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Elucidating the crosstalk between condensin subunits and its relevance in chromosome condensation

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Cell Cycle Regulation and Chromosome Structure Unit

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Résumé

Au cours de la division cellulaire, l'ADN subit une série de transformations structurelles complexe qui entraîne sa compaction en chromosomes mitotiques par un processus appelé la condensation des chromosomes. Le complexe chromosomique condensine est un acteur majeur de la condensation. Ce complexe protéique, pentamérique, est formé de deux sous-unités catalytiques SMC (Structural Maintenance of Chromosome) et de trois sous-unités de régulation, hautement conservés de la levure jusqu'à l'homme. Chez la levure *Saccharomyces cerevisiae*, il est composé des sous-unités Smc2 et Smc4, et de trois protéines non-SMC appelées Brn1, Ycs4 et Ycg1. Malgré son importance, le mécanisme d'action de condensine reste largement inconnu. Le but de ces travaux est de chercher à mieux comprendre le mécanisme d'action de condensine et comment les interactions entres les différentes protéines composant le complexe peuvent réguler sa fonction. Au cours du cycle cellulaire le locus ADNr (ADN ribosomique- la sequence d'ADN qui code pour ribosomes) subit une restructuration dépendante de condensine. Cette thèse a permis de mettre en évidence quatre types de morphologies au locus ADNr lors des différentes phases du cycle cellulaire. Par ailleurs, afin de déterminer le rôle de l'interaction entre les sous-unités catalytiques et régulatrices de condensine dans la régulation du complexe, nous avons identifié et muté six résidus positifs sur l'extrémité C-terminale de Brn1. Ce mutant présente des défauts dans la formation du complexe condensine ainsi que des défauts de condensation. De plus ce mutant est co-létale avec des mutants affectant la tubuline ce qui suggère que ces résidus ont un rôle important dans la régulation de condensine. Ensemble, nos résultats suggèrent un modèle de régulation de condensine par l'interaction entre les sous-unités de condensine.

Mots-clés: complexe condensine, condensation des chromosomes, cycle cellulaire, régulation de condensine ADNr, BRN1, SMC4

Abstract

DNA undergoes a series of complex structural transformations during cell division, resulting in its compaction into intact mitotic chromosomes called chromosome condensation. The pentameric condensin complex has been strongly implicated as a major effector of this phenomenon. It is a multi-subunit protein complex with two catalytic "Structural maintenance of chromosome" (SMC) subunits and three regulatory subunits, highly conserved from yeast to humans. The condensin complex in *Saccharomyces cerevisiae* is made up of two SMC subunits (Smc2 and Smc4) and three regulatory non-SMC proteins (Brn1, Ycs4, Ycg1). Despite its importance, the mechanism of action of condensin remains largely unknown. The purpose of this work is to try to better understand the mechanism of action of condensin and how interactions between different proteins composing the complex can regulate its function. During the cell cycle the rDNA locus (ribosomal DNA- DNA sequence that codes for ribosomes) undergoes condensin-dependent restructuring. This thesis has highlighted four types of morphologies of the rDNA locus during different phases of the cell cycle. In order to determine the role of the interaction between the catalytic and regulatory subunits of condensin in the regulation of the condensin complex, we have identified and mutated six positive residues on the C-terminus of Brn1 which affect complex formation, condensation activity. This mutant has defects in forming the condensin complex defects and condensation. Furthermore, this mutant is co-lethal with tubulin mutants suggesting that these residues play an important role in regulating condensin. Together, our results suggest a model for regulation of the condensin complex by the interplay between its subunits.

Key words: condensin complex, chromosome condensation, cell cycle, condensin regulation, rDNA, BRN1, SMC4

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List of Abbreviations

Co-IP	Co- Immuno Precipitation
C-terminal	Carboxy Terminal
FACS	Fluorescence Activated Cell Sorter
FISH	Fluorescence *In Situ* Hybridization
FITC	Fluorescien Iso ThioCyanate
NLS	Nuclear Localization Signal
N-terminal	Amino terminal
PAGE	PolyAcrylamide Gel Electrophoresis
PI	Propidium Iodide
rDNA	ribosomal Deoxyribo Nucleic Acid
S.cerevisiae	*Saccharomyces cerevisiae*
SDS	Sodium Dodecyl Sulphate
SMC	Structural Maintenance of Chromosomes
TCA	TriChloro Acetic Acid
YEPD	Yeast Extract, Peptone, Dextrose

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INTRODUCTION

Cell Cycle

The cell divides into two equal daughter cells in a timed series of events including the duplication of the genome. The cell cycle is divided into interphase and mitosis (Flemming, 1882). Interphase is the period of preparation during which the cell readies itself for division. It consists of G1 phase, where the cell synthesizes all the nutrients, enzymes and proteins required for replication and S phase (Howard and Pelc, 1951) where the DNA replicates and all the chromosomes are duplicated. During mitosis, the cell growth stops and the cellular energy is invested in separating the duplicated chromosomes into two equal halves.

The mitotic spindle is the subcellular structure that is instrumental in the segregation of chromosomes during mitosis (Jordan et al, 2000). Microtubules attach to the chromosomes via the kinetochore. Microtubules are repeating units of motor proteins α and β-tubulins. Dimers of α and β-tubulins polymerize in a GTP dependent manner with α- tubulin at the plus end, β-tubulin at the minus end of the microtubule which possesses GTPase activity (Downing and Nogales, 1998). Once all the chromosomes are attached to microtubules from opposite poles (bi-orientation), depolymerisation of the microtubules generates tension which subsequently results in chromosome separation (Zhou et al, 2002).

Chromosome Condensation

With the entry of cells into mitosis, interphase chromatin fibres undergo a series of complex structural transformations resulting in compaction into intact mitotic chromosomes. This is a key step in the cell cycle and essential to the maintenance of genomic integrity during cell division (Boveri, 1888). This process is tightly controlled during the cell cycle to allow effective segregation of chromosomes in late mitosis. Chromosome condensation is not a simple process of transforming a linear DNA molecule into rod-like chromosome. It is important in the organization of the chromatin to resolve tangles between sister chromatids, prior to anaphase (Koshland and

Strunnikov, 1996). It is vital for segregation of the sister chromatids to ensure equal division of genetic content into the two daughter cells.

Hierarchal Levels of Folding

The DNA double helix self-organizes hierarchically by protein interaction, establishing higher order structural features in chromatin. In eukaryotes, DNA is arranged in the cell nucleus with the help of packaging proteins called histones (Van Holde, 1989). These proteins provide structural strength to the DNA. The basic level of DNA compaction is the nucleosome (Kornberg, 1974), where the DNA double helix is wrapped around the histone octamer containing two copies each of histones H2A, H2B, H3 and H4. DNA between nucleosomes is bound by linker histone H1 which facilitates packaging of the 10 nm "beads on the string" nucleosomal chain into a more condensed 30 nm fiber (Rippe et al., 2008). During cell division, chromatin compaction increases even more to form the classical chromosome, which can cope with large mechanical forces dragging them into each of the two daughter cells (Van Holde, 1989). The condensation of chromatin into thread-like chromosomes gives mitosis its name (in Greek mitos refers to thread). It is one of the most striking morphological events of the cell cycle. Since the observation of mitotic chromosomes by Flemming (Flemming, 1882), the mechanistic explanation for the process of chromosome condensation remains elusive.

Evidence supporting a mitosis-specific condensing agent was brought out by classic experiments of cell fusion in the 1970s, where it was shown that fusion of interphase HeLa cell and mitotic cells resulted in prematurely condensed chromosomes (Johnson and Rao, 1979). In the *Xenopus* cell-free system, interphase nuclei incubated with mitotic extracts resulted in assembled metaphase chromosomes (Lohka and Maller, 1985; Newport and Spann, 1987). Subsequent studies in budding and fission yeasts (Strunnikov et al, 1995; Saka et al, 1994) demonstrated the role of members of a ubiquitous protein family- SMC- in mitotic chromosome condensation. Seminal studies in *Xenopus* egg extracts (Hirano and Mitchison, 1994) and chicken cells (Saitoh et al, 1994) led to the identification of the heterodimer core, Smc2 and Smc4, of the condensin complex, as catalytic proteins with a major role in chromosome condensation and segregation. The condensin

complex was first isolated from *Xenopus levis* egg extracts as 13S and 8S sedimentation coefficients containing complexes (Hirano and Mitchison, 1994).

Condensin

Condensin is a hetero-pentameric chromosomal ATPase, composed of two Structural Maintenance of Chromosomes complex subunits (SMC) and three non-SMC regulatory subunits. The primary structure of SMC proteins is shared from bacteria to humans, suggesting that the activity of condensin is evolutionarily conserved. It consists of five distinct domains - two nucleotide-binding motifs, the Walker A and Walker B motifs, located in the highly conserved N-terminal and C-terminal domains, respectively; the central domain, composed of a moderately conserved "hinge" sequence that is flanked by two long coiled-coil motifs which are arranged in an antiparallel fashion to make a two-armed, symmetrical structure (Melby et al. 1998, Figure 1). The central hinge is flexible and allows opening and closing of the two arms (Hirano et al. 2001). The SMC heterodimer associates with the non-SMC regulatory subunits to form the fully functional holocomplex. Both SMC and non-SMC subunits are essential for the function of the complex.

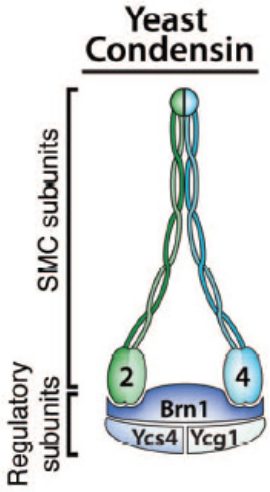


Figure 1: Condensin complex in *Saccharomyces cerevisiae* (Bazile et al., 2010)

Condensin in *Saccharomyces cerevisiae* consists of the catalytic core comprised of the Smc2-Smc4 heterodimer. The regulatory subunits, Brn1, Ycs4 and Ycg1 bind to the heterodimer (Figure

1). Brn1 is a member of the kleisin family of proteins, which binds to Smc2 at its amino terminus and Smc4 at its carboxy terminus to form a closed ring (Onn et al, 2007). Ycs4 and Ycg1 share a structural motif called the HEAT repeats important in protein recognition (Neuwald and Hirano, 2000). They bind to the amino and carboxy terminus of Brn1 respectively (Figure 1). All the three non-SMC regulatory subunits are required for binding to chromatin and condensation activity of the condensin complex (Hirano et al, 1997).

Higher vertebrates contain two different types of condensin complexes- condensin I and II, which are similar in their SMC subunits (SMC2/CAP-E and SMC4/CAP-C), but differ in regulatory non-SMC subunits (CAP-D2, CAP-G, and CAP-H in Condensin I and CAP-D3, CAP-G2, and CAP-H2 in condensin II). They have different roles in mitosis and are subjected to different spatiotemporal regulation (Ono et al, 2003).

Regulation of Condensin

Subcellular localization pattern of condensin varies according to cell cycle events in different organisms. In *Saccharomyces cerevisiae*, condensin always associates with nucleus (Freeman et al, 2000), but in *Saccharomyces pombe,* condensin localizes to cytoplasm during interphase and migrates into nucleus in a Cdk1 dependent manner during the onset of mitosis (Sutani et al, 1999). Similarly in vertebrates, condensin I is localized to cytoplasm and migrates into nucleus, when the nuclear envelope breaks down; in contrast the localization of condensin II is always limited to nucleus (Ono et al, 2004). Though condensin in yeast demonstrates strong sequence similarity to condensin I, its localization and function are more similar to condensin II. *C. elegans* is known to have a third type of condensin- Condensin-I^{DC} involved in dosage compensation (Csankovszki et al, 2009).

It is essential to know how the condensin complex is regulated and how this complex achieves chromosomal condensation. Studies have suggested that condensin is regulated by post-translational modifications, specifically phosphorylation of its subunits at different time points in the cell cycle (Sutani et al, 1999; Kimura et al, 1998, St-Pierre at al, 2009). The other known mechanism is by regulating supercoiling activity of condensin. It is reported that condensin uses

ATPase activity to constrain positive supercoils on purified plasmid DNA (Kimura and Hirano, 1997).

It is well studied that condensin molecule is phosphorylated by different kinases. In human cells, Cdk1/Cdc2 kinase phosphorylates the condensin in mitotic specific manner and casein kinase-II phosphorylates it in interphase (Takemoto et al, 2004). Consistent with this, the supercoiling activity of condensin *in vitro* increases in mitosis and it is suppressed during interphase. Aurora-B also phosphorylates the condensin molecule in *S. pombe*, *Drosophila* and *C.elegans* (Tada et al, 2011; Giet et al, 2001; Lipp et al, 2007). Studies in budding yeast showed that the condensin is phosphorylated by Cdk1 kinase and polo like kinase (Cdc5) *in vitro* and by Cdc5 *in vivo* during anaphase (St-Pierre et al, 2009). It has been proposed that chromosomal condensation can be achieved by phosphorylation of condensin molecule by three different kinases during cell cycle in *Saccharomyces cerevisiae* (Bazile et al, 2010). Cdk1 acts on condensin during entry into mitosis. Later during anaphase when Cdk1 levels drop, condensin is phosphorylated by Cdc5. Upon this, a third kinase might phosphorylate the condensin to achieve proper chromosomal condensation. This third kinase could be Aurora-B or some other kinase, as Aurora-B does not phosphorylate condensin *in vitro* in the yeast *S. cerevisiae* (Bazile et al, 2010).

Visualization of chromosome structure

Amorphous interphase DNA undergoes multiple-fold compaction to form the mitotic chromosome. Several techniques are available to visualize chromosome structure and condensation. Human chromosomes can be easily visualized by microscopy because of the large size of the genome. Visualizing the chromosomes is technically challenging in yeasts *S.cervisiae* and *S.pombe* since they possess relatively smaller genome of 12Mb. *S.pombe* genome consists of only 3 chromosomes. Hence, chromosome condensation is monitored in these organisms by measuring the distance between two genomic loci at different stages of the cell cycle. However, for the same size of genome, *S.cerevisiae* has 12 chromosomes. Hence, the structure of chromosomes during the cell cycle can be visualized by FISH. FISH is a powerful cytogenetic tool that is used to detect and localize the presence of specific DNA sequences on chromosomes. FISH takes advantage of fluorescent labelled probes that bind to very specific parts of the chromosome with which they show a high degree of sequence complementarity. Fluorescent probes bound to the chromosomes are visualized by

fluorescence microscopy. The yeast rDNA locus is an ideal system to study the effect of condensin on chromosome reorganisation during mitosis. It is an approximately 1-2 Mb region on the right arm of chromosome XII consisting of 100-200 tandem copies of a 9.1 kb repeat (Petes, 1979), which contains the genes encoding the 5S, 5.8S, 25S and 18S rRNAs. Because of the presence of multiple repeats and homogeneity of this locus, FISH has been used routinely to monitor chromosome structure at the rDNA in *S.cerevisiae* (Guacci et al, 1994). It has been shown that the rDNA undergoes cell-cycle dependent changes in chromosome organization resulting in distinct morphology (Lavoie et al, 2004).

Rationale

Though the structural features of condensin are well established, its mechanism of action during chromosome condensation is not yet understood. My graduate work focused on elucidating the interaction between the regulatory and catalytic subunits of condensin with the following objectives:

1. To define the kinetics of chromosome condensation

FISH is a useful cytogenetic technique to monitor the structure of rDNA through the cell cycle. Previous studies have monitored morphological changes in whole chromosomes (Manders et al, 1999) and defined genomic regions (Guacci et al, 1994; Lavoie et al, 2004) suggesting that the well conserved chromosome compaction is not a single-step mechanism but a complex pathway involving multiple intermediates. Lavoie *et al* described cell-cycle dependent transformation of the rDNA from diffuse "puff" signal in G1 to a distinct "loop" signal in metaphase (Lavoie et al, 2004). The authors also described transient clusters and lines during the time course of the cell-cycle. I intend to study the dynamics of chromosome condensation kinetics at lower temperatures using cold sensitive mutants. The hypothesis is that lower temperature decreases the rate of cell cycle progression which provides a unique opportunity to identify and characterize transient cell cycle-dependent condensation intermediates.

The focus of this study is to develop a FISH system that monitors the cell-cycle progression of rDNA morphology in synchronous cultures at 16˚C. We will use a cold-sensitive mutant- *tub2-*

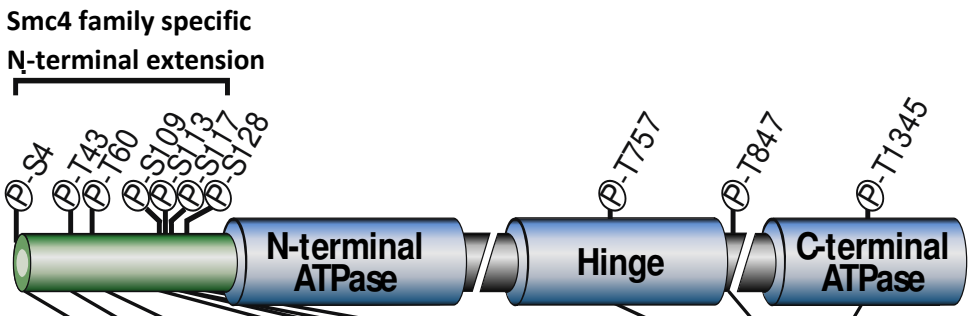
311 which is derived from a substitution (G92E) in β-tubulin resulting in depolymerisation of microtubules. This blocks the cells in metaphase due to activation of spindle assembly checkpoint at the restrictive temperature of 16˚C.

2. To determine the role of Smc4 N-terminal extension in interaction with Brn1.

Phosphorylation sites, identified on Smc4 in budding yeast, are mostly confined to N-terminal part (Bazile et al, 2010). Structurally, the N-terminal extension of Smc4 is close to the regulatory subunits, specifically Brn1. The Brn1 subunit is placed strategically in the complex in that it interacts with all other subunits of the complex (Onn et al, 2007). Since the C-terminal of Brn1 binds to the N-terminal ATPase domain, phosphorylation of Smc4 may play a role in the interaction between Smc4 and Brn1 thereby regulating complex formation and loading of condensin onto chromatin.

Mass spectrometry analysis of *in vivo* purified condensin complex confirmed the presence of five Cdk1 consensus phosphorylation sites on Smc4, all located in the N-terminus extension. Alignment of the C-terminal Brn1 with the N-terminal Smc4 reveals positively charged residues against the consensus sites of Cdk1 phosphorylation on Smc4 (Figure 2). Also, the N-terminal extension of Smc4 is highly disorganised. Based on these initial results, I hypothesize that upon phosphorylation of Smc4, Brn1 interacts with Smc4 to form a closed ring complex thereby facilitating loading onto chromatin. If the positively charged residues are replaced by negatively charged or neutral residues, it might affect the dynamics of interaction between condensin subunits and thereby chromatin loading on condensin.

A)



Robellet et al, (unpublished data)

B)

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Cter-Brn1
N-ter-Smc4

QDDEDHKFPIRENKVTYSRVSKKVDVRRLKKNVWRSINNLIQEHDSRKNREQSSNDSET
NNNNNNSNKVPSLQLLELRRTPSRGPSRPPSQSYSKHDRGRSSTKPPQLNPVHLSTTTS

HTEDESTKELKFSDIIQGISKMYSDDTLKDISTSFCFICLLHLANEHGLQITHTENYND
NTPQSFAYRNEGSSLVLKRPTYSKSYSAELAPLDPEPTNESLNVRNEVQSDEEADGQDL

LIVNYEDLATTQAAS
SLEPEQASKRKQRKSLPSDSM
```

C)

Homo sapiens	T	A	K	K	M	D	M	K	K	L	K	Q	S	M	W
Bos Taurus	T	A	K	K	M	D	M	K	K	L	K	Q	S	M	W
Mus musculus	T	A	K	K	M	D	M	K	K	L	K	Q	S	M	W
S.Pombe	R	A	K	K	V	D	V	R	V	L	K	E	K	L	W
S.Cerevisiae	V	S	K	K	V	D	V	R	R	L	K	K	N	V	W

Figure 2: A) Phosphorylation sites on N-terminus extension of Smc4. B) Alignment of C-terminus of Brn1 with the N-terminal extension of Smc4. C) Conserved positively charged residues on the C-terminus of Brn1 across species.

Study design

The choice of organism for this study is budding yeast *Saccharomyces cerevisiae*. This model organism has unique and essential tools for this kind of study. It has a haploid life cycle, which is useful to recover recessive mutations. The cell cycle can be followed in yeast by monitoring changes in the cellular morphology. The ovoid cell in G1 phase of the cell cycle begins to form a bud during S phase which grows in size through G2 and mitosis after which the daughter cells divide. It is amenable to mutant construction and other genetic manipulations. Yeast genome codes for a single condensin complex as compared with higher eukaryotes, which have 2 condensin complexes. Therefore it is an ideal system to study functional analysis of condensin through the interactions between the regulatory and catalytic subunits of condensin complex.

MATERIALS AND METHODS

S.cerevisiae culture

All the strains used in this study were derived from the W303 genetic background. The genotypes of the strains are described in Table 1. Cells were grown in YEPD at 23˚C unless mentioned otherwise. Cells were synchronized in G1 using α-factor, a mating pheromone, and blocked in metaphase by nocodazole, a spindle depolymerizing drug. Standard procedures were used for yeast culture. Conditions for growth and cell cycle blocks were performed as described (D'Amours et al, 2004).

Cell preparation for fluorescent *in situ* hybridization

Detection of the rDNA array was performed by FISH, essentially as described (Guacci et al, 1994; Lavoie et al, 2004). For arresting the cell cycle at G1, cells were incubated at 23˚C for 2.5 hours in 5 µg/ml α-factor. Cells were filtered with 0.45 µm membrane filter and washed with fresh YEPD medium and released into medium at restrictive temperature. Upon removal of α-factor, cells were released into 23˚C for 30 minutes and then shifted to the restrictive temperature of 16˚C. Wildtype cells were incubated in 15 µg/ml nocodazole for 4 hours. Tubulin mutants were arrested in metaphase by incubation at 16˚C for 4 hours. Samples were taken every 30 min.

Cells were fixed in 3.6% formaldehyde for 2 hours at 23˚C, washed 3X with distilled water at resuspended in 1 M Sorbitol, 20 mM KPO_4, pH 7.4 (cells can be stored overnight at 4˚C). Spheroplasts were digested with 3 mg/ml Zymolyase 100T for 1 hour at 23˚C and transferred to polylysine-coated slides and incubated for 15 min at room temperature to allow them to adhere to the slides. Unbound spheroplasts were removed from the wells and replaced with 0.5% SDS for 10 min due to which the cells appear swollen and transparent. Slides were then submerged in 3:1 methanol/acetic acid in a coplin jar for 5 min at room temperature and allowed to air dry overnight at room temperature.

In situ hybridization

Rnase A (100 µg/ml in 2X SSC) was added to each slide well containing fixed cells, and the slides were incubated in a humid chamber for 2 hours at 37˚C. The slides were washed four times by submersion in coplin jars with 2X SSC at room temperature at 2 min/wash and dehydrated by submersion in increasing concentrations (70, 80 and 95%) of ethanol at -20˚C and allowed to air dry. To denature chromosomal DNA, the slides were incubated in a denaturing solution containing 70% formamide, 2X SSC at 70˚C for 2 min and immediately dehydrated by a series of ethanol washes (70, 80, 90 and 100%) at -20˚C at 1 min/wash. The denatured DNA was then treated with Proteinase K (200 µg/ml in 20 mM Tris, pH 7.8 and 2 mM $CaCl_2$) for 15 min in a humid chamber at 37˚C and dehydrated by a series of ethanol washes (70, 80, 90 and 100%) at -20˚C at 1 min/wash. rDNA probes were denatured in 50% formamide, 10% dextran sulphate, 400 µg/ml salmon sperm DNA, 2X SSCP (0.3 M NaCl, 0.03 M Sodium citrate, 0.04 M NaH_2PO_4 at pH 6.0) for 10 min at 70˚C and incubated on ice for 5 min. Typically, 2 ng of probe was added to 10 µl of hybridization mix. 5 µl of denatured probe mix was added to each well. Hybridization was done overnight at 35˚C in a humid chamber. Slides were then washed with 60% formamide, 2X SSC at 37˚C for 20 min, twice in 2X SSC (37˚C, 5 min/wash) and once in PBD (0.1 M NaH_2PO_4, 0.1 M Na_2HPO_4, 0.1% Igepal) to remove non-specific hybridization. Hybridized digoxigenin labelled probes were detected by serial incubations with 1:250 dilutions of mouse anti-digoxigenin antibody, goat anti-mouse antibody conjugated with FITC and anti-goat antibody conjugated with FITC for 20 min each at 37˚C in a humid chamber with 3 washes with PBD (room temperature, 2 min/wash) after each step of antibody incubation. Chromosomal DNA was stained with propidium iodide. Images were visualized using the DeltaVision microscope.

Probe for *In situ* Hybridization

The rDNA locus was cloned into a centromeric plasmid. The probe was prepared by amplifying this rDNA by PCR, gel filtration of the 9 kb PCR product and labelling of the probe with digoxigenin. Probes were labelled with digoxigenin by nick translation using the BioNick Translation kit from Invitrogen. 100 ng of probe was mixed with Nick translation buffer (1M Tris

HCl, $MgCl_2$, BSA, β-mercaptoethanol), digoxigenin, enxyme mix and water to make a 50 µl reaction and incubated at 16˚C for 1 hour.

The reaction was stopped with 5 µl of 0.5 M EDTA. Labelled probe was precipitated with 10 µl sonicated salmon sperm DNA, 6 µl 3 M sodium acetate and 120 µl cold ethanol by incubation at -80˚C for 15 min. The pellet was obtained after centrifugation at 13,200 rpm for 15 min at 4˚C and resuspended in 100 µl distilled water. The labelled probe was re-precipitated in 10 µl 3 M sodium acetate and 200 µl cold ethanol by incubation at -80˚C for 15 min. The pellet was obtained after centrifugation at 13,200 rpm for 15 min at 4˚C, washed with 1 ml cold 70% ethanol, air dried and resuspended in 25 µl of 1X TE.

Co-immunoprecipitation

Yeast cells were grown till mid-log phase in YEPD and incubated at restrictive temperature of 37˚C for 2 hours. The cells were pelleted and washed with distilled water. The pellet was resusupended in equal volume of IP buffer (100 mM KCl, 100 mM NaF, 50 mM Tris-HCl pH 7.5, 1 mM EDTA, 1 mM EGTA, 0.1% Tween 20, 10% Glycerol).

Standard procedures with minor modifications were followed as described (Brar et al, 2006). The cell suspension was frozen in liquid nitrogen as tiny droplets and lysed using the Freezer mill. The lysate was thawed, protease inhibitors were added and centrifuged at 13,200 rpm for 30 min at 4˚C. The supernatant was then added to 20 µl of protein G coupled-sepharose beads, pre-washed twice in 250 µl of IP buffer and rotated for 30 min at 4˚C to remove non-specific binding proteins. The lysate was centrifuged for 5 min at 13,200 rpm at 4˚C and divided into three equal volumes. 1 µg of primary antibody was added per 100 µl of lysate, rotated for 1 hour at 4˚C. 20 µl of Sepharose beads, pre-washed twice in 250 µl of IP buffer was added to each volume and rotated for 1 hour at 4˚C to obtain the antigen-antibody complex. The beads were centrifuged for 5 min at 2,200 rpm at 4˚C and washed thrice with 500 µl IP buffer, resuspended in 30 µl sample buffer and analysed by SDS-PAGE and Western Blot.

SDS-PAGE analysis

Protein extracts were prepated by TCA-glass bead lysis procedure (Foiani et al, 1994). Protein samples were separated using a 10% resolving gel (30% acrylamide, 20% SDS, 1 M Tris pH 8.8, 10% APS, TEMED in 10 ml of H_2O). Samples were diluted with 2X SDS-PAGE buffer and denatured at 100˚C for 5 min before loading onto the gel. Unstained gels were analyzed by Western blots.

Western blot analysis

Protein run on 10% gel was transferred onto a PVDF membrane using the iBlot dry blotting system from Invitrogen. After transfer, the membrane was blocked with 10 ml of PBST (1X PBS, 0.1% Tween-20) with 2% skimmed milk and 1% BSA for 1 hour at room temperature with gentle shaking and then exposed to primary antibody (mouse antibody diluted 1:1000 in PBST with 2% skimmed milk and 1% BSA) for 2 hours at room temperature. The membrane was rinsed with PBST and then exposed to secondary antibody (anti-mouse HRP diluted 1:10,000 in PBST with 2% skimmed milk and 1% BSA) for 1 hour at room temperature. The membrane was washed with PBST thrice with 10 min gentle agitation at room temperature. The blot was developed using the enhanced chemiluminiscent substrate from Perkin and Elmer.

Mutant generation by site-directed mutagenesis

Primers were designed to introduce the *brn1-6A* and *brn1-6E* mutations using the QuikChange kit from Stratagene. Mutants were constructed by replacing the positively charged residues with neutral (alanine) (K643A, K644A, R648A, R649A, K651A and K652A) and negatively charged (glutamate) residues (K643E, K644E, R648E, R649E, K651E and K652E) of Brn1 protein using site-directed mutagenesis approach.

YCplac, a single-copy plasmid containing wildtype *BRN1* that can be integrated in *BRN1* genomic locus of yeast, was used as a template to mutagenize the 6 residues on C-terminus of Brn1. The bacterial strands were digested by incubating with DpnI enzyme for 2 hours at 37˚C. DpnI is a

restriction enzyme which digests methylated GATC residues. The mutagenized product was then transformed into competent *E.coli* cells to obtain the mutant plasmid.

Yeast Transformation

The plasmid containing *brn1-6A* and *brn1-6E* were then digested with Acc651, PvuI and SalI to linearize the plasmid. The 4.2 kb band containing the mutant BRN1 followed by HA tag and URA marker was transformed into wildtype yeast cells by heat shock method. 50 ml of yeast cells were grown till mid-log phase, pelleted down and washed with 50 ml distilled water and 1 ml of 1 M LiAc. To the cell pellet, 240 µl of 50% PEG, 36 µl of 1M LiAc, 10 µl of 10 mg/ml sonicated salmon sperm DNA and 74 µl of linearized plasmid was added and incubated at 30˚C for 30 min and subjected to heat shock at 42˚C for 15 min. The cells were centrifuged at 3000 rpm for 2 min, resuspended in 60 µl of sterile water and plated on solid medium deficient in uracil and incubated at 23˚C for 2 days. Transformation of the mutant allele was confirmed by sequencing of the genomic locus.

RESULTS

Cell-cycle dependent rDNA intermediates

To look at transient intermediates of rDNA condensation, we monitored the morphology of the rDNA locus through the cell cycle by FISH with a wildtype strain and a tubulin mutant, *tub2-311.* Yeast cells were grown till mid-log phase, blocked in G1 with α-factor and synchronously released into cell cycle at 16˚C. Wildtype strain was blocked in metaphase by incubating with nocodazole, a spindle poison, which depolymerizes microtubules, for 4 hours. *tub2-311*, a β-tubulin mutant cold sensitive strain was blocked in metaphase by incubating at 16˚C for 4 hours. Samples were taken every 30 min and analysed for rDNA morphology by FISH (Figure 3).

At G1 most of the rDNA was seen as diffuse puff signals. 60 min after release into 16˚C, the most abundant species was that of a tightly packed line/crescent. During time intervals 120 min and 210 min after release into 16˚C, we observed the complex convoluted phenotype which we termed the 'arabesque' owing to its pattern which is similar to artistic motifs in Arabic art (Robellet et al, unpublished data). At 240 min post G1 release, most cells had resolved their rDNA into the metaphasic loops.

To monitor the cell cycle progression, we checked the budding index which showed that the mutant progressed slightly faster than the wildtype (Figure 4A). FACS analysis indicated that these phenotypes could be cell-cycle dependent- puff at G1, crescent at S, arabesque at G2 and loop at metaphase (Figure 4B and 4C).

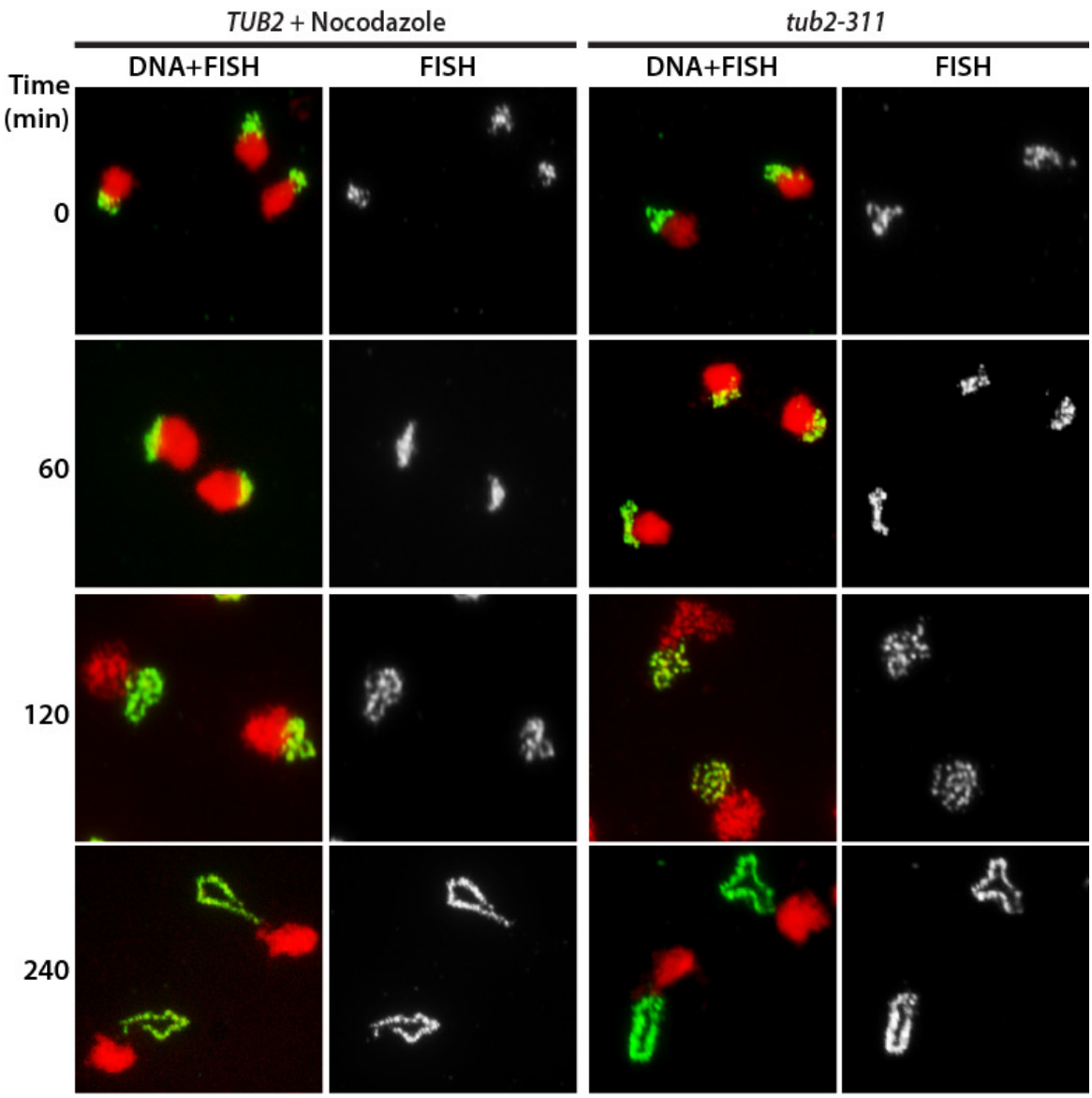


Figure 3: Cell-cycle-dependent intermediates in rDNA condensation.

Fluorescent *in situ* hybridization to monitor rDNA condensation during cell cycle progression. D1 (wildtype) and D3582 (*tub2-311*) strains were used. Cells were synchronized in G1 using α-factor and released into restrictive temperature of 16˚C. Samples were collected at time-intervals of 30 minutes. Following release to the permissive temperature, nocodazole was added to the wildtype culture 1 hour post release to prevent cycling beyond the first cell cycle. Micrographs of FISH of the yeast rDNA (FITC, green) and chromosomes (PI, red). At each time point, a representative micrograph of the most prominent or newly emerging species is shown.

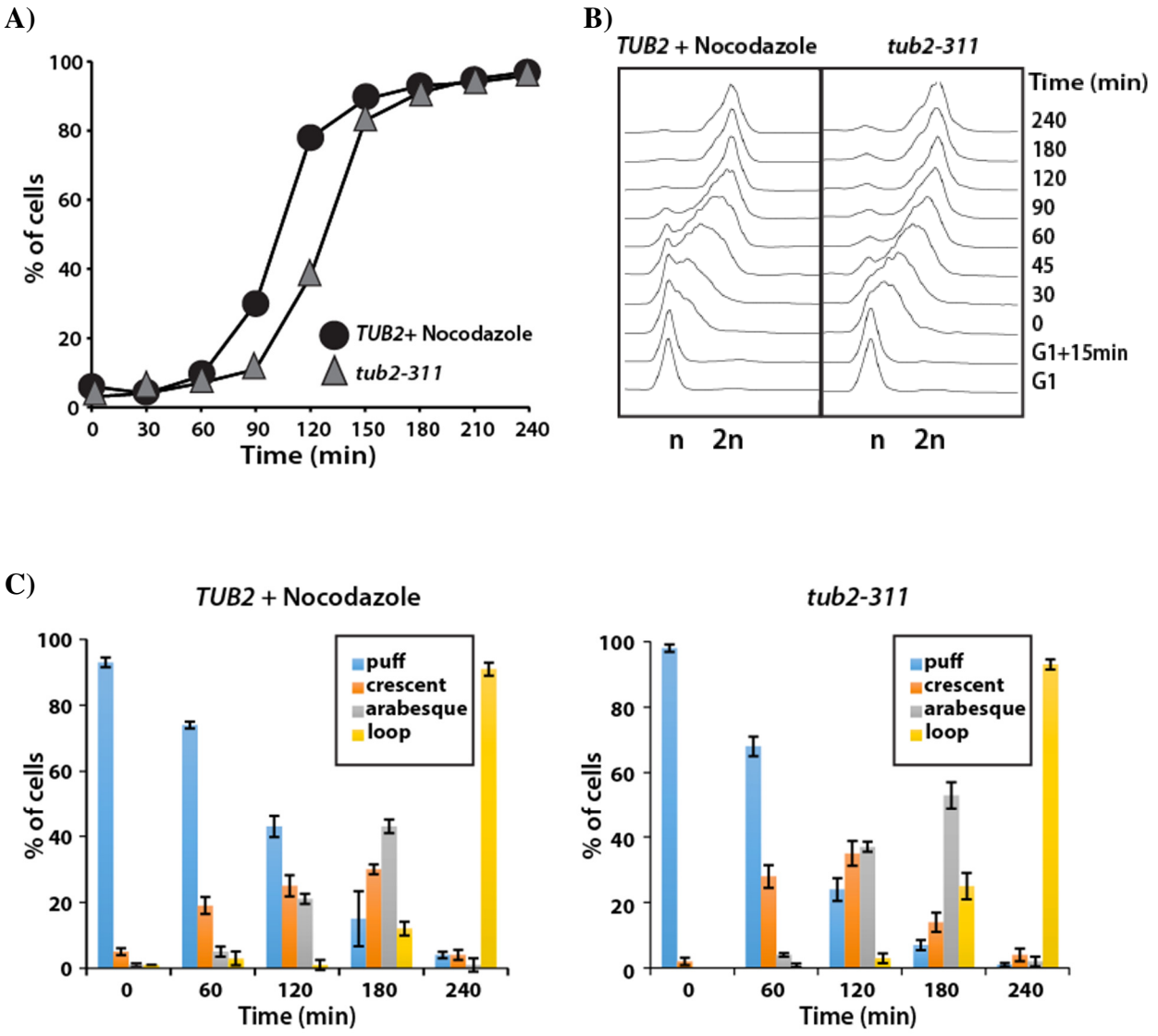


Figure 4: Kinetics of cell-cycle-dependent intermediates in rDNA condensation

Time points were taken from the experiment shown in Figure 3, and cells were processed for A) Budding index and B) Flow cytometric analysis. The quantitation of the most prominent species is shown in panel C. At least 100 nuclei were scored per time point.

rDNA intermediates are condensin-dependent

To demonstrate that these rDNA intermediates were condensin-dependent, we screened condensin mutants for cold sensitivity at 16˚C (Figure 5). Several condensin mutants such as *smc4-757*, *smc4-3* and *ycg1-521* demonstrated a cold-sensitive phenotype. Since the phenotype of *ycg1-521*, a Cdc5 phospho-mutant of the Ycg1 subunit (St-Pierre et al, 2009) was more severe, it was chosen for further analyses and was crossed with *tub2-311* to obtain a *tub2-311*, *ycg1-521* double mutant. FISH was performed as described before in the double mutant, which resulted in the "puff" phenotype throughout the cell cycle (Figure 6). This showed that the transformation of rDNA locus from puff to loop was condensin-dependent. Cell cycle progression was normal in both control and condensin mutant cells (Figure 7).

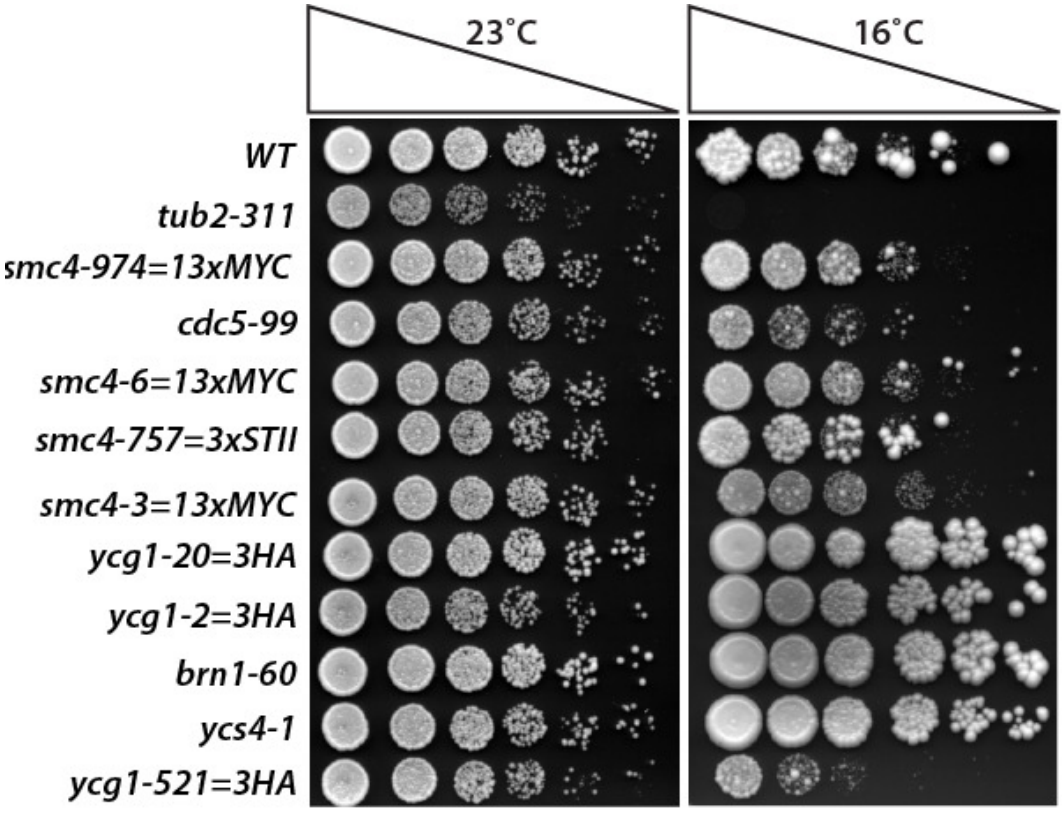


Figure 5: Screening for cold sensitive condensin mutants

Five-fold dilution series of condensin mutant strains were spotted on YEPD plates to check viability at permissive (23˚C) and restrictive (16˚C) temperatures.

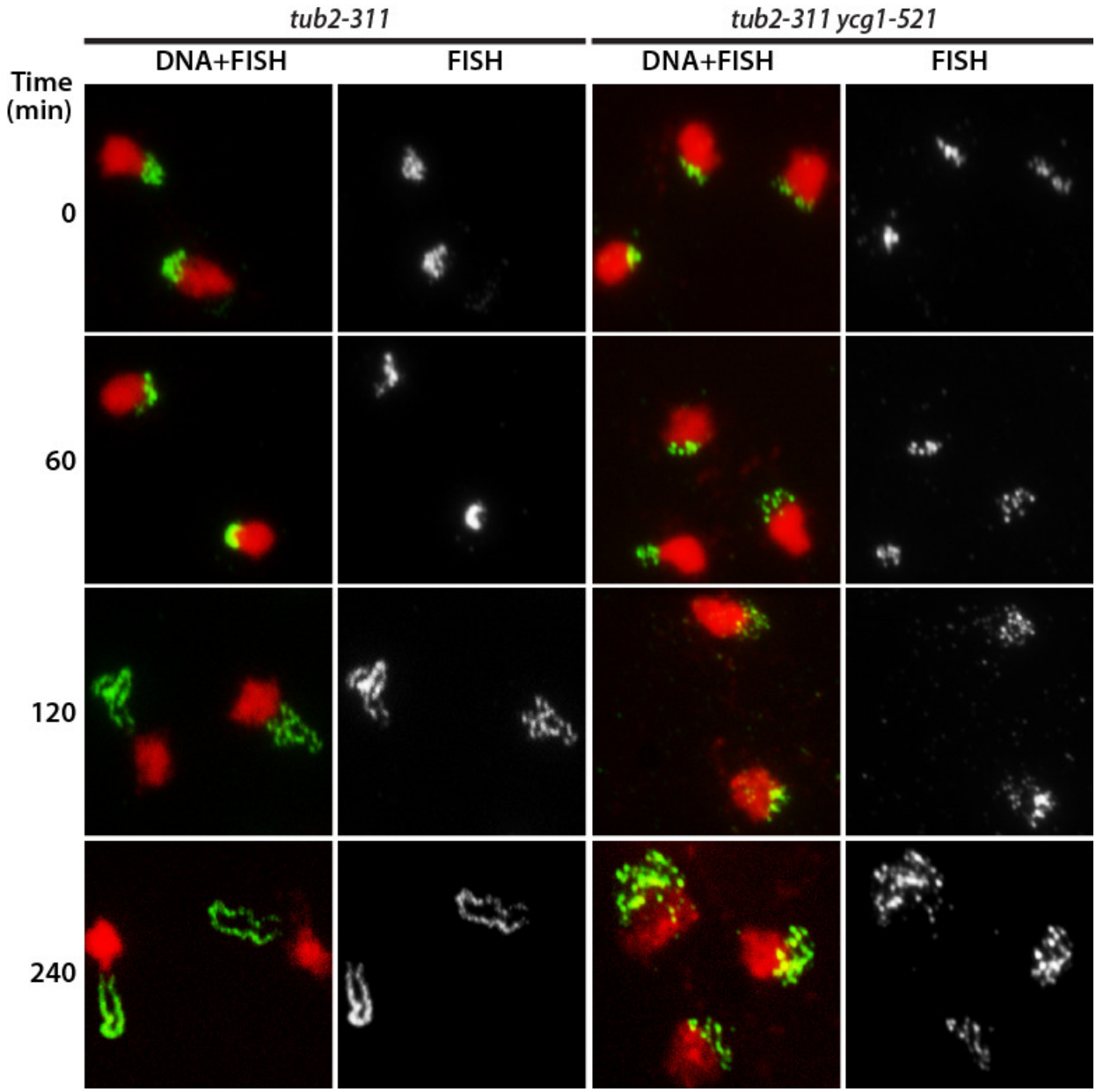


Figure 6: Cell-cycle-dependent rDNA intermediates in tubulin-condensin double mutant

Fluorescent in situ hybridization to monitor rDNA condensation during cell cycle progression. D3582 (*tub2-311*) and D4092 (*tub2-311 ycg1-521*) double mutant strains were used. Cells were synchronized in G1 using α-factor and released into restrictive temperature of 16˚C. Samples were collected at time-intervals of 30 minutes. Micrographs of FISH of the yeast rDNA (FITC, green) and chromosomes (PI, red). At each time point, a representative micrograph of the most prominent or newly emerging species is shown.

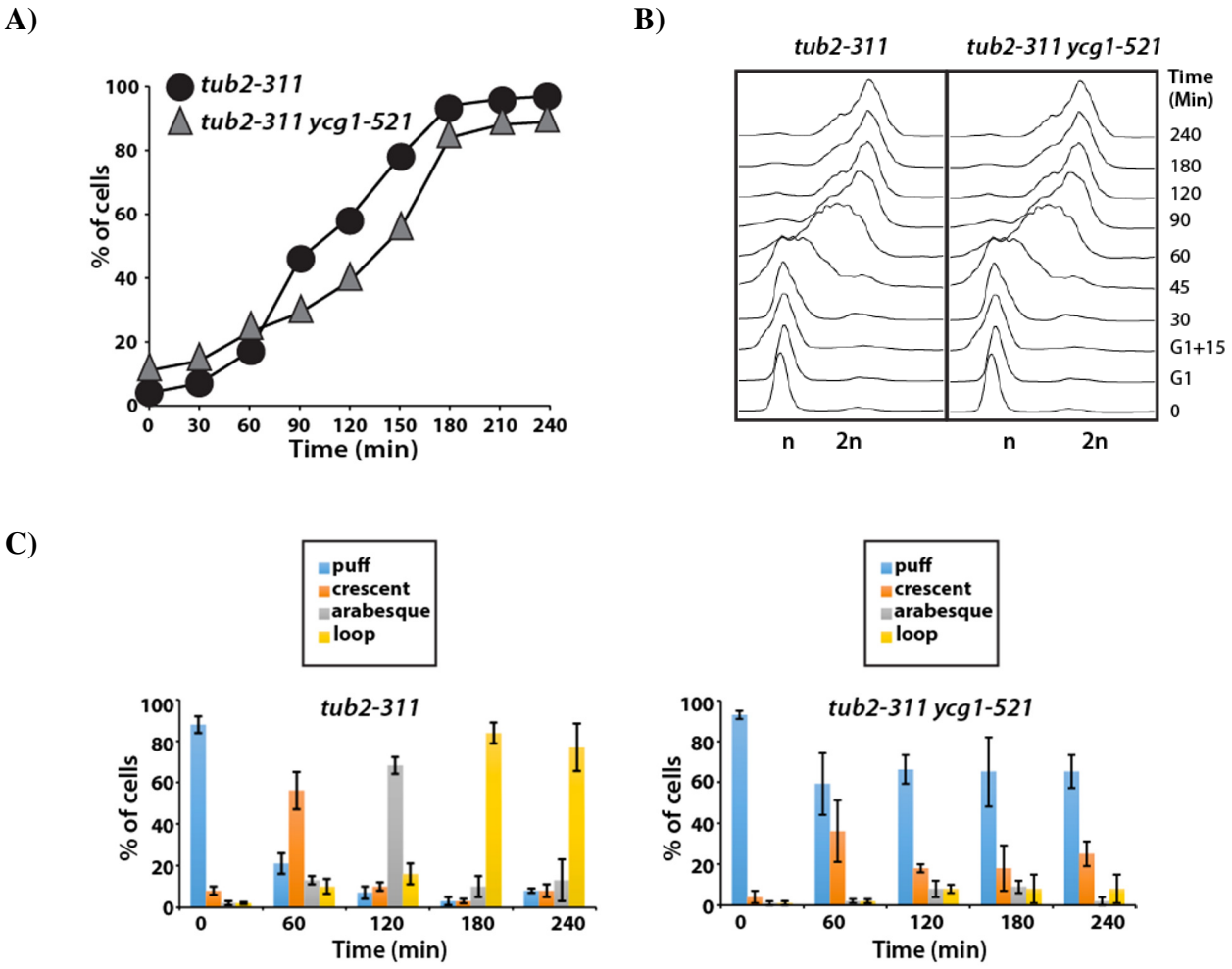


Figure 7: Kinetics of cell-cycle-dependent rDNA intermediates in tubulin-condensin double mutant.

Time points were taken from the experiment shown in Figure 6, and cells were processed for A) Budding index and B) Flow cytometric analysis. The quantitation of the most prominent species is shown in panel C. At least 100 nuclei were scored per time point.

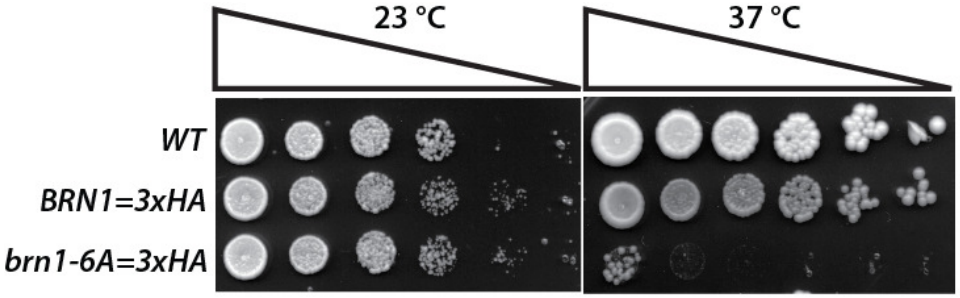
Characterization of the cellular phenotype of the *brn1* mutants

Phosphorylation sites, identified on Smc4 in budding yeast, are mostly confined to N-terminal part (Bazile et al, 2010). Alignment of the C-terminal Brn1 with the N-terminal Smc4 reveals positively charged residues against the consensus sites of Cdk1 phosphorylation on Smc4 (Figure 2B). These positively charged residues are highly conserved across species from *Saccharomyces cerevisiae* to *Homo sapiens* (Figure 2C). To understand the dynamics of interaction between the regulatory (Brn1) and catalytic (Smc4) subunits of condensin, *brn1* mutants were generated. Mutants were constructed by replacing a series of positively charged residues with neutral (alanine) (K643A, K644A, R648A, R649A, K651A and K652A- *brn1-6A*) and negatively charged (glutamate) residues (K643E, K644E, R648E, R649E, K651E and K652E- *brn1-6E*) using site-directed mutagenesis approach. If these residues are important, the *brn1-6A* mutant, which results in disruption of the charge should be thermosensitive and *brn1-6E,* in which there is complete inversion of the charge should demonstrate a more severe phenotype. In the *brn1-6A* mutant, the positive charge was neutralized and as predicted, *brn1-6A* was thermosensitive (Figure 8A) at the restrictive temperature of 37˚C.

A heterozygous diploid strain with *BRN1::URA3* and *brn1-6E::URA3* alleles was induced to sporulate and tetrads were dissected on YEPD plates. The genotype of viable segregants was scored on synthetic medium lacking uracil. In the *brn1-6E* mutant, replacement of the positive charge with negatively charged glutamate resulted in lethality (Figure 8B).

To characterize the cellular phenotype of the *brn1-6A* mutant, FISH was performed on the rDNA locus (Figure 9A). Cells were synchronized in G1 with the use of α-factor and released into permissive and restrictive temperature of 37˚C. Cells were blocked in metaphase by nocodazole, a spindle depolymerizing drug. Cells were processed for FISH as described previously. The *brn1-6A* mutant demonstrated the "puff" phenotype in metaphase-blocked cells at 37˚C indicating the inability of the condensin complex to organise the rDNA locus into the 'loop' phenotype. Quantification of the cells showed that in the wildtype, about 70% of cells had loops whereas in the *brn1-6A* mutant, 80% cells retained the puff phenotype. This shows that these residues are important to promote the condensation activity (Figure 9B).

A)



B)

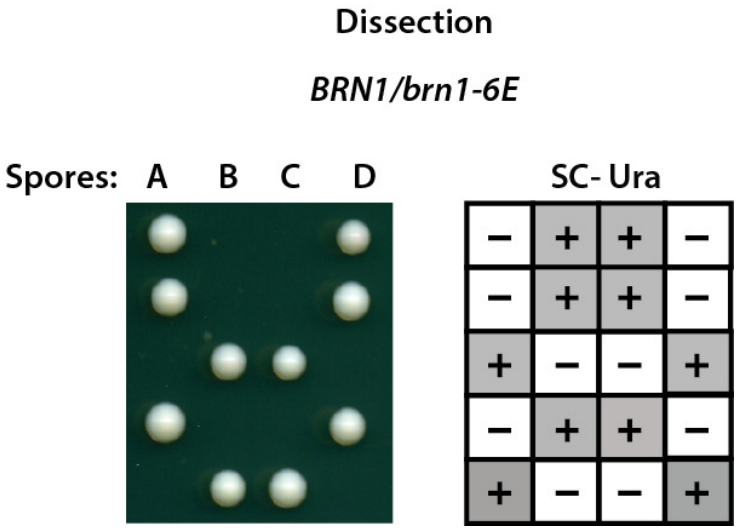


Figure 8: Characterization of cellular phenotype of *brn1* mutants

A) Five-fold dilution series of *brn1-6A* mutant strain were spotted on YEPD plates to check viability at permissive (23˚C) and restrictive (37˚C) temperatures. B) A heterozygous diploid strain with *BRN1::URA3* and *brn1-6E::URA3* alleles was induced to sporulate and tetrads were dissected on YEPD plates. The genotype of viable segregants was scored on synthetic medium lacking uracil. Segregants viable on synthetic medium lacking uracil are denoted by **'+'** and inviable segregants on synthetic medium lacking uracil are denoted by **'-'.**

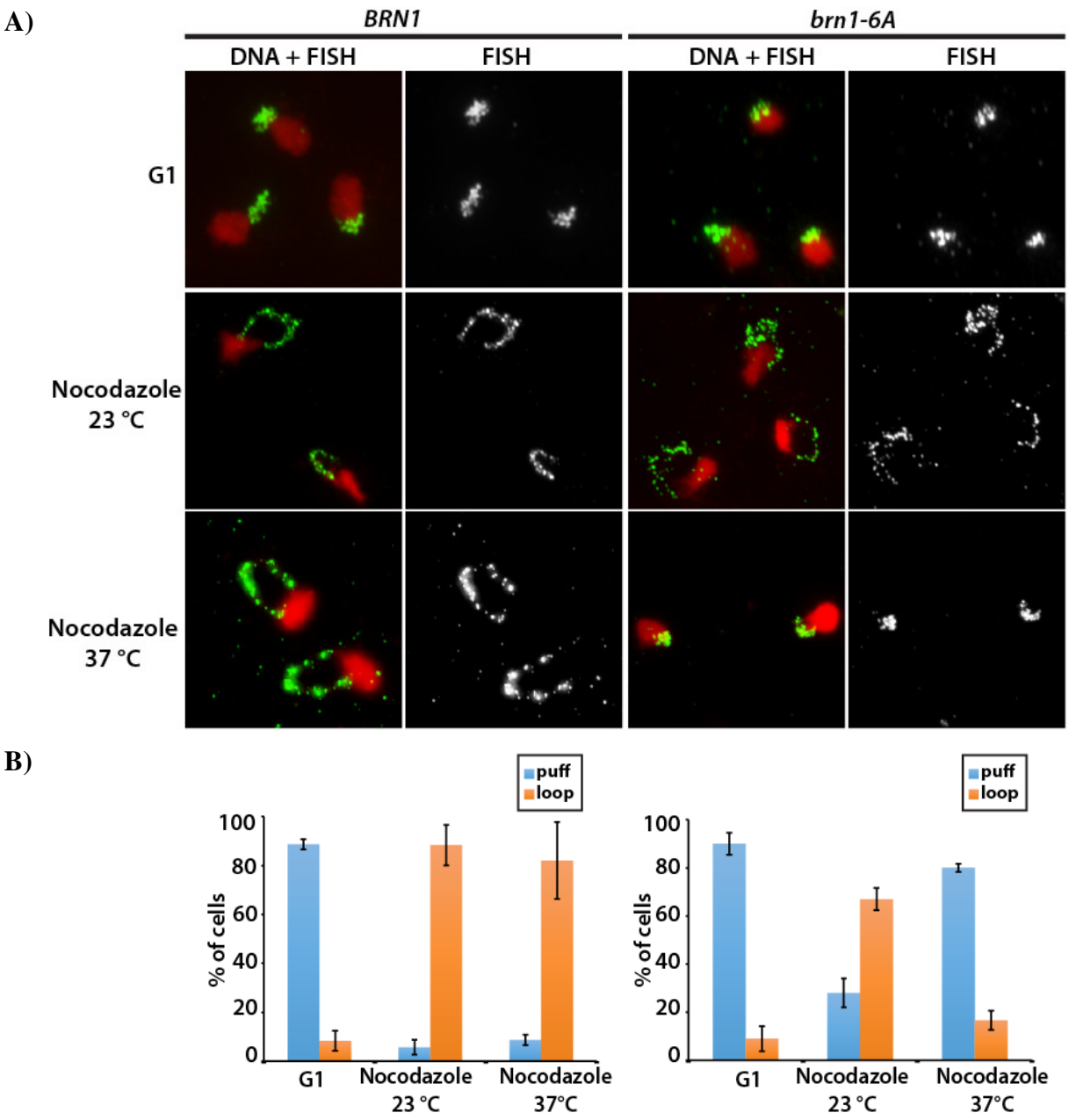


Figure 9: Condensation defects in *brn1-6E*

(Figure legend on Page 32)

A) Fluorescent in situ hybridization to monitor rDNA condensation during cell cycle progression. Wildtype and *brn1-6A* strains were used to synchronize the cells in G1 using α-factor and release into at permissive (23˚C) and restrictive (37˚C) temperatures. Following release to the permissive temperature, nocodazole was added to the culture to arrest the cell cycle in metaphase. Micrographs of FISH of the yeast rDNA (FITC, green) and chromosomes (PI, red). At each time point, a representative micrograph of the most prominent or newly emerging species is shown.

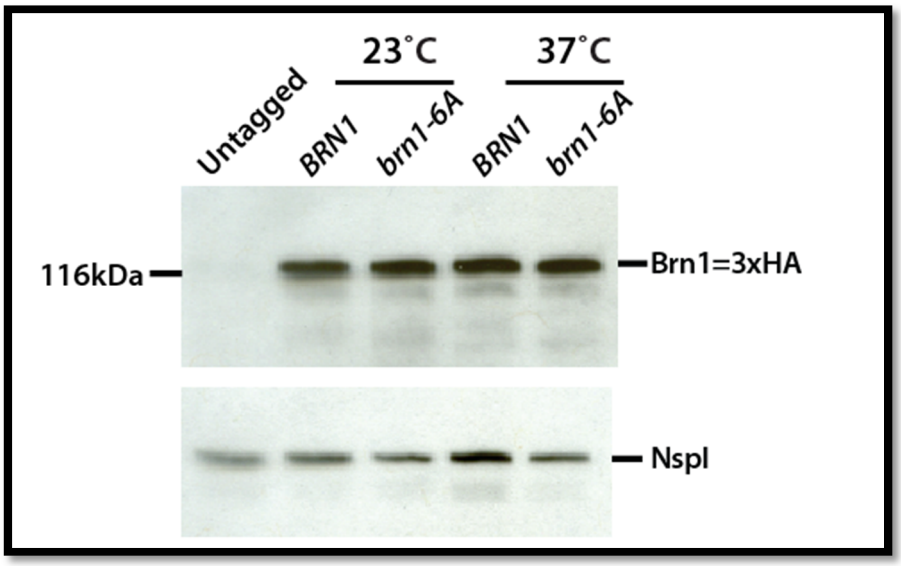
B) The quantitation of the most prominent species at G1 and Nocodazole arrest at at permissive (23˚C) and restrictive (37˚C) temperatures.

Characterization of the biochemical interaction between Smc4 and Brn1

Since *brn1-6A* mutant showed a thermosensitive phenotype at 37˚C, these residues may be important in complex formation or contributing to the stability of the protein. Hence, mutating them could result in disruption of the complex or an unstable Brn1 protein. So, we looked at the expression levels of Brn1 in the mutant. There was no significant difference in the level of Brn1 expression between wildtype and mutant backgrounds at both permissive (23˚C) and restrictive (37˚C) temperatures (Figure 10A).

To demonstrate that the interaction between Smc4 and Brn1 depends on the charged residues on Brn1 corresponding to the Cdk1 consensus sites on Smc4, and to confirm the complex formation in this mutant, we performed Co-IP of *brn1-6A* with Smc4 at the restrictive temperature of 37˚C which showed a significant reduction in pull-down suggesting that the complex formation is affected in the *brn1-6A* mutant (Figure 10B).

A)



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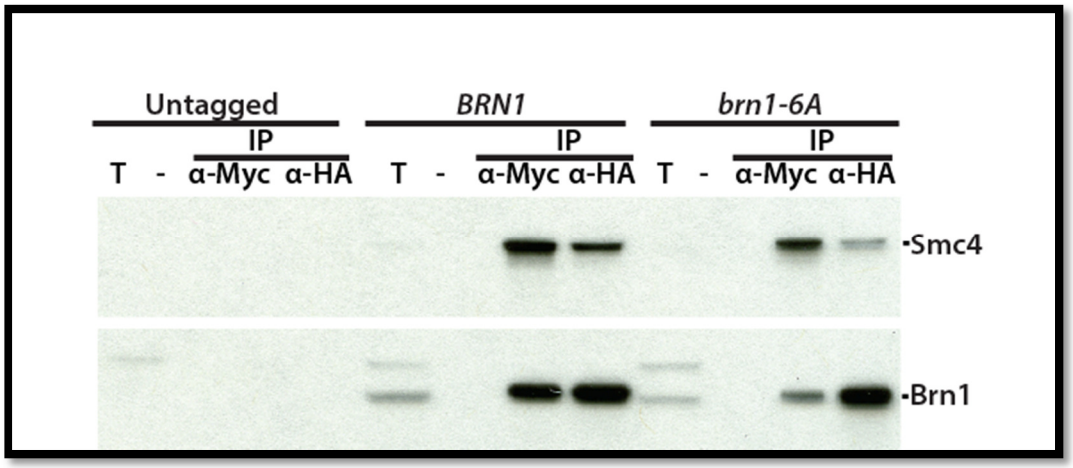


Figure 10: Stability of *brn1-6A* mutant

A) Lysates from D515 (*BRN1-3HA*) and D4011 (*brn1-6A-3HA*) were prepared from cells at permissive (23˚C) and restrictive (37˚C) temperatures 2 hours post G1 release and processed for western analysis using anti-HA antibody. B) Lysates from D1 (*BRN1, SMC4*), D4130 (*BRN1-3HA, SMC4-13Myc*) and D4106 (*brn1-6A-3HA, SMC4-13Myc*) were prepared from cells at restrictive temperature (37˚C) 2 hours post G1 release. Brn1-3HA and Smc4-13Myc were immunoprecipitated from these lysates and processed for western analysis using anti-HA and anti-Myc antibodies.

To demonstrate that the interaction is specific to the N-terminus of Smc4 and C-terminus of Brn1, we developed short constructs of N-terminal fragment of Smc4 and C-terminal fragment of Brn1. The N-terminal extension of Smc4 is unique to condensin complex as the other Smc proteins, Smc3 in cohesin and Smc6 in the Smc5/6 complex do not have the N-terminal extension. It has been shown that this extension contains 7 Cdk1 consensus sites, which might be the initiation switch for condensin activation.

Brn1 C-terminal fragment of two lengths were designed, one with 635-831 amino acids and another with 395-831 amino acids of the C-terminus of the Brn1 protein but, upon transformation there was no stable expression in *Saccahromyces cerevisiae.* The short version containing 635-831 amino acids was not detected by western-blot. The second construct with 395-831 amino acids was transformed into wildtype strain. Clones showed some bands on gel at the expected size of 46 kDa and a possible degradation product at 30 kDa (Figure 11). Brn1 is a large protein of 116 kDa and the short version might be subjected to degradation and difficult to express *in vivo*. Thus, it was not possible to express a truncated version of Brn1 containing the C-terminal fragment.

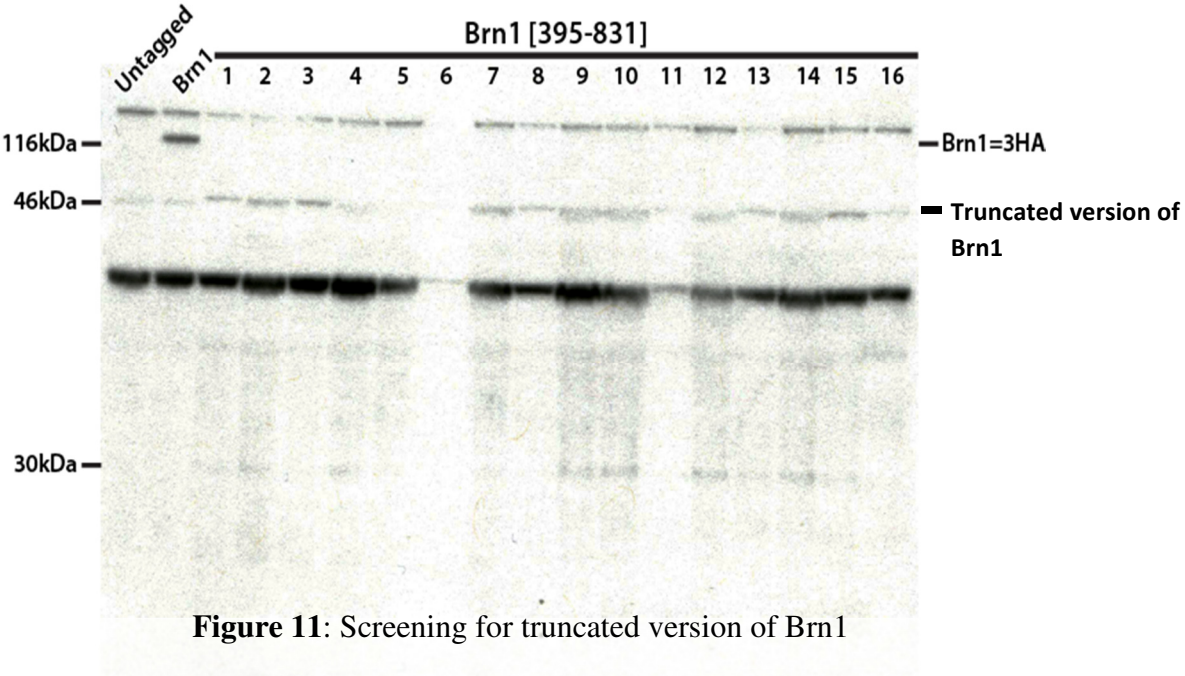


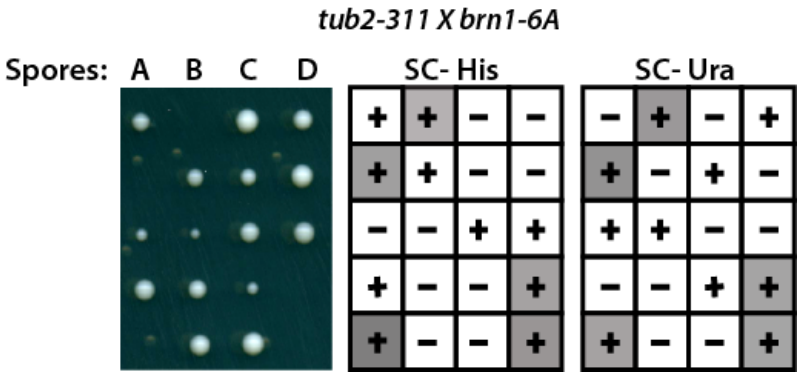
Figure 11: Screening for truncated version of Brn1

Plasmid p1120 (YCplac22-NLS-Brn1 (395-831)-3xHis3-HA-URA3) was transformed into wildtype strain and transformant clones were selected on synthetic medium lacking uracil. Lysates were prepared from 16 clones and processed for western analysis using anti-HA antibody.

BRN1 interaction with *TUB2*

To examine if these positive residues of Brn1 are involved in interaction with microtubules, (Laflamme et al., 2014), we checked for the genetic interaction of *BRN1* with *TUB2*, a gene that encodes for β-tubulin. A cross between *brn1-6A* and *tub2-311* mutants resulted in synthetic lethality (Figure 12A), indicating that these two genes are involved in a similar pathway. Interestingly, zygotic mating of another *BRN1* mutant allele, *brn1-570* with *tub2-311* did not show lethality (Figure 12B). *brn1-570* is a Cdc5 phosphomutant of *BRN1*. The *ycg1-521* allele used to show condensin dependence of rDNA intermediates is also a Cdc5 phosphomutant. The *ycg1-521* and *brn1-570* phosphomutants are deficient in phosphorylation of Ycg1 and Brn1 subunits respectively during anaphase (St-Pierre et al, 2009). Phosphorylation of a subunit adds positive charge on these subunits. Since microtubules are made up of negatively charged polymers of α/β tubulins, interaction with positive residues on may stabilize the attachment of microtubules on centromere. This suggests that these positive residues of Brn1 may play a role in condensin-microtubule attachment.

A)



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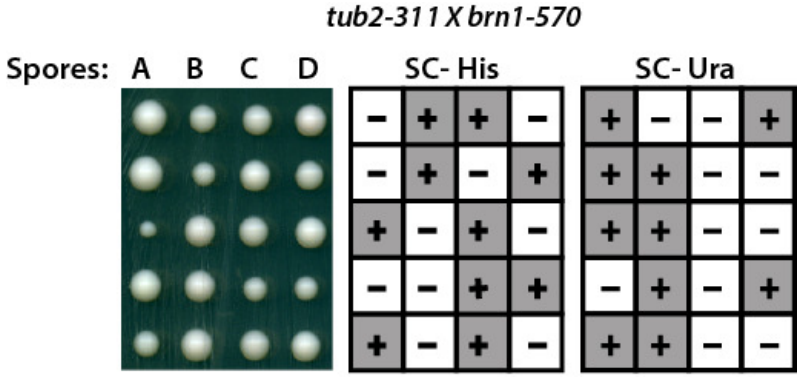


Figure 12: Genetic interaction of *BRN1* with *TUB2*

A) A heterozygous diploid strain with *tub2-311::HIS3* and *brn1-6A::URA3* alleles was induced to sporulate and tetrads were dissected on YEPD plates. The genotype of viable segregants was scored on synthetic medium lacking uracil and histidine. B) A heterozygous diploid strain with *tub2-311::HIS3* and *brn1-570::URA3* alleles was induced to sporulate and tetrads were dissected on YEPD plates. The genotype of viable segregants was scored on synthetic medium lacking uracil and histidine. Segregants viable on synthetic medium lacking uracil and histidine are denoted by **'+'** and inviable segregants on synthetic medium lacking uracil and histidine are denoted by '-'.

DISCUSSION

rDNA morphology during cell cycle

There have been considerable efforts to understand the mechanism of initiation and maintenance of chromosome condensation during the cell cycle. With the current understanding of how condensin mutants result in loss of chromosome compaction and segregation, it is evident that the condensin complex is a major contributor involved in alteration of chromosome structure. It is regulated by multiple kinases at different time points in the cell cycle. The molecular details of chromosome condensation remains largely uncharacterized and thus we have tried to address this essential process using *Saccharomyces cerevisiae* as a model organism.

Chromosome condensation is an essential prerequisite for the maintenance of genomic integrity and viability. In this study, we defined four distinct species or intermediates during the process of mitotic condensation of the rDNA locus during the time course of a single cell cycle: large and small puffs in G_1, lines in S phase, arabesque during early mitosis and loops in M phase. Since the rate of cell cycle was decreased by lower temperatures, we were able to identify and characterize transient species. These *in vivo* rDNA species are likely representative of progressive levels of chromosome organization. Indeed, rDNA lines are significantly more compact than the pre-mitotic puff phenotype, but are transient. With progression of the cell cycle, rDNA further resolves into arabesques and finally loops, which are clearly resolved away from the bulk of yeast chromosomes. We propose that these different rDNA morphologies reflect *in vivo* kinetic intermediates that are an inherent component of the condensation process. Consistent with this, the transformation from one species to another is dependent on condensin, a well-established effector of the condensation phenomenon.

The progression of rDNA intermediates from puffs into loops may represent an ordered sequence of reactions that are required for mitotic chromosome folding. Such a multistep process is a common feature observed in other higher-order nucleoprotein complex assembly reactions. Condensin-dependent chromosome intermediates can be modeled as steps in higher-order nucleoprotein complex assembly. First, condensin binds its substrate DNA, wrapping chromatin

around a protein core as has been observed *in vitro* with naked DNA (Bazett-Jones et al. 2002). Since the rDNA is composed of tandem repeats, it seems likely that condensin would be regularly distributed throughout the rDNA array, demonstrated by protein localization studies (Freeman et al. 2000; Bhalla et al. 2002). Subsequent higher-order complex formation would involve interactions between distant, condensin-bound DNA sites on the rDNA locus. This transient assembly step could be stimulated by cohesins bound within each rDNA repeat (Laloraya et al. 2000), consistent with the suggestion that cohesins play a role as *cis*-acting factors in condensation (Lavoie et al. 2002). Further resolution of the chromosomes would occur generating line and loop morphologies, with possible oligomerization reactions between condensin molecules. If the higher-order chromatin organization and assembly of condensins proceeds in a co-operative manner, with sterical constraints, high affinity and low dissociation, then the function of condensin might also be to provide rigidity to the chromosomes as suggested by studies in *C. elegans* (Stear and Roth, 2002). Consistent with this notion, the transition we observe from rDNA puffs to loops involves the formation of a more extended rDNA structure, which protrudes away from the nucleus rather than folding randomly onto itself. These features of loops are strongly suggestive that the chromosomes become rigid.

It remains to be determined whether the condensation intermediates of the *rDNA* locus of chromosome XII on the *S.cerevisiae* genome and the ensuing model for condensation will prove a faithful model for euchromatic chromosomes in yeast and other organisms. Although *S.cerevisiae* condensation employs the same machinery as all other eukaryotes, the rDNA is a specialized substrate consisting of a 1 Mb region of repeated structure. The uniformity and size of the rDNA locus likely facilitates the detection of intermediate species yet to be reported at euchromatic sites in yeast and other organisms. However, additional levels of regulation may make aspects of these intermediates specific to this locus or to repetitive DNA. The rDNA appears to remain condensed later in mitosis than euchromatic sites (Guacci et al. 1994). This difference may suggest that specific regions of chromosomes undergo condensation at different rates and different times. The requirement for condensin for both rDNA and euchromatic chromosome condensation argues that the fundamental mechanism of condensation is likely to be conserved. Because the enzymatic and structural role for condensin in condensation is likely to remain unchanged with different DNA substrates, it is essential to understand the mitotic rDNA structure to provide significant insight

into the fundamental mechanism of condensin function *in vivo* that will be applicable to euchromatic sequences in yeast and other organisms.

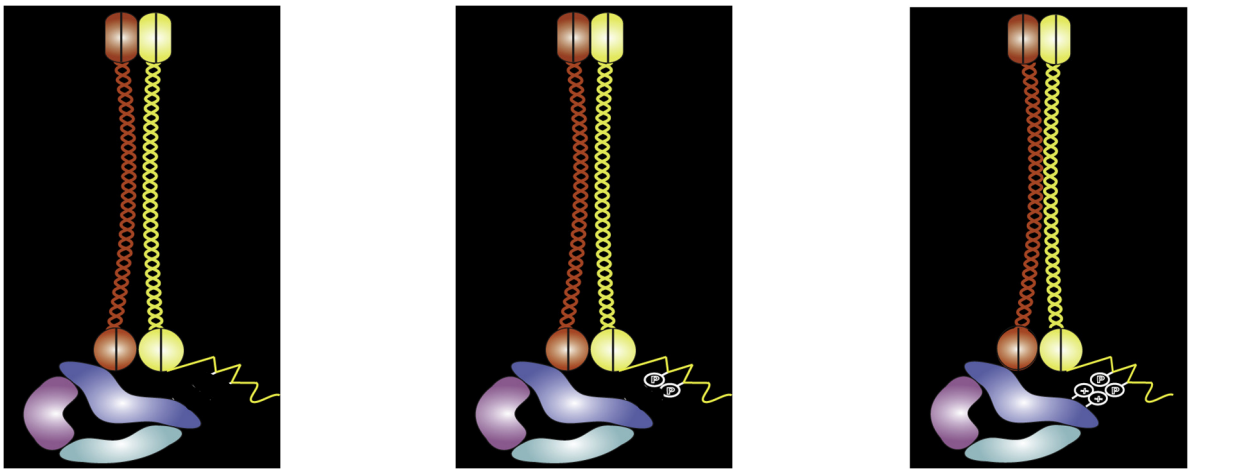
Interaction between condensin subunits

Eukaryotic cells possess another SMC protein complex, known as cohesin, that plays a major role in sister chromatid cohesion during mitosis and meiosis (Nasmyth and Haering, 2005; Losada and Hirano, 2005). The cohesin complex consists of Smc1, Smc3, a kleisin subunit (Scc1) and a fourth subunit (Scc3) with no obvious structural motifs. Biochemical analyses have shown that the N-terminal and C-terminal domains of Scc1 associate with the head domains of Smc3 and Smc1, respectively. This has led to the prediction that the cohesion functions through the formation of a tripartite 'ring-like' structure (Haering *et al*, 2002).

Consistently, an electron microscopic study showed that cohesin complexes purified from vertebrate cells display a ring-like shape in which the non-SMC subunits apparently bridge the two head domains of the SMC dimer (Anderson *et al*, 2002). Previous studies have shown that the kleisin subunit is indispensable in the assembly of the condensin I holocomplex suggesting that the architecture of condensins and cohesin is conserved at the level of the SMC dimer–kleisin interactions (Onn et al, 2007). Since cohesion activity is regulated by opening and closing of the ring, a similar mechanism may regulate condensin loading and maintenance on chromatin. Consistent with this, *brn1-6A* showed thermosensitivity and *brn1-6E* was lethal implying that these residues are essential for viability. Complex formation in the *brn1-6A* was significantly reduced suggesting that the closed ring structure was achieved by interaction between the Smc4 N-terminus extension and positive residues on C-terminus of Brn1. Condensation defects in the *brn1-6A* demonstrated that these residues are important for condensation activity.

All the available data suggest that condensin is phosphorylated by different kinases, so there must be a definite advantage of adding more negative charges on the condensin molecule with respect to DNA-protein interactions. Unpublished work from our lab demonstrates that phosphorylation of Smc4 by Cdk1 at specific residues initiates condensin activation. One possibility might be that the negative charge on the Smc4 subunit alters the conformation of the complex such that it is

more accessible to loading onto chromatin. This theory is supported by the analysis of Brn1 sequence in different species, revealing a number of positively charged residues on the C-terminus, which are evolutionarily conserved. *In silico* sequence alignment of the N-terminal extension of Smc4 with the C-terminus of Brn1 shows that these positively charged residues likely interact with the Cdk1 phosphorylation consensus sites, suggesting a role in regulation of the condensin complex (Figure 13). To further dissect and elucidate the structure and function of condensins, reconstitution of the sub- and holocomplexes from recombinant subunits is essential.



Open conformation of condensin

Phosphorylation of Smc4

Interaction of Smc4 and Brn1 to form the closed ring

Figure 13: Proposed model for condensin regulation by interaction of catalytic and regulatory subunits

In the current study, we tried to express recombinant condensin subunits to study the specific interaction between Smc4 and Brn1. It has been demonstrated that using the full length Smc4 for coimmunoprecipitation results in the pull down of all condensin subunits (Onn et al, 2007). To demonstrate that the interaction is specific to the N-terminus of Smc4 and C-terminus of Brn1, we developed short constructs of N-terminal fragment of Smc4 and C-terminal fragment of Brn1. It has been shown that amino acids 635-812 are essential for interaction with Smc4 (Onn et al, 2007). The N-terminal extension of Smc4 is unique to condensin complex as the other Smc proteins, Smc3 in cohesin and Smc6 in the Smc5/6 complex do not have the N-terminal extension.

It has been shown that this extension contains 7 Cdk1 consensus sites, which might be the initiation switch for condensin activation. But, transformation of plasmids containing the truncated version of Brn1 could not result in stable expression as the protein could not be detected by Western blot analysis. Brn1 is a large protein of 116 kDa and the short version might be subjected to degradation and difficult to express *in vivo*.

Condensin I is shown to demonstrate pseudo-symmetric architecture in which the kleisin subunit is important in that the N-terminal half of CAP-H links the first HEAT subunit (CAP-D2) to SMC2, whereas it's C-terminal half links the second HEAT subunit (CAP-G) to SMC4. Also, recent structural studies show that N- and C-terminal domains of kleisins contain a common structural fold although the two domains share virtually no sequence similarity (Haering *et al*, 2004; Fennell-Fezzie *et al*, 2005). Thus, it is suggested that each half of the condensin I complex is composed of an SMC subunit, a common structural domain (from the kleisin subunit) and a HEAT subunit. Hence, loss of the N-terminal structural domain might hinder the expression of a truncated version of the protein. A possible alternative would be to express and purify the two proteins in a bacterial system and perform biochemical interaction studies.

Interaction of Brn1 with Tubulin

In addition to chromosome condensation, chromosome attachment to microtubules prior to anaphase is essential for faithful segregation of sister chromatids. The proper attachment of kinetochores on sister chromatids to microtubules from opposite poles is called biorientation. Recent studies have shown that condensin is a regulator of biorientation (Verzijlbergen et al, 2014). The authors show that Brn1 is enriched at the centromere concurrent with proper spindle morphology at the kinetochore. Since microtubules are made up of negatively charged polymers of α/β tubulins, interaction with positive residues on Brn1 may stabilize the attachment of microtubules on centromere. Consistent with this, *brn1-6A* demonstrated synthetic lethality with *tub2-311,* while another allele of *BRN1 brn1-570* crossed with *tub2-311* remained viable. This suggests that these positive residues on the C-terminus of Brn1 might play a role in biorientation.

CONCLUSION AND PERSPECTIVES

Using the rDNA locus as a substrate, we identified cell cycle dependent morphologies-puff in G1, crescent in S, arabesque in early mitosis and loop in mitosis. The transformation of the rDNA from puff to loop is orchestrated by condensin. This system can be used to identify novel condensin mutants.

We have identified six positive residues on the C-terminus of Brn1 which affect complex formation, condensation activity and interaction with tubulin. This suggests that these residues have a role in condensin regulation. In future, we could screen truncated versions of Brn1 of different lengths to look at interaction with Smc4. Since *brn1-6A* showed synthetic lethality with *tub2-311*, it would be worthwhile to look at spindle morphology and microtubule binding in the *brn1-6A* background. Also, mass spectrometric analysis of Brn1 would help in identifying putative post-translational modifications at these specific residues.

Cell cycle events are tightly regulated and failure or defects in chromosome segregation leads to various types of cancers or lethality. Understanding of cell cycle events is necessary for the design of therapeutic approaches against cancers. These cell cycle events are interconnected and post-translational modifications play an important role in regulating these various cellular activities. One such complex process during cell cycle is chromosomal condensation, which is poorly understood. Studying the condensation process, which is one of the first cytological events during mitosis, would allow us to understand more about the mechanism and how it is connected with other processes of the cell.

TABLES

Table 1: List of strains used in this study

Figure #	ID	Genotype
3, 4,6,7	D3582	MATa, *tub2-311*::*Tadh1::HIS3MX6*
6,7	D4092	MATa, *tub2-311*::*Tadh1::HIS3MX6*, *ycg1-521-3HA::kanMX6*
8	D4238	MATa/MATx, *BRN1/brn1-6E-3HA-12His::URA3*
10	D515	MATa, *BRN1-3HA*
10	D4011	MATa, *brn1-6A-3HA*
10	D4130	MATa, *BRN1*-3HA, *SMC4-13Myc*
10	D4106	MATa, *brn1-6A*-3HA, *SMC4-13Myc*

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