Published in final edited form as:
Cell Cycle. 2004 July ; 3(7): 960–967.

Cdc14p/FEAR Pathway Controls Segregation of Nucleolus in *S. cerevisiae* by Facilitating Condensin Targeting to rDNA Chromatin in Anaphase

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Abstract

The condensin complex is the chief molecular machine of mitotic chromosome condensation. Nucleolar concentration of condensin in mitosis was previously shown to correlate with proficiency of rDNA condensation and segregation. To uncover the mechanisms facilitating this targeting we conducted a screen for mutants that impair mitotic condensin congression to the nucleolus. Mutants in the *cdc14*, *esp1* and *cdc5* genes, which encode FEAR-network components, showed the most prominent defects in mitotic condensin localization. We established that Cdc14p activity released by the FEAR pathway was required for proper condensin-to-rDNA targeting in anaphase. The MEN pathway was dispensable for condensin-to-rDNA targeting, however MEN-mediated release of Cdc14p later in anaphase allowed for proper, albeit delayed, condensin targeting to rDNA and successful segregation of nucleolus in the *slk19* FEAR mutant. Although condensin was physically dislodged from rDNA in the *cdc14* mutant, it was properly assembled, phosphorylated and chromatin-bound, suggesting that condensin was mis-targeted but active. This study identifies a novel pathway promoting condensin targeting to a specific chromosomal address, the rDNA locus.

Keywords

condensin; rDNA; nucleolus; chromatin; Cdc14; chromosome segregation; anaphase

INTRODUCTION

Proliferating cells must execute precise transmission of genetic information during cell division to ensure genetic integrity of species and cell lineages. An assembly of multiple protein factors controls this essential process, partially through maintaining the evolutionarily conserved and highly ordered three-dimensional organization of chromatin. One particular level of this high-order chromatin structure is mitotic chromosome condensation, which establishes the inter-domain contacts within the single chromatid, allowing its successful segregation in anaphase.[1] Recent studies established that the essential role of chromosome condensation is not in making chromosomes merely "shorter and thicker," but instead is in compartmentalization of sister chromatids to facilitate anaphase.[2–4]

Mitotic chromosome structure is intimately dependent on activity of the condensin complex,[1] even though additional/alternative systems evidently contribute to complete mitotic chromosome compaction in higher eukaryotes.[3,4] In *S. cerevisiae* these elusive additional mechanisms, which can largely compensate for the loss of condensin function in higher cells,

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are either absent or inactive. Therefore, loss of condensin activity in *S. cerevisiae* leads to completely impaired chromosome condensation.[2,5] Budding yeast also lack condensin paralogues that form an alternative condensin complex in vertebrates.[6] Thus, condensin function in *S. cerevisiae* is more transparent to molecular analysis, especially taking into account the relative simplicity of yeast genome organization.

Previously, we demonstrated that the condensin complex exists in *S. cerevisiae* and that its composition is conserved, as compared to *X. laevis* and human cells. *S. cerevisiae* condensin includes two SMC proteins: Smc2p and Smc4p [2,7] as well as three additional non-SMC subunits: Brn1p, Ycs4p and Ycs5p/Ycg1p.[2,8,9] All five condensin subunits were shown to be essential for cell viability, indicating that *S. cerevisiae* condensin acts as an integral complex in vivo. Genetic analysis demonstrated that mutant alleles of genes encoding condensin subunits phenocopy the originally described *smc2* mutant allele with respect to their role in chromosome condensation.[2,5,8,9]

Localization of condensin in yeast cells with GFP tagging of Smc4p and chromatin immunoprecipitation analysis (ChIP) revealed that rDNA chromatin is the primary target of condensin binding throughout the cell cycle.[2] Moreover, around the metaphase-to-anaphase transition condensin undergoes an even more extreme compartmentalization to the nucleolar region.[2] This process is likely homologous to the state of maximal mitotic chromosome condensation in human and fission yeast cells. Thus, the mitotic concentration of condensin in the nucleolar region was validated as a non-invasive in vivo assay for rDNA chromatin condensation in *S. cerevisiae*.[10] Genetic analysis of conditional ts-mutants in condensin subunits confirmed that rDNA is the main target of condensin activity. Chromosomes carrying rDNA were sensitive to impaired condensin function and were lost at a high rate in the *smc2* and *smc4* mutants.[2] Furthermore, rDNA did not segregate in condensin mutants at a non-permissive temperature, even as other chromosomes proceeded through anaphase.[2] In addition to the block in rDNA segregation under non-permissive conditions, a delay in segregation of long chromosomal arms (*ADE8* and *MET16* loci) and YACs was also observed (see Fig. 5 in ref. 2), indicating that long chromosomal arms and chromatin saturated with repeats could bear additional sites for condensin activity. Existence of some essential secondary sites for condensin binding was corroborated by the fact that *S. cerevisiae* strains lacking the chromosomal rDNA array (complemented by an episomal rDNA)[11] still had an essential requirement for condensin, even though Smc4p-GFP was no longer seen concentrated in the nucleolus in mitosis.[2]

Thus, studies on chromosome partition in condensin mutants and the demonstration that mitotic chromatin compaction per se is not a prominent process in *S. cerevisiae* have established that the primary biological role of condensin (and probably chromosome condensation driven by it) is to facilitate separation of sister chromatids in anaphase. However, the nature of condensin preference for rDNA chromatin remains unknown. It is unlikely to be a simple direct pathway.[12] For example, it was shown that both SUMO deconjugation machinery[10] and sister chromatid cohesion establishment (Strunnikov A, unpublished) are required for condensin targeting to rDNA in mitosis. To expand our knowledge about the mechanism of condensin targeting to rDNA we undertook a screen for conditional mutants that impair targeting of condensin to rDNA in mitosis. This screen resulted in identification of a novel pathway, which is required for condensin targeting to rDNA, but probably does not regulate the enzymatic activity of condensin. We demonstrated that activity of Cdc14p in early anaphase is responsible for proper condensin binding to rDNA chromatin in mitosis.

MATERIALS AND METHODS

All yeast genetic techniques were according to standard protocols.[32,33] The genotypes of yeast strains are shown in Table 1. Yeast cell cycle arrest and release experiments were conducted as described.[7,34] The quality of arrest was monitored microscopically and by FACS (not shown). For Figures 1 and 3 arrests were 4 hr at 37°C in SD-uracil media; for Figures 4A, B and Figure 5, 2.5 hr in YPD; and for Figure 4C, 2 hr in YPD.

Microscopy was performed with the AxioVert (Zeiss) microscope with epifluorescence. The images were captured by integrated 0.2 μm-step Z-axis scanning with cooled CCD camera. The pLF640 plasmid and gene-targeting protocol used to construct the Smc4p-GFP fusion in vivo has been described.[2] The *SIK1:*mRFP*::kanMX* DNA fragment was amplified by PCR with *SIK1*-flanking primers from the *SIK1:*mRFP*::kanMX* strain DNA[25] and used to transform yeast strains described in Table 1. Replacement of *SIK1* with *SIK1:*mRFP*::kanMX* was confirmed by PCR analysis. Before microscopy cells were fixed with formaldehyde (5 min, 3.7%), washed with PBS and kept on ice.

Anti-condensin antibodies have been described.[2] Chromatin-binding assays (Fig. 4B) were performed according to reference [35] with slight modifications. Immunoprecipitations (Fig. 4A) were performed with the same buffer (EBX) supplemented with DNase I in capped 2-ml columns (BioRad). Cell lysates were incubated (4°C) with anti-HA antibodies (12CA5, Roche) and coupled to CNBr-Sepharose (Amersham). The antibody-bound proteins were eluted from the column-packed beads with 2% SDS (90°C). The Ycs5 protein tagged with 3xHA and 6xHis tags and the in-vivo-replacement tagging procedure has been described.[2] Two strains (6cAS453 and 3aAS453) were used to partially purify and concentrate the tagged Ycs5 protein by IMAC. After cells were released from alpha-factor arrest the cultures were split in half and immediately shifted to 37°C. To one of the halves nocodazole was added within minutes after release. After 2 hr at 37°C or at 37°C with nocodazole cells were harvested and immediately resuspended/disrupted (with glass beads) in a chaotropic extraction buffer (6 M Guanidine-HCl, 50 mM Tris pH8.0, 1xPBS, 0.35 M NaCl) to prevent loss of phosphorylation during extraction. Extracts were clarified by centrifugation and incubated with Ni-NTA-Superflow resin (Quiagen) for 12 hr at 4°C. The resin suspension was passed through a 2-ml disposable BioRad column to form an open column bed. After several washes (with the extraction buffer, with a similar buffer where Guanidine-HCl was replaced by 6 M urea and with the urea buffer supplemented with 20 mM imidazole) the bound proteins were collected in 100 μl of stripping buffer (2% SDS, 20 mM EDTA, 1 mM β-mercaptoethanol). Ycs5p-HA was visualized by Western blotting after separation by PAGE (100V, 2 hr) in the 3–8% gradient Tris-acetate gel (Invitrogen). These conditions allowed for resolution of phosphorylated and non-phosphorylated forms of Ycs5p-HA, which were not resolved by other PAGE conditions (4–12% Bis-Tris/MOPS gels, 200V, 45 min) used in this work (Fig. 4A and B).

ChIP assays for Figure 5A were performed using the following protocol. Cells from 50-ml cultures were harvested and cross-linked in 1% formaldehyde for 30 min at room temperature. After cross-linking, cells were washed twice in Tris-Buffered Saline (TBS, pH 7.4). Cells were suspended in 400 μl of Lysis Buffer (50 mM HEPES-OH pH 7.5, 0.75 M NaCl, 5 mM EDTA, 1% Triton X-100, 0.1% Na Deoxycholate) with Complete protease inhibitors (Roche). An equal amount of glass beads was added and the cells were lysed in a mini bead beater for 10 minutes at 4°C. The lysate was collected and sonicated to an average fragment size of less than 1 kb. The lysate was collected by centrifugation for 5 min in a microfuge at 4°C. For the Brn1p-HA ChIP experiment, the lysate was divided in two halves. The anti-HA antibody (Roche) (0.5 μg) was added to one half of the sample, while the other half was used as the “no antibody” control. The samples were then incubated on a mini-rotator overnight at 4°C. 15 μl of Protein G beads suspension (Pierce) was added to each sample and sample was incubated on a mini-

rotator for 3 hours at room temperature. After the beads settled down the buffer was removed. The beads were washed five times using 1 ml of lysis buffer (0.75 M NaCl) with protease inhibitors, and washed three times using the lysis buffer (0.5 M NaCl). The beads were then washed once using wash solution 3 (10 mM Tris-HCl pH 8.0, 0.25 M LiCl, 0.5% NP-40, 0.5% Na Deoxycholate, 5 mM EDTA) and TE (pH 8.0), respectively, and were resuspended in 20 μl of TE (pH 8.0). Samples were incubated at 65°C for 6 hours to reverse cross-links and placed at 95°C for 20 min. For input DNA, 100 μl TE with 1% SDS was added to 20 μl of lysate. After reversing cross-links at 65°C for 6 hours, 100 μg Proteinase K was added and the samples were incubated at 37°C for 2 hr. Samples were extracted once with phenol: chloroform:isoamyl-alcohol and once with chloroform. 200 μl ethanol, 10 μl Na acetate and 5 mg glycogen were added and sample was precipitated at −20°C overnight. Precipitated and washed DNA was dissolved in 100 μl of TE. After PCR analysis (1 cycle of 95°C for 5 min, followed by 25 cycles of 95°C for 1 min, 55°C for 30 sec, and 72°C for 1 min) samples were run on 1.5 % agarose gel at 125 V for 45 min. For duplex PCR reactions, *TUB2* (which does not bind condensin in vivo) PCR product was used as a negative control for ChIP and an internal control for input DNA. The linear ranges of PCR amplification were estimated by serial dilutions of immunoprecipitated DNA.

The quantitative ChIP protocol was essentially the same as described above. Quantitative PCR reactions were performed by the Real-time PCR System (Stratagene MX3000P). PCR reactions (50 μl) contained 1 μl template DNA (ChIP or Input), 25 μl 2x SYBR Green master mix (Stratagene), and 50 nM primers. PCR parameters were 1 cycle of 95°C for 10 min, followed by 35 cycles of 95°C for 1 min, 55°C for 30 sec, and 72°C for 1 min. Primer concentrations and PCR cycle parameters was optimized to eliminate formation of primer dimers. Primers for rDNA sequences were the same as used in reference [36]. Primers #1 and #33 were routinely excluded from the quantitative PCR set to allow analysis of all samples for a given strain in a single 96-well PCR plate. No notable condensin binding was found to these two sites in a separate experiment (not shown). PCR products were quantified using the MX3000P software (Stratagene) in the cycle corresponding to the linear range of amplification (cycles 32 and 33). The enrichment ratio was determined by calculating the ratio of rDNA-ChIP to rDNA-input (total chromatin) and normalizing the data to the “no antibody” control background. All the rDNA-ChIP to rDNA-input ratios were calculated as: $2^{\{Ct\ (Input) - Ct\ (ChIP)\}}$ divided with {dilution rate (ChIP)/dilution rate (Input)}. Ct values of all PCR products were determined by the MX3000P software.

RESULTS

Our original finding that the essential biological function of condensin is to segregate tandem repeat-containing sister chromatids[2] allowed us to develop several new approaches to study molecular mechanics and regulation of condensin in budding yeast. One of these approaches was to monitor the nucleolar accumulation of Smc4p-GFP in mitosis as an in-vivo assay for chromosome condensation.[10] We undertook a systematic screening of conditional mutants that arrested in mitosis to identify the candidate genes encoding potential condensin regulators and/or rDNA-targeting factors. This collection also included some mutants defective in mitotic rDNA segregation (e.g. *cdc14–1*). We replaced the *SMC4* gene with a fully functional *SMC4*-GFP fusion in more than forty conditional mutants and monitored localization of Smc4p-GFP after a three-hour conditional shift (37°C in most cases). In some cases we used synchronous release from a G_1 arrest into non-permissive conditions in the presence of nocodazole (e.g. for *esp1–1*). After completion of the initial screening we focused our attention on the *cdc14–1* mutant, as it showed the most striking defect in condensin localization to nucleolus in mitosis (Fig. 1A), especially as compared to the *cdc15-1* mutant, which arrests at a similar point in late anaphase (Fig. 1B). The *cdc14* mutant is known to have decondensed rDNA, while in *cdc15* rDNA is properly condensed.[13]

We first tested whether the enzymatic activity of Cdc14p was required for proper condensin localization to nucleolar chromatin. It is an important consideration, as Cdc14p, similarly to condensin, is present in the nucleolus, but in the enzymatically inactive state (RENT complex).[14,15] Moreover, condensin mis-localization in the *cdc14-1* mutant could not be reversed by returning the cells to the permissive temperature (data not shown). This potentially indicates that either the Cdc14p activity per se is not required for condensin concentration at the rDNA locus, or that condensin relocalization from rDNA to other sites is itself irreversible (at least in late anaphase). To address this question we ectopically expressed the mutant *cdc14*–Δ(1–374) allele which encoded protein defective in phosphatase activity[16] in the *cdc14–1* cells. This phosphatase-deficient Cdc14p did not rescue the *cdc14–1* defects in condensin localization (data not shown). Thus, mutations in *CDC14* specifically impair the condensin-to-rDNA-targeting pathway, revealing a new role for this multifunctional phosphatase.

Two other mutants analyzed in the original screen showed similarly de-localized condensin: *esp1-1* and *cdc5-1* both displayed diffuse nuclear Smc4p-GFP signals. Interestingly, both the *ESP1* and *CDC5* genes are linked with *CDC14* in two mitotic pathways. *ESP1* and *CDC5* genes are components of FEAR network,[17–19] while *CDC5* is also required for MEN pathway.[20,21] As both the FEAR and MEN pathways involve Cdc5p-controlled release of Cdc14p activity from a sequestered (nucleolar) form, we assumed that the Cdc14 protein was the main effector controlling condensin targeting. It seemed unlikely, however, that the MEN pathway would mediate condensin targeting, as condensin concentrates in the rDNA locus between metaphase and anaphase, long before exit from mitosis. Indeed analysis of Smc4p-GFP localization in the *cdc15-1* (included in the original screening), as well as in the *tem1*-Δ and *net1*-Δ MEN mutants demonstrated that condensin was properly concentrated in the nucleolus (Fig. 1B).

The FEAR network, on the other hand, facilitates the earliest known release of Cdc14p activity in the cell cycle and thus could be responsible for the Cdc14p-controlled step in condensin targeting. We tested whether components of FEAR network were involved in condensin targeting. With the *esp1–1* mutant it was difficult to assess the dynamics of condensin targeting, as *esp1–1* cells arrest in mitosis only for a short period of time and then proceed to exit mitosis with unsegregated sister chromatids.[22,23] Thus, we focused our attention primarily on the *slk19*–Δ mutant. The *slk19*–Δ cells had a notable delay in mitosis and allowed monitoring of condensin targeting with higher precision. Microscopic analysis of asynchronous *slk19*–Δ population revealed that high proportion of cells were delayed in late anaphase with properly targeted condensin, however no cells had singular nucleolus-concentrated condensin in large-budded cells (not shown), morphology characteristic for metaphase-to-anaphase transition (Fig. 4E in ref. 2). This observation was consistent with the hypothesis that failure to release the Cdc14 protein early in mitosis results in improper condensin localization, however subsequent release of Cdc14 by MEN allows eventual proper targeting of condensin and successful segregation of rDNA chromatids. In order to verify this hypothesis we monitored condensin localization to nucleolus following synchronous release from a G_1 arrest. We followed the budding pattern and bud size in conjunction with the position and concentration of the Smc4p-GFP signal (Fig. 2). The wild-type ($Slk19^+$) cells proceeded synchronously though the cell cycle (Fig. 2A) and demonstrated nucleolar concentration of Smc4p-GFP at the metaphase-to-anaphase transition and during anaphase, as expected. The *slk19*–Δ cells proceeded through anaphase with a slight delay, but without displaying cells with a singular concentrated condensin signal. Instead, the *slk19*–Δ cells had a new class of cells, virtually absent in the wild-type population. These *slk19*–Δ cells were apparently undergoing anaphase with condensin diffusely distributed in the nucleus (red, Fig. 2A; arrow 1, Fig 2B), instead of condensin being concentrated in the nucleolus. However, this cell class was diminished by the end of the time-course, giving way to cells with two separate concentrated Smc4p-GFP signals (dark blue, Fig. 2A; arrow 2, Fig 2B). Thus *slk19*–Δ and FEAR network are required for the

early anaphase condensin concentration at the nucleolus, but are dispensable in the mid-to-late anaphase, the time when Cdc14p is released by the MEN network.

The putative mechanism of condensin de-localization in *cdc14p* mutants could be either dislodging of condensin from its proper location in rDNA chromatin or diffusion of nucleolar chromatin itself with condensin, presumably inactive, but still bound. The latter seemed less likely as the nucleolus was reported to be relatively compact, albeit enlarged, in the arrested *cdc14* mutant cells.[24] Nevertheless, we monitored the position of nucleolus with an alternative fluorescent marker to exclude the possibility that rDNA chromatin was stretched out in the *cdc14–1* cells to give the appearance of improperly targeted condensin. We utilized the recently described Sik1p-mRFP fusion[25] as a constitutive nucleolar marker.

We investigated whether Smc4p-GFP was properly colocalized with Sik1p-mRFP to rDNA during anaphase arrest mediated by the *cdc14-1* mutation. The *cdc14–1* and *cdc15–1* mutants expressing Smc4p-GFP were shifted to 37°C and condensin localization was compared to Sik1p localization (Fig. 3). In the arrested *cdc15-1* cells, used as a control, the Smc4p-GFP fusion was fully colocalized with Sik1p-mRFP at two polar sites in mother and daughter cells, respectively (Fig. 3A and B). In the majority of arrested *cdc14-1* cells, the nucleolar Sik1p-RFP signal was found undivided, in the mid-zone of anaphase cells (Fig. 3C and D). This localization was consistent with the reported failure of nucleolar segregation in *cdc14* mutants[24] and was reminiscent of condensin-mutant induced rDNA non-segregation.[2] However, the Smc4p-GFP fusion did not colocalize with Sik1p-RFP (Fig. 3C and D). These results strongly suggest that condensin is not enriched in the rDNA at the *cdc14-1*-mediated anaphase arrest. Moreover, in some cells, at higher magnification condensin appeared to be excluded, at least partially, from the nucleolar area (Fig. 3E).

Thus, while it is evident that condensin is more uniformly distributed throughout the nucleus in the *cdc14-1*-arrested cells, the mechanism of condensin depletion/exclusion from the nucleolus is not clear. It could involve disassembly of the condensin complex, release of condensin from chromatin, relocalization of condensin to alternative chromosomal sites or inactivation through inadequate posttranslational modification.

In order to assess the degree and mechanism of condensin's inability to target rDNA in the *cdc14-1* cells, we first compared the integrity and chromatin association of condensin in the *cdc14* and *cdc15* mutants. Condensin immunoprecipitation via the HA-tagged Brn1p demonstrated that condensin was fully assembled in both mutants at 37°C and that there were no significant variations in the relative subunit amounts (Fig 4A). Moreover, the condensin complex was also fully associated with chromatin in both mutants at the restrictive temperature, suggesting that condensin was redistributed to other genomic sites in the arrested *cdc14* cells (Fig 4B). Finally we tested whether condensin phosphorylation was perturbed in the arrested *cdc14* cells. Indeed, targeting of condensin to rDNA may be coupled to its activation via post-translational modification, as it is the case in *X. laevis*.[26] Recently an important role of Ycs5p phosphorylation for condensin activity in yeast mitosis has been substantiated.[12] The anaphase-specific increase in the phosphorylation level of Ycs5p was attributed to activity of Ipl1p kinase.[12] Cdc14p, in its turn, controls some aspects of Ipl1p localization.[27] To test whether this post-translational modification of condensin is dependent on Cdc14p we assessed phosphorylation of Ycs5p in the *cdc14*-arrested cells. Ycs5p-6xHis-3HA was purified for following strains and conditions: asynchronous wild-type at 37°C, *cdc14* arrested at 37°C, wild-type arrested at 37°C with nocodazole and, finally, cdc14 released from G_1 into 37°C in the presence of nocodazole (Fig. 4C). Nocodazole treatment resulted in reduction of unphosphorylated Ycs5p in wild-type cells but had little effect on the Ycs5p-6xHis protein purified from *cdc14* cells, as Ycs5p was likely fully phosphorylated both at the late-anaphase

cdc14-specific arrest and at the nocodazole-mediated arrest. Thus, condensin appears to be functional, albeit mis-targeted, in the arrested *cdc14* cells.

As the amount of condensin in the cell appeared to be equivalent in the *cdc14* and *cdc15* mutants we applied the chromatin immunoprecipitation approach to determine the relative amounts of condensin bound to rDNA chromatin. First we assessed the occupancy of the RFB region in the rDNA (Fig. 5B), which was shown to be a hot-spot for condensin binding.[2,10] Comparison of the RFB occupancy by Brn1p-HA in the *cdc14* and *cdc15* mutants at the non-permissive temperature indicated that virtually no condensin was bound to RFB in the *cdc14* mutant (Fig. 5A). To verify this with a more rigorous method we combined Brn1p-HA chromatin immunoprecipitation with quantitative real-time PCR analysis. This approach allowed us to delineate both qualitative and quantitative changes in condensin binding across the rDNA repeat in the arrested *cdc14* cells. As Figure 5C shows, the *cdc14* rDNA has only two condensin-binding peaks, out of six observed in the *cdc15* cells. These results conclusively confirmed that condensin complex was mis-localized in the *cdc14*-arrested cells. The nearly complete loss of condensin from rDNA could explain the rDNA de-condensation (see ref. [13]) and rDNA non-segregation (Fig. 3 and ref. [24]) phenotype of the *cdc14* mutants.

DISCUSSION

While the composition, in vitro enzymatic activity and general molecular architecture of condensin have been elucidated to a great degree,[1] the molecular mechanism of the chromatin condensation reaction in vivo remains a mystery. Solving the two following problems is necessary to understand the molecular nature of condensin activity in vivo. First, as condensin undergoes cell cycle dependent changes in localization and activity levels, its function should be regulated in a cell cycle-dependent fashion. However, data from different systems suggest that either condensin regulation is not conserved in evolution or all regulatory mechanisms have not yet been found. Second, the existing data on condensin supercoiling activity with naked DNA[28] cannot be extrapolated to accommodate a chromatin fiber as a substrate. Thus, while accepting the paradigm that the basic activity of condensin is to change the superhelical state of chromatin, we have yet to identify a natural substrate for condensin activity in vivo and to understand the mechanism of affinity between condensin and chromatin in order to elucidate the molecular mechanism of chromosome condensation.

As condensin is not a sequence-specific DNA-binding complex, there are two possible alternative scenarios for specific recognition of chromatin motifs by condensin: either condensin recognizes a specific chromatin structure (such as repeats, positioned nucleosomes, histone modifications) or some auxiliary proteins (facilitators) attract condensin and, possibly, anchor it at a specific place. In either case, loss of some of the factors involved in determining the specificity of condensin binding should recapitulate the condensin mutant phenotype. Conversely, mutations in proteins that directly regulate condensin activity, e.g., through post-translational modification, should also manifest a condensin mutant-like phenotype.

Based on these assumptions combined with our previous findings that the rDNA repeats is the primary binding target for condensin in vivo[2] and that condensin undergoes relocalization to the rDNA chromatin at the metaphase-to-anaphase transition we designed some specific tests for condensin activity and chromatin-binding in *S. cerevisiae*.[2,10] Using these genetic and cell biology assays (condensin targeting to the nucleolus and mis-segregation of the rDNA-containing chromosomes) we conducted screening of candidate mutants for defects in chromosome condensation regulation and/or condensin targeting to chromatin. This approach previously resulted in identification of at least two pathways essential for condensin targeting to rDNA in mitosis: the sister chromatid cohesion (SCC) pathway (Strunnikov A, unpublished) and the SUMO/Smt3p-deconjugation pathway.[10]

Here, using the Smc4p-GFP rDNA-targeting assay we identified the third pathway essential for condensin targeting to rDNA. We demonstrated (Figs. 1, 2 and 3) that the Cdc14p activity specifically released by the FEAR,[15] but not the MEN[29] network, is required for proper condensin targeting to nucleolar chromatin in mitosis (Fig. 6). This finding allowed us for the first time to determine the specific point at which condensin is concentrated in the nucleolus in the normal cell cycle. While previously we concluded that this concentration is an attribute of both metaphase and anaphase,[2] the role of the FEAR/Cdc14p pathway in this process indicates that maximal condensin accumulation in the nucleolus probably happens in early anaphase, when FEAR is activated. We also found that late-anaphase release of Cdc14p (by MEN) can compensate the condensin-targeting defect when the FEAR pathway is inactivated, thus allowing mutants in FEAR genes to survive (Fig. 2).

The exact protein target of the Cdc14p phosphatase activity that mediates the early-anaphase condensin concentration in the nucleolus/rDNA chromatin is not yet known. We prefer the hypothesis that some nucleolar protein controlled by Cdc14p is required for condensin targeting to rDNA. The alternative explanation, that Cdc14p directly acts on condensin seems unlikely, as we show (Fig. 4) that condensin is fully assembled, properly phosphorylated and apparently chromatin-bound in the *cdc14* mutant. Moreover, as the non-rDNA chromosomes segregate properly in the *cdc14* mutant, with condensin still bound to chromatin (Fig. 4B), it seems quite probable that condensin is still active but mis-targeted in the *cdc14* cells at the non-permissive temperature. Our analysis of the double *cdc14-1* and *smc2-8* mutant alleles shows that such cells are viable at 23°C, but display blocked segregation of all chromosomes, not just the rDNA-containing, at the non-permissive temperature (data not shown). This suggests that the mutant condensin, which is re-localized due to the *cdc14* mutation, impairs segregation of the non-rDNA sister chromatids in this double mutant. Such a feature, i.e. the existence of active but mis-targeted condensin, makes the mutants in the *CDC14* pathway unique, in comparison to the *smt4* and SCC mutants (also defective in condensin targeting), as in these mutants segregation of all chromosomes is impaired. Identification of the non-rDNA condensin-binding sites and analysis of their occupancy by condensin at the *cdc14* arrest would lend conclusive proof to the theory that Cdc14p is involved in nucleolar targeting but not in activation of the condensin complex per se. Moreover, characterization of such a unique enzymatic pathway, which is responsible for specific targeting of condensin to a well-characterized chromatin domain, should allow comprehensive investigation of the mechanisms attracting condensin to rDNA in mitosis.

As a result of our work, we come very close to understanding the mechanism that prevents nucleolar segregation in the *cdc14* mutants.[24,30,31] Using a highly sensitive and accurate quantitative ChIP approach we demonstrated that condensin is largely absent from the rDNA repeats at the *cdc14*-mediated arrest (Fig. 5). This inevitably leads to inability of sister chromatids (in the rDNA region) to segregate (Fig. 6), i.e. recapitulation of the classic condensin-mutant phenotype.[2] Loss of condensin from rDNA is not, however, a feature of late anaphase, as in the *cdc15* mutant condensin was properly bound to the rDNA repeats, as was determined by microscopic analysis (Figs. 1 and 3) and by quantitative ChIP (Fig. 5). Thus, the *cdc14* mutant's failure to segregate sister rDNA regions and the similar phenotype of condensin mutants[2] are both mediated by the inability of condensin to perform its function. However the mechanisms in the two cases are significantly distinct: in case of *cdc14* condensin does not bind the rDNA repeats, while in several tested condensin mutants the complex still occupies the rDNA sites (data not shown), yet presumably is inactive.

Figure 6 recapitulates our current view of condensin regulation by the FEAR/Cdc14 pathway. We propose that the main regulatory role of FEAR in condensin targeting to rDNA in early anaphase is to liberate the sequestered Cdc14p activity. Cdc14p, however, is the key player in this pathway, as we demonstrated that the enzymatically inactive Cdc14p cannot complement

the condensin mis-targeting phenotype. Moreover, we suggest that the late-anaphase release of Cdc14p (by MEN), that still occurs in the FEAR mutant *slk19*, is able to correct the absence of Cdc14p in early anaphase by facilitating condensin targeting to rDNA later in anaphase. The *cdc14* mutant, however, has a permanent defect in condensin targeting to rDNA and in condensin-dependent segregation of the rDNA sister chromatids. Irreversibility of the *cdc14* rDNA non-segregation and condensin mis-targeting phenotypes indicates that condensin cannot return to the rDNA chromatin after a certain cell cycle point. While the molecular nature of this turning point is unknown, it seems likely that condensin becomes immobile at the end of anaphase, possibly due to yet uncharacterized decondensation pathway.

Relationship of the Cdc14 pathway to two other pathways controlling condensin targeting, the SCC and SUMO-deconjugation pathway remains to be established. The recent finding that the SCC function controls the pre-anaphase step in rDNA compaction[12] may indicate that this pre-anaphase condensation is a prerequisite for condensin targeting at the onset of anaphase. However, as the observed FEAR-mediated condensin targeting is an anaphase event, it seems unlikely that cohesion plays a significant role at that point of the cell cycle. The Smt4p-controlled pathway[10] for condensin targeting could potentially overlap with the Cdc14p-mediated pathway described here, as no SUMO/Smt3p modifications essential for mitosis have been identified yet. However, neither condensin nor Cdc14p itself are hyper-sumoated in the *smt4*-Δ mutant (data not shown), indicating that over-sumoation of these proteins is not responsible for condensin mis-targeting observed in the *smt4*-Δ nuclei.[10]

Acknowledgements

We would like to thank numerous colleagues who supplied the strains and gave advice for the original Smc4p-GFP screening; Ray Deshaies, who, before FEAR was discovered, has brought to our attention that some process other than the MEN should control condensin targeting via Cdc14p; L. Aragon, L. Freeman, N. Dhillon and R. Kamakaka for helpful discussions; M. Lichten, for advice on large-scale ChIP; Kei Ko, for technical help.

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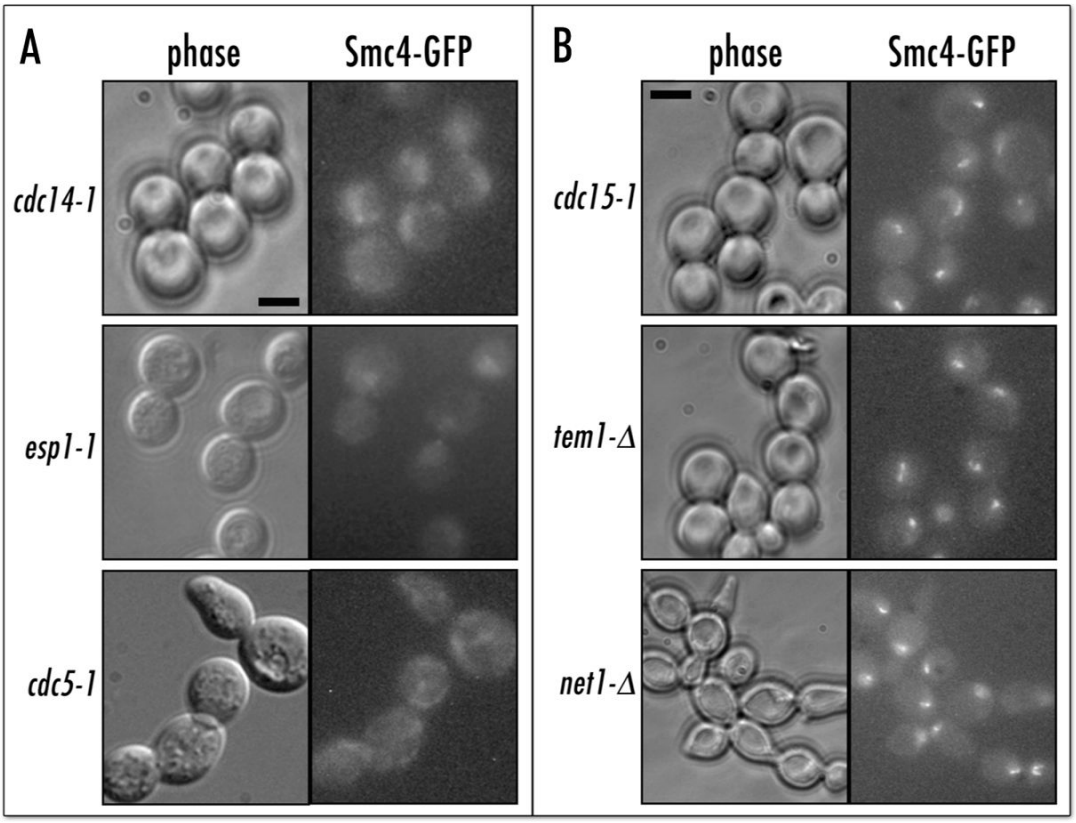

Figure 1.
Condensin is delocalized in FEAR but not MEN mutants. (A) Smc4p-GFP signal is delocalized in FEAR mutants at 37°C. The *esp1–1* strain (Table 1) was kept in mitosis with nocodazole. Scale bar 5 μm. (B) MEN mutants properly localize condensin to rDNA. Arrest conditions: *cdc15-1* (37°C), *tem1*-Δ(3-hr shut-off of pGAL:*NET1* at 23°C), *net1*-Δ (mitotic delay at 23° C). Strain genotypes in Table 1.

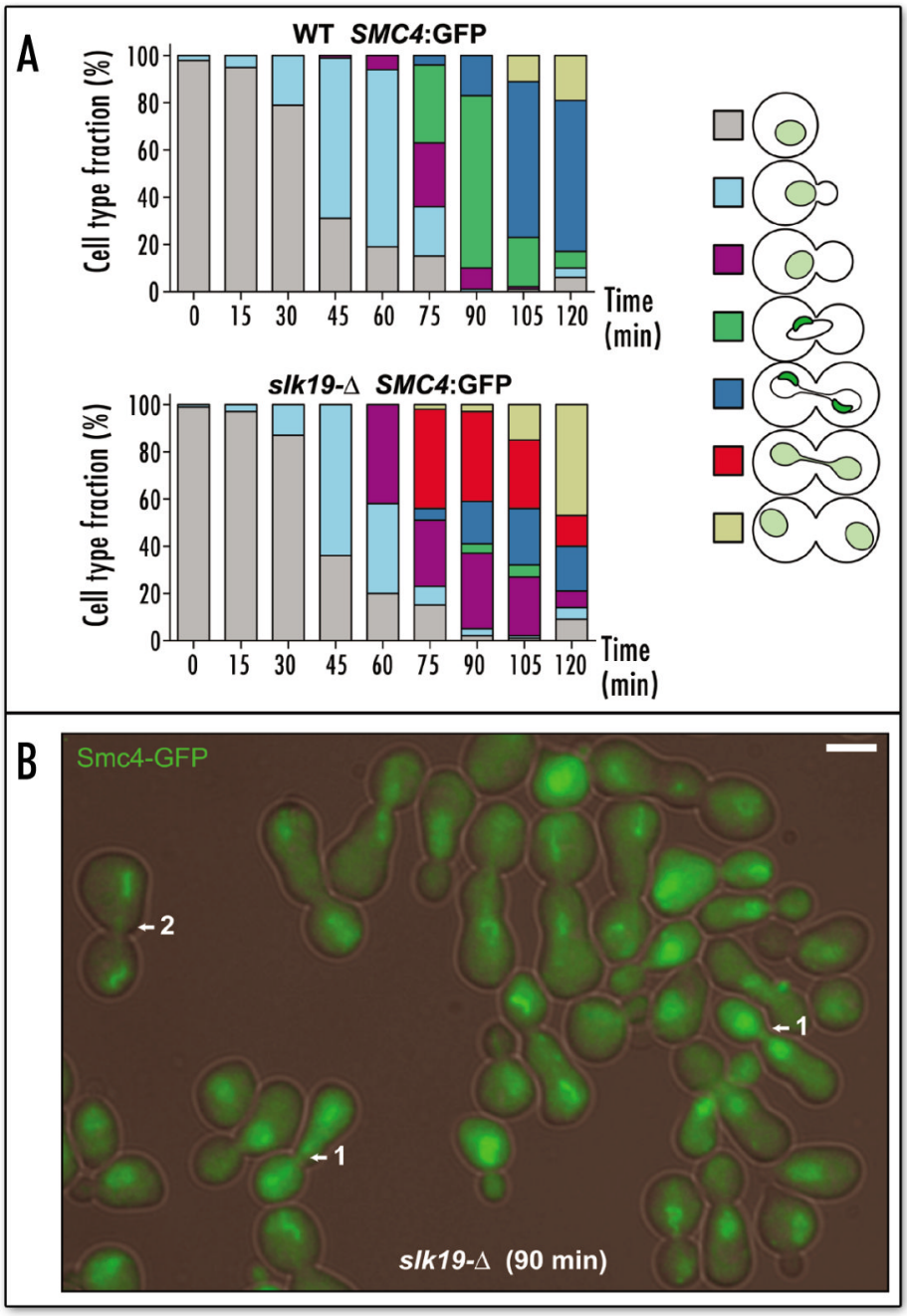

Figure 2.

FEAR mutant *slk19* is defective in anaphase concentration of condensin. (A) Condensin-to-nucleolus concentration in the synchronous cell cycle. Parallel analysis of 640-BY4741 (WT) and 640-4002451-BY4741 (*slk19*-Δ) strains released from alpha-factor arrest allowed comparative monitoring of Smc4p-GFP dynamics in the nucleus. Diffuse condensin, light green nucleus; nucleolus-targeted condensin, crescent-shaped dark-green subnuclear structure. (B) Micrograph of the 90-min point in the time-course analysis of the *slk19*-Δ strain. Arrow 1, unique class of cells undergoing anaphase with mistargeted condensin; Arrow 2, mid-to-late anaphase recapture of condensin by nucleolus. Scale bar 5 μm.

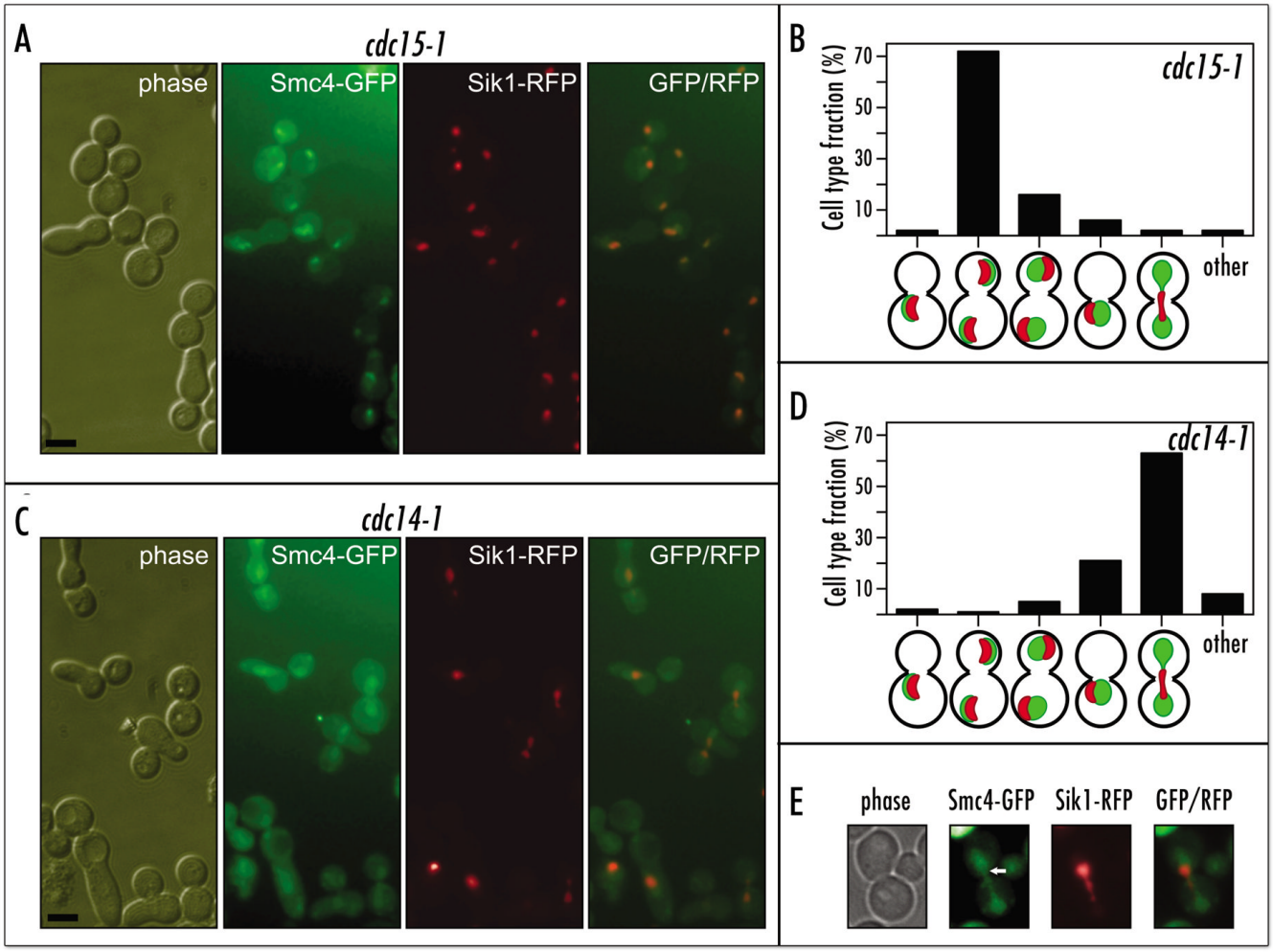

Figure 3.

Diffuse condensin distribution in the *cdc14* mutant correlates with a nucleolus segregation defect. (A) Micrograph and (B) quantification of relative distribution of Smc4p-GFP and Sik1p-mRFP in the 640-3AS426/*SIK1:mRFP::kanMX* cells after a temperature shift. Scale bar 5 μm. (C) Micrograph and (D) quantification of relative distribution of Smc4p-GFP and Sik1p-mRFP in the 640-1AS432/*SIK1:mRFP::kanMX* cells after a temperature shift. Scale bar 5 μm. (E) Same as in (C), with higher magnification.

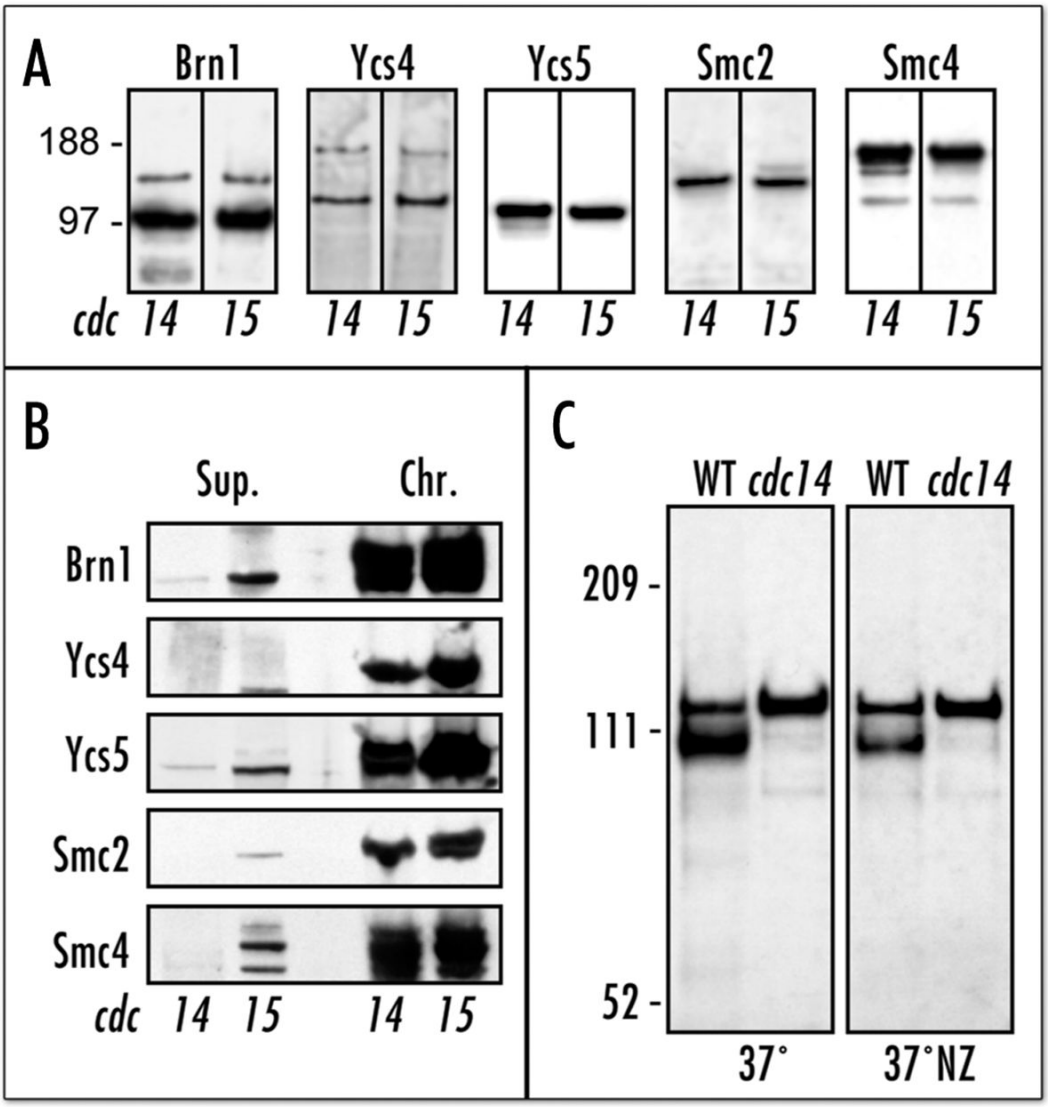

Figure 4.

Mistargeted condensin is functional. (A) Western-blot of Brn1p-HA-precipitated condensin complex, showing comparison of condensin composition in *cdc14* and *cdc15* mutants at non-permissive temperature. Strains: Table 1. (B) Western-blot comparing chromatin-bound and soluble condensin fractions[35] in *cdc14* and *cdc15* mutants at non-permissive temperature. Supernatant fractions (Sup.) are equivalent to 1/5 of the chromatin (Chr.) fractions. The strains are identical to (A). (C) Western-blot (anti-HA) analysis of Ycs5p-6xHis-3HA IMAC eluates from 3aAS453 (*cdc14–1*) and 6cAS453 (WT) incubated at two different conditions (See Materials and Methods). The upper Ycs5p band is a phosphorylated from. Mock purification (no 6xHis tag) produces no anti-HA-reactive bands at these stringent conditions (not shown).

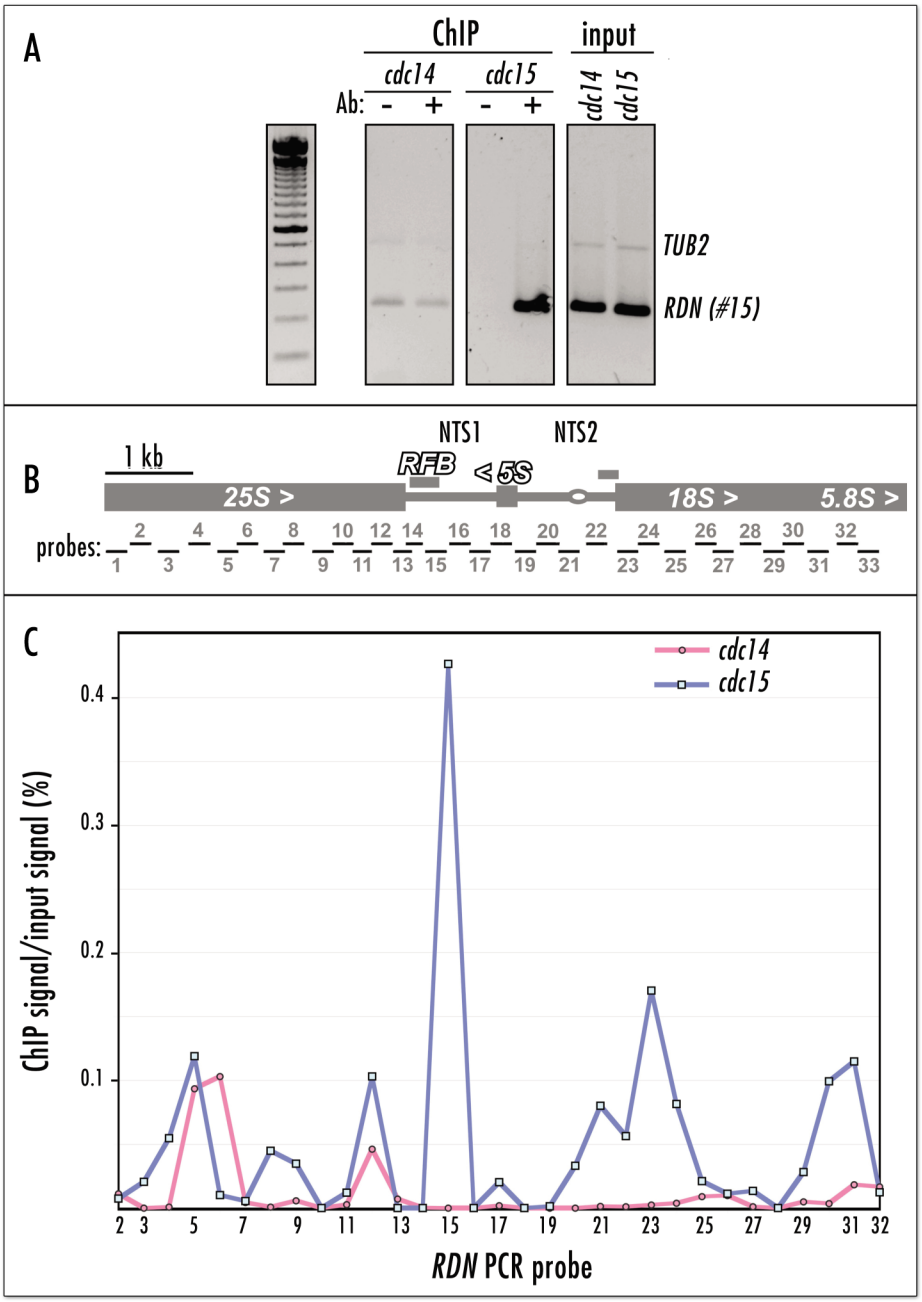

Figure 5.

Condensin is physically de-localized from rDNA in the *cdc14* cells. (A) Chromatin IP analysis of *cdc14-1 BRN1*:HA and *cdc15-1 BRN1*:HA strains arrested at 37°C for 2.5 hr. The RFB region, PCR probe #15 in (B), a known hot-spot of condensin localization to the rDNA repeat, [2,10] is used to assess the relative enrichment for condensin binding (Brn1p-HA ChIP). The *TUB2* gene probe, which contains no binding sites for condensin (unpublished), is a negative control for duplex PCR. (B) Positions of PCR probes used for quantitative PCR analysis of ChIP in (C). PCR primer sequences were exactly as in reference [36]. (C) Quantitative PCR analysis of Brn1p-HA ChIP from *cdc14-1* and *cdc15-1* strains arrested at 37°C for 2.5 hr. The

quantitative PCR values from cycle 32 (see Materials and Methods) are plotted as ratios of precipitated DNA to total DNA (in %).

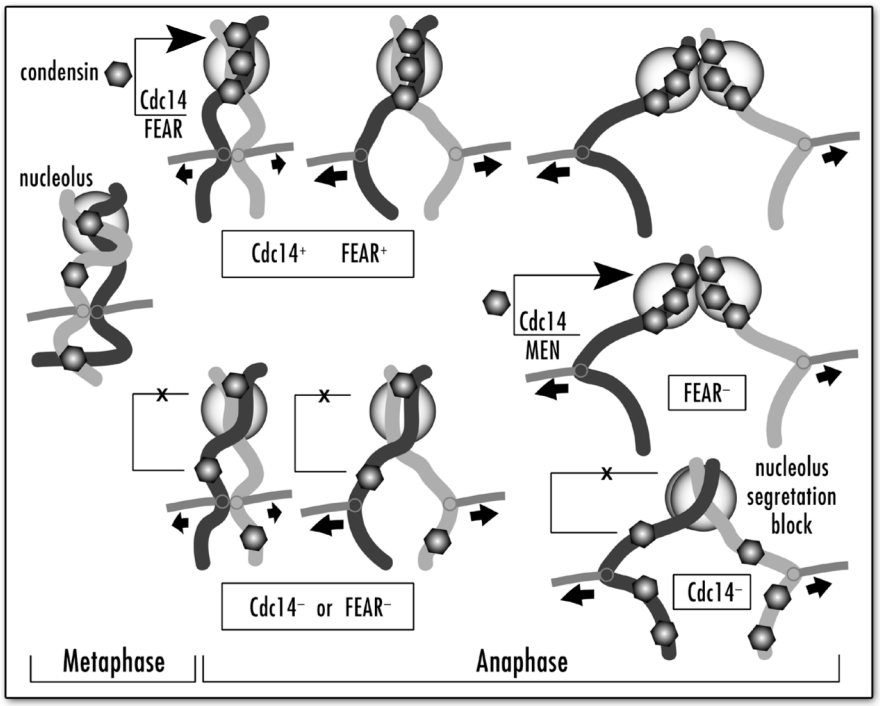

Figure 6.

Interplay between Cdc14p, FEAR and MEN pathways in condensin localization to nucleolus in anaphase. Apparent chromosomal condensin distribution and corresponding success of rDNA/nucleolus segregation are shown for normal cell cycle, FEAR-deficient and Cdc14p deficient cells. Also shown is a supplementary role of MEN in condensin targeting when FEAR is inactivated.

Table 1

Yeast Strains

Strain name	Relevant genotype	Original source
3cAS451	*MATa ade2 leu2 lys2 trp1 ura3 cdc14–1 pep4-Δ::HIS3 BRN1:6His:3HA::URA3*	this work
1cAS452	*MATa leu2 trp1 ura3 cdc15–1 pep4–Δ::HIS3 BRN1:6His:3HA::URA3*	this work
3aAS453	*MATa ade2 leu2 trp1 ura3 cdc14–1 YCS5:6His:3HA::URA3*	this work
6cAS453	*MATa leu2 lys2 ura3 YCS5:6His:3HA::URA3*	this work
640-1AS432	*MATα ade2 his3 leu2 trp1 ura3 cdc14–1 SMC4:GFP::URA3*	this work
640-3AS426	*MATα his3 leu2 ura3 cdc15–1 SMC4:GFP::URA3*	this work
640-8AS491	*MATa ade2 his3 trp1 lys2 leu2 met15 ura3 esp1-1 SMC4:GFP::URA3*	this work
640-K291-9C	*MATa his3 ura3 cdc5–1 SMC4:GFP12::URA3*	L. H. Johnston
640-WY46	*MATa ade2 his3 leu2 trp1 ura3 tem1–Δ::pGAL:UPL-NET1::TRP1 bar1 SMC4:GFP12::URA3*	W. Shou
640-WY69	*MATa ade2 his3 leu2 trp1 ura3 bar1 net1–Δ::his5sp SMC4:GFP12::URA3*	W. Shou
640-BY4741	*MATa his3 leu2 met15 ura3 SMC4:GFP::URA3*	ATCC
640-4002451-BY4741	*MATa his3 leu2 met15 ura3 slk19–Δ::kanMX SMC4:GFP::URA3*	ATCC