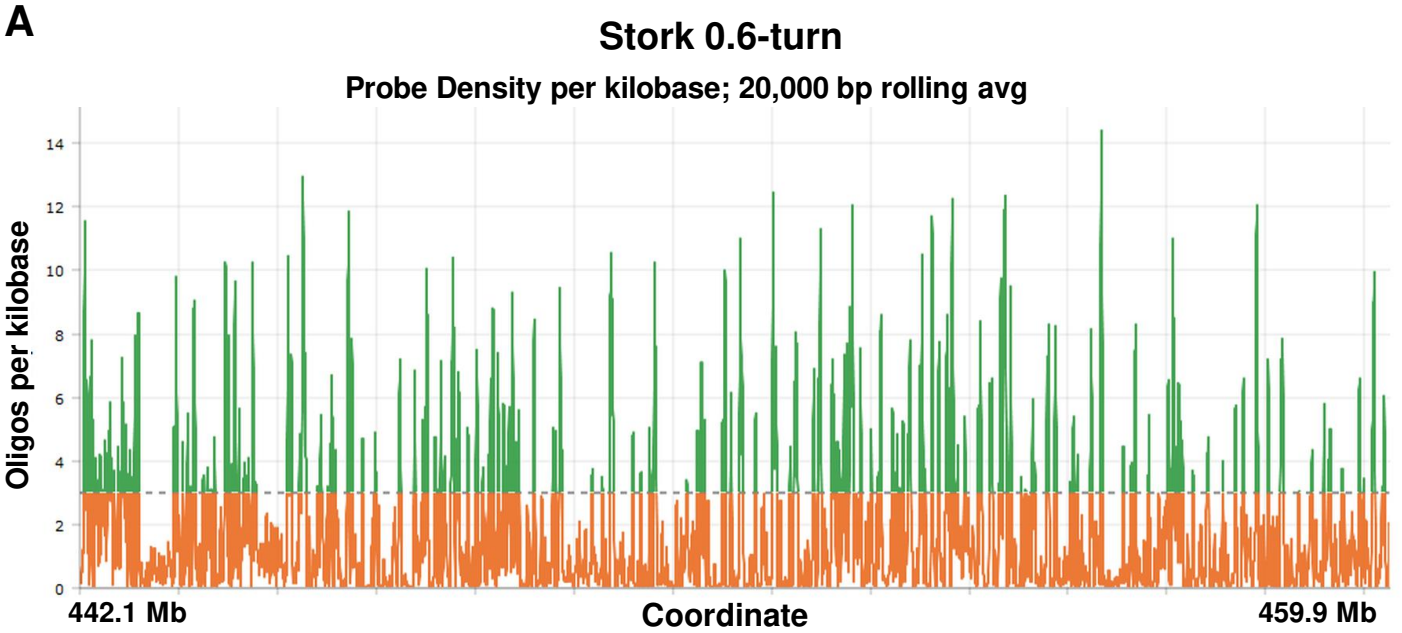

1H
2H
3H
4H
6H
7H
No. of oligos per 40 Mb bin (×1000)
Position in MorexV2 reference (Mb)

Figure S5


A
Stork 0.6-turn
Probe Density per kilobase; 20,000 bp rolling avg
Oligos per kilobase
0
2
4
6
8
10
12
14
442.1 Mb
Coordinate
459.9 Mb

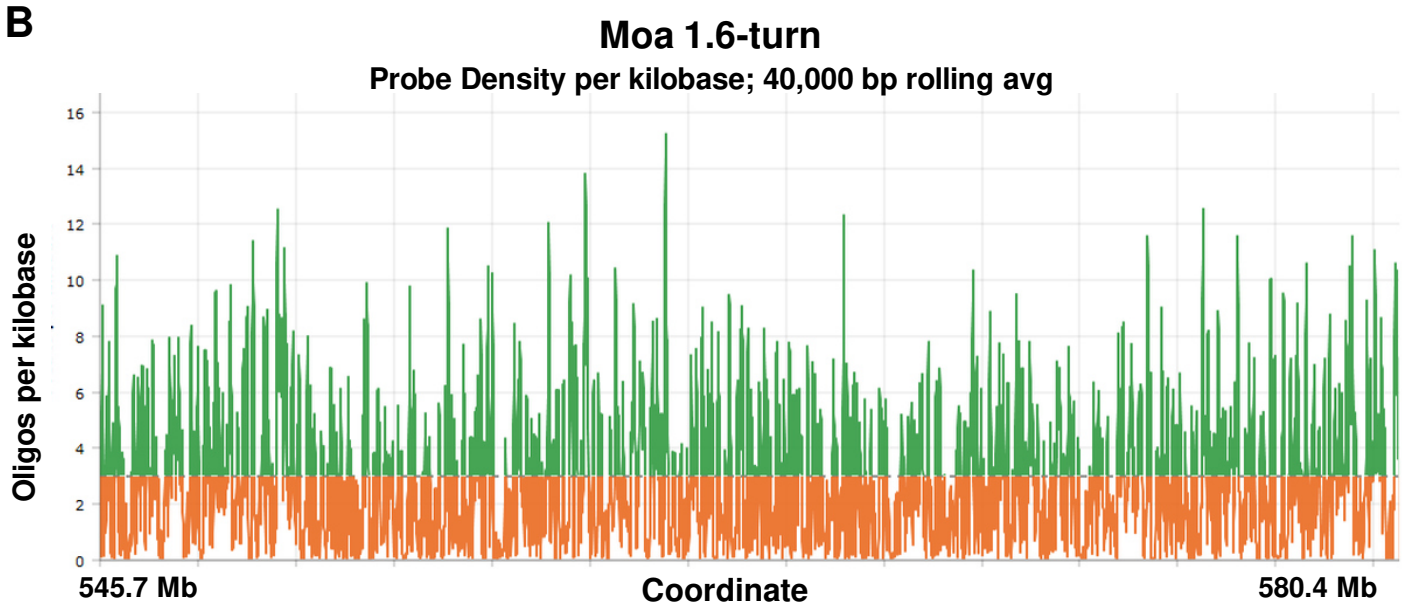

B
Moa 1.6-turn
Probe Density per kilobase; 40,000 bp rolling avg
Oligos per kilobase
0
2
4
6
8
10
12
14
16
545.7 Mb
Coordinate
580.4 Mb

Figure S6

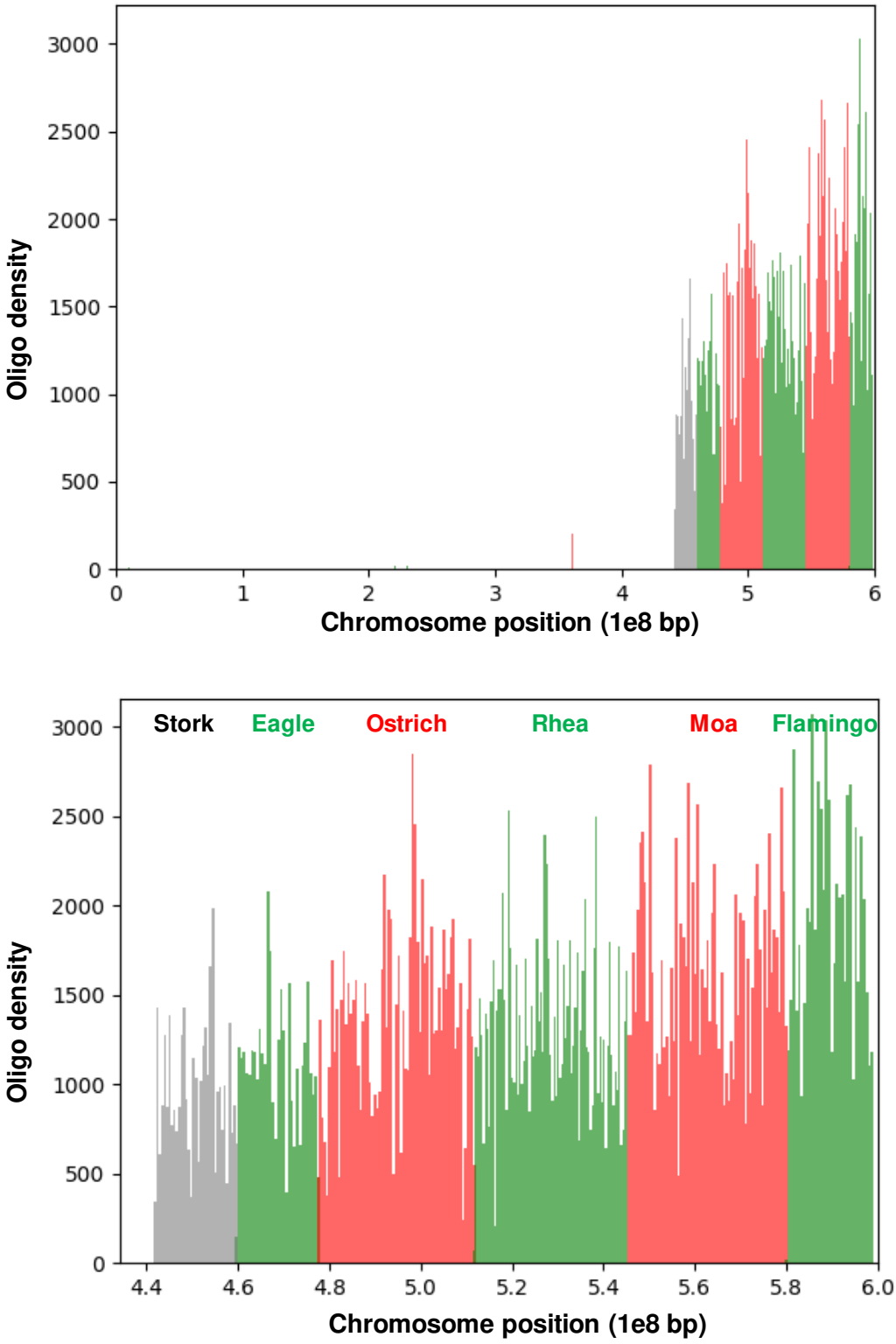

Oligo density
Chromosome position (1e8 bp)
Stork
Eagle
Ostrich
Rhea
Moa
Flamingo

Figure S7

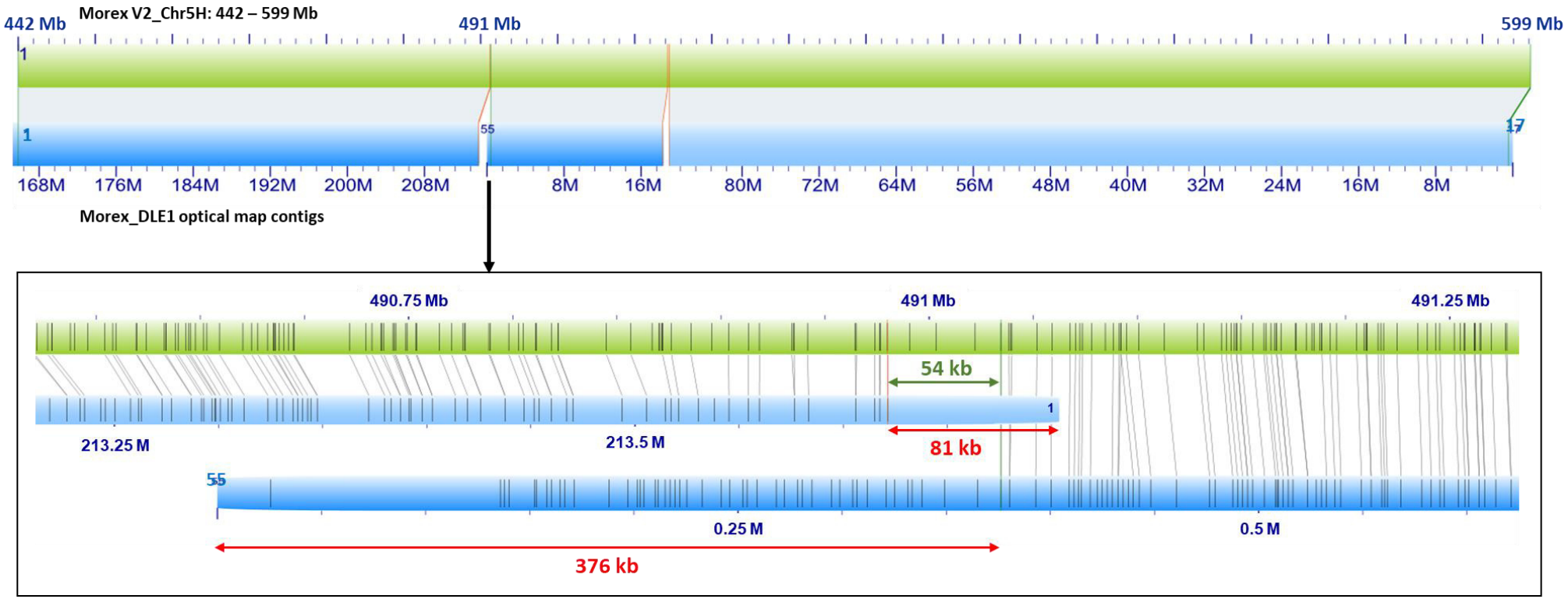

Morex V2_Chr5H: 442 – 599 Mb
442 Mb
491 Mb
599 Mb
1
1
55
17
168M
176M
184M
192M
200M
208M
8M
16M
80M
72M
64M
56M
48M
40M
32M
24M
16M
8M
Morex_DLE1 optical map contigs
490.75 Mb
491 Mb
491.25 Mb
54 kb
1
213.25 M
213.5 M
81 kb
55
0.25 M
0.5 M
376 kb

Figure S8

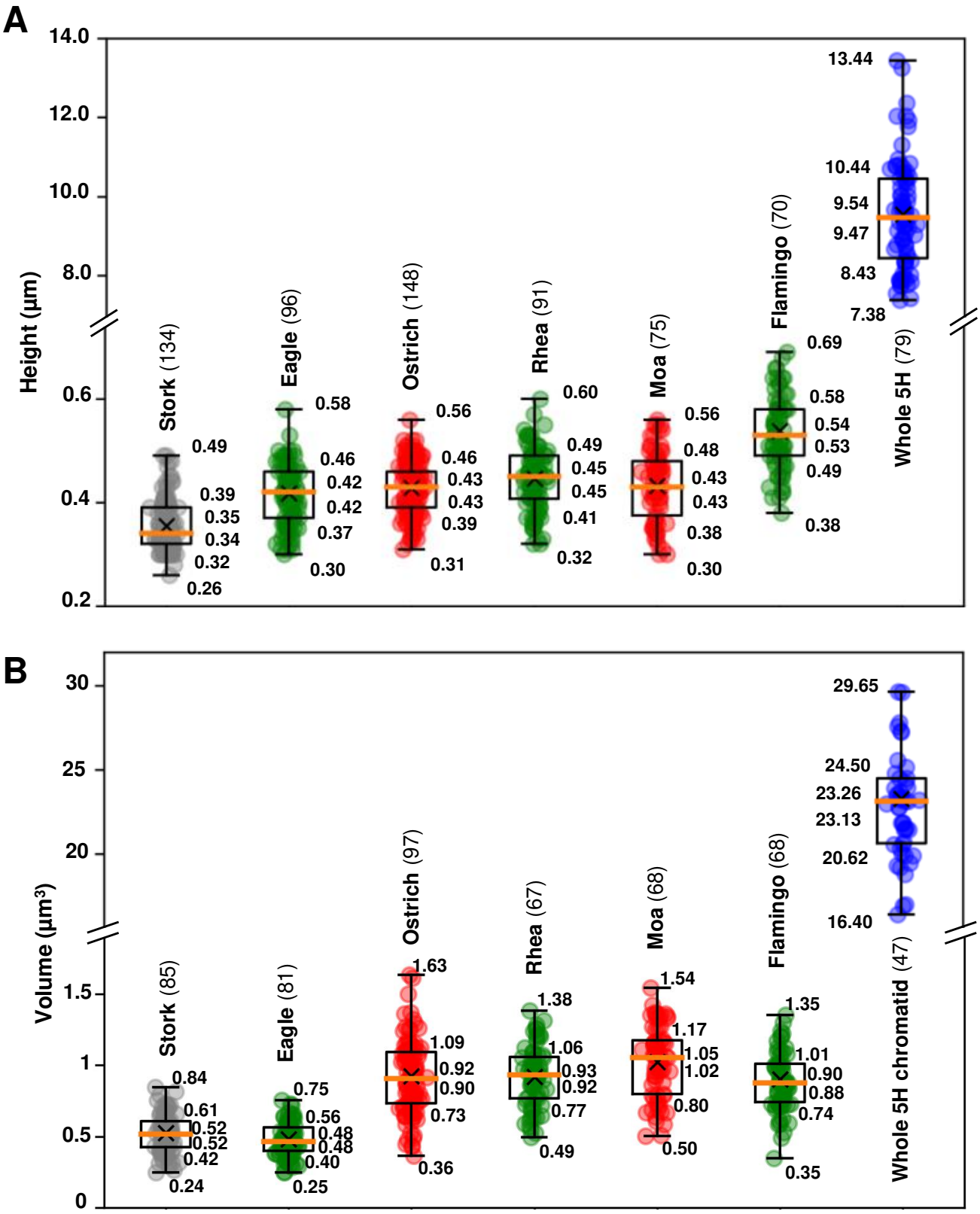

A
Height (µm)
14.0
12.0
10.0
8.0
0.6
0.4
0.2
Stork (134)
0.49
0.39
0.35
0.34
0.32
0.26
Eagle (96)
0.58
0.46
0.42
0.42
0.37
0.30
Ostrich (148)
0.56
0.46
0.43
0.43
0.39
0.31
Rhea (91)
0.60
0.49
0.45
0.45
0.41
0.32
Moa (75)
0.56
0.48
0.43
0.43
0.38
0.30
Flamingo (70)
0.69
0.58
0.54
0.53
0.49
0.38
Whole 5H (79)
13.44
10.44
9.54
9.47
8.43
7.38
B
Volume (µm³)
30
25
20
1.5
1
0.5
0
Stork (85)
0.84
0.61
0.52
0.52
0.42
0.24
Eagle (81)
0.75
0.56
0.48
0.48
0.40
0.25
Ostrich (97)
1.63
1.09
0.92
0.90
0.73
0.36
Rhea (67)
1.38
1.06
0.93
0.92
0.77
0.49
Moa (68)
1.54
1.17
1.05
1.02
0.80
0.50
Flamingo (68)
1.35
1.01
0.90
0.88
0.74
0.35
Whole 5H chromatid (47)
29.65
24.50
23.26
23.13
20.62
16.40

Figure S9

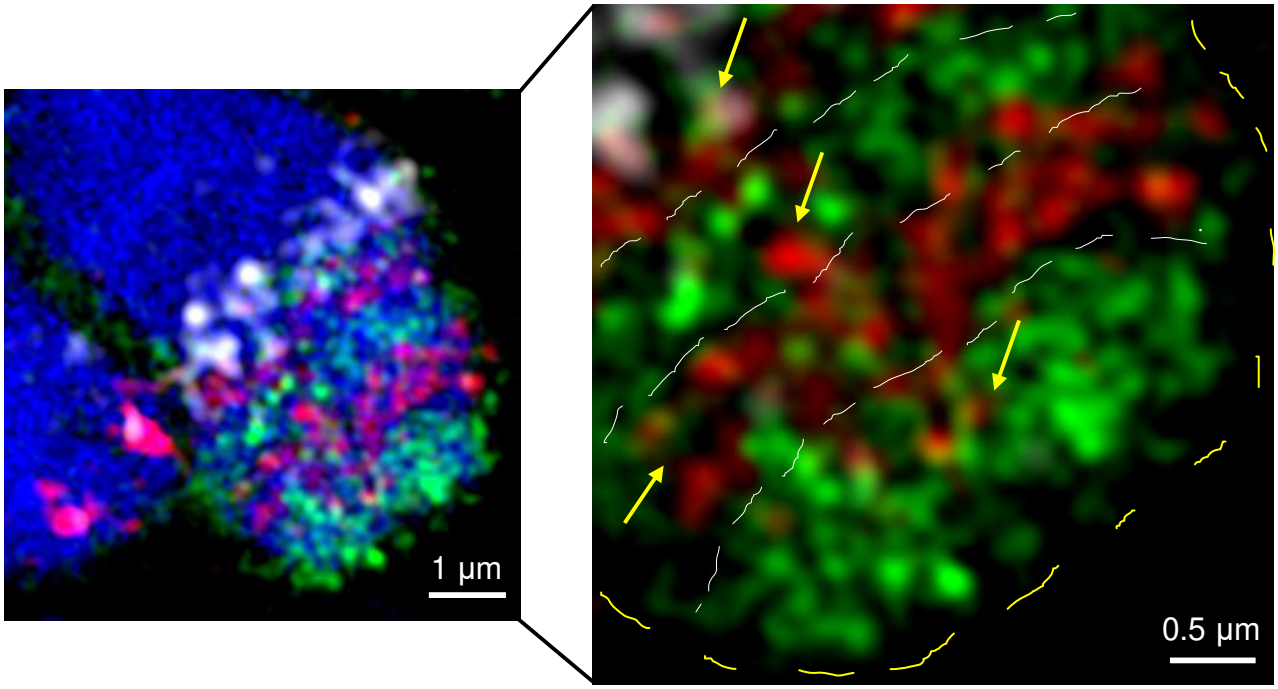


Figure S10

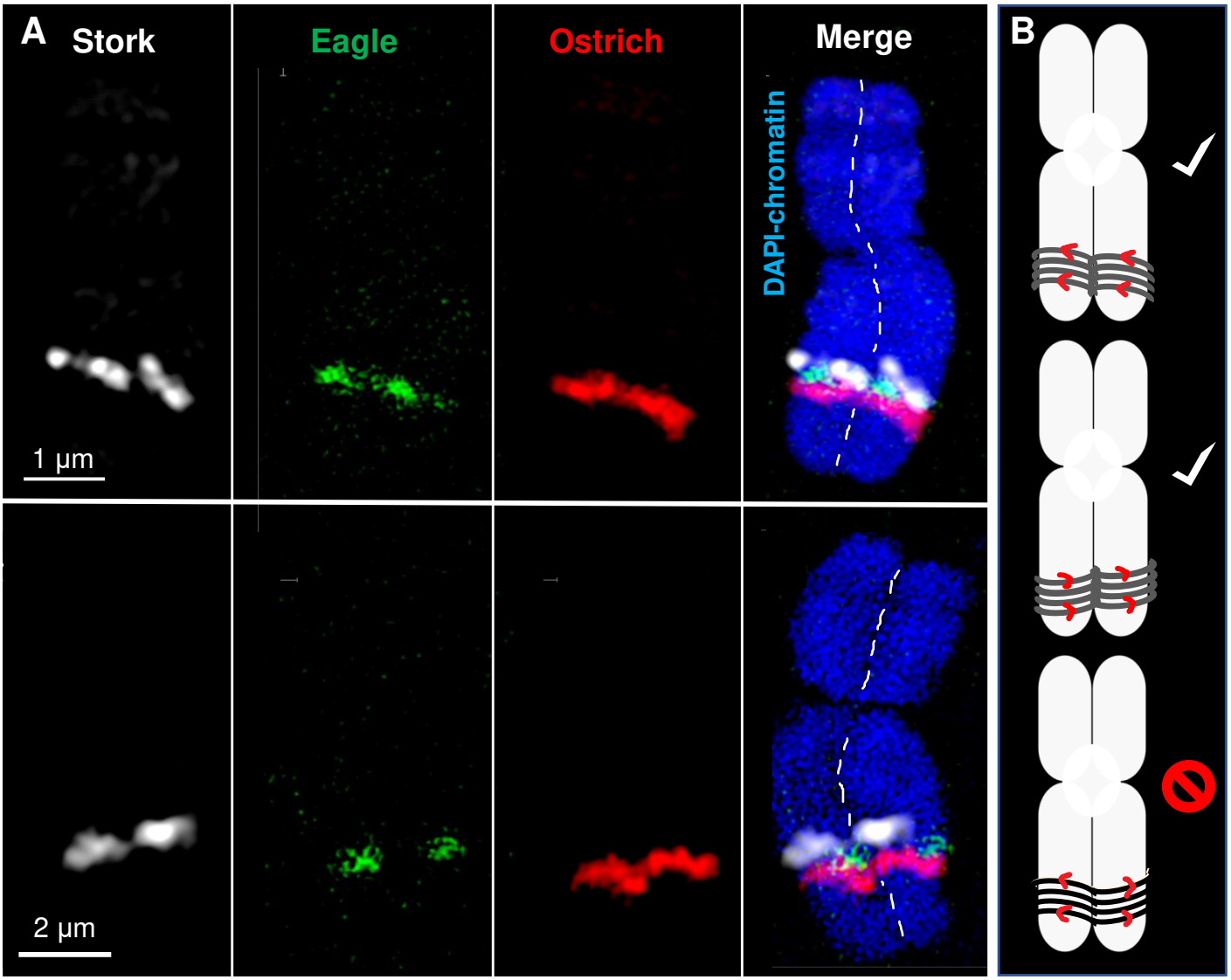
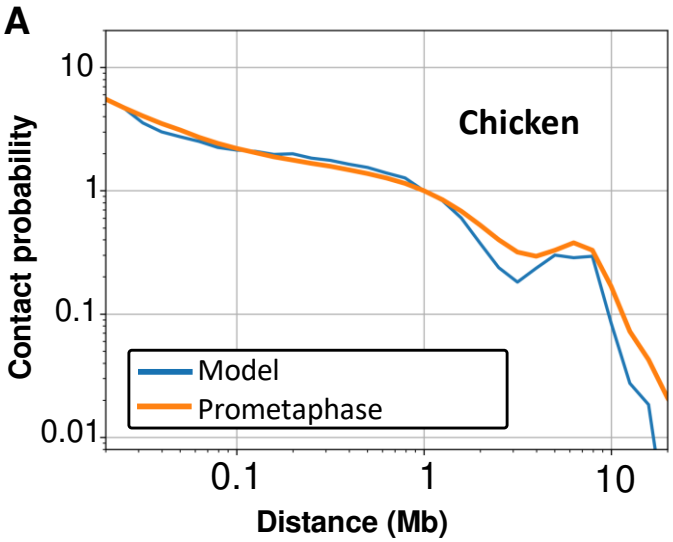
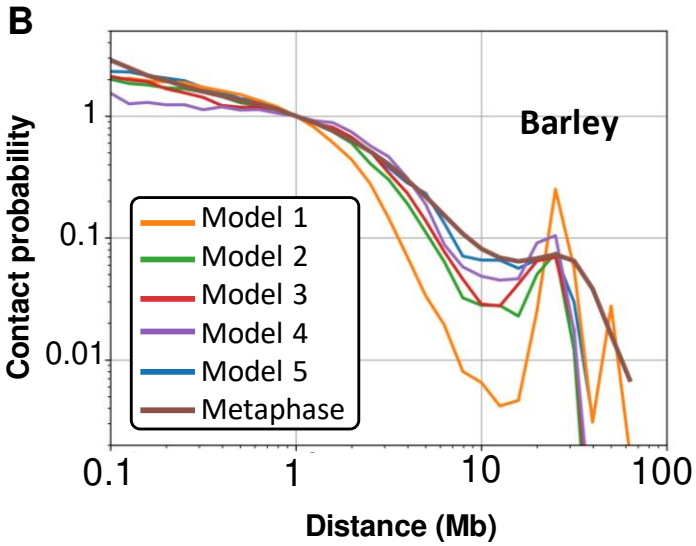


Figure S11



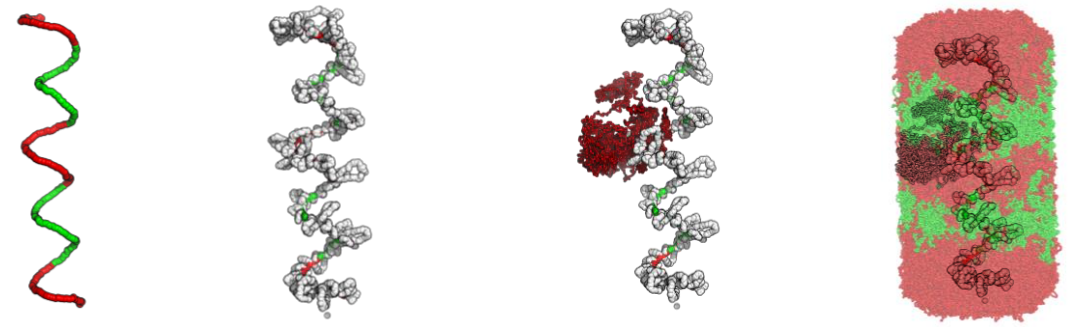


C

Simulated model parameters for barley

	Turn length (Mb)	Turn height (nm)	Major loop size (kb)	Minor loop size (kb)
Model 1	30	150	400	80
Model 2	30	400	400	80
Model 3	30	400	800	80
Model 4	30	400	800	160
Model 5	30	400	3,000	500

D



E

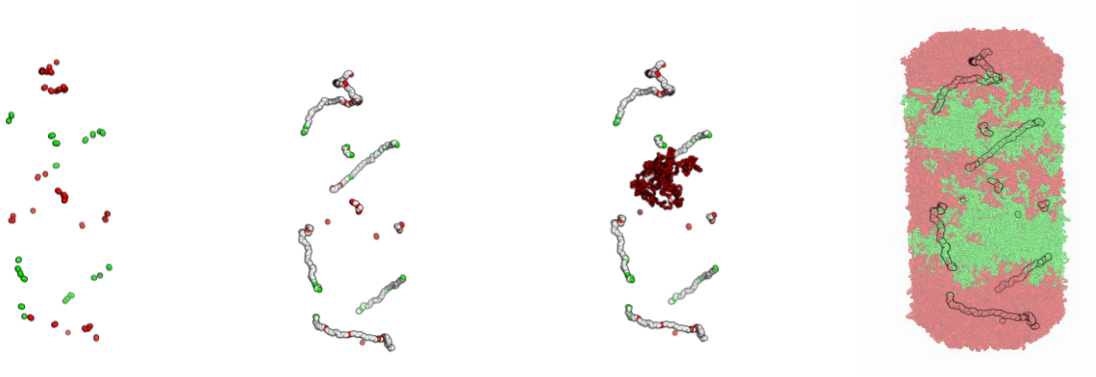


Figure S12

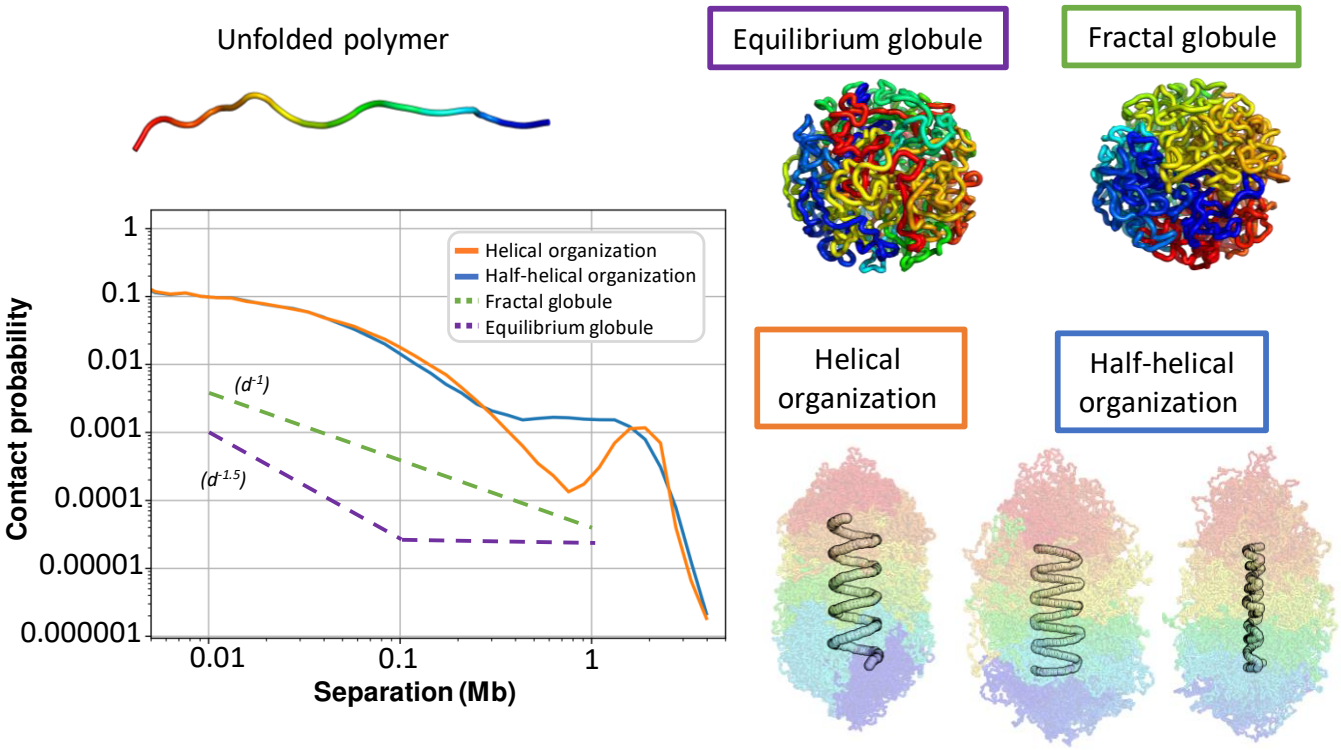

Unfolded polymer
Equilibrium globule
Fractal globule
Helical organization
Half-helical organization
Contact probability
Separation (Mb)
Helical organization
Half-helical organization
Fractal globule
Equilibrium globule
(d-1)
(d-1.5)
1
0.1
0.01
0.001
0.0001
0.00001
0.000001
0.01
0.1
1

Figure S13

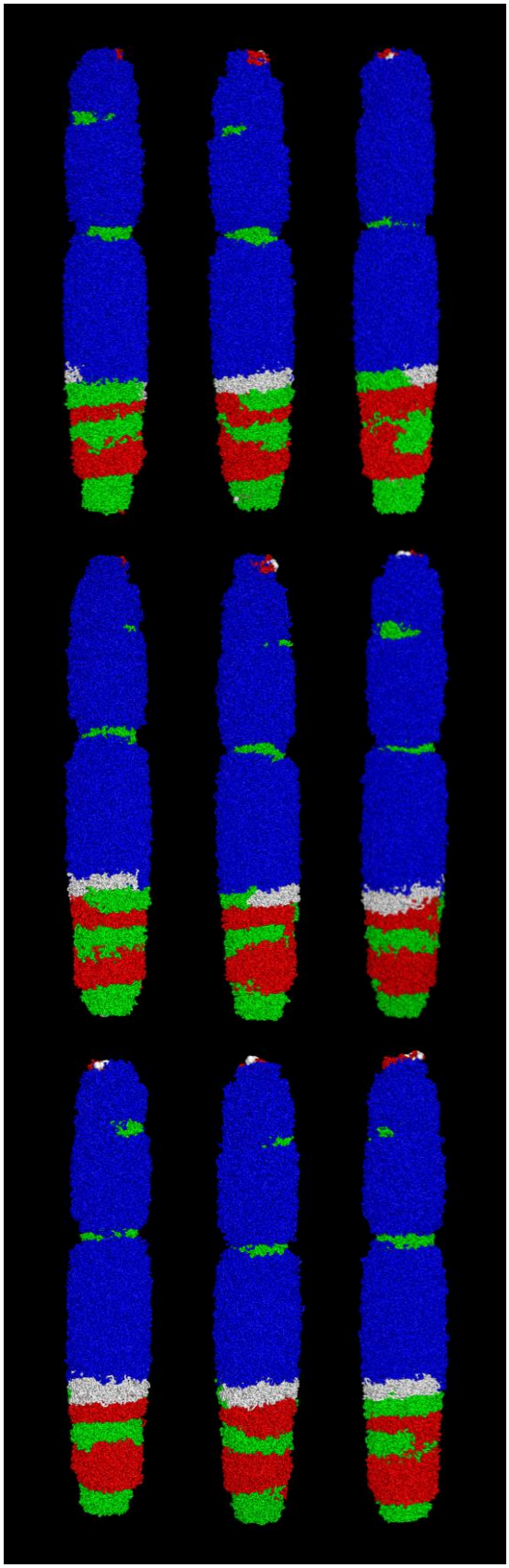
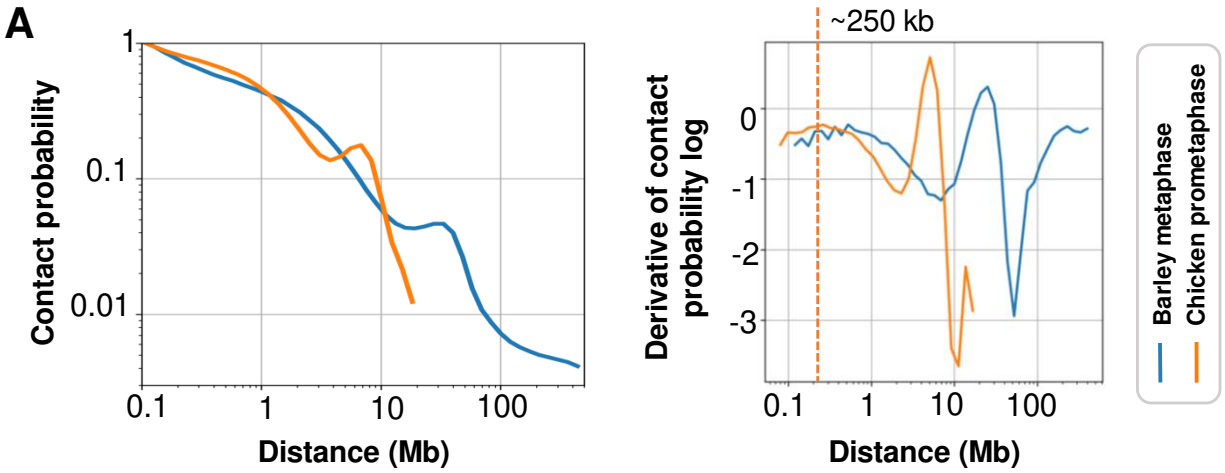
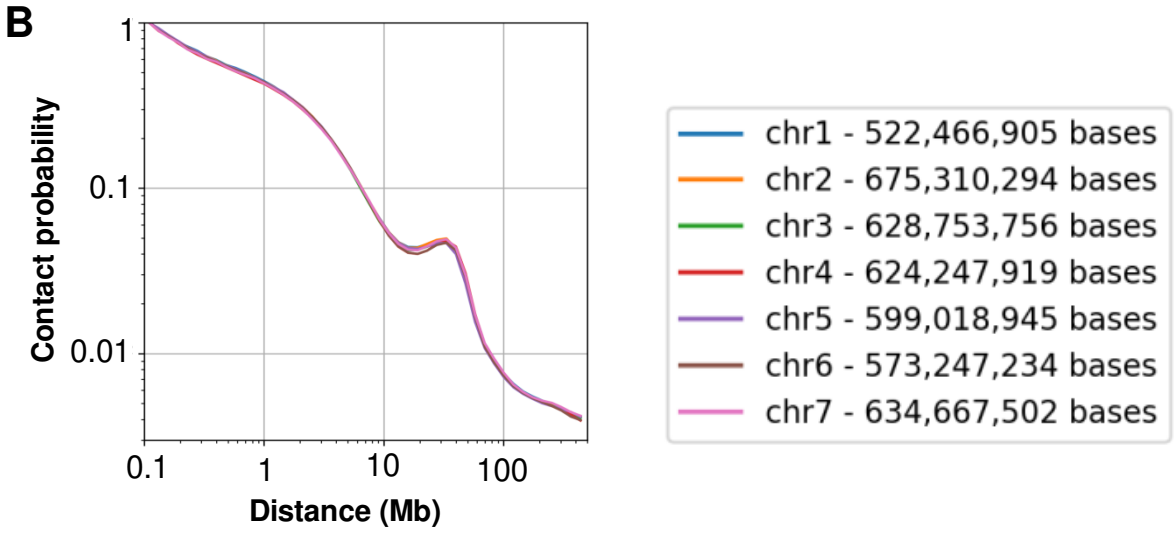


Figure S14


A
Contact probability
1
0.1
0.01
0.1
1
10
100
Distance (Mb)
~250 kb
Derivative of contact probability log
0
-1
-2
-3
0.1
1
10
100
Distance (Mb)
Barley metaphase
Chicken prometaphase


B
Contact probability
1
0.1
0.01
0.1
1
10
100
Distance (Mb)
chr1 - 522,466,905 bases
chr2 - 675,310,294 bases
chr3 - 628,753,756 bases
chr4 - 624,247,919 bases
chr5 - 599,018,945 bases
chr6 - 573,247,234 bases
chr7 - 634,667,502 bases

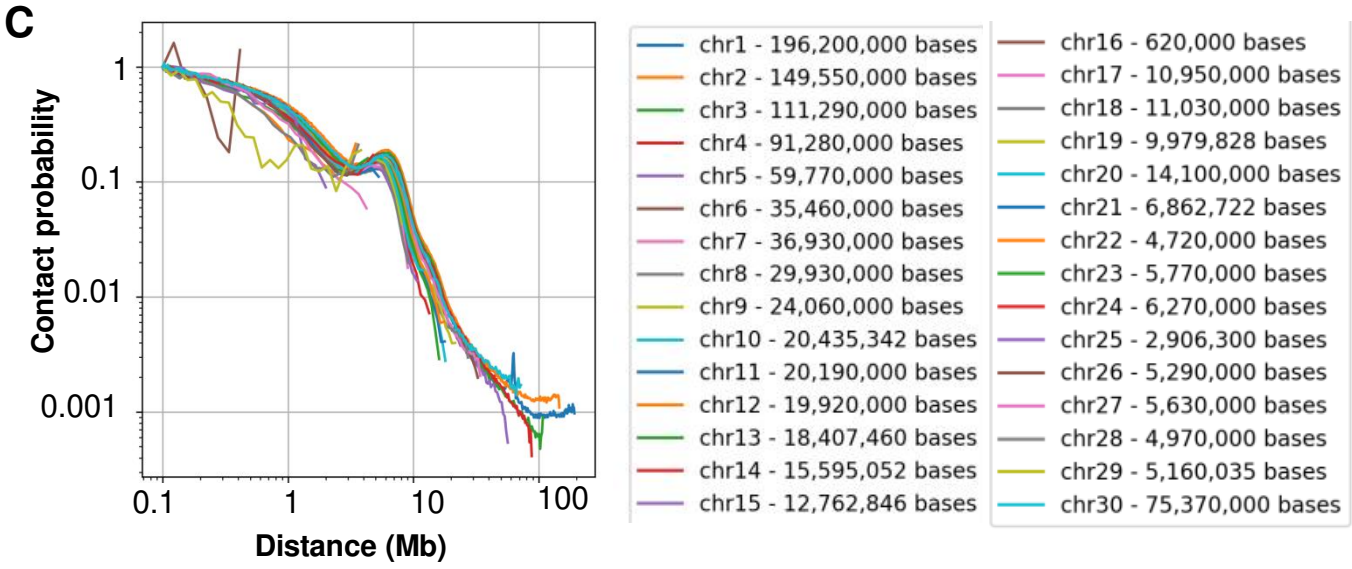

C
Contact probability
1
0.1
0.01
0.001
0.1
1
10
100
Distance (Mb)
chr1 - 196,200,000 bases
chr2 - 149,550,000 bases
chr3 - 111,290,000 bases
chr4 - 91,280,000 bases
chr5 - 59,770,000 bases
chr6 - 35,460,000 bases
chr7 - 36,930,000 bases
chr8 - 29,930,000 bases
chr9 - 24,060,000 bases
chr10 - 20,435,342 bases
chr11 - 20,190,000 bases
chr12 - 19,920,000 bases
chr13 - 18,407,460 bases
chr14 - 15,595,052 bases
chr15 - 12,762,846 bases
chr16 - 620,000 bases
chr17 - 10,950,000 bases
chr18 - 11,030,000 bases
chr19 - 9,979,828 bases
chr20 - 14,100,000 bases
chr21 - 6,862,722 bases
chr22 - 4,720,000 bases
chr23 - 5,770,000 bases
chr24 - 6,270,000 bases
chr25 - 2,906,300 bases
chr26 - 5,290,000 bases
chr27 - 5,630,000 bases
chr28 - 4,970,000 bases
chr29 - 5,160,035 bases
chr30 - 75,370,000 bases

Figure S15

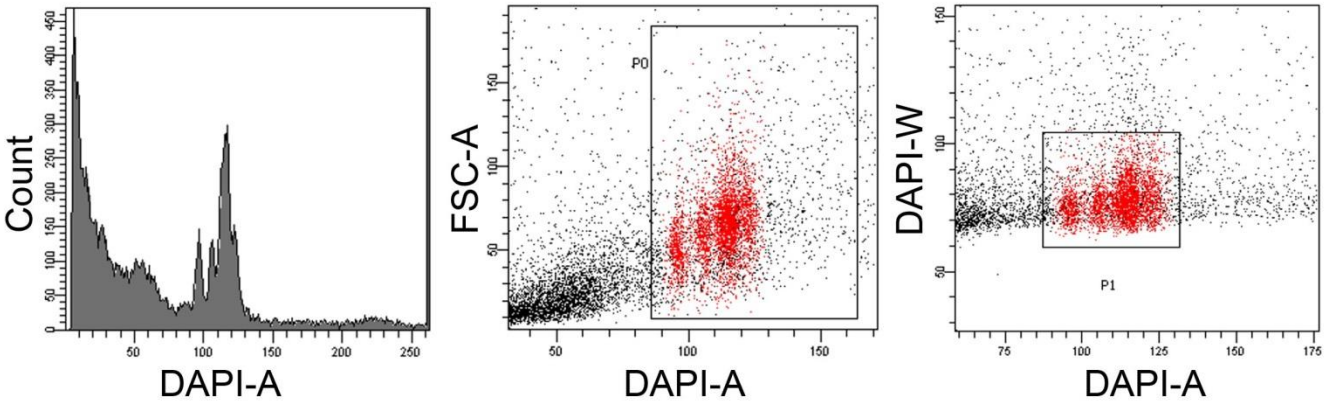


Figure S16

Table 1: Parameters of helically organized somatic metaphase chromosomes of barley compared to other species. Chromatid helix turn sizes and nested loop sizes are based on Hi-C data and a bottle brush-like model except for mouse and human.

Species	n	Haploid genome size (Gb)	Chromosome size (Mb)	Chromonema thickness (nm)	Turn length (Mb)	Nested loop length (Mb)	References
Barley	7	5	522 - 675	400	20-38	500[4]	This work
Onion/garlic	8	14.9/16.2	1800-2300	400-600[1], 400-550[2]	n. d.	n. d.	Niu et al., 1989; Hao et al., 1990, 1994
Axolotl	14	35	660 - 2,960	~250[5]	35	600[4]	Callan, 1966; Smith et al., 2019; Schloissnig et al., 2021
Chicken	34	1	0.6 - 196	150 - 200	12[5]	80[2, 6] (150[2, 4])	Gibcus et al., 2018
Mouse	21	2.6	61 - 195	200-300, 400	n. d.	n. d.	Rattner and Lin, 1985; Summer, 1998
Human	23	3.1	47 - 247	150-200[3]	16.6[5]	60[6]	Bak et al., 1977; Ohnuki ,1968; Pienta and Coffey, 1984; Rattner and Lin, 1985; Takayama and Taniguchi, 1986; Gibcus et al., 2018; Hayashida et al., 2021

[1]In prometaphase
[2]In telophase
[3]Not Hi-C study
[4]First peak in the contact probability log derivative
[5]Calculated from the longest mitotic chromosome length
[6]Corresponds to minor loop size
n. d. - not determined

Table S1: Values of local turn length of each chromosome calculated for 5 Mb bins, as described in supplementary materials and Figure S4. These values are graphically shown in Figure 1E. The three first lines display the sequence length of each chromosome, and the positions of the primary (centromere) and secondary constriction (NOR) at the short arms of 5H and 6H, as predicted from Hi-C data.

	1H	2H	3H	4H	5H	6H	7H
Chromosome size (Mb)	522.5	675.3	628.7	624.4	599.0	573.2	634.7
Centromere position (Mb)	205.5	305.8	271.9	282.4	206.0	260.0	328.8
NOR position (Mb)	-	-	-	-	50.3	79.5	-
Position (Mb)				**Turn length (Mb)**			
2.5-7.5	6.01674	3.41191	10.0504	5.60E-08	5.96904	4.30E-08	5.47E-09
7.5-12.5	3.3989	3.39141	1.24E-07	2.38075	19.6863	6.14956	5.96E-07
12.5-17.5	13.6668	13.0618	11.0963	12.0694	22.1876	13.5131	6.32619
17.5-22.5	19.7629	17.18	18.8189	18.0119	24.3624	21.5796	15.2778
22.5-27.5	25.619	19.5063	23.289	22.9052	25.8212	25.1436	20.3829
27.5-32.5	26.3202	22.5116	26.0755	25.7573	26.2016	27.3088	22.4413
32.5-37.5	28.6499	24.3615	27.7775	27.5784	27.6527	29.2685	23.6694
37.5-42.5	29.4102	25.5515	29.5156	29.6878	26.864	30.6588	25.1945
42.5-47.5	30.4477	26.9644	30.6173	30.8019	13.6946	31.102	25.793
47.5-52.5	30.447	28.0789	31.1308	31.3201	3.90E-08	30.6038	25.6596
52.5-57.5	30.3814	28.9819	32.008	31.8613	1.22E-07	29.8517	25.8668
57.5-62.5	30.7721	29.178	32.1477	32.8573	25.2976	27.7301	25.6354
62.5-67.5	30.6446	29.6516	32.7131	33.4771	22.4426	27.0497	26.2038
67.5-72.5	30.8399	30.7569	33.2433	33.8482	26.4577	26.0902	26.9197
72.5-77.5	31.2262	31.1721	32.958	34.242	29.1869	25.5726	28.3646
77.5-82.5	32.7546	31.5567	33.5336	34.3294	30.456	1.99E-12	28.5584
82.5-87.5	33.2509	31.2866	33.3311	34.6787	30.8924	1.54E-07	29.7553
87.5-92.5	34.091	31.3926	33.6411	34.8001	32.0145	25.3131	30.1972
92.5-97.5	34.819	31.5472	34.1038	35.2321	32.6105	25.6364	30.681
97.5-102.5	34.9206	31.4086	33.9768	35.0999	32.5178	27.1352	30.9678
102.5-107.5	35.4487	31.6383	34.6804	34.9774	32.1115	28.9428	31.1725
107.5-112.5	35.2697	31.4884	34.6192	35.2558	31.4494	30.3431	31.8339
112.5-117.5	34.7301	31.8003	34.8457	35.4576	30.6382	31.6458	32.1062
117.5-122.5	34.4552	31.733	35.1639	35.6888	31.0143	32.8772	32.3051
122.5-127.5	33.6035	31.9941	35.1249	36.0441	30.4393	33.6562	32.4979
127.5-132.5	32.8825	32.1363	35.4935	36.2429	29.8152	34.4415	33.0943
132.5-137.5	32.0877	32.9942	35.6706	36.4408	29.8695	34.5958	33.4276
137.5-142.5	30.6648	33.1226	35.3801	36.8475	29.2471	34.7721	33.8254
142.5-147.5	30.5385	33.591	35.0965	37.2356	29.4943	34.8878	33.4321
147.5-152.5	29.2401	33.6246	34.9161	37.4198	31.0086	35.2648	32.5287
152.5-157.5	28.9405	33.6886	34.9754	37.4845	30.27	36.1125	36.2879
157.5-162.5	28.4541	33.7891	34.8313	37.2513	29.1436	36.69	37.0673
162.5-167.5	27.8497	33.3587	34.8597	37.298	28.0698	37.1208	36.0702
167.5-172.5	26.2151	33.4029	34.3964	37.2553	25.5765	37.2073	31.7659
172.5-177.5	23.036	34.0477	34.0597	36.7538	22.9311	37.364	33.1757
177.5-182.5	18.3452	34.2014	33.3938	35.8105	13.1703	37.2536	33.0345
182.5-187.5	3.23232	34.2238	32.9613	35.0615	8.88E-09	36.7446	32.3794
187.5-192.5	5.77E-11	34.066	32.8202	34.7704	3.68E-08	36.3706	35.1794
192.5-197.5	4.26E-08	34.0345	32.7427	33.9533	1.34E-07	35.8814	37.3129
197.5-202.5	9.58E-10	33.9191	32.4727	32.2715	6.15E-09	34.4791	37.6781
202.5-207.5	1.14E-08	34.0749	32.1912	31.6223	9.65E-08	33.876	33.5086
207.5-212.5	4.94E-09	33.8319	31.8995	33.0899	1.26E-07	32.5153	34.6433
212.5-217.5	4.44E-15	33.7478	31.137	32.4271	1.36E-08	30.1152	35.8472
217.5-222.5	2.10E-07	33.3611	29.9599	31.3009	4.68E-07	28.3225	36.1778
222.5-227.5	7.7988	32.7032	29.071	31.0981	4.35708	26.2381	36.2295
227.5-232.5	20.0756	32.9118	27.7213	32.0108	22.6128	23.3928	36.4219
232.5-237.5	24.9688	32.6444	26.2091	30.793	26.6324	14.8782	36.5365

	1H	2H	3H	4H	5H	6H	7H
Position (Mb)				**Turn length (Mb)**			
237.5-242.5	27.9133	32.9266	21.9091	30.7244	28.6671	2.99E-07	36.2472
242.5-247.5	30.6028	32.0568	18.0876	28.7014	30.7917	7.12E-10	36.0978
247.5-252.2	32.3382	32.0024	7.76712	25.1582	32.184	1.36E-08	36.3516
252.5-257.5	33.1497	31.8705	7.42E-08	22.4684	33.04	7.06E-09	36.0632
257.5-262.5	33.7869	30.3695	2.14E-09	12.6957	33.606	1.45E-09	35.4676
262.5-267.5	34.3179	29.3664	5.56E-07	4.58E-11	33.6956	4.81E-08	35.0643
267.5-272.5	34.2778	27.3048	2.19E-06	3.78E-10	31.9944	2.80618	34.5308
272.5-277.5	34.3777	24.7343	1.70E-08	5.06E-09	32.4848	1.60E-07	33.081
277.5-282.5	34.6652	19.9785	2.99E-08	3.56E-08	33.2126	8.64977	31.6357
282.5-287.5	34.6182	12.539	1.21E-09	8.74E-07	34.098	20.4693	30.8667
287.5-292.5	34.519	2.44E-13	6.19E-12	2.85E-06	34.7032	25.0188	28.3714
292.5-297.5	34.1255	1.19E-06	10.0299	6.00E-13	34.7975	27.3963	27.165
297.5-302.5	33.8657	9.33E-13	23.7339	14.3295	34.5394	28.6435	22.2139
302.5-307.5	33.0197	8.39E-10	26.1005	21.6783	34.7994	29.3851	17.3955
307.5-312.5	33.1925	6.59E-10	30.108	14.0763	34.4565	33.7904	2.33809
312.5-317.5	33.4299	1.79E-08	32.716	22.1995	34.907	34.8424	3.94E-07
317.5-322.5	33.4936	7.70E-10	34.2683	26.508	35.0075	33.2338	4.64E-07
322.5-327.5	33.2605	9.41484	35.4378	27.5564	34.6288	34.2273	1.83E-06
327.5-332.5	32.7137	19.3441	36.6982	28.5718	34.7095	34.1128	19.4349
332.5-337.5	32.6454	22.8902	37.4345	30.9083	34.6917	34.5335	5.71E-08
337.5-342.5	32.088	26.9874	37.5309	31.8386	34.3407	34.5175	1.43E-09
342.5-347.5	31.8489	27.8013	36.8835	32.6608	34.2121	34.2827	1.48E-10
347.5-352.5	31.4395	31.0436	36.1659	33.8463	33.9803	34.5684	6.32615
352.5-357.5	31.0148	31.8322	35.7429	32.9708	34.01	34.6865	18.7447
357.5-362.5	31.3222	33.8545	35.2958	33.1092	34.0151	34.7757	24.8626
362.5-367.5	31.0258	34.6337	34.8958	32.6703	33.9587	34.9235	26.6516
367.5-372.5	30.9657	34.973	34.3256	33.118	33.9184	34.394	28.9271
372.5-377.5	30.9108	35.6196	33.4739	32.3202	33.6151	34.6627	31.3187
377.5-382.5	30.6854	35.6359	32.4056	33.0433	33.2048	34.3154	32.6222
382.5-387.5	30.726	35.6518	32.246	32.7307	33.4495	33.848	34.0566
387.5-392.5	30.9995	35.5138	33.215	31.5889	33.2956	33.5464	34.4456
392.5-397.5	31.3994	35.1389	33.804	32.0617	33.2773	33.8538	34.8513
397.5-402.5	31.0889	34.4437	34.0328	33.5815	33.1836	33.8478	35.2768
402.5-407.5	30.4093	33.7706	33.9275	34.0165	32.954	33.9513	35.0858
407.5-412.5	29.8711	33.7733	34.2662	34.1398	32.5537	33.8335	34.8808
412.5-417.5	29.1366	32.7707	34.1763	34.3838	32.1396	33.6421	34.9159
417.5-422.5	29.1749	32.1647	34.0588	34.8112	32.1213	34.6381	34.4724
422.5-427.5	28.6555	32.9012	33.7376	34.6444	31.8305	33.6526	34.0792
427.5-432.5	28.9366	32.9395	33.8082	35.519	31.2156	34.2223	34.1023
432.5-437.5	28.2459	33.4327	33.6258	34.8364	31.0672	34.1226	33.8582
437.5-442.5	28.9684	33.7228	33.1357	34.2773	30.6198	33.7286	33.8694
442.5-447.5	28.2041	33.89	32.7506	33.8143	30.3953	32.994	34.0414
447.5-452.5	28.2515	34.0404	32.2745	33.3632	30.0231	33.0358	33.8713
452.5-457.5	27.5916	34.4113	32.2374	33.5331	29.8501	32.2564	33.3055
457.5-462.5	26.291	34.2249	32.136	32.7541	29.6158	31.5048	33.2865
462.5-467.5	25.9303	34.5612	31.6487	32.743	29.184	32.3898	33.4259
467.5-472.5	25.4411	34.3868	31.4897	33.4705	29.0229	31.822	33.2463
472.5-477.5	23.9437	34.1271	31.4121	32.9942	29.1223	31.0097	33.3311
477.5-482.5	23.3546	34.209	30.8674	33.3189	27.7607	31.2104	33.4033
482.5-487.5	22.6197	34.0958	30.4565	33.4761	26.0865	31.0838	33.3216
487.5-492.5	21.7581	34.1659	29.9014	33.1369	25.7346	31.4538	33.0992
492.5-497.5	20.3533	34.0828	29.4672	33.0742	24.8074	30.3505	33.0381
497.5-502.5	17.3684	34.4562	28.3903	32.7133	23.9356	29.4287	32.731
502.5-507.5	12.2531	33.7769	28.2051	32.3605	22.5968	29.4079	32.3883
507.5-512.5	3.78151	33.7908	26.9858	32.0161	22.4535	29.1924	31.7152
512.5-517.5	12.0529	33.1738	26.6734	31.5706	20.9526	28.1931	32.112
517.5-522.5	6.13769	32.9463	26.9733	30.9844	21.5121	27.3251	32.3378
522.5-527.5		32.4902	26.0259	30.59	21.265	27.2698	31.6343
527.5-532.5		31.8125	26.2868	30.093	21.0415	26.2253	31.6827

	1H	2H	3H	4H	5H	6H	7H
Position (Mb)				**Turn length (Mb)**			
532.5-537.5		31.5727	26.074	29.7905	20.4688	25.9744	31.4727
537.5-542.5		30.5718	25.5686	30.3023	21.187	25.6962	31.7087
542.5-547.5		29.8406	25.6092	29.9643	21.6532	23.5544	31.4402
547.5-552.5		29.2722	25.4209	29.2289	22.4012	21.8487	30.7147
552.5-557.5		29.0004	24.5428	28.6189	24.049	17.465	30.7752
557.5-562.5		27.9736	23.806	28.1934	23.1059	11.6061	30.1034
562.5-567.5		27.0287	24.2538	27.9885	23.353	6.49198	29.6873
567.5-572.5		27.4258	23.9249	27.339	21.6832	3.90E-07	29.1887
572.5-577.5		26.6489	23.1854	26.4052	19.8346		28.5508
577.5-582.5		25.0741	24.0151	25.3589	17.2077		28.2294
582.5-587.5		23.595	23.3074	24.1774	7.7922		26.6481
587.5-592.5		23.6982	22.2953	23.2408	8.17889		26.2341
592.5-597.5		24.8433	20.5223	23.2727	1.51E-06		24.7005
597.5-602.5		24.6947	20.2942	20.5142			23.5091
602.5-607.5		24.7661	18.5698	17.4219			21.548
607.5-612.5		23.5646	16.2229	11.4805			19.0632
612.5-617.5		22.9534	13.6966	5.22E-09			15.5532
617.5-622.5		22.9203	14.5255	3.97E-08			5.2426
622.5-627.5		21.6953	10.2715				1.24672
627.5-632.5		21.5698					1.01E-11
632.5-637.5		22.3165					
637.5-642.5		22.1064					
642.5-647.5		21.342					
647.5-652.5		20.4072					
652.5-657.5		17.5867					
657.5-662.5		10.5045					
662.5-667.5		4.48304					
667.5-672.5		7.678					
672.5-677.5		13.348					

Table S2: Coordinates and lenghts of the oligo-FISH probes (named according to birds) adjacent to the 5HL subtelomere based on the barley cv. Morex v2 genome assembly.

Probe	No. oligos	Start (Mb)	End (Mb)	Length (Mb)	Average Turn size (Mb)	Number of turns*
Stork	33987	442.1	459.9	17.7	30.1±0.4	0.6
Eagle	35062	459.9	477.5	17.6	29.2±0.2	0.6
Ostrich	91061	477.4	511.8	34.4	24.8±1.8	1.4
Rhea	105984	511.8	545.3	33.5	21.3±0.5	1.58
Moa	108831	545.3	580.2	34.9	21.6±2.1	1.62
Flamingo	62181	580.1	599.0	18.8	8.3±6.1	2.3

* Length of the probe divided by the average turn size in its region.

Table S3: Volumes of the barley satellite chromosomes 5H and 6H (identified based on their secondary constriction and centromere position) and NOR-free (1H-4H, 7H) chromosomes. The measurements were done on polyacrylamide embedded spatially preserved chromosomes.

Chromosome	n	Mean±SD (µm³)
5H	2	49.5±2.3
6H	3	55.0±12.8
1H-4H, 7H	10	56.1±9.8
1H-7H	15	55.0±10.1

Table S4: Statistics of the metaphase Hi-C datasets. Seven libraries were analyzed: three replicates from interphase libraries, three replicates from metaphase libraries and one more metaphase library generated with an alternative protocol, which yields less unspecific contacts, for comparison with polymer modelling. For each library we computed: total number of contact reads (all reads), percentage of PCR duplicated reads (duplicated), number of non-duplicated reads with a mapping quality (MAPQ) of at least 10 (high-quality reads), number of intra-chromosome contacts separated by more than 1 kb (1 kb separation), intra-chromosome contacts separated by more than 1 Mb (1 Mb separation), and number of inter-chromosome contacts (inter-chromosome). * percentage of all reads: ** percentage relative to number high-quality reads; *** percentage of the reads separated by more than 1 kb.

LIBRARY	ALL READS	DUPLICATED (%*)	HIGH-QUALITY READS (%*)	1KB SEPARATION (%**)	1MB SEPARATION (%***)	INTER-CHROMOSOME (%***)
INTERPHASE_1	260,968,878	13.21	39,558,947 (15.16)	16,036,382 (40.54)	7,110,811 (44.34)	4,138,138 (25.8)
INTERPHASE_2	204,856,338	1.19	31,097,110 (15.18)	18,557,444 (59.68)	8,604,498 (46.37)	4,567,769 (24.61)
INTERPHASE_3	219,294,615	1.49	36,292,924 (16.55)	35,634,600 (98.19)	12,389,518 (34.77)	18,915,266 (53.08)
METAPHASE_1	509,196,403	6.06	91,401,051 (17.95)	62,357,229 (68.22)	12,764,657 (20.47)	48,635,089 (77.99)
METAPHASE_2	735,344,585	8.28	92,941,157 (12.64)	69,241,898 (74.5)	23,853,444 (34.45)	41,427,153 (59.83)
METAPHASE_3	579,559,917	7.09	80,301,755 (13.86)	60,884,103 (75.82)	17,350,884 (28.5)	40,922,614 (67.21)
METAPHASE_AGAR	122,636,722	50.74	12,194,950 (9.94)	9,809,014 (80.43)	4,771,893 (48.65)	3,254,155 (33.17)