[Frontiers in Bioscience 13, 5838-5846, May 1, 2008]

Unreplicated DNA in mitosis precludes condensin binding and chromosome condensation in *S. cerevisiae*

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1. ABSTRACT

Condensin is the core activity responsible for chromosome condensation in mitosis. In the yeast *S. cerevisiae,* condensin binding is enriched at the regions where DNA replication terminates. Therefore, we investigated whether DNA replication completion determines the condensin-binding proficiency of chromatin. In order to fulfill putative mitotic requirements for condensin activity we analyzed chromosome condensation and condensin binding to unreplicated chromosomes in mitosis. For this purpose we used pGAL:*CDC6 cdc15*-ts cells that are known to enter mitosis without DNA replication if *CDC6* transcription is repressed prior to S-phase. Both the condensation of nucleolar chromatin and proper condensin targeting to rDNA sites failed when unreplicated chromosomes were driven in mitosis. We propose that the DNA replication results in structural and/or biochemical changes to replicated chromatin, which are required for two-phase condensin binding and proper chromosome condensation.

2. INTRODUCTION

Mitotic chromosome condensation is driven by consorted activities of structural chromatin proteins and enzymes, which enable a highly ordered compaction of sister chromatids into condensed chromosomes, thus preparing them for segregation. The condensin complex (1) is the major enzymatic activity required for this process (2, 3). Condensin complex is present in all eukaryotic cells and is composed of five subunits: the Smc2/Smc4 heterodimer and three non-SMC subunits (1, 4, 5). In higher eukaryotes, there are two distinct sets of these non-SMC subunits, which form two condensin complexes (condensin I and II) upon association with the same SMC dimer (6, 7). In vertebrates, the condensin-depleted sister chromatids are universally defective in separation during anaphase (8-10), as was originally reported in yeast (5, 11).

The molecular mechanisms of condensin activity remain obscure, however, mainly due to the inaccessibility of highly condensed chromatin in higher cells to

Table 1. Yeast strains

Strain (background)	Genotype	Origin
640-EY0987 (S288c)	*MATalpha his3 leu2 lys2 ura3 SIK1:mRFP::kanMX SMC4:GFP::URA3*	This work
622-YPH499-4 (S288c)	*MATa ade2 bar1 his3 leu2 lys2 trp1 ura3 SIR2:GFP12::HIS3 SIR4 42::URA3*	This work
5757 (W303-1A)	*MATa his3 leu2 trp1 cdc15-2 cdc6::hisG ura3::URA3::pGAL:CDC6*	K.Nasmyth
SLJ127 (W303-1A)	*MATa ade2 his3 bar1 leu2 trp1 ura3 can1 cdc15-2*	D.Morgan

biochemical and molecular analyses. Therefore, it is still unknown how condensin interacts with chromatin fiber - its natural substrate. Recent data (12) suggests that condensin does not need to be regulated (e.g. by posttranslational modification) in order to display its basic enzymatic activity. Therefore, the regulation of condensin activity *in vivo* largely modulates condensin accessibility and affinity to chromatin, e.g. through nuclear envelope breakdown (6), nuclear import (4) or inhibitory phosphorylation (12). Directing condensin binding to specific (and probably invariant) chromatin sites (13, 14) has to be an important component of this pathway, as judged from the highly ordered mitotic chromosome structure (and the corresponding regularity of the condensin binding pattern), which appears to be identical in every cell cycle (15, 16). In the absence of an *in situ* molecular assay for condensin activity, targeting condensin to specific chromosomal sites served as an important tool to identify several molecular pathways, which facilitate condensin function *in vivo*. The requirement of functional cohesin for condensin regulation in *S. cerevisiae* was one of the first such pathways identified (17, 18). After the characterization of the first specific binding sites for condensin in rDNA (5) it was shown that condensin fails to bind rDNA in cohesin mutants (A.S. unpublished). Several additional pathways controlling condensin targeting to mitotic chromatin were identified subsequently, by using the rDNA locus as a test system. Among these pathways are: condensin regulation by the SUMO pathway (19), the Cdc14 phosphatase control (13, 20) and replication fork barrier (RFB) dependence (21, 22). Moreover, it was recently shown that the degree of condensin binding to rDNA significantly affects the whole-genome pattern of condensin association (23). An analysis of the genome-wide pattern of condensin binding brought additional insights into the phenomenology and putative mechanisms determining the pattern of condensin distribution along chromosomal arms. The strongest correlation in this respect was found with the sites of DNA replication termination (14), which suggested the possibility that condensin binding is directly dependent on replicon layout. The nature of this dependence is only beginning to be elucidated (24, 25), however it could be either direct, i.e. DNA replication termination is a physical requirement for condensin loading, or indirect: an epigenetic cohabitation of condensin and DNA replication sites.

We investigated the functional link between condensin binding and replication function in budding yeast. *S. cerevisiae* provide a good cellular model for this study, as in this system mitotic chromosome compaction is significantly reduced as a result of both DNA underreplication (26) and the dysfunction of condensin (5), the second - unlike in cells of Metazoa (9, 10). Moreover, yeast cells can be forced into mitosis with unreplicated (27) or underreplicated (28) chromosomes. This provides a unique experimental setting to study the dependence of mitotic condensin binding on chromatin from the completion of DNA replication. In this work we proposed and tested the hypothesis that the completion of DNA replication is the fundamental requirement for condensin binding to specific chromatin sites and the resulting chromosome condensation.

3. MATERIALS AND METHODS

All genetic techniques were standard (43, 44). Yeast strain genotypes are shown in Table 1. Cell-cycle experiments were conducted as previously described in a number of our publications. FISH was performed as in (26) with slight modifications; rDNA probes, the arrest protocol and quantification of the FISH signal were as in (5). In short, for FISH analysis, (Figure 3) cells were arrested in G1 with alpha-factor, transferred to 37˚C and then released from the G1 arrest into nocodazole-containing media. This protocol facilitates the comparison of mutant and wild-type cells at the same point in mitosis. The quality of arrest was monitored microscopically and by FACS. For Figure 3A the 5757 (*cdc15-2 pGAL:CDC6*) cells (27) were arrested in telophase (YPGal, 37˚C, 2hr) and released at 23˚C in either YPD (2% dextrose) or YPGal (2% galactose). Cells were collected at 30' min intervals and processed for FACS and FISH. The 150-min point after the *cdc15* arrest release corresponded to the telophase of the next mitosis. For Figs. 2A, 3B and 3C, nocodazole (15μg/ml) was added to the cultures after their release from 37˚C. An isogenic wild type *CDC6* strain SLJ127 (*cdc15-2*) (45) was used as a control under the same protocols in Figs. 2C and 3C.

Routine microscopy was performed with the AxioVert (Zeiss) microscope with epifluorescence. The images were captured with a Hamamatsu cooled CCD camera. The Smc4-GFP, Sir2-GFP and Sik1-mRFP localization assays (Figure 1) have been described (5, 13). The deconvolution microscopy and 3-D segmentation was done using a Deltavision system. 64 optical planes with 0.15 micron step were collected per field. At least 70 cells were quantified for each experiment.

Chromatin-binding assays were performed as in (33) under the growth regimen described in Results. Anti-condensin antibodies have been described (5). Chromatin IP reactions were as described in (13), using the Smc2 protein tagged with the HA-tag. Quantitative PCR reactions were run using the Stratagene MX3000P real-time PCR system. The design of primer sets was the same as in (46). qPCR reactions (50 microliters), containing 1 microliter of template DNA (ChIP or input), 25 ml of 2x SYBR-Green master mix (Stratagene), and 50nM of primers were run for 1 cycle of 95˚C (10 min) and then for 40 cycles of 95˚C for 1 min, 60˚C for 30 sec, followed by 72˚C for 45 sec. PCR

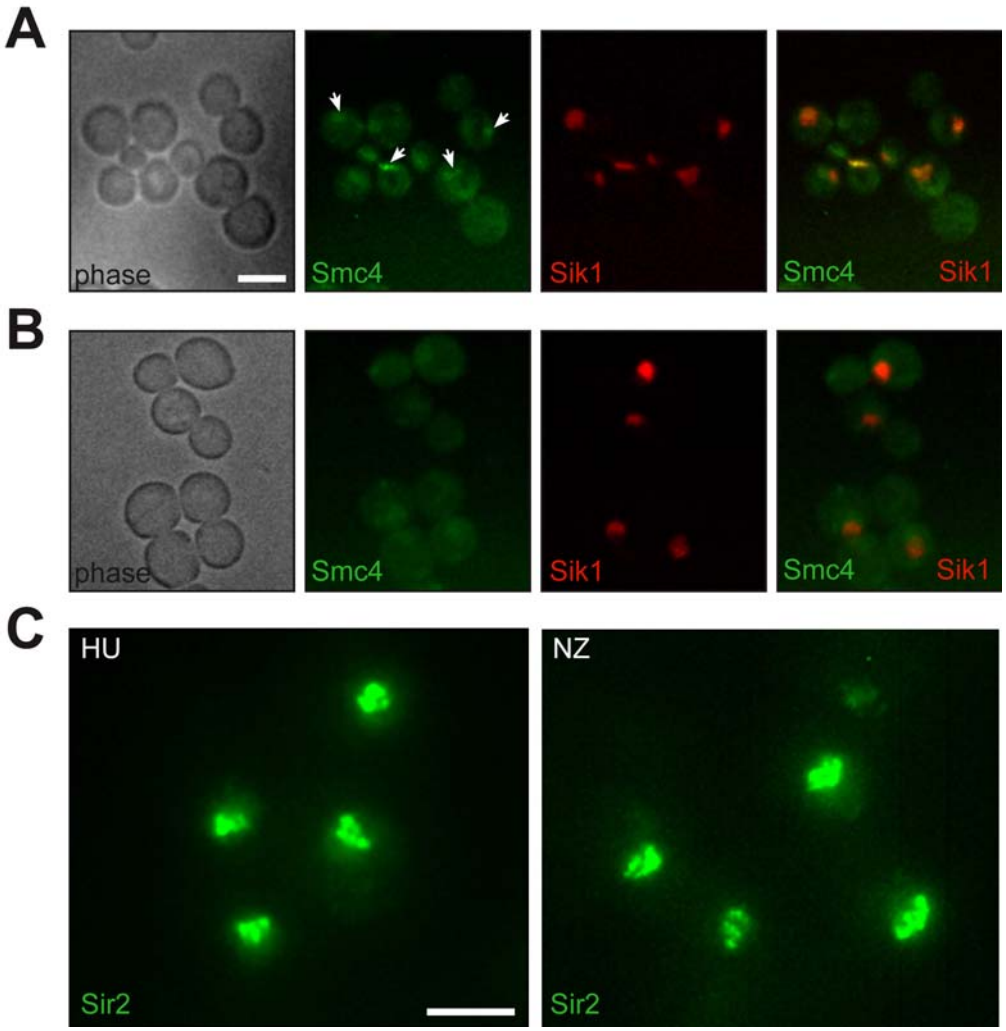

Figure 1. Condensin targeting to the nucleolus is impaired in cells arrested by DNA replication checkpoint. (A) Condensin enriches nucleoli in nocodazole-arrested cells. The strain expressing both Smc4-GFP (condensin subunit) and Sik1-mRFP (a nucleolar marker) (640-EY0987) was arrested with nocodazole for 3 h at 30˚C. Arrows indicate the colocalization of condensin-enriched areas with nucleoli. Scale bar 5 μm. (B) Condensin fails to target to nucleoli in hydroxyurea-arrested cells. Conditions the same as in (A), except hydroxyurea (200mM) was used to arrest cells. (C) rDNA chromatin is not dispersed in hydroxyurea-arrested cells. The *SIR4-42* strain expressing Sir2-GFP (622-YPH499-4) was arrested with hydroxyurea or nocodazole for 3 h at 23˚C. In this strain Sir2p is exclusively bound to rDNA chromatin (47). The images were deconvolved and 3-D segmentation was conducted (see Results) using a DeltaVision workstation. Scale bar 5 μm.

quantification was performed on the basis of three independent experiments, using Ct values determined by the MxPro software (Stratagene). The enrichment ratio was determined by calculating the ratio of ChIP qPCR to input qPCR as following: $2^{[Ct\ (Input)\ -\ Ct\ (ChIP)]}$ divided by the corresponding dilution ratios for ChIP and Input, respectively. The mock ChIP with no antibody and the *TUB2* gene ChIP were used as negative controls.

4. RESULTS

It has been previously shown that the rDNA locus in yeast is compacted upon metaphase arrest induced by nocodazole (26). At the same time, cells arrested with *cdc6* and *cdc17* mutations, which impair the DNA replication, have decondensed rDNA (26). Even though the pathways inhibiting the spindle elongation are distinct in DNA replication inhibition (hydroxyurea) and nocodazole arrests (29, 30), the absence of condensation upon DNA replication block is somewhat surprising, as there is a consensus that yeast cells arrested with hydroxyurea are in mitosis-like physiological state (31). Therefore, the absence of rDNA condensation without DNA replication must reflect some fundamental requirement for condensin function, which is not satisfied when DNA is underreplicated.

We monitored condensin association with the nucleolus in cells arrested by hydroxyurea and nocodazole. While condensin binding to the nucleolar chromatin is not

as complete in nocodazole arrested cells as in unchallenged metaphase (5), which is consistent with the partial decondensation of chromosomes upon spindle assembly checkpoint activation (32), the nucleolar enrichment with Smc4-GFP is quite obvious (Figure 1A). In contrast, the same strain arrested with hydroxyurea did not show preferential enrichment of the nucleolus with condensin, although condensin did not appear to be completely excluded from the nucleolar area (e.g. as in *cdc14* mutants (13)). This result suggests that the distinct levels of rDNA condensation under these two conditions could be directly related to the corresponding differences in condensin binding to rDNA. However, as rDNA is supposed to be decondensed in hydroxyurea-arrested cells, this may mimic the apparent diffusion of condensin from nucleolar area. Therefore, we measured the nucleolar chromatin condensation in living cells using the *SIR4-42 SIR2:*GFP strain (5). The result of deconvolution and three-dimensional segmentation analysis of rDNA chromatin (Figure 1C) showed that in living cells the space occupied by nucleolar chromatin is comparable between hydroxyurea- and nocodazole-arrested cells (median values are 72 and 59 voxels, respectively). Considering that there is 2-fold less rDNA in hydroxyurea-arrested cells, this ratio roughly corresponds to 2.5-fold condensation in nocodazole; however these numbers also indicate that condensin diffusion in hydroxyurea-arrested cells is not due to the dispersion of nucleolar chromatin. Thus, it is likely that there is incomplete condensin loading onto rDNA chromatin in the hydroxyurea-arrested cells.

Unlike condensin I in vertebrates, the yeast condensin is constantly nucleus and the commitment to mitosis results in significant relocalization of its pool to rDNA (5, 21), as well as to centromeric regions (14). One possible explanation for the condensin's failure to enrich nucleoli in hydroxyurea-arrested cells could be the general defect of condensin binding to chromatin. We tested this possibility by assaying the condensin-chromatin binding biochemically, using the established fractionation assay (33). In order to overcome possible physiological and protein level differences in metaphase arrests caused by drugs, (e.g. as in Figure 1) we obtained cells with unreplicated DNA at the exactly same stage of the cell cycle as the control cells, by employing an assay that drives unreplicated chromatids into mitosis. We used a *cdc15–2* strain carrying the *CDC6* gene under the control of the *GAL* promoter (27). In essence, the cells were arrested in late mitosis (*cdc15*-ts) by shifting to a non-permissive temperature and then released from the arrest under one of two conditions: either induced or repressed expression of *CDC6* (Figure 2A). The latter enables cells with unreplicated chromatids (see confirmation by FACS analysis below) to enter mitosis (27). The chromatin fractionation assay revealed that condensin was still bound to bulk chromatin in the absence of DNA replication (Figure 2A). This result demonstrates that condensin is fully expressed and chromatin-bound in mitotic cells with unreplicated DNA.

Even if condensin is fully bound to unreplicated chromatin, under the conditions we used its binding to the rDNA locus can be reduced and/or have a different pattern. In order to quantify condensin binding to rDNA we analyzed the Smc2 condensin subunit binding within the rDNA repeat by ChIP assay, under conditions similar to Figure 2A, except that an isogenic *cdc15–2* strain with a wild-type *CDC6* was used as a control (to avoid possible differences related to two carbon sources in Figure 2A). For ChIP analysis, cells of two strains (*cdc15–2 CDC6* and *cdc15–2 pGAL:CDC6*) were both released from the *cdc15* arrest into glucose media and proceeded through the cell cycle (with the addition of nocodazole delayed by 30 min), until both were arrested in the first mitosis. We monitored condensin association with four rDNA sites (Figure 2B). Two of them, the 5' 35S precursor site (probe #23) and the RFB site (probe #15), are strong condensin-binding sites in mitosis, while two neighboring sites (probes # 27 and #12) are virtually condensin-free throughout the cell cycle (21). ChIP analysis reproducibly showed that the mitotic condensin binding pattern changed in unreplicated rDNA. The RFB site had significantly lower occupancy than wild type, while the promoter-proximal 5' 35S site had abnormally higher enrichment (Figure 2C). It is believed that both condensin binding to RFB (13, 22) and condensin association with Pol I-transcribed regions (21) are essential for rDNA segregation. However, the pattern observed in unreplicated chromosomes is distinct from other previously tested conditions: *cdc14* mutants (no condensin binding to either site) (13), transcriptionally hyperactivated rDNA (no 5' 35S site binding) or *FOB1* deletion (reduction in condensin binding to both sites) (21). The reasonable conclusion is that, despite condensin's presence in unreplicated rDNA chromatin (Figure 2C), it is apparently nonfunctional, explaining the rDNA FISH results in replication mutants (26).

In order to test directly whether condensin is indeed functionally impaired in unreplicated mitotic chromatin, we assayed rDNA condensation by FISH under these conditions. Using the experimental design similar to Figure 2A (except no nocodazole addition), in a time-course analysis we showed that $Cdc6^-$ cells failed to replicate their DNA and frequently entered anaphase, resulting in reductional chromosome segregation with respect to rDNA (Figure 3A). In order to quantify rDNA condensation for *cdc15–2 pGAL:CDC6* cells and for the control (*cdc15–2 CDC6* cells), both strains were arrested in mitosis with nocodazole and analyzed by FISH. In brief, cells exited the *cdc15*-mediated arrest and proceeded normally through the cell cycle in the presence of nocodazole, resulting in the mitotic arrest. Cells not expressing Cdc6p (*pGAL:CDC6,* glucose media) were arrested by nocodazole at the same point in mitosis as other cultures, but with unreplicated chromosomes. The cultures were split and processed for rDNA FISH (Figure 3B). FISH analysis demonstrated that there was a 2.5-fold increase in the area of mitotic rDNA chromatin in the absence of replication (Figure 3C). This rDNA decondensation can be attributed to the rDNA replication failure ($Cdc6^-$), as compared to the cells that did undergo DNA replication ($Cdc6^+$) (Figure 3C). This dramatic difference could potentially translate into an even more significant decrease in rDNA condensation, if the absence

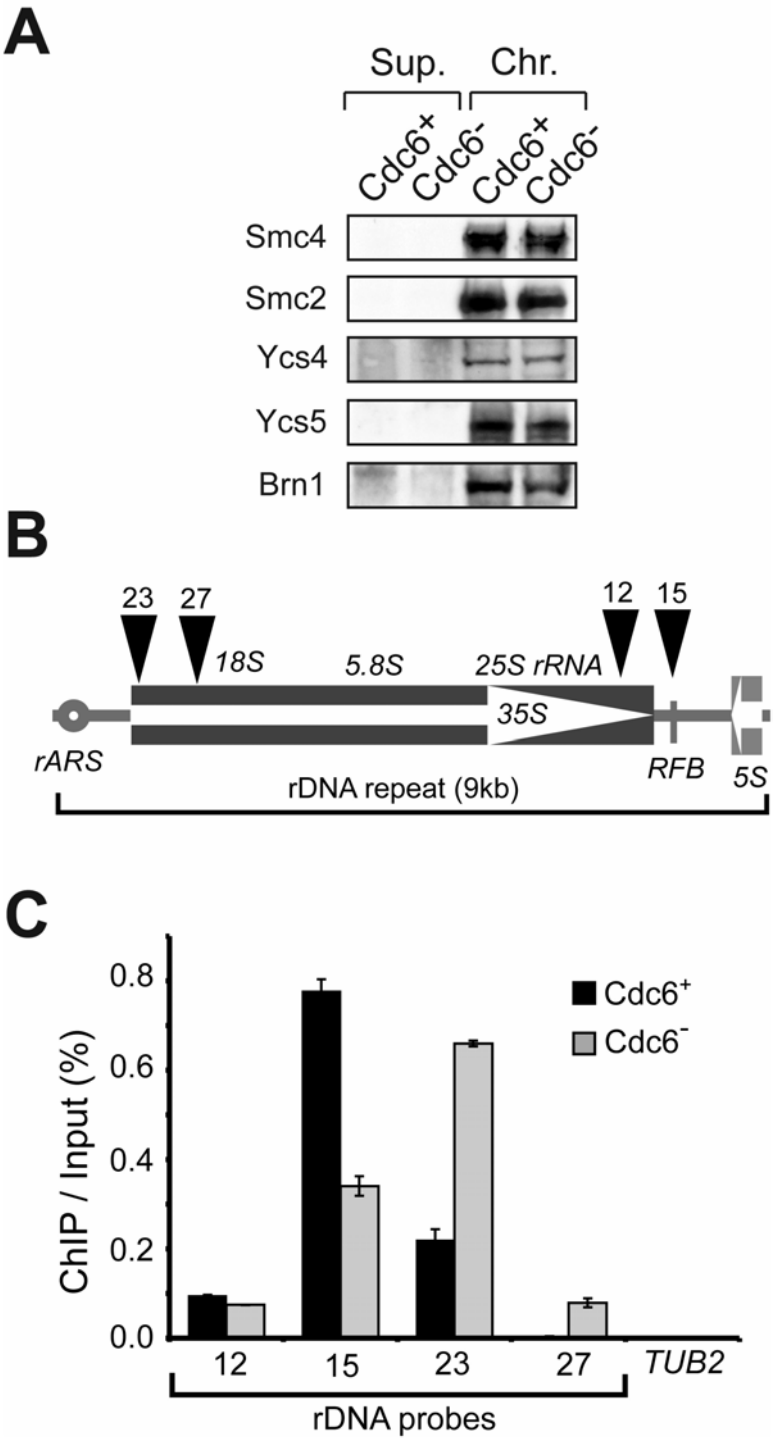

Figure 2. Condensin binds chromatin in the absence of replication, but with altered pattern in rDNA. (A) Western blot analysis of the chromatin-bound fraction of condensin subunits at the replicated ($Cdc6^+$) and unreplicated (Cdc6-) chromatin prepared as in (33). The *cdc15-2 pGAL:CDC6* strain (5757) (27) was grown in the presence of galactose (Cdc6+) or dextrose (Cdc6-) and arrested with nocodazole. (B) The layout of condensin-binding qPCR ChIP probes in the rDNA repeat. Probes design and numbering are as in (46). (C) Condensin has an altered distribution pattern in unreplicated rDNA in mitosis. After the release from *cdc15* arrest the *pGAL:CDC6* (5757, Cdc6-) (27) and *CDC6* (SLJ127, Cdc6+) strains were grown as described in Results in the presence of or dextrose (Cdc6-) and arrested with nocodazole. The qPCR ChIP results were averaged from three independent experiments. *TUB2* PCR was a negative control.

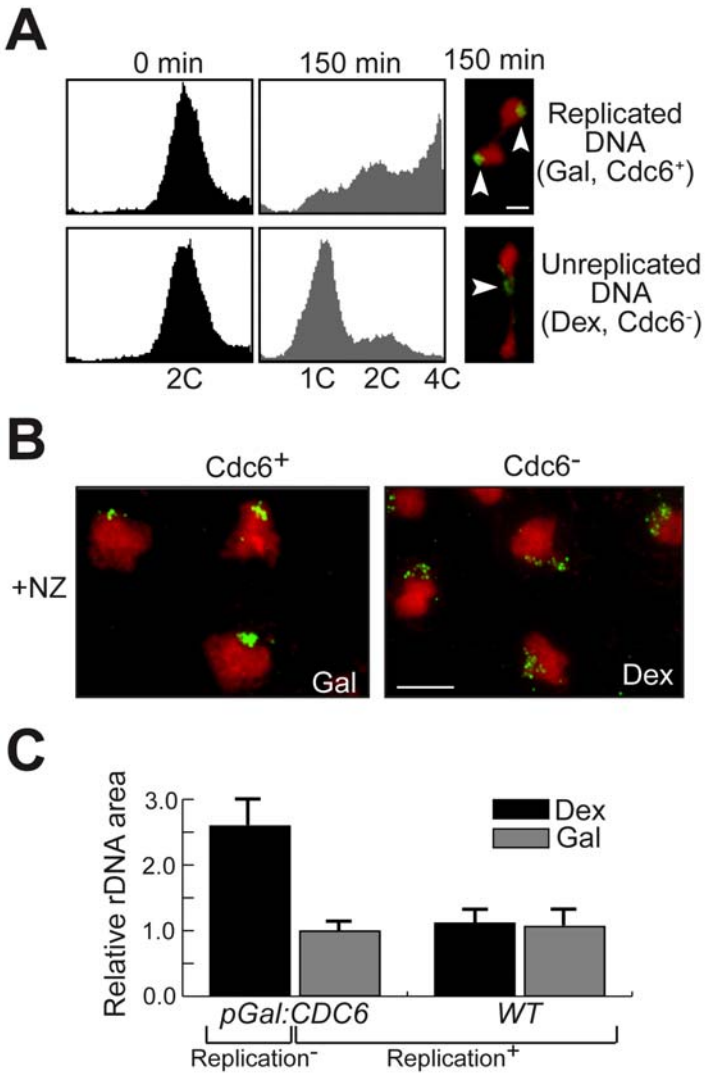

Figure 3. rDNA condensation fails in mitotic cells without DNA replication. (A) FACS and FISH analysis of the *cdc15-2* pGAL:*CDC6* strain (5757) (27) released from the *cdc15* arrest (t=0 min) and assayed at the next mitosis (t=150 min). Haploid cells arrested at the *cdc15-2*-specific point show 2C DNA content, probably due to delayed cytokinesis, and thus many cells reach apparent 4C DNA content in the next cycle. However, similarly arrested *cdc15-2* cells, progressing through the first cell cycle with depleted Cdc6p, show their true 1C DNA content after 150 min. Arrows in the upper rDNA-specific FISH panel show replicated, segregated and condensed rDNA signals (green) in the anaphase Cdc6+ cell. Propidium iodide (red) stains the bulk of nuclear DNA. The lower panel arrow points to the unsegregated decondensed rDNA signal in the Cdc6-anaphase. Scale bar 5µm. (B) rDNA FISH analysis of *cdc15-2* pGAL:*CDC6* strain (5757) grown in the presence of galactose (Cdc6+) or dextrose (Cdc6-) and arrested with nocodazole. Colors are as in (A). Scale bar 5µm. (C) rDNA condensation fails in the absence of replication. Relative rDNA compaction was calculated on the basis of experiments identical to (B), as a ratio between the rDNA FISH signal area and propidium iodide – stained area, according to (5). pGAL:*CDC6* – 5757 cells, *CDC6* - SLJ127 cells.

of half of the rDNA (i.e. sister chromatid) could be accounted for. The isogenic control strain with the wild-type *CDC6* gene did not show any variation in rDNA compaction, regardless of the carbon source used in the media (Figure 3C), thus validating experiments comparing dextrose media to galactose (as in Figure 2A).

The result in Figure 3C shows that DNA replication is required for chromosome condensation, even when unreplicated chromatids are placed in the adequate mitotic environment. This condensation failure correlates with decreased condensin occupancy at the RFB (Figure 2C). This, for the first time, demonstrates that DNA replication termination, but not the RFB DNA itself, is directly required for rDNA condensation. Thus, it is likely that the DNA replication process per se and/or the resulting presence of paired sister chromatids is a necessary prerequisite for the formation of higher order chromosome structure in mitosis.

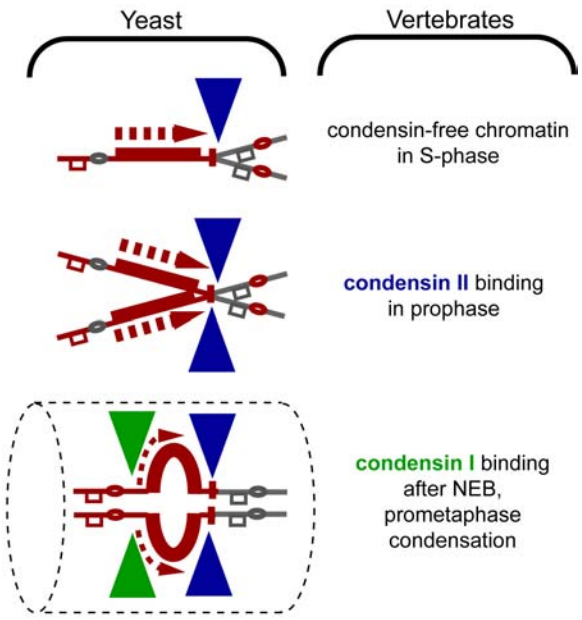

Figure 4. Hypothetical parallel between the two-site condensin loading in yeast rDNA and sequential loading of condensins I and II in vertebrates. The transcriptionally active repeat is shown for simplicity, with origin firing downstream, based on (48). Shaded box – the 35S RNA gene, open box – the 5S rRNA gene, open circle – *rARS* (origin of replication), vertical line – the RFB site, dotted line – Pol I transcription. Blue triangles – condensin loading (or condensin-chromatin contacts) resulting from replication fork termination. Green triangles – condensin loading (or condensin-chromatin contacts) to 35S promoter junction enabled by transcription downregulation in mitosis. The kinetics of condensin I and condensin II loading is according to (34, 35); color coding denotes the match to hypothetically equivalent yeast condensin pool).

5. DISCUSSION

The regulation of condensin loading onto chromatin is extremely important for its biological function, yet it is apparently not conserved in different species, or between different condensin variants. For example, condensin I and II in vertebrates have drastically distinct chromatin loading patterns and cell cycle dynamics (34, 35). Such dramatic differences remain puzzling, they cannot be adequately explained without defining what constitutes a condensin site in chromatin. The recently completed surveys of genomic localization of condensin in budding yeast (14, 23) show that condensin distribution along the chromosome does not appear to compulsorily adhere to known chromosomal landmarks. At the same time, some correlation with the functional sites of chromosomes is evident in several systems: condensin is enriched at pericentromeric regions (14, 34, 36, 37), condensin is enriched at telomeres under some conditions (23, 38) and condensin binding notably coincides with the regions of DNA replication termination in yeast (14). The latter is a good candidate for a universal law determining condensin location and possible loading mechanism in all eukaryotes.

The degree of DNA replication involvement in condensin loading could be at several levels. First, condensin loading to some sites may be directly dependent on the arrival of a replication fork there. It this case, condensin might either "ride" the replication fork or recognize the replication swivel proteins. Alternatively, condensin may be prebound at the DNA replication termination zones epigenetically, and thus be involved in positioning these zones. This latter hypothesis appears unlikely now, as our results show that condensin does not enrich RFB in mitosis if DNA replication does not occur (Figure 2C). Therefore, it is more probable that condensin binding is physically dependent on the replication fork termination.

In our work we used the well-characterized binding of condensin to the rather specialized rDNA locus and the RFB site, as a model for functional condensin dependence on DNA replication. The focus on rDNA is due to the lack of comprehensive genome-wide characterizations of other individual condensin sites. While our results conclusively demonstrate that DNA replication is the basic requirement for chromosome condensation, the mechanism of this requirement remains unclear. The complexity of the problem, even in such a seemingly simple experimental model, stems from the fact that there are two strong condensin-bound sites in rDNA: in the RFB region and at the promoter-gene junction for the 35S rRNA precursor. Moreover, it is possible that condensin binding to these two rDNA sites is not entirely independent: first, condensin binding to the 35S 5' region is reduced upon disruption of the *FOB1* gene, which encodes the RFB-binding protein; second, condensin binding to both sites is eliminated in *cdc14* mutants (13). The crosstalk between the two sites appears even more likely, as the spacing between them nearly matches the positions of the Pol I enhancer and promoter (39, 40), which must interact in some repeats. As condensin is a very large complex (41), it could plausibly "crosslink" two chromatin sites, either physically or functionally. In support of this logic, our results show that rDNA condensation does not occur when condensin binding to the rDNA repeat is altered (not eliminated, as hydroxyurea arrest results would suggest, Fig, 1B), if DNA replication is blocked. Most surprisingly, rDNA remains decondensed under these conditions (Figure 3C), despite the notable and increased presence of condensin at the 35S gene 5' site (Fig, 2C). There is no data to indicate that condensin accumulated at the 35S 5' site in nocodazole-arrested Cdc6$^-$ cells is inactive. On the contrary, the recent study on *Xenopus* suggests that condensin can universally condense chromatin, if binding to chromatin fiber is facilitated (12). Thus, based on the results of the present work, we can hypothesize that productive binding of condensin to both rDNA sites (and possibly the presence itself of a sister chromatid) is necessary for condensation, while binding to either site alone is not sufficient.

However, it seems difficult to adapt this model to higher eukaryotes, due to the fact that condensin I binds to

chromatids long after completion of DNA replication. Nevertheless, the results of the present work invoke a compelling parallel with probable functional cooperation (6, 35) between condensins I and II in vertebrates. Namely, it is plausible that the initial loading of yeast condensin (and possibly condensin II in vertebrates) occurs at the DNA replication termination sites (Figure 4). Later, in mitosis, an additional pool of yeast condensin (and the condensin I in vertebrates) enriches the sites that were unavailable due to active transcription, which is decreased (in yeast) or shut down (in vertebrates) in mitosis. The coordinated activity of yeast condensin (and condensin I and II in vertebrates) at these two types of sites (one determined by DNA replication and another by transcription repression) is then needed to compact chromosomes both axially and radially. In support of this two-step model, an earlier revealing study in *Xenopus* showed that chromatin is condensed, but the chromosomal axis is not formed, if chromosomes are allowed to enter the mitotic phase in the absence of replication (42). The "sequential loading and cooperation" model (Figure 4) fits well with our knowledge of requirements for condensin binding sites in rDNA for its condensation and segregation (in budding yeast), as well as with data from cell-free *Xenopus* extracts (6). However, it needs more supporting data for non-rDNA sites in yeast and for condensin I and II cooperation in vertebrates.

6. ACKNOWLEDGEMENTS

We thank Mary Dasso for helpful discussion, T. Karpova and J. Sedat for Deltavision advice and help. This work was supported by the Intramural Program of the NIH (USA). L.A. was supported by the MRC (UK).

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Key Words: DNA Replication, Chromosome Condensation, Condensin, rDNA, Nucleolus

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