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Assays for mitotic chromosome condensation in live yeast and mammalian cells

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Abstract The dynamic reorganization of chromatin into rigid and compact mitotic chromosomes is of fundamental importance for faithful chromosome segregation. Owing to the difficulty of investigating this process under physiological conditions, the exact morphological transitions and the molecular machinery driving chromosome condensation remain poorly defined. Here, we review how imaging-based methods can be used to quantitate chromosome condensation *in vivo*, focusing on yeast and animal tissue culture cells as widely used model systems. We discuss approaches how to address structural dynamics of condensing chromosomes and chromosome segments, as well as to probe for mechanical properties of mitotic chromosomes. Application of such methods to systematic perturbation studies will provide a means to reveal the molecular networks underlying the regulation of mitotic chromosome condensation.

Keywords chromosome condensation · cell division · condensin · live cell imaging · quantitative image analysis · GFP

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Abbreviations

DAPI	4′,6-diamidino-2-phenylindole dihydrochloride
EGFP	enhanced green fluorescent protein
GFP	green fluorescent protein
Sir2	silent information regulator 2
Yku 70	70 kDa subunit of the DNA-end binding Ku complex

Introduction

Chromosomes, the physical building blocks of the genome, are spatially confined to discrete territories that fill the space of the interphase cell nucleus. When a cell divides, chromosomes need to reorganize into compact rod-shaped bodies to permit the segregation of their replicated sister chromatids to opposite spindle poles. The structural chromatin dynamics underlying the formation of mitotic chromosomes have been defined as mitotic chromosome condensation.

Mitotic chromosomes need to meet a number of criteria. First, they require significant compaction to provide sufficient space for their individualized movements on the mitotic spindle. It is essential that their DNAs are completely disentangled from each other, and that their surfaces are non-adherent to neighboring chromosomes. The replicated sister chromatids need to be aligned in parallel compact rods to support bipolar attachment to the mitotic spindle.

Mitotic chromosome arms need to be shorter than half of the spindle length to permit complete segregation to opposite spindle poles prior to cytokinesis. Finally, chromosomes need to mechanically withstand the spindle forces, which act mostly on confined chromosomal sub-regions, the kinetochores. Neither the precise structure of interphase chromatin, nor its spatial arrangement in mitotic chromosomes is understood in much detail. Thus, it is not surprising that the process of mitotic chromosome condensation remains poorly defined.

Mitotic chromosomes are structured along their axis by a defined pattern of chromosomal bands, and they enrich specific protein components at the core of their axis. This initially led to a model for mitotic condensation based on the assembly of radial chromatin loops along a central axis. However, micromanipulation experiments suggested that this axis may not provide any mechanical or structural contribution to mitotic chromosomes (Poirier and Marko 2002). Furthermore, recent studies found higher-order chromatin fibers of various sizes within mitotic chromosomes, which favors a model of hierarchical folding of chromatin into fibers of different scales (discussed in Swedlow and Hirano 2003; Kireeva et al. 2004; Belmont 2006).

Molecular dissection of the machinery driving mitotic chromosome condensation has been limited by the difficulty of experimentally assaying this process. Many traditional approaches relied on fixed cell preparations, which can strongly perturb native chromatin organization (e.g., as discussed in Belmont 2006 and Gassmann et al. 2004). Moreover, the synchronization or staging methods used in such fixed-cell approaches provide insufficient detail on the exact kinetics of chromosome condensation. These limitations can be overcome by appropriate methods for assaying chromosome condensation in live cells. Here, we review methods for measuring chromosome condensation *in vivo*. We focus on three main aspects of chromosome condensation: general morphological dynamics of uniformly labeled chromatin, intra-chromosomal restructuring visualized by labeled chromosomal segments, and mechanical integrity of condensed chromosomes. We discuss how such methods have been implemented in different model systems, including animal tissue culture cells, and budding yeast.

Probing chromosome condensation with general chromatin markers

Quantitating chromatin texture

Mitotic chromosome condensation in animal tissue culture cells is apparent on phase-contrast microscopy without specific markers (e.g. Sarkar et al. 2002; Mikhailov et al. 2004). These morphological dynamics can in principle be quantitated (Sarkar et al. 2002), but this requires manual definition of nuclear regions and it is rather sensitive to the precise image conditions and general cell morphology.

Chromatin can be efficiently visualized in live cells by expression of core histones fused to fluorescent proteins (e.g., histone 2B fused to green fluorescent protein, H2B-GFP (Kanda et al. 1998)). Overexpressed fluorescent core histones have been found to be non-toxic and are very suitable for stable expression in animal cell lines. This allows one to follow prophase condensation under the light microscope with high spatial and temporal resolution, as illustrated in Fig. 1A.

The structural dynamics of fluorescently labeled chromatin can be quantitated by statistical analysis of the image texture (Beaudouin et al. 2002; Kaitna et al. 2002). For example, the standard deviation of the pixel intensities on chromatin regions provides one such morphometric feature, which increases about 2-fold during condensation (Fig. 1B; for comparison, the mean intensity measured in the same region remains constant; Fig. 1C). Texture-based quantitation of condensation could be refined by using a larger set of texture features (Conrad et al. 2004; Glory and Murphy 2007). Quantitative feature extraction allows one to reliably and automatically annotate the timing and extent of prophase condensation, and is thus suitable for high-throughput analysis of systematic perturbation experiments. However, the statistical features are often difficult to link with specific aspects of condensation, e.g., simple chromatin compaction versus its re-organization into rod-like structures.

In addition to texture analysis, the shape of individual chromosomes can be measured directly. For example, changes in the chromosome arm diameters have been used to assay one specific aspect of condensation in fixed (Kireeva et al. 2004) and live cells (Gerlich et al. 2006) (Fig. 1D, E). While arm width is an informative parameter of chromosome

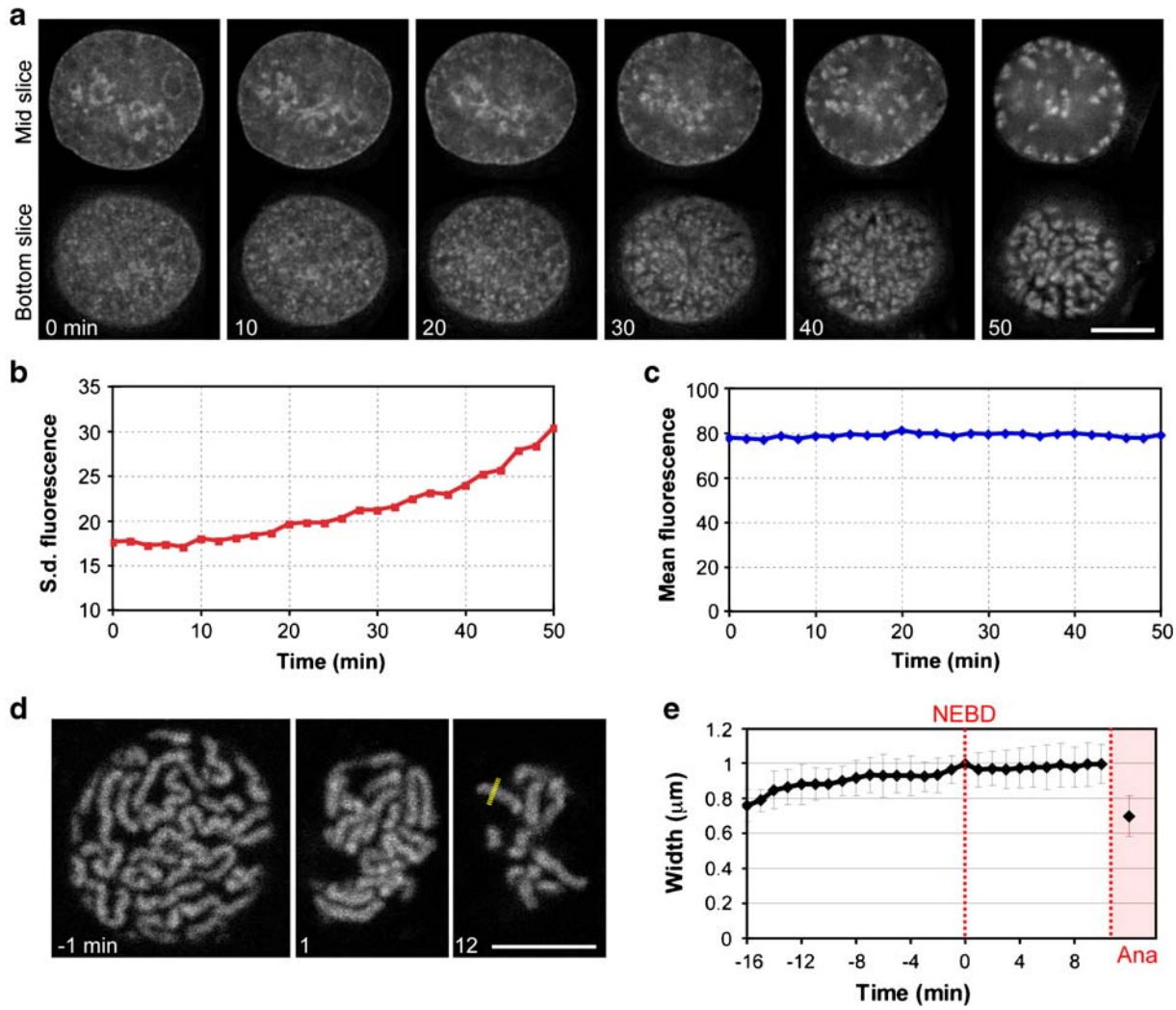

Fig. 1 Prophase chromosome condensation visualized in live cells by fluorescent core histones. **a** Three-dimensional time-lapse imaging of a human cervix carcinoma (HeLa) cell stably expressing H2B-EGFP. Mid- and bottom *z*-slices of selected time points are shown; original data is 35 *z*-slices at 300 nm step, 26 time points at 2 min time-lapse. Imaging was on a Delta Vision deconvolution microscope. Bar represents 10 µm. **b** Kinetics of the standard deviation (s.d.) of fluorescent pixel intensities in chromatin regions as a measure of chromosome condensation of the same cell shown in (A). **c** Kinetics of mean fluorescence for the same chromatin region shown in (B). **d** Live imaging of a normal rat kidney (NRK) cell stably expressing H2B-EGFP. Selected *z*-sections and time points of a 3D movie recorded on a confocal laser scanning microscope. Chromosome width can be measured as indicated by the yellow bar, representing 10 µm. **e** Kinetics of chromosome width (mean ± s.d.) shows that condensation is completed before nuclear envelope breakdown (t=0; defined by the loss of a defined nuclear boundary). **d** and **e** are reprinted from Gerlich et al. (2006) with permission from Elsevier

condensation, it is difficult to measure automatically because of the complex orientation of chromosomes.

Volume measurements of chromatin regions

Chromosome condensation can also be assayed by the nuclear space occupied by chromatin. This requires three-dimensional time-lapse imaging of the entire nucleus labeled by fluorescent chromatin. The volume of chromatin regions can then be determined by fluorescence intensity thresholding. Volumes detected by intensity thresholding depend on the threshold definition, and this method is therefore not suitable for obtaining precise absolute values for chromatin

volumes. However, fixed threshold settings for the analysis of entire time-series can be used to determine relative volume changes over time without strong bias by the threshold definition (Gerlich et al. 2001). This approach was applied to cells that decondensed chromatin during mitotic exit, because at this stage chromatin is relatively evenly distributed throughout the newly reformed nuclei (Gerlich et al. 2001). The complex pattern of condensing chromatin foci during prophase makes it more difficult to determine the exact boundaries of chromatin regions. The reliability of volume measurements in prophase cells can thus be improved by the a priori knowledge about the constant overall amount of chromatin (and thus fluorescence signal). Based on this, intensity thresholds can be automatically adjusted for each time point to derive equal total fluorescence intensity contained in the detected regions. This allows the establishment of robust volumetric measurements of chromatin in movies of cells progressing through all stages of mitosis (Mora-Bermudez and Ellenberg 2007; Mora-Bermudez et al. 2007).

Visualizing chromosome condensation in yeast

In budding yeast, the visualization of condensing chromosomes by general chromatin markers is much more difficult, owing to the small size of the cells and chromosomes, and potentially also to a lower degree of mitotic compaction. Also, the closed configuration of yeast mitosis complicates the identification of individual chromosome arms, because of the confinement to the small nuclear space. Direct visualization of condensing chromosomes in budding yeast has thus been limited to meiotic preparations (Kuroiwa et al. 1984).

The fission yeast *S. pombe* has fewer and larger chromosomes than *S. cerevisiae*; thus chromosome condensation can be followed by general chromatin markers such as DAPI. Changes in the shape of the nucleus during the cell cycle (Toda et al. 1981), as well as chromosome morphology (Uemura et al. 1987), served as quantifiable parameters of chromosome condensation.

Condensation of labeled chromosomal subregions

The determination of the exact folding path of mitotic chromosomes and its dynamic assembly from interphase chromatin is a key open question of chromosome biology. High-resolution analysis by a combination of light microscopy and electron microscopy on fixed samples of cells at different prophase stages suggested a hierarchical folding model of mitotic chromosome assembly (Kireeva et al. 2004). Investigation of the dynamics of chromatin folding at the subchromosomal scale *in vivo* requires labeling of subchromosomal regions, which has been achieved by a number of different approaches.

Labeling of individual sister chromatids

To improve the spatial discrimination between neighboring chromosomes, single sister chromatids can be labeled by incorporation of fluorescent nucleotide analogues in a single round of chromosome replication preceding live imaging of mitosis (Manders et al. 1999). This labeling method allowed resolution of individual chromosomes in microscopic images from early prophase cells, and was used to track the folding dynamics of heterochromatic foci with higher fluorescence signal. This quantitative study of condensing chromatin motion suggested that chromosomes assemble from pre-formed interphase heterochromatin arrangements.

Visualizing arrays of chromosomal loci

To follow the dynamics of more confined subchromosomal regions, the Belmont laboratory engineered chromosomes to contain arrays of bacterial lac operators, which can be visualized by expression of a GFP-tagged lac repressor (Robinett et al. 1996). Detailed light microscopy and electron microscopy showed that the lac arrays assembled into higher-order chromatin structures and did not perturb chromosome morphology, and thus provide a valid marker for native chromatin dynamics. This approach allowed visualization in live cells of chromatin fibers of about 100 nm diameter (Robinett et al. 1996), which had previously been observed only in electron microscopic images as basic chromosome folding units (Belmont and Bruce 1994). It is possible to place the lac operator into specific DNA sequence context. This approach questioned the relevance of scaffold associated regions (SARs) in their contribution to axial positioning of chromatin within mitotic chromosomes (Strukov et al. 2003). Nevertheless,

reproducible axial positioning of specific chromatin regions was observed (Dietzel and Belmont 2001), indicating that the chromatin context must contribute to axial organization.

Photo-labeling of chromosome arm regions

An alternative approach to visualization of chromosomal subregions is based on photo-labeling. This method exploits the fact that the photophysical properties of fluorescent proteins can be altered by strong laser illumination. Conventional fluorescent proteins can be irreversibly switched to a dark state by strong illumination at the normal excitation wavelength (bleaching), while new variants of fluorescent proteins have been designed that can be activated (Patterson and Lippincott-Schwartz 2002; Habuchi et al. 2005), or altered in their spectral emission properties (Ando et al. 2002; Chudakov et al. 2004; Wiedenmann et al. 2004; Matsuda et al. 2008) by illumination at specific wavelengths. Many chromatin markers, including fluorescently tagged core histones like H2B, are very stably associated with the DNA during all stages of the cell cycle (Gerlich et al. 2003). This allows establishment of patterns of photo-labeling as landmarks on chromosomes (Gerlich et al. 2003; Walter et al. 2003; Mora-Bermudez et al. 2007) for study of the dynamics of chromosome condensation *in vivo*. While this method does not provide sufficient spatial control to establish marks at distinct axial chromosome positions, it is particularly suitable for revealing longitudinal dynamics along the chromosome axes. This approach contributed to the discovery of a chromatin compaction step by axial shortening during anaphase (Mora-Bermudez et al. 2007).

Labeling ribosomal DNA regions in budding yeast

In budding yeast, a highly repetitive ribosomal DNA (rDNA) region, which makes up a stretch about half the size of the largest chromosome, served as a target region to visualize a subchromosomal structure. A simple way to stain rDNA is by fluorescent in-situ hybridization (FISH) (Guacci et al. 1994). However, this requires harsh fixation and staining conditions and thus may significantly perturb chromatin organization. The rDNA region can also be visualized in live cells by expression of the fluorescently tagged nucleolar protein Net1 (Machin et al. 2005). rDNA labeling revealed distinct morphologies at different stages of the cell cycle. In metaphase, rDNA localized to a well-defined loop (Guacci et al. 1994; Lavoie et al. 2004), the length of which was used to quantify condensation in metaphase and anaphase cells (Sullivan et al. 2004; Machin et al. 2005). As an alternative quantitation of rDNA condensation, the area of a minimal circle around the fluorescent signal was measured (Freeman et al. 2000). Expression of fluorescently labeled nucleolar proteins has also been used to analyze nucleolar structure in living fission yeast cells (Win et al. 2004). Analysis of the rDNA locus has provided important insights into chromosome condensation in budding yeast. However, this region of DNA is highly specialized: its sequence is highly repetitive, and it contains elevated levels of condensin complex during G_2/M (Freeman et al. 2000). Furthermore, it was reported to undergo hypercondensation during anaphase (Machin et al. 2005). It therefore remains unclear to what extent conclusions from the rDNA apply to non-ribosomal DNA.

Visualizing non-rDNA chromosome regions in budding yeast

Subchromosomal labeling in budding yeast outside of rDNA regions can be achieved by FISH probes to specific genomic loci. Labeling of two distinct loci on a single chromosome allows measurement of the axial compaction of intermediate chromatin (Guacci et al. 1994). Alternatively, FISH probes can be designed to visualize a continuous region of chromosomal DNA, which allows quantitation of the area of the fluorescent signal (Scherthan et al. 1992). Labeling of non-rDNA chromosomal regions *in vivo* has been achieved by integration of bacterial lac- and tet-operator sequences at specific loci, which can be visualized by expressing the respective DNA-binding protein (lac— or tet- repressor) tagged with fluorescent protein (Straight et al. 1996; Michaelis et al. 1997). This method has been simplified by a recently developed toolbox (Rohner et al. 2008). Axial chromosome compaction can then be determined by distance measurements between the labeled loci. This approach revealed that distinct regions on a single chromosome condense to different extents (Vas et al. 2007), highlighting the importance of considering local effects on chromosome condensation. Even though initially used only in staged cultures of

living cells, the method is in principle suitable for real-time imaging.

Measuring mechanical properties of mitotic chromosomes

Mechanical stability is an essential feature of mitotic chromosomes, because spindle forces that act locally at kinetochores need to move the entire chromosome. It is generally believed that mechanical resistance of mitotic chromosomes is established during chromosome condensation, but only few molecular components that contribute to mechanical stability have been identified, and the mechanism by which they stabilize chromosomes remains poorly defined. Assays to measure mechanical properties of mitotic chromosomes will be key to a better understanding of how chromosome condensation shapes functional mitotic chromosomes.

Deformation of chromosomes by mechanical micromanipulation

Mechanical properties of chromosomes can be determined from their deformation in response to external forces applied by glass micropipettes. This chromosome stretching approach allows one to calculate the Young's modulus as a measure of the elasticity of chromatin. It was applied to chromosomes isolated from cells (Claussen et al. 1994; Hliscs et al. 1997; Poirier et al. 2000; Poirier and Marko 2003), as well as to chromosomes inside live cells (Nicklas 1963; Nicklas 1983; Li and Nicklas 1995), revealing that chromosomes consist of soft and highly elastic chromatin. Interestingly, the local elasticity of mitotic chromosomes correlates with the chromosome banding pattern (Hliscs et al. 1997). While the elegant approach of mechanical micromanipulation has greatly enhanced our understanding of basic biophysical properties of mitotic chromosomes, it is technically challenging and has been established only in cells with particularly large dimensions. It therefore cannot be applied to some of the canonical model cell systems, including yeast, *Drosophila*, and human cells. Micromanipulation-based methods for study of chromosome mechanics have recently been discussed in detail (Marko 2008).

Measuring axial stiffness of chromosomes based on thermal bending

Mechanical features of mitotic chromosomes can also be derived directly from quantitative live imaging, without the need for mechanical micromanipulation. One such parameter is the bending stiffness of chromosomes along their longitudinal axis. This method is based on the fact that any type of elastic rod deforms in solution because of its thermal excitation. Measuring the length over which thermally exited bends occur at a given constant temperature allows direct calculation of the bending stiffness of a given polymer (Gittes et al. 1993). This approach has allowed determination of the bending stiffness of isolated mitotic newt chromosomes (Poirier et al. 2002), and of *in vitro* assembled *Xenopus* chromosomes (Houchmandzadeh and Dimitrov 1999). Application of thermal bending measurements to live cells is complicated by the fact that spindle forces also contribute to chromosome deformations. This problem can be solved by disassembling the mitotic spindle using microtubule poisons, which allow measurement of the bending stiffness of mitotic chromosomes visualized inside live *Drosophila* cells by fluorescent core histones (Marshall et al. 2001). Thermal bending measurements rely on precise shape measurements of individual chromosomes. In many cell types it is difficult to determine the precise boundaries of chromosomes based on general chromatin markers, because of their large overall number and crowded arrangement in the metaphase plate. Visualization of individual chromosomes in live cells could be improved by using subchromosomal markers specifically localizing to the central axis of condensed chromosomes, e.g., tagged condensin (Gerlich et al. 2006) or topoisomerase II (Mo et al. 1998).

Spindle force-dependent centromere deformations for assay of chromatin rigidity

An alternative imaging-based approach to address the mechanical stability of mitotic chromosomes is based on deformation measurements of chromatin in response to mitotic spindle forces. In many cell types, force-generating spindle attachments at the kinetochores concentrate on pairs of confined chromo-

somal regions. Time-lapse recordings of human cells overexpressing a fluorescently labeled kinetochore component (CENP-B; Shelby et al. 1996) revealed that spindle force-dependent stretching fluctuated over time. Abrogating spindle forces blocked oscillatory interkinetochore motion and lead to compaction of inner centromeres, which can be quantitated on the basis of the reduction of interkinetochore distance. This method is straightforward to implement, and allows efficient comparison between different experimental conditions, as has been shown by addressing the function of distinct condensin complex subunits (Fig. 2A–D; Gerlich et al. 2006). Measuring mean interkinetochore distances both in the presence of an intact mitotic spindle as well as after drug-induced spindle disassembly, allows differentiation of pure 'chromatin compaction' phenotypes from 'mechanically unstable' phenotypes (Gerlich et al. 2006; Oliveira et al. 2005). Because the spindle forces acting on kinetochores are not directly quantitated, it is not possible to derive the absolute values for elasticity modules with this approach. In addition, experimental perturbations designed to target chromatin condensation factors could in principle also affect spindle forces, and thereby potentially bias calculated chromatin elasticity measures. Finally, this approach investigates only mechanical properties of centromeric chromatin, which contains specialized components and therefore might differ from the general status of chromatin condensation at other chromosomal regions.

Mitotic spindle force-dependent oscillations of interkinetochore centromeric regions can also be assayed in budding yeast, using kinetochore markers, or by visualizing centromere-proximal DNA regions with the lac operator/GFP-lac repressor system (Pearson et al. 2001) (Fig. 2E, F). Because of their attachment to a single microtubule, the spindle forces on interkinetochore centromeric chromatin are well defined, which allows calculation of absolute values of the Young's modulus (Bouck et al. 2008). By labeling chromosomal regions at distinct distances from the centromere in a set of reporter cell lines, it was also possible to investigate chromosome stretching at the onset of anaphase (Pearson et al. 2001). This assay was based on differences in the relative kinetics of spindle elongation and segregation onset of the labeled chromosome region.

Spindle force-dependent deformations of non-centromere chromatin regions

As with the spindle-force assays in animal cells (see above), these approaches permit only the investigation of mechanical properties of centromeric chromatin, which localizes between the sister kinetochores. For analysis of mechanical properties of non-centromeric regions, yeast chromosomes can be engineered to conditionally assemble a second kinetochore on a single chromosome (dicentric chromosome). This can be achieved by integration of a second centromere that can be conditionally inactivated by an adjacent inducible promoter (Hill and Bloom 1989). Because the two kinetochores will randomly attach to the two spindle poles, it is expected to obtain 50% of dicentric chromosomes with the kinetochores pulled to opposite poles. In these cells, stretching of the chromatin between the centromeres can be assayed by the deformation of a 10 kb lac operator array integrated between the two centromeres. By this strategy, two heterochromatin-regulating factors, *Sir2* and *Yku70,* were found to contribute to the mechanical stability of chromosomes, apparent from the decondensed appearance of the lac array in the respective mutants (Thrower and Bloom 2001).

Conclusions

Fluorescence live cell microscopy and quantitative image analysis provide efficient means to assay chromosome condensation *in vivo*, which, owing to the difficulty of preparing native chromatin fixations, can be essential to reveal physiologically relevant functions of candidate condensation factors.

The key limitation in any of the assays discussed above is the resolution limit of the light microscope, typically around 200 nm in the imaging plane, and about 600–800 nm along the optical axis. This allows one to monitor only the highest level of chromatin folding dynamics. The recent development of super-resolution light microscopy methods (Betzig et al. 2006; Schermelleh et al. 2008; Huang et al. 2008; Westphal et al. 2008) will boost our understanding of chromosome condensation at smaller scales.

Another opportunity to improve the resolution of condensation assays lies in the development of new

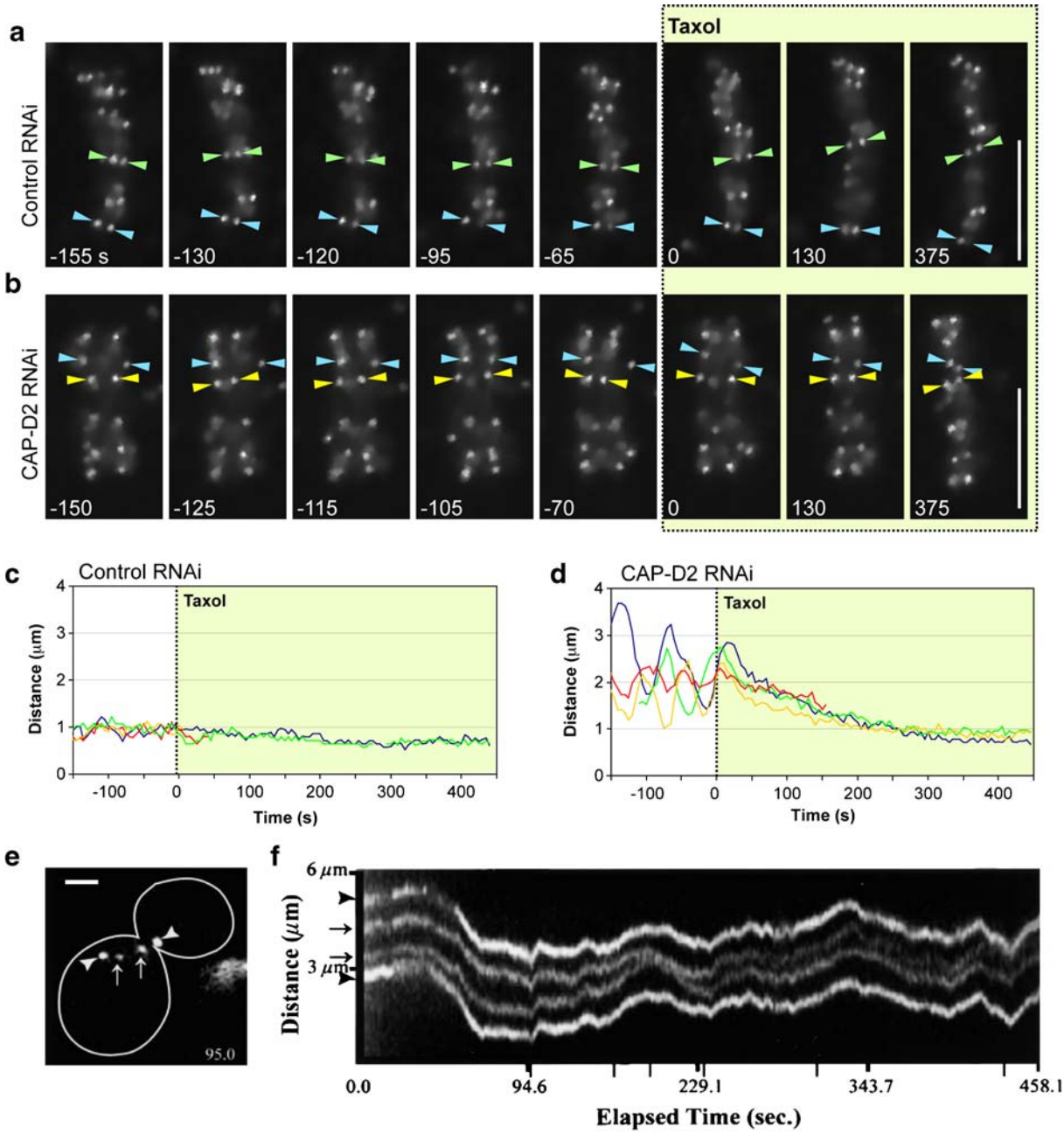

Fig. 2 Elastic deformation of centromeric chromatin in response to spindle forces. **a** Centromere dynamics in control metaphase cell. Time-lapse recording of unperturbed EGFP-CENP-A-expressing HeLa cell (t=−155 to 0 s). After inhibition of spindle forces by 10 μM Taxol at t=0 s, the interkinetochore distance decreases. Green and blue arrowheads correspond to quantitative measurements in (C). Bar represents 10 μm. **b** Spindle force-dependent increase in centromere dynamics in condensin I-depleted cell. Time-lapse recording of CAP-D2-depleted cells. After inhibition of spindle pulling forces by 10 μM Taxol at t=0 s, centromere oscillations cease and they recompact similar to control cells. Yellow and blue arrowheads correspond to quantitative measurements in (D). **c–d** Quantitative analysis of interkinetochore distance dynamics. Individual plots correspond to the centromeres highlighted in (A) and (B) and additional centromeres not highlighted in the images. **a–d** are reprinted from Gerlich et al. (2006) with permission from Elsevier. **e, f** Dynamic separation and oscillations of centromere proximal lac operator sites in budding yeast at preanaphase. **e** The lac operator marker was integrated 1.1 kb from CEN11, and visualized by GFP-lac repressor (arrows). In addition, spindle poles were labeled with Spc72-GFP (arrowheads). Bar represents 2 μm. **f** A kymograph sequence of a time-lapse recorded at 0.9 s intervals. **e**, **f** © Pearson et al. (2001). Originally published in *The Journal of Cell Biology.* 152:1255–1266

probes. The rapid progress in the development of *in vivo* probes that report on conformational changes, posttranslational modifications, or protein-protein interactions will hopefully soon allow monitoring of chromatin condensation at the molecular scale *in vivo*.

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