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Mitotic chromosome size scaling in Xenopus

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As rapid divisions without growth generate progressively smaller cells within an embryo, mitotic chromosomes must also decrease in size to permit their proper segregation, but this scaling phenomenon is poorly understood. We demonstrated previously that nuclear and spindle size scale between egg extracts of the related frog species *Xenopus tropicalis* and *Xenopus laevis* but show here that dimensions of isolated mitotic sperm chromosomes do not differ. This is consistent with the hypothesis that chromosome scaling does not occur in early embryonic development when cell and spindle sizes are large and anaphase B segregates chromosomes long distances. To recapitulate chromosome scaling during development, we combined nuclei isolated from different stage *Xenopus laevis* embryos with metaphase-arrested egg extracts. Mitotic chromosomes derived from nuclei of cleaving embryos through the blastula stage were similar in size to replicated sperm chromosomes but decreased in area approximately 50% by the neurula stage, reproducing the trend in size changes observed in fixed embryos. Allowing G_2 nuclei to swell in interphase prior to mitotic condensation did not increase mitotic chromosome size, but progression through a full cell cycle in egg extract did, suggesting that epigenetic mechanisms determining chromosome size can be altered during DNA replication. Comparison of different sized mitotic chromosomes assembled in vitro provides a tractable system to elucidate underlying molecular mechanisms.

Introduction

If a mitotic chromosome exceeds half the length of the spindle axis at telophase, proper genome segregation will be impaired, highlighting the importance of coordinating mitotic chromosome length with spindle length. Artificially lengthening chromosomes in plants severely reduces fertility and viability of the organism due to loss of DNA, as chromosomes fail to separate completely.[1,2] This phenomenon has also been observed in animal cells where axial compaction of chromosomes is maximal during anaphase and depends on Aurora B kinase-dependent phosphorylation of condensin I.[3,4] However, mechanisms regulating mitotic chromosome architecture and size are poorly understood, and the problem of mitotic chromosome scaling has not been studied systematically.

Embryonic development of *Xenopus laevis* frogs provides a powerful system to investigate size control of cellular structures, as the ~1 mm diameter fertilized egg undergoes rapid cleavages without growth to generate smaller and smaller cells. Whereas nuclei decrease in size gradually and linearly between stages 5 and 11 (16 cells to gastrula),[5] mitotic spindles remain large for a longer time then shrink more sharply starting at stage 8 (the mid-blastula transition, approximately 4,000 cells).[6] Do mitotic chromosomes scale with nuclear size, spindle size or independently? Previous analyses of chromosome size have been problematic, because individual chromosome dimensions are difficult to measure in situ, and chromosome fixation techniques utilizing acetic acid or treatments that arrest cells in mitosis can lead to chromosome distortion or hyper-condensation. Published mitotic chromosome measurements in *X. laevis* have been limited to egg extracts, in which individual chromosomes range in length from 15–20 μm,[7] and acetic acid-fixed embryos, in which average chromosome length is seen to decrease from around 11.5 μm to 8 μm between the blastula and tailbud stages.[8]

Here we have established an in vitro system to assess the size of mitotic chromosomes during development that recapitulates the trend observed in fixed embryos and allows us to manipulate the cell cycle and test for conditions that alter chromosome dimensions. Our experiments illustrate how chromosome segregation mechanisms change with cell size and provide a novel approach to identify chromosome scaling factors.

Results

Sperm chromosomes assembled in egg extracts do not exhibit interspecies scaling. We showed previously that compared with *Xenopus laevis,* egg extracts from the smaller, related frog *Xenopus tropicalis* assemble smaller interphase nuclei and mitotic spindles in vitro.[5,9] Both nuclear and spindle scaling between species were seen to depend on cytoplasmic factors, and were much less affected by ploidy differences, with diploid *X. tropicalis* sperm nuclei containing 10 chromosomes, while pseudotetraploid *X. laevis* has 18. To test for differences in mitotic chromosome size, sperm nuclei from both species were added to cytostatic factor (CSF) metaphase-arrested egg extracts, cycled through interphase to induce DNA replication and then arrested

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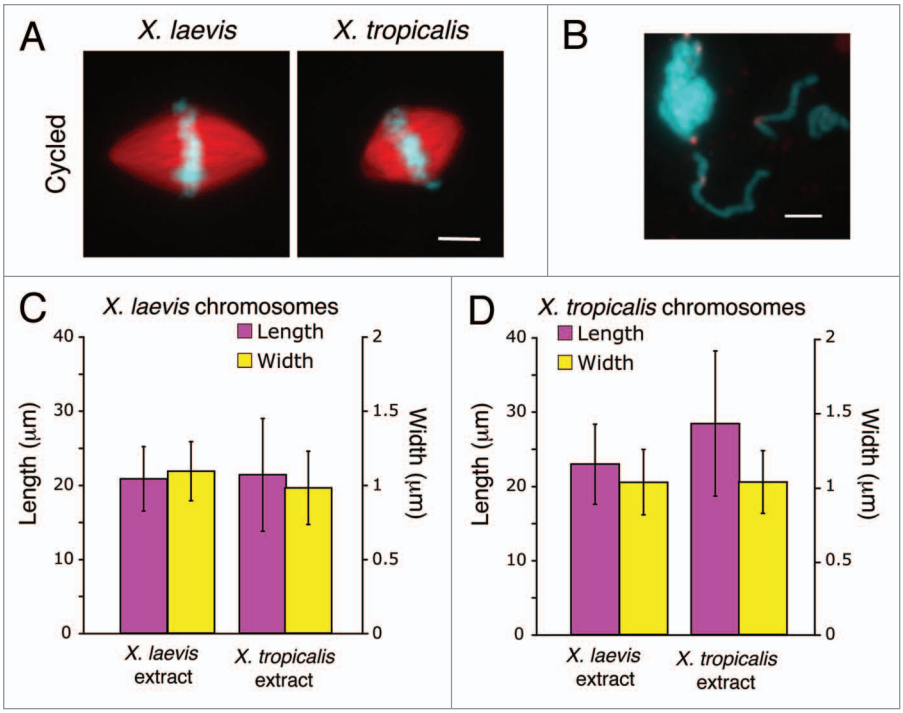

Figure 1. *X. laevis* and *X. tropicalis* mitotic chromosomes are similar sizes. (A) Images of spindles assembled in *X. laevis* or *X. tropicalis* egg extract around duplicated *X. laevis* sperm chromosomes. Scale bar = 10 μm. (B) Representative image of chromosomes isolated from metaphase egg extract. Kinetochores were stained by immunofluorescence for INCENP (red) and chromosomes labeled with Hoechst dye (blue). A large cluster of mitotic chromosomes is visible (upper left corner) as well as two individual chromosomes (upper right and bottom) Scale bar = 5 μm. Single, duplicated mitotic chromosomes were identified by a large INCENP spot between two sister chromatids. (C) Graph showing length (pink bars and left Y-axis) and width (yellow bars and right Y-axis) of *X. laevis* chromosomes in *X. laevis* (left set) or *X. tropicalis* egg extract (right set). Size differences are not significant (n = 3 extracts, ≥ 50 chromosomes, $p > 0.3$). (D) Bar graph showing length (pink bars and left hand Y-axis) and width (yellow bars and right Y-axis) of *X. tropicalis* chromosomes in *X. laevis* (left set) or *X. tropicalis* extract (right set). *X. tropicalis* mitotic chromosomes are significantly longer ($p < 0.005$) but not thinner ($p > 0.9$), in *X. tropicalis* extract than in *X. laevis* extract.

in metaphase (**Fig. 1A**). Chromosomes clustered together at the metaphase plate and were not resolvable from one another until dilution and fixation in a formaldehyde-containing buffer that stabilized them but depolymerized spindle microtubules. Fixed chromosomes were then centrifuged through a glycerol cushion onto coverslips, stained and imaged (**Fig. 1B**).[7,10] Even with this procedure, most mitotic chromosomes clumped together, with approximately 5–10% resolved well enough for measurement, which was performed manually for each condition in three separate egg extracts. Standard deviations were high, reflecting the variability of DNA content among Xenopus chromosomes.[11] Only fully replicated chromosomes recognizable by the appearance of two sister chromatids and inner centromere staining of INCENP were quantified (**Fig. 1B**).[7,12]

Comparison of mitotic chromosome dimensions did not reveal any striking differences between species (**Fig. 1C and D**). Replicated *X. laevis* sperm chromosomes were not significantly different in length in the two egg extracts (20.8 ± 4.4 μm in *X. laevis* and 21.3 ± 7.6 μm in *X. tropicalis*), although they were slightly thinner (1.09 ± 0.19 μm vs. 0.97 ± 0.25 μm) in *X. tropicalis* extract. *X. tropicalis* mitotic chromosomes were slightly longer than *X. laevis* chromosomes in both extracts (22.9 ± 5.4 μm in *X. laevis* extract and 27.8 ± 8.9 μm in *X. tropicalis*), exceeding the average *X. tropicalis* spindle length of about 22 μm.[9] *X. laevis* chromosomes also frequently exceeded half the length of the metaphase spindle of 33 μm (data not shown).[9] Thus, duplicated sperm chromosomes are large and of a similar size when isolated from egg extracts of the two frog species.

Recapitulation of mitotic chromosome scaling in vitro. We wondered why mitotic sperm chromosomes were so large in egg extracts, and considered several possible explanations. Perhaps egg extract preparation removed activities necessary to assemble bona fide mitotic chromosomes in vitro. A second systematic problem could be the fixation and isolation techniques, which could distort mitotic chromosome dimensions. A third explanation is that chromosomes are indeed big, but that anaphase B mechanisms function to segregate them long distances in the early embryo. Following chromosome segregation, large asters at the spindle poles migrate to the center of daughter cells, separating daughter nuclei by tens or hundreds of microns depending on cell size.[13]

To address these possibilities, we first attempted to visualize and measure unperturbed chromosomes in situ and in squashed embryos with gentle fixatives using DNA dyes or by expression of GFP-histone H2B, but we were hindered by high background fluorescence and the tight clustering of chromosomes during mitosis. To evaluate whether our fixation method was capable of preserving relative chromosome size differences, we took advantage of the Xenopus egg extract system, which has been proven to faithfully reconstitute mitotic events, and tested whether mitotic chromosomes from different stages of development could be generated and compared in vitro. Nuclei isolated from embryos that had been arrested in G_2 at various developmental stages with cycloheximide were added to *Xenopus laevis* CSF metaphase-arrested egg extracts (**Fig. 2A**). For comparison, *X. laevis* sperm nuclei were added directly to CSF extracts to form unreplicated chromatids, or incubated in interphase egg extracts and then cycled into metaphase arrest, generating replicated sperm chromosomes.[14] Both length and width were measured to calculate average chromosome areas (**Fig. 2B**). Consistent with their cell cycle status and previous results, unreplicated chromatids were longer (24.1 ± 8.7 μm for CSF vs. 20.4 ± 4.7 μm for cycled), but approximately two thirds the width of replicated chromosomes (0.56 ± 0.11 μm for CSF vs. 0.86 ± 0.15 μm for cycled), yielding significantly

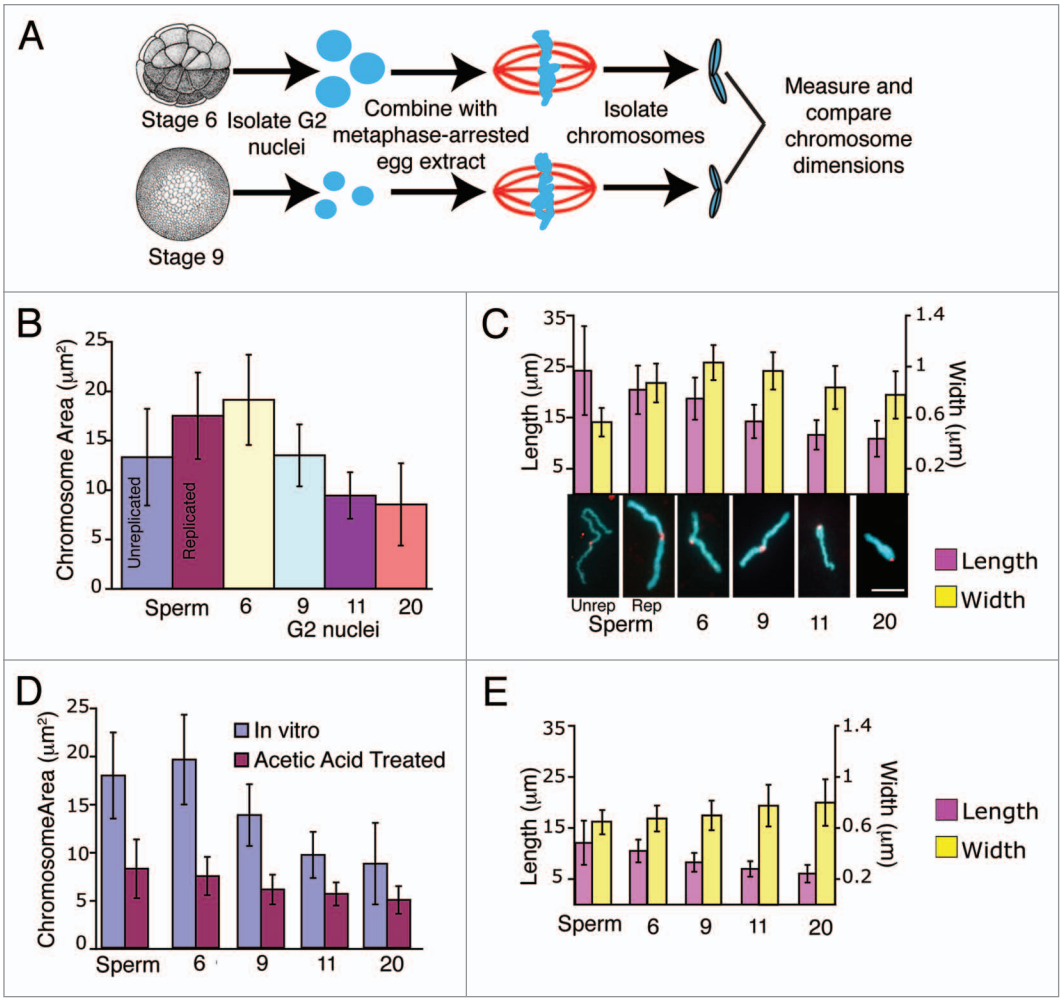

Figure 2. Measurement of mitotic chromosomes assembled in *X. laevis* egg extract from sperm or embryo nuclei. (A) Schematic of method to compare size of mitotic chromosomes condensed in vitro. G_2 nuclei from different stages of development were prepared and combined with metaphase-arrested egg extract. Following embryo nuclear envelope break down, mitotic spindles formed. Chromosomes were allowed to condense fully and then isolated, measured and compared. (B) Graph of mitotic chromosome areas from nuclei at different stages of development. Mitotic chromosome area reduced significantly at stage 9. (n = at least 3 extracts, ≥ 33 chromosomes for each condition). (C) Graph showing mitotic chromosome length (pink bars and left Y-axis) and width (yellow bars and right Y-axis). Changes in chromosome length are significant ($p \leq 0.01$) throughout development. Chromosome width is significantly greater early in development, stages 3–6 ($p \leq 0.001$), unchanged between stages 9 and 17 ($p \geq 0.05$) and significantly reduced at stage 20 ($p \leq 0.01$). Representative images of chromosomes isolated for each stage are shown. Scale bar = 5 μm. (D) Graph comparing the area of mitotic chromosomes condensed in vitro (blue bars) to those prepared from embryos in the presence of acetic acid by a chromosome spreading technique (dark pink bars, n = 3 embryos, ≥ 50 chromosomes). Replicated sperm chromosomes were prepared as usual but treated with 60% acetic acid for 5 min prior to methanol fixation. Chromosomes were significantly smaller when treated with acetic acid in all cases ($p \leq 0.001$). (E) Graph showing length (pink bars and left Y-axis) and width (yellow bars and right Y-axis) of chromosomes in embryo chromosome spreads. Chromosome size changes followed the same trend as in (C). They became significantly and progressively shorter during development ($p \leq 0.001$). Cycled sperm, stage 6 and stage 9 chromosomes became significantly thinner ($p \leq 0.001$) after acetic acid treatment but stage 11 and stage 20 did not ($p \geq 0.09$).

smaller average chromosome area (13.3 ± 4.8 μm² compared with 17.5 ± 4.4 μm²).[7,10] At 19.1 ± 4.6 μm² in area, mitotic chromosomes assembled from G_2-arrested cleavage stage 6 were similar in size to replicated sperm chromosomes. However, a significant decrease in chromosome area became apparent at blastula stage 9 (13.5 ± 3.1 μm²), and further reductions were evident by midgastrula (stage 11, 9.4 ± 2.3 μm²) and late neurula (stage 20, 8.5 ± 4.1 μm²). Closer inspection of chromosome lengths and widths revealed that chromosomes assembled from G_2 embryo nuclei from early stages (stages 3–7) were slightly larger than sperm chromosomes in egg extracts. Between stages 7 and 11, chromosomes decreased significantly in length but not width, which subsequently also decreased (**Fig. 2C** and data not shown). These observations indicate that our in vitro system can recapitulate mitotic chromosome size changes that occur during embryogenesis[8] and indicate that chromosomes are quite large during early development. Since G_2 embryo nuclei would be expected to contain the factors required to establish higher order chromosome architecture, our results also suggest that normal mitotic chromosome morphology is being reproduced in egg extracts.

Embryo chromosome dimensions are altered by fixation techniques. The trend in developmental chromosome

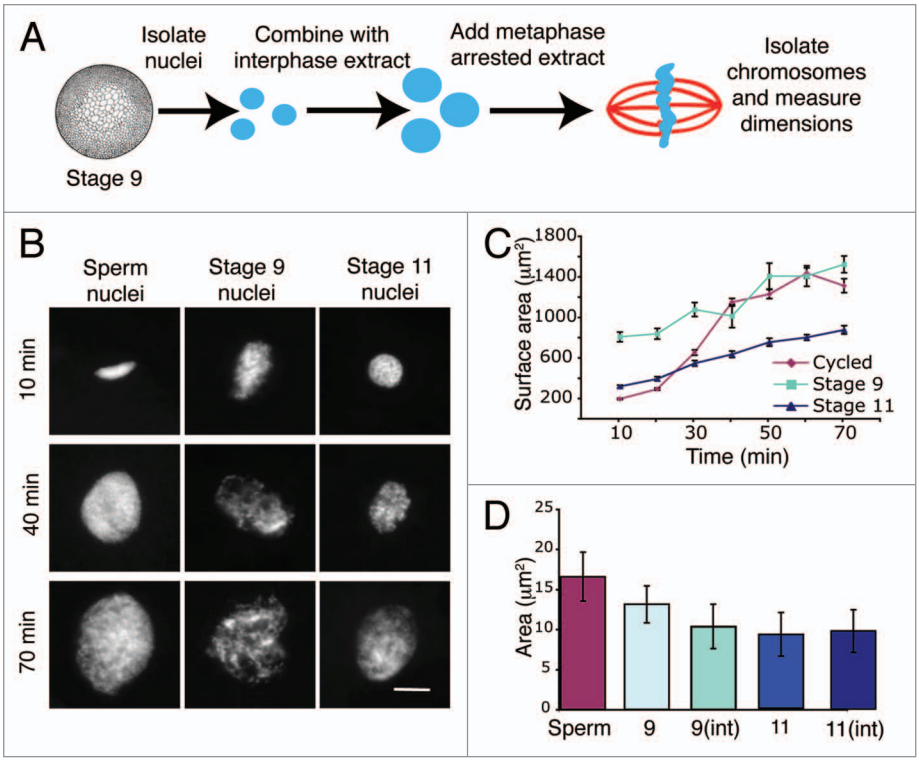

Figure 3. Nuclear expansion in interphase prior to chromosome condensation does not alter mitotic chromosome size. (A) Schematic of assay in which G_2 nuclei isolated from embryos were combined with egg extract then released and arrested in interphase by addition of calcium and cycloheximide. Nuclei expanded for 70 min, and reactions were then returned to metaphase by addition of fresh CSF extract, and mitotic chromosomes isolated and measured. (B) Representative images of sperm, stage 9 and stage 11 nuclei incubated in interphase egg extracts at several time points. Scale bar = 10 μm. (C) Graph of nuclear growth during the interphase incubation (n = 3 extracts, ≥ 35 nuclei per condition). (D) Average areas of mitotic chromosomes (n = 3 extracts, ≥ 35 chromosomes per condition). Chromosomes from expanded nuclei were either the same size (stage 11, $p \geq 0.3$) or significantly smaller in area (stage 9, $p \leq 0.001$) than controls.

size changes observed in egg extracts was similar to that previously documented in fixed embryos,[8] but overall the size of mitotic chromosomes assembled in vitro appeared larger. A major difference between the in vitro and embryo conditions is the fixation method used. Whereas mitotic chromosomes in egg extracts were first fixed with formaldehyde and post-fixed in methanol after spinning onto coverslips, harsh acetic acid treatment was necessary to separate embryo chromosomes from one another. Consistent with previous studies in references 15 and 16, we found that acetic acid treatment of embryo and egg extract chromosomes reduced their size. Replicated sperm and embryo chromosomes were reduced by 50% or more in area compared with chromosomes assembled in vitro (**Fig. 2D**) and were of similar widths regardless of their lengths (**Fig. 2E**). Importantly, both in vitro and embryo measurements exhibited the same trend in chromosome length changes during development. Therefore, the in vitro mitotic chromosome assembly assay described here provides a novel approach to monitor chromosome size changes during development without harsh fixation techniques.

Mitotic chromosome remodeling in egg extract requires progression through the cell cycle. Although the in vitro system recapitulated chromosome size changes observed during development, we wondered to what extent chromosomes were being remodeled and resized by egg cytoplasm. Prolonged incubation in metaphase egg extract did not alter the dimensions of sperm or embryo chromosomes, which reached a steady-state within 45 min (**Fig. S1**). We first considered whether mitotic chromosome dimensions were influenced by nuclear size, which also decreases during development.[5] However, whereas nuclei were seen to decrease in size gradually and linearly between stages 5 and 11 (16 cell to gastrula), mitotic chromosomes did not appear significantly smaller until blastula stage 9 (**Fig. 2**). Therefore, nuclear size reduction observed during early development is not accompanied by proportional changes in mitotic chromosome dimensions as measured in vitro and in embryos.

The in vitro system allowed us to test whether altering nuclear size nevertheless affects mitotic chromosome size. G_2 embryo nuclei or replicated sperm for comparison were added to interphase-arrested egg extracts for 70 min before inducing mitotic entry and arrest in metaphase. Mitotic chromosome dimensions were measured 45 min later (**Fig. 3A**). All nuclei expanded during this interphase incubation, although replicated sperm nuclei and stage 9 nuclei grew larger than stage 11 nuclei (**Fig. 3B and C**). Surprisingly, mitotic chromosomes from expanded G_2 nuclei were either the same size or slightly smaller in area than those that entered directly into metaphase (**Fig. 3D**). These data indicate that mitotic chromosomes are not influenced by nuclear size in vitro, but attain normal in vivo dimensions.

Thus, embryo chromosomes are refractory to remodeling by interphase or metaphase-arrested cytoplasm, indicating that stable epigenetic mechanisms operate to establish their size. However, we noted one example in which chromosomes are rapidly remodeled in vivo during a single cell cycle (**Fig. 4A**). Whereas female meiotic chromosomes prepared by acetic acid fixation from unfertilized eggs were tiny, with an average length of 2.4 ± 0.6 μm, chromosomes prepared just 60 min following fertilization, during the first mitotic division were about four times longer, with dimensions corresponding to those from early embryos (≥ 10 μm, **Fig. 2E**). To investigate whether progression through the cell cycle in vitro could also modify mitotic chromosome dimensions, we replicated embryo nuclei in egg extracts (**Fig. 4B**). G_2 nuclei isolated from embryos at stage 7 or 17 were combined with metaphase-arrested egg extracts to assemble mitotic chromosomes, released into anaphase and

subsequent interphase by addition of calcium and then cycled back into metaphase 90 min later by addition of CSF-arrested egg extract (**Fig. 4B**). Mitotic chromosomes from the first and second metaphase were measured and compared (**Fig. 4C and D**). Following passage through a complete cell cycle in vitro, there was no change in length for cycled sperm or stage 7 mitotic chromosomes, which were similar in size to each other in the first metaphase. However, mitotic chromosomes from stage 17 embryo nuclei became significantly longer. Chromosomes from both of the embryonic stages were slightly thinner after DNA replication. Thus, passage through an entire cell cycle in an egg extract does not significantly alter the size of large chromosomes, but remodels small embryo chromosomes into larger chromosomes resembling those of the early embryo.

Discussion

Our study is the first to systematically investigate the question of mitotic chromosome scaling during *X. laevis* development. In vitro assembled chromosomes followed the same trend in size changes as those of fixed embryos, but were treated with gentle fixation conditions more likely to preserve in vivo chromosome morphology. Although it is possible that our methodology leads to an overall expansion and/or distortion of chromosomes, their relative sizes can nevertheless be compared. Painting a single chromosome for live imaging in vivo would be an ideal approach to quantitatively assess size changes during development, but such technology has not yet been developed.

Initially, we found it remarkable that metaphase egg cytoplasm did not resize embryo chromosomes. We conclude that unlike spindle and nuclear size, which are governed by cytoplasmic factors, mitotic chromosome architecture is intrinsically determined and depends on the embryonic stage of the nucleus. Since the genome itself does not change during development, epigenetic factors must operate with specific proteins and/or modifications leading to different sized chromosomes. Possibilities include core and linker histone isoforms, which are known to change during development,[7,17,18] or DNA or histone modifications. Differences in lower order chromosome organization could subsequently affect determinants of higher order mitotic chromosome architecture, such as condensin or topoisomerase II.[19-21] Particularly intriguing is the recent finding that the relative levels of condensin I and II alter chromosome shape in egg extracts.[22] The molecular nature and function of chromosome scaling factors as well as the effects of altering them in vivo are exciting topics for further investigation.

Establishing an assay to monitor mitotic chromosome scaling in vitro allowed us to manipulate the cell cycle and test for conditions that alter chromosome

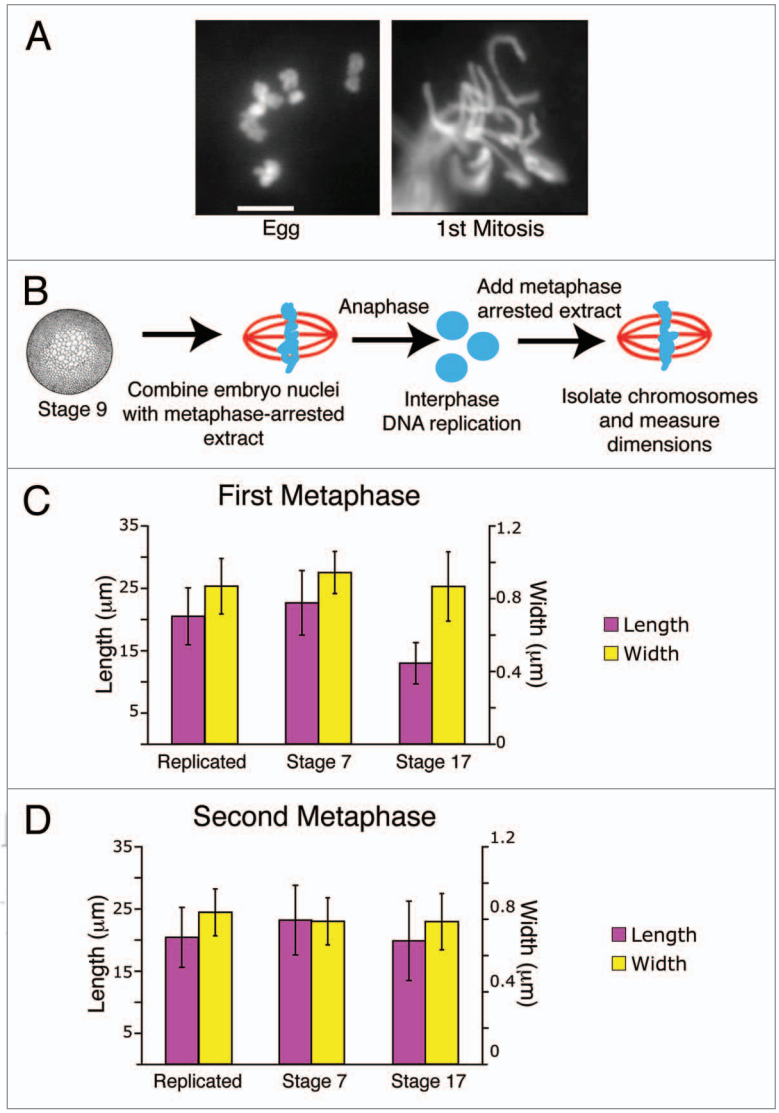

Figure 4. Progression through the cell cycle can remodel chromosomes. (A) Chromosome spread images of meiotic egg chromosomes and zygote chromosomes at the first mitotic division. Meiotic chromosomes appeared significantly shorter (average length 2.4 μm) than mitotic chromosomes typical of the early embryo (approximately 10 μm long, see **Fig. 2E**). Both types of chromosomes were isolated using the acetic acid spreading technique. Scale bar = 5 μm. (B) Schematic of assay in which G_2 nuclei isolated from embryos are combined with egg extract to assemble metaphase chromosomes then cycled into interphase by addition of calcium. After allowing 90 min for DNA replication, reactions were cycled back into metaphase by addition of fresh CSF extract, and mitotic chromosomes isolated and measured. (C) Graph showing the length (pink bars and left Y-axis) and width (yellow bars and right Y-axis) of sperm, stage 7 and stage 17 mitotic chromosomes at the first metaphase (n = 3 extracts, ≥ 85 chromosomes per condition). (D) Graph showing the length and width of mitotic chromosomes isolated from reactions that had cycled through interphase into a second metaphase. (n = 3 extracts, ≥ 38 chromosomes per condition). Sperm mitotic chromosomes were not significantly different in either length or width in the second metaphase ($p \geq 0.1$). Stage 7 mitotic chromosomes were not significantly different in length ($p \geq 0.6$) but were thinner ($p \leq 0.02$). Stage 17 mitotic chromosomes were both longer ($p \leq 0.001$) and thinner ($p \leq 0.007$) in the second metaphase.

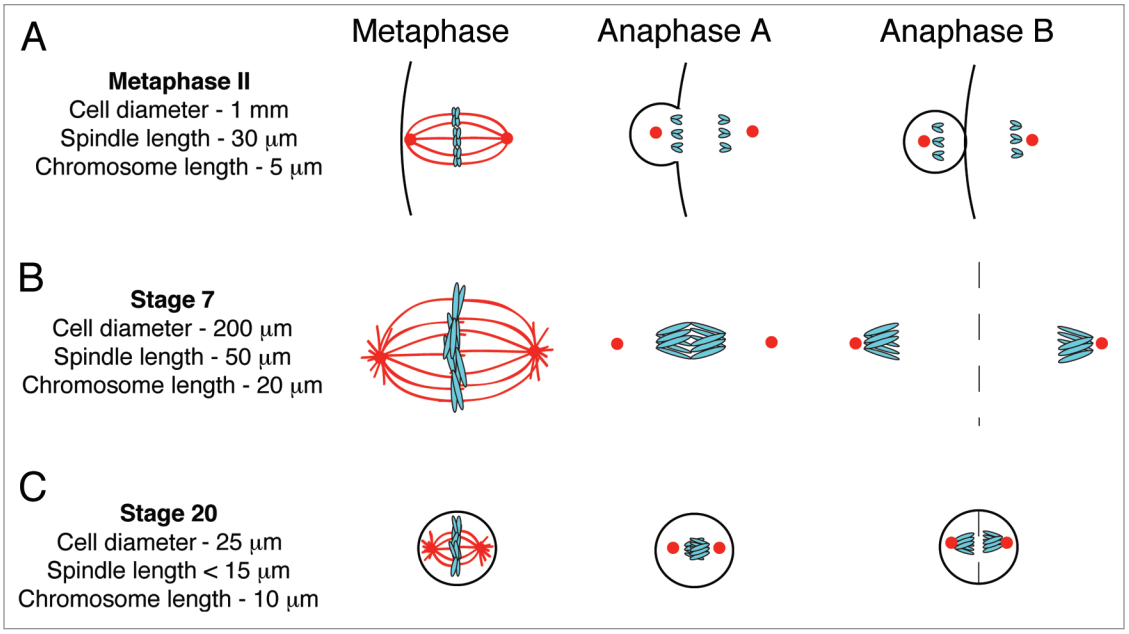

Figure 5. Models of chromosome segregation at different stages of development based on size measurements. Left-most panels show metaphase spindles and chromosomes; center and right panels show just the chromosomes and the position of spindle poles (red dots) during anaphase. (A) Metaphase II-arrested eggs are very large (1 mm diameter) relative to the meiotic spindle (30 μm long) and small chromosomes (predicted to be ~5 μm long without acetic acid treatment). The spindle lies just adjacent to the cell cortex and the cell division is asymmetric, producing one very small polar body and one very large egg. The small polar body restricts the distance that one half spindle can move, so anaphase movements and chromosomes are small to facilitate their complete segregation. (B) Stage 7 embryos cells (~200 μm diameter, not depicted) are smaller than the egg but contain much larger chromosomes (~20 μm long) and larger spindles (~50 μm long). Extended anaphase B movements occur to deliver segregated chromosomes to the center of daughter cells, thus promoting the segregation of large chromosomes. (C) Later in development, stage 20 embryos have small cells (~25 μm diameter), small spindles (≤ 15 μm long) and small chromosomes (~8 μm long). The small cell size limits the distance anaphase B can move chromosomes, which are shorter in length to permit their complete segregation.

dimensions. Whereas nuclear expansion during interphase did not alter chromosome size, passage through an entire cell cycle in egg extract was sufficient, consistent with the observation that small female meiotic chromosomes are converted into long mitotic chromosomes during the first cell cycle of the fertilized egg (**Fig. 4A**). Our assay provides an approach to determine what factors contribute to the remodeling, which likely occurs during DNA replication.

Our analysis facilitates comparison of chromosome size with that of other cellular structures known to scale during development, the interphase nucleus[5] and the mitotic spindle,[6] and sheds light on how chromosome segregation mechanisms adjust to differences between meiosis and mitosis, and the subsequent rapid cell size changes that occur during embryogenesis (**Fig. 5**). Developmental chromosome size changes do not scale with reductions in nuclear size, but are more closely correlated with spindle size, which is logical considering that components of the cell division apparatus must be coordinated to permit proper chromosome segregation in different sized cells. The anastral spindle at the cortex of an egg in metaphase of meiosis II is very small compared with the size of the cell, and meiotic chromosomes are very short, permitting their segregation into a small polar body with minimal loss of cytoplasm (**Fig. 5A**). From the first mitotic division until the midblastula transition at stage 8, mitotic spindles are up to twice the length of the meiotic spindle,[6] and mitotic chromosomes prepared from early embryos are also large, frequently exceeding half the metaphase spindle length. However, anaphase B mechanisms can likely accommodate long chromosomes, because asters at the spindle poles move hundreds of microns apart to the center of daughter cells (**Fig. 5B**).[13] As cell size decrease,s the spindle eventually shortens to adjust and can no longer accommodate large chromosomes, because anaphase B movements are constrained (**Fig. 5C**). In addition to differences in spindle architecture and dynamics, cell cycle regulation of chromosome condensation factors such as condensin,[23] as well as Aurora B activity at the spindle midzone during anaphase provide other mechanisms to ensure complete chromosome separation.[3,4,24]

It is worth noting that although the Xenopus egg extract system recapitulates meiotic spindle assembly; sperm nuclei do not assemble into female meiotic chromosomes, but rather resemble early embryo mitotic chromosomes. This may help explain the long-standing observation that anaphase chromosome segregation is not very robust in this system, because chromosome and spindle types do not match. Interestingly, *X. tropicalis* egg extracts assemble smaller meiotic spindles, but mitotic chromosome sizes do not scale between the two species, presumably because anaphase B mechanisms accommodate large chromosomes during early development of both frogs and even though their cell sizes differ, the distance between the center of daughter cells is still long enough for complete chromosome segregation.

In conclusion, Xenopus facilitates the development of powerful in vitro systems to address size control mechanisms for a

variety of cellular structures including mitotic chromosomes. Since the determinants of higher order chromosome packing are poorly understood, the question of whether and how chromosome architecture is altered to accommodate cell size changes has rarely been considered. In addition to shedding light on scaling mechanisms, studies in Xenopus may provide fundamental insight into how higher order chromosome architecture is attained and how it contributes to the fidelity of cell division.

Materials and Methods

Egg extract, spindle and embryo nuclei preparation. *X. laevis* and *X. tropicalis* CSF (cytostatic factor) metaphase-arrested egg extracts and sperm nuclei were prepared as described in references 9 and 25, as were mitotic spindle assembly reactions.[9] In vitro fertilization of *Xenopus laevis* eggs was by standard procedures.[26] Embryos were arrested in G_2 with cycloheximide, and G_2 nuclei were isolated as previously described in references 5 and 27. For stages 7 and earlier, embryos were crushed at 9,000 g for 10 min at 16°C instead of 10,000 g.[5] This increased the yield by reducing loss of these large nuclei into the heavy membrane pellet. Glycerol was added to a concentration of 8% and embryo nuclei were stored at -80°C in 25 μl aliquots.

Isolation and staining of mitotic chromosomes. Cycled sperm chromosome reactions were set up as previously described in references 7, 14 and 25. Reactions were incubated at RT to begin nuclear assembly. After about 70 min (once the extract entered prophase and DNA started to condense) 1 volume of fresh CSF extract was added to cycle the extract back into metaphase. At this point, for experiments using embryo nuclei, previously isolated embryo nuclei were slowly thawed on ice and washed in 1 ml of egg lysis buffer [ELB: 250 mM sucrose, 50 mM KCl, 2.5 mM $MgCl_2$ and 10 mM HEPES (pH 7.8)] to remove residual cycloheximide. Embryo nuclei were spun at 1,600 g for 3 min at 4°C to concentrate them. Fresh CSF extract of equal volume to the cycled sperm nuclei reaction was added to the concentrated embryo nuclei and mixed well. Reactions were then processed in the same way.

When mitotic structures formed, the reactions were diluted 10-fold with chromosome dilution buffer [1x XBE2 (10 mM HEPES, 100 mM KCl, 2 mM $MgCl_2$, 0.1 mM $CaCl_2$, 5 mM EGTA, 50 mM sucrose), 4% formaldehyde, 0.25% Triton X-100] and mixed by inverting the tube. Reactions were fixed for 15 min are RT, and then the entire volume was loaded on a 5 ml cushion of 1x XBE2 + 30% glycerol. Reactions were then spun through the cushion at 18,000 g for 15 min at 16°C on to coverslips. Coverslips were fixed in -20°C methanol for 30 sec and washed with PBS-NP40 (1x PBS, 0.1% Nonidet P-40). Coverslips were blocked for 1 h in PBS-BSA (1x PBS, 3% BSA), then incubated with rabbit-anti-INCENP primary antibody (Abcam, 1:1,000 in PBS-BSA) for 1 h, followed by PBS-NP40 washes and incubation with secondary goat-anti-rabbit-alexa-568 antibody (Molecular Probes, 1:1,000 in PBS-BSA) for 1 h. Coverslips were washed as before and mounted on slides with a small drop of Vecta-shield with DAPI to stain the DNA.

Imaging and quantification of chromosome size. Chromosomes were imaged using identical exposures on an epifluorescence microscope (Olympus, model BX51) with CCD camera (Hamamatsu, model C4742–98), shutter controller (Sutter Instrument Co., model Lambda 10-2), and Metamorph software (Universal Imaging Corp.) using a 100x oil (Olympus, N.A. 1.3) objective. For morphological quantification, images were manually scaled and thresholded, cut where necessary, and subjected to Integrated Morphometry Analysis (Metamorph). For statistical analysis a two-tailed Student t-test was performed.

Swelling and analysis of nuclei in interphase extract. Interphase-arrested extract[7] (50 μl) was added to embryo nuclei or sperm nuclei. Squashes[25] of each reaction were taken every 10 min for 70 min. Reactions were cycled back to metaphase by the addition of 50 μl of fresh CSF extract. Mitotic chromosomes were isolated as described above. Nuclei were imaged and analyzed as chromosomes above but with a 40x dry (Olympus, N.A. 0.75) objective.

Embryo chromosome spreads. Embryo chromosome spreads were conducted as previously described (faculty.virginia.edu/xtropicalis/KaryotypeXtropicalis.htm) with modifications. Embryos at the appropriate stage of development were placed into 1 ml of ddH_2O for 20 min. Embryos were then transferred into 1 ml of 60% acetic acid in ddH_2O for 5 min. Individual embryos were placed on to slides (two embryos per slide) and excess acetic acid mixture poured off. 24 x 60 coverslips were then firmly pressed on the embryos to squash them completely. Slides were then placed on dry ice until the acetic acid solution was completely frozen, about 20 min. The coverslips were then pried up using a razor blade. Slides were dipped into -20°C methanol for 5 sec. Excess methanol was allowed to run off the slide. Spreads were allowed to dry completely and then washed 3x with ddH_2O. Again, spreads were dried completely and then stained with a 5 μg/ml Hoechst in H_2O for 5 min. Hoechst was drained off the slides and 15 μl of Vecta-shield was placed on each spread, covered with a 22 x 22 coverslip and the edges sealed with nail polish. Imaging of chromosomes was as described above.

Treatment of cycled sperm with acetic acid. Mitotic chromosomes were allowed to form, then were fixed and spun down onto coverslips as above. Before methanol fixation, coverslips were treated with 60% acetic acid in water for 5 min. The acetic acid mixture was removed and coverslips immediately fixed in methanol. Similar results were obtained if acetic acid treatment was performed after methanol fixation.

Cycling extract into second mitosis. Reactions were set up as described above. When mitotic structures in both cycled sperm and embryo nuclei had formed, calcium (10 mM in water) was added at 500 μM to release the reactions into anaphase. After 30 min, the calcium concentration was increased to 700 μM to drive the reactions fully into interphase. After another 60 min, 1 volume fresh CSF extract was added to each cycled reaction to drive the reaction into a second metaphase. Mitotic structures were allowed to form and treated as above for fixation, isolation and analysis.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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Note

Supplemental material can be found at:
www.landesbioscience.com/journals/cc/article/17975

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